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Worthington Enzyme Manual

Enzymes and Related Biochemicals

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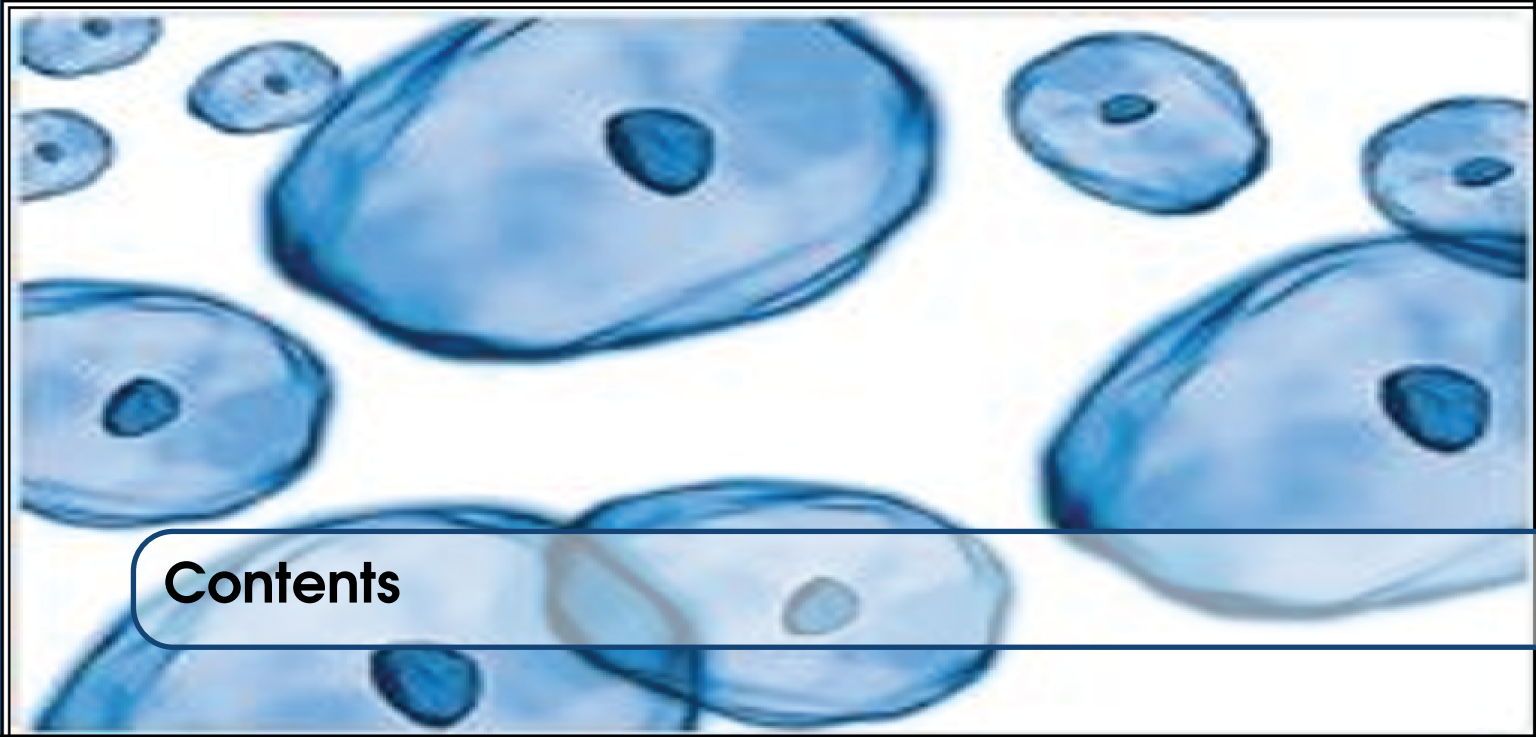

Contents

1. Actin

Actin is a protein that functions in the contractile system of skeletal muscle, where it is found in the thin filaments. In muscle, fibrous actin (F-actin) is a helical polymer of a globular polypeptide chain, G-actin. Actin is present in all eukaryotes and has a highly conserved protein sequence (Pollard 1990).

Actins of non-muscle cells are encoded by different genes than those of muscle, and have been found to be involved in platelet shape (Lefebvre *et al*. 1993), endocytosis (Robertson *et al*. 2009), cell motility (Lazarides and Weber 1974), cell division (Hill *et al*. 1996), metastasis (Gabbiani *et al*. 1975), and cell signaling (Liu *et al*. 1990).

Actin is usually isolated as G-actin, which may be reversibly transformed into a viscous polymerized fibrous form, F-actin, by the addition of neutral salts and at neutral, or slightly alkaline, pH (Chantler and Gratzer 1975). The reaction, which involves bound nucleotide is: Actin is usually isolated as G-actin, which may be reversibly transformed into a viscous polymerized fibrous form, F-actin, by the addition of neutral salts and at neutral, or slightly alkaline, pH (Chantler and Gratzer 1975). The reaction, which involves bound nucleotide is:

$$\text{G-actin-ATP} \rightarrow \text{F-actin-ADP} + \text{Pi} \tag{1.1}$$

History

While there has been some debate regarding the official discoverer of actin, it is believed that in 1887 Halliburton was the first to make an extract that contained crude actin, and not until 1942 that Straub (Straub 1942) first isolated pure actin. Straub's original method of isolating actin is relatively similar to the procedures used today (Pollard 1990). At that time, however, little was known about muscle structure at the submicroscopic level.

The 1950s brought a greater understanding of the filamentous structure of actin. The sliding filament model was developed, and remains essentially unchanged since then. This theory explains that the thick and thin filaments within the sarcomere slide past one another, shortening the entire length of the sarcomere. In order to slide past one another, the myosin heads will interact with the actin filaments and, using ATP, bend to pull past the actin (Huxley 2004).

During the 1960s and 1970s, two dimensional X-ray patterns of the actin structure were obtained and studied (Huxley 2004). In the 1980s, the first interpretable electron density maps of the actin molecule were acquired (Hirono *et al*. 1989), and the ATP hydrolysis and phosphate dissociation was characterized (Kono 1988).

The involvement of actin and its associated proteins' involvement in metastasis was investigated in the 1990s, and the crystal structure was solved (Kabsch *et al*. 1990). Recent research has focused on the functions of actin in endocytosis (Robertson *et al* 2009), and it has been proposed that actin is a component of chromatin remodeling complexes in RNA biogenesis (Percipalle 2009).

The structure contains four domains. Two of these domains are similar alpha/beta domains, which contain the ATPase catalytic site. The F-actin helix consists of 13 molecules of G-actin in six turns of the helix, which repeat every 360 Angstroms (Branden and Tooze 1999). Each actin molecule consists of five sulfhydryl groups. Actin is associated with tropomyosin and the troponin complex in muscle. Actin also contains a myosin binding site, where it forms temporary complexes with myosin during muscle contraction, and permanently during rigor mortis (Pollard 1990).

Molecular Characteristics

Actin consists of 376 amino acids. The high proportion of proline and glycine (4.9 and 7.5%, respectively) residues contributes to G-actin's globular shape. Six actin genes are expressed in mammals and birds, and these sequences all share a great deal of homology (Vandekerckhove and Weber 1984).

Composition

The structure contains four domains. Two of these domains are similar alpha/beta domains, which contain the ATPase catalytic site. The F-actin helix consists of 13 molecules of G-actin in six turns of the helix, which repeat every 360 Angstroms (Branden and Tooze 1999). Each actin molecule consists of five sulfhydryl groups. Actin is associated with tropomyosin and the troponin complex in muscle. Actin also contains a myosin binding site, where it forms temporary complexes with myosin during muscle contraction, and permanently during rigor mortis (Pollard 1990).

CAS

- 51005-14-2

Protein Accession Number

- P68135

CATH: Classification (v. 3.3.0)

Class:

- Alpha Beta

Architecture:

- 2-Layer Sandwich and Alpha-Beta Complex

Topology:

- Nucleotidyltransferase; domain 5, and Actin; Chain A, domain 4

Molecular Weight

- 41.8 kDa

Isoelectric Point

- 5.23

Extinction Coefficient

- 43,840 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 10.48

Applications

- Binding studies
- Endogenous internal controls to normalize gene expression studies

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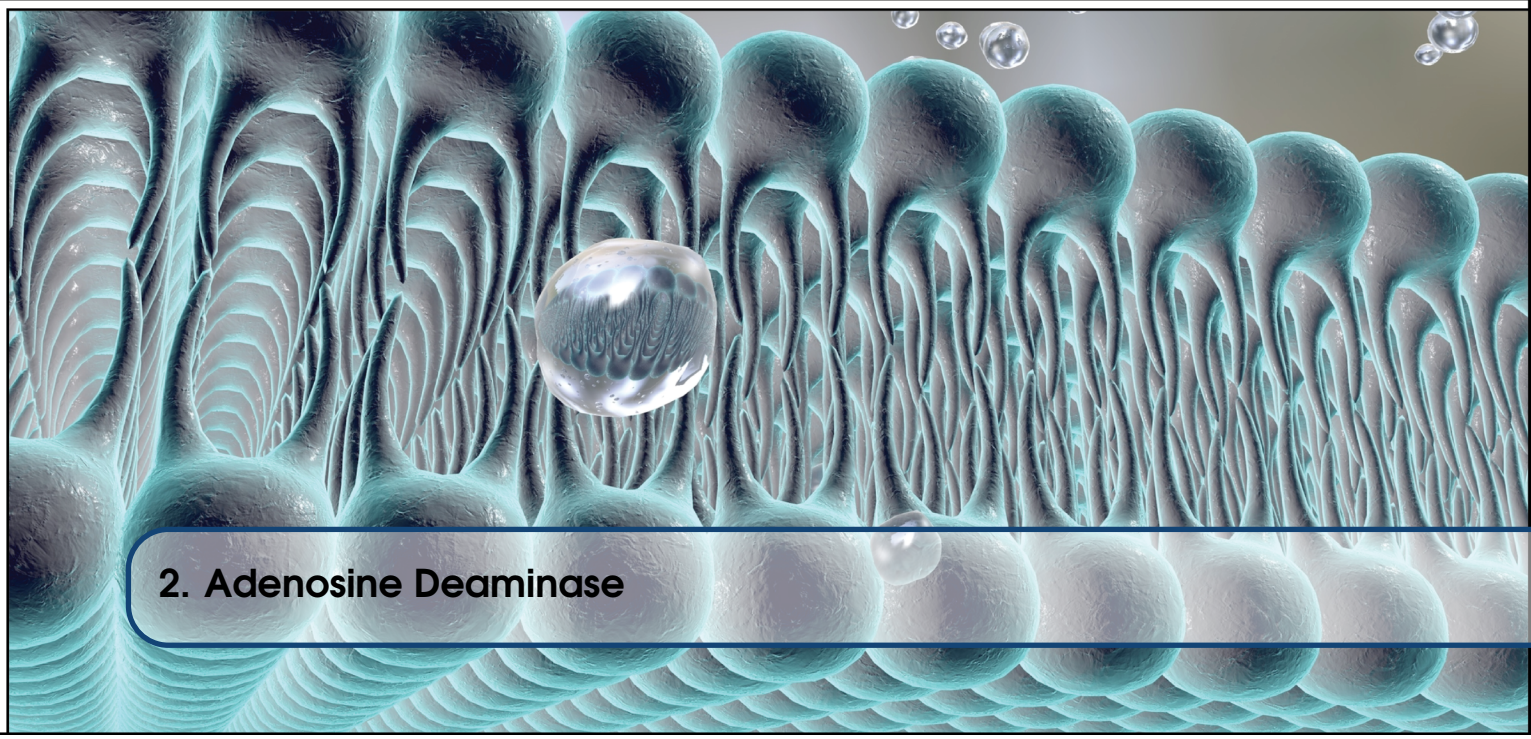

2. Adenosine Deaminase

Adenosine deaminase catalyzes the irreversible deamination of 2'-deoxyadenosine and adenosine to deoxyinosine and inosine. It is found in a wide variety of prokaryotes and eukaryotes in different forms (Pospisilova and Frebort 2007).

$$\text{Adenosine} + H_2O \rightarrow \text{Inosine} + NH_3 \tag{2.1}$$

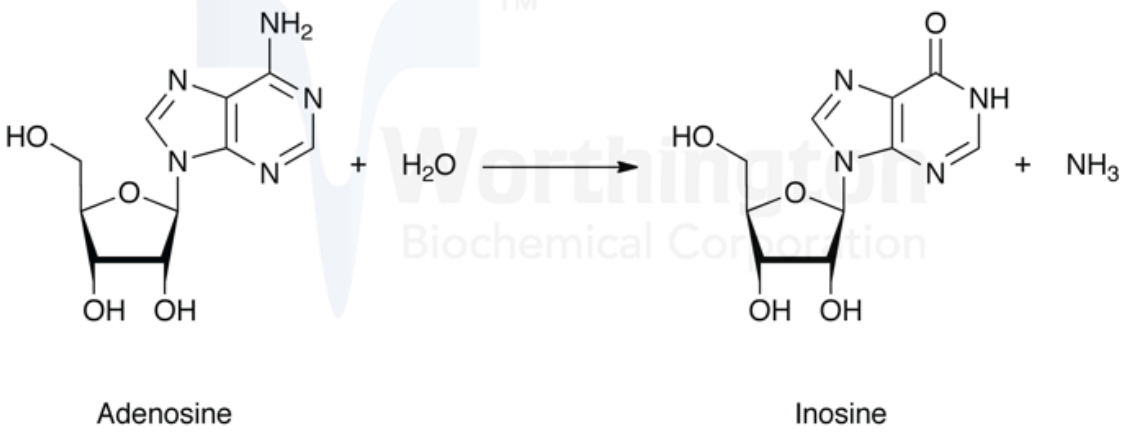

Figure 2.1: Enzymatic Reaction - Adenosine Deaminase

History

In 1928 and in 1932, Schmidt first showed the high specificity of adenosine deaminase (Schmidt 1928 and 1932). Conway and Cook found that unlike other deaminases studied at the time, adenosine deaminase was not as affected by tissue inhibitors, phosphate, or bicarbonate (Conway and Cook 1939). It was later discovered that the tissue Schmidt had been using was a very poor source of adenosine deaminase, and Conway and Cook discovered the high activity in calf mucosa.

Throughout the 1940s and 1950s, the reaction mechanism was further investigated, and adenosine deaminases from different sources were purified and studied (Kaplan 1952 and 1955).

In the 1960s, the preparation of calf spleen adenosine deaminase was developed and refined, which led to a greater understanding of its enzymatic properties (Pfronger 1967 a and b). In the late 1960s, adenosine deaminases from different species were compared (Wolfendental 1968, Akedo *et al.* 1970, and Edwards *et al.* 1971), and into the 1970s research began to focus on adenosine deaminase from humans to better understand its connection to immunodeficiencies (Meuwissen and Pollara 1974, and Hirschhorn *et al.* 1976).

Research of the 1980s increased the use of adenosine deaminase as a diagnostic tool in a variety of illnesses including meningitis and tuberculosis (Ribera *et al.* 1987, and Segura *et al.* 1989). Inhibitors of adenosine deaminase were investigated in the 1990s (Weitberg and Corvese 1990), and in 1991 the crystal structure of mouse adenosine deaminase was resolved (Wilson *et al.* 1991).

Today, human adenosine deaminase is widely studied because of its importance in the medical field. Gene regulation and gene therapy are are being investigated to understand the clinical implications of ADA gene regulation (Aiuti *et al.* 2009, and Wang 2009).

Molecular Characteristics

In higher eukaryotes, two different isozymes are encoded by different genes (Maier *et al.* 2005). In humans, ADA1 is a single-chain Zn-binding protein and almost all activity is attributed to this protein. ADA2 is believed to be produced by monocytes and is found in negligible quantities. Mutations in the ADA1 gene, where expression is blocked, cause immunodeficiency, whereas mutations that cause overexpression cause hemolytic anemia (Pospisilova and Frebort 2007).

The enzyme has a wide phylogenetic distribution and its sequence is highly conserved from bacteria to humans (Chang 1991). The genes from lower eukaryotes contain no introns, whereas 95% of the DNA sequence in mammalian ADA genes are occupied by introns (Pospisilova and Frebort 2007). Gene sequences have been reported for cattle, mouse, human, *Escherichia coli*, *Saccaromyces cerevisiae*, and *Streptomyces virginiae*. The amino acid residues around the active site are highly conserved in mammals (Cristalli *et al.* 2001). Human and bovine adenosine deaminases are 93% identical (Kelly *et al.*1996), and human and mouse adenosine deaminases are 83% superimposible (Wiginton *et al.* 1984, Daddona *et al.* 1984, and Yeung *et al.* 1985). The bacterial enzyme shares 33% identity with mammalian adenosine deaminase (Kelly *et al.* 1996).

Specificity

Adenosine deaminase has evolved to act upon adenosine, and in the lab on its derivatives (Sharoyan *et al*. 2006). Additionally, adenosine deaminase acts on carbon-nitrogen bonds other than peptide bonds, in cyclic amidines (White and White 1997).

Composition

Adenosine deaminase has a $(\text{beta/alpha})_8$ barrel structural motif, and contains a zinc atom in the catalytic pocket. A single, bound, divalent cation (zinc or cobalt) is required for catalytic activity (Cristalli *et al*. 2001).

IUB

- 3.5.4.4

CAS

- 9026-93-1

Protein Accession Number

- P56658

CATH: Classification (v. 3.3.0)

Class:
- Alpha Beta

Architecture:
- Alpha-Beta Barrel

Topology:
- TIM Barrel

Molecular Weight

- 40.8 kDa

Optimal pH

- 5.0-9.0 (Rossi and Lucacchini 1974)

Isoelectric Point

- 5.33

Extinction Coefficient

- 43,960 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$

Active Residue

- Histidine (H214)
- Cysteine (C262)
- Aspartic acids (D295 and D296)

Activators

- Zn^{2+}

Inhibitors

- Ag^{+}, Hg^{2+}, Cu^{2+}
- TPCK, DFP
- Sulfhydryl reagents
- Erythro-9-(2-hydroxy-3-nonyl)adenine and Erythro-9-(2-hydroxy-3-nonyl)-3-deazaadenine
- 9-(p-aminobenzyle)adenine and p-chloromercuribenzoate
- Purine riboside

Applications

- Elevated levels are a diagnostic test for various human diseases (Ribera *et al.* 1987, and Segura *et al.* 1989)
- Inosine, deoxyinosine and dideoxyinosine synthesis (Sheid 1996)
- Determination of adenosine in biological fluids (Piras 1973)
- Determination of 5'-nucleotidase in blood (Blackburn *et al.* 1992)

Assay Information

Method

- The reaction velocity is measured by a change in absorbance at 265 nm resulting from the deamination of adenosine. One unit converts one micromole of adenosine to inosine per minute at 25°C and pH 7.4 under the specified conditions.

Reagents

- 1.4 mM Adenosine in 0.05 M Phosphate buffer, pH 7.4
- 0.05 M Phosphate buffer, pH 7.4

Enzyme

- Dilute immediately before use in ice cold 0.05 M phosphate buffer, pH 7.4 to a concentration of 0.2 - 0.8 $\frac{\text{units}}{\text{ml}}$.

Procedure

Spectrophotometer Settings: Wavelength: 265 nm Temperature: 25°C

Pipette into cuvettes as follows:

- 0.05 M Phosphate buffer, pH 7.4 - 2.88 ml
- 1.4 mM Adenosine - 0.1 ml

Incubate in spectrophotometer for 3-5 minutes to achieve temperature equilibration and establish blank rate, if any. At zero time add 0.02 ml enzyme solution and mix thoroughly. Record decrease in A_{265} for 2-3 minutes. Use the initial rate to calculate $\frac{\Delta A_{265}}{\text{min}}$.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{265}}{\text{min}}}{\frac{8.1 \text{ x mg enzyme}}{\text{ml reaction mixture}}}$$

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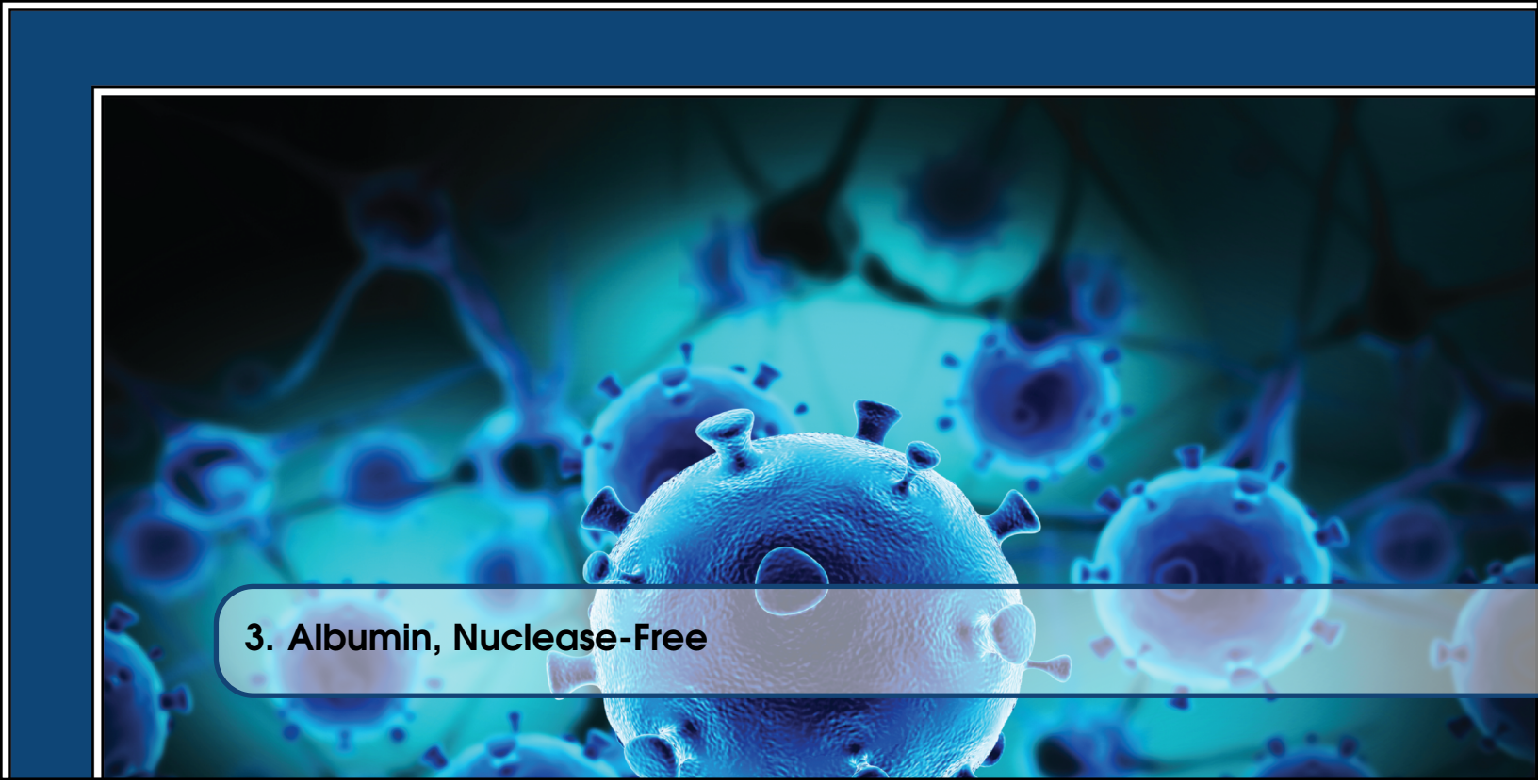

3. Albumin, Nuclease-Free

Serum albumin is the most abundant protein of the circulatory system and functions to bind and transport long chain fatty acid anions, to remove bilirubin, and as back-up transport of thyroid and steriod hormones (Murayama and Tomida 2004, and Peters 1977).

History

The albumin name is derived from the Latin word albus, which means white, referring to its presence in egg white.

In 1921, Howe demonstrated the differences in the albumin/globulin ratio of albumin sera from newborn and adult animals (Howe 1921). In 1947 Pederson showed that in calves this difference was due to the presence of a fetal-specific alpha-globulin (Pederson 1947). In the 1950s, and into the 1960s, further differences in various plasma proteins of fetal and adult animals were shown in a number of species (Andreoli and Robbins 1962, Pantelouris and Hale 1962, and Wise *et al.* 1963).

In the 1960s, the effects of low pH on albumin were studied (Bloomfield 1966, and Slayter 1965), and in the 1970s Albumin's biosynthesis was investigated, which led to the determination of the N-terminal amino acid sequences (Peters 1977, and McGillivray *et al.* 1979).

In the 1980s and 1990s, the conformational changes that occur upon thermal denaturation (Takeda *et al.* 1989) The primary structure of bovine serum albumin was determined in 1990 (Hirayama *et al.* 1990).

Today, albumin is primarily used in the research and medical communities for molecular studies (Kreader 1996). The polymorphisms of albumin are also being studied, which has led to the discovery of new albumin variants in cattle (Ibeagha-Awemu *et al.* 2004).

Molecular Characteristics

The protein is synthesized as a precursor, preproalbumin. Residues 19-24 are the proregion. The signal sequence of bovine preproalbumin has shown considerable homology with that of rat preproalbumin; however, the sequence has shown no homology with other bovine proteins (MacGillivray *et al*. 1979, and Fujiwara and Amisaki 2006).

Composition

Bovine serum albumin consists of nine loops connected by 17 disulfide bridges that are protected in the core of the protein (Restani *et al*. 2004). Bovine serum albumin is very soluble in water yet it is relatively resistant to digestion.

CAS

- 9048-46-8

Protein Accession Number

- P02769

CATH: Classification (v. 3.3.0)

Class:
- Mainly Alpha

Architecture:
- Orthogonal Bundle

Topology:
- Serum Albumin; Chain A, Domain 1

Notes:
- (Based on human serum albumin, which is 76% identical to bovine)

Molecular Weight

- 66.4 kDa

Optimal pH

- 5.0-7.0 (El Kadi *et al*. 2006)

Isoelectric Point

- 5.6

Extinction Coefficient

- 41,180 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280} = 6.20$

Applications

- Albumin is used in applications requiring inhibition or reduction of non-specific protein-protein interactions and interactions between proteins and biological macromolecules in techniques such as:
- Western, Northern, Southern, and dot blots (Sambrook 2001)
- PCR and RT-PCR reactions (Kreader 1996, and Pandya *et al*. 1994)
- Enzymatic reactions (Gianfreda and Scarfi 1991, and Chang 1994 and 1995)
- Nucleic acid hybridizations (Sambrook 2001)

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confirmed the presence of Zinc metal (Vallee and Hoch 1955a).

In the 1960s the inhibitors of alcohol dehydrogenase were studied (Atkinson *et al*. 1967), along with the roles of specific structural components (Auricchio and Bruni 1969). Structural and kinetic studies (Blackwell *et al*. 1974) continued on into the 1970s when conformational changes associated with binding were investigated (Abdallah *et al*. 1975), along with the isozymes (Berger *et al*. 1974).

During the 1980s the genetics, biochemistry, and developmental regulation of ADH in various species, including mice (Watabiki *et al*. 1989) and pig (Keung and Fong 1988), were investigated. The 1990s brought better understanding of the role of the zinc metal (Magonet *et al*. 1992), and the discovery of additional inhibitors (Pereira *et al*. 1992, Sachan and Cha 1994, and Shiao *et al*. 1994).

Recent research has focused on obtaining alcohol dehydrogenases with higher catalytic activity, and a better understanding ADH gene regulation (Larroy *et al*. 2002a and b, Lertwattanasakul *et al*. 2007, and Park 2009).

Molecular Characteristics

Seven genes for *Saccharomyces cerevisiae* have been identified (Lertwattanasakul *et al*. 2007). ScADH1 is expressed in large amounts in the presence of glucose, and it encodes for the fermentative enzyme that produces ethanol (Bennetzen and Hall 1982). ScADH2 is negatively regulated by glucose and encodes the isozyme that converts ethanol to acetaldehyde (Ciriacy 1975, Wills and Jornvall 1979, and Russel and Hall 1983). The ScADH3 gene is suppressed by glucose and the mature form of the protein is found the mitochondria (Young and Pilgrim, 1985). ScADH4, ScADH5, and ScADH6 encode for the ScADH4, ScADH5, and ScADH6 proteins. ScADH6 and ScADH7 are thought to contribute to the balance of NADP/NADPH (Larroy *et al*. 2002a and b).

Specificity

Yeast ADH has a more narrow specificity than that of the liver enzyme. It accepts ethanol and is somewhat active on the straight chain primary alcohols. It acts to a very limited extent on certain secondary and branched chained alcohols (Dickinson and Dalziel 1967a).

Composition

Alcohol dehydrogenase is a tetramer with each subunit containing one zinc atom (Vallee and Hoch 1955). Per subunit, there are two distinct active site sulfhydryl groups which can be distinguished on the basis of differential reactivity with iodoacetate and butyl isocyanate (Twu, Chin, and Wold 1973). A histidine residue has an essential role (Dickenson and Dickinson 1975 and LeBrun *et al*. 2004).

IUB

- 1.1.1.1

CAS

- 9031-72-5

Protein Accession Number

- P00330

CATH: Classification (v. 3.3.0)

Class:

- Alpha Beta

Architecture:

- Alpha-Beta Complex and 3-Layer (aba) Sandwich

Topology:

- Quinone Oxidoreductase; Chain A, domain 1 and Rossmann fold

Molecular Weight

- 146.8 kDa

Optimal pH

- 5.4 (Shore and Theorell 1966)

Isoelectric Point

- 6.23

Extinction Coefficient

- 189,320 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 12.89

Activators

- Sulfhydryl activating reagents
- Mercaptoethanol
- Dithiothreitol
- Cysteine
- Heavy metal chelating agents (White and White 1997)

Inhibitors

- Heavy metals and -SH reagents
- Thiourea
- Purine and pyrimidine derivatives
- Chloroethanol and fluoroethanol
- N-alkylmaleimides
- Iodoacetamide
- 1,10-phenanthroline

- 8-hydroxyquinoline
- Beta-NAD analogs (White and White 1997)

Applications

- Enzymatic determination of primary alcohols, and aldehydes
- Synthesis of chiral compounds
- Spectrophotometric assay of plasmalogenase
- Enzymatic catalysis in organic solvents
- Studies of NAD^+, NADH, $NADP^+$, and NADPH

Assay Information

Method

- The reaction velocity is determined by the method of A Vallee and Hoch (1955) in which the rate of absorbance at 340 nm resulting from reduction of NAD is measured. One unit reduces one micromole of NAD per minute at 25°C under the specified conditions.

Reagents

- 0.1 M Sodium pyrophosphate buffer, pH 9.2
- 2 M Ethanol. Dilute 12.12 ml of 95% ethanol to 100 ml with reagent grade water.
- 0.025 M NAD. Note: NAD may vary in salt form and degree of hydration. Care should be exercised to use an analytical grade and the correct molecular weight.
- 0.1 M Phosphate buffer, pH 7.5
- 0.1% Albumin (BSA)

Enzyme

- Dissolve lyophilized enzyme at one $\frac{\text{mg}}{\text{ml}}$ in 0.1 M phosphate buffer, pH 7.5.
- Immediately prior to use, dilute all enzymes to a concentration of 0.05-0.25 $\frac{\text{units}}{\text{ml}}$ in 0.1% albumin.
- For protein determinations, dissolve enzyme in 0.1 M phosphate buffer, pH 7.5.

Procedure

Spectrophotometer Settings: Wavelength: 340 nm Temperature: 25°C

Pipette into each cuvette:

- 0.1 M Pyrophosphate buffer - 1.5 ml
- 2.0 M Ethanol - 0.5 ml
- 0.025 M NAD - 1.0 ml

Incubate in spectrophotometer for 3-4 minutes at 25°C to achieve temperature equilibrium and establish blank rate, if any. At zero time, add 0.1 ml of appropriately diluted enzyme to the cuvette and record the A_{340} for 3-4 minutes. Calculate the $\frac{\Delta A_{340}}{\text{min}}$ from the initial linear portion of the curve.

Calculation

- $\frac{\text{Units}}{\text{ml}} = \frac{\frac{\Delta A_{340}}{\text{min}} \text{ x cuvette volume x enzyme dilution}}{\text{6.22 x sample volume}}$

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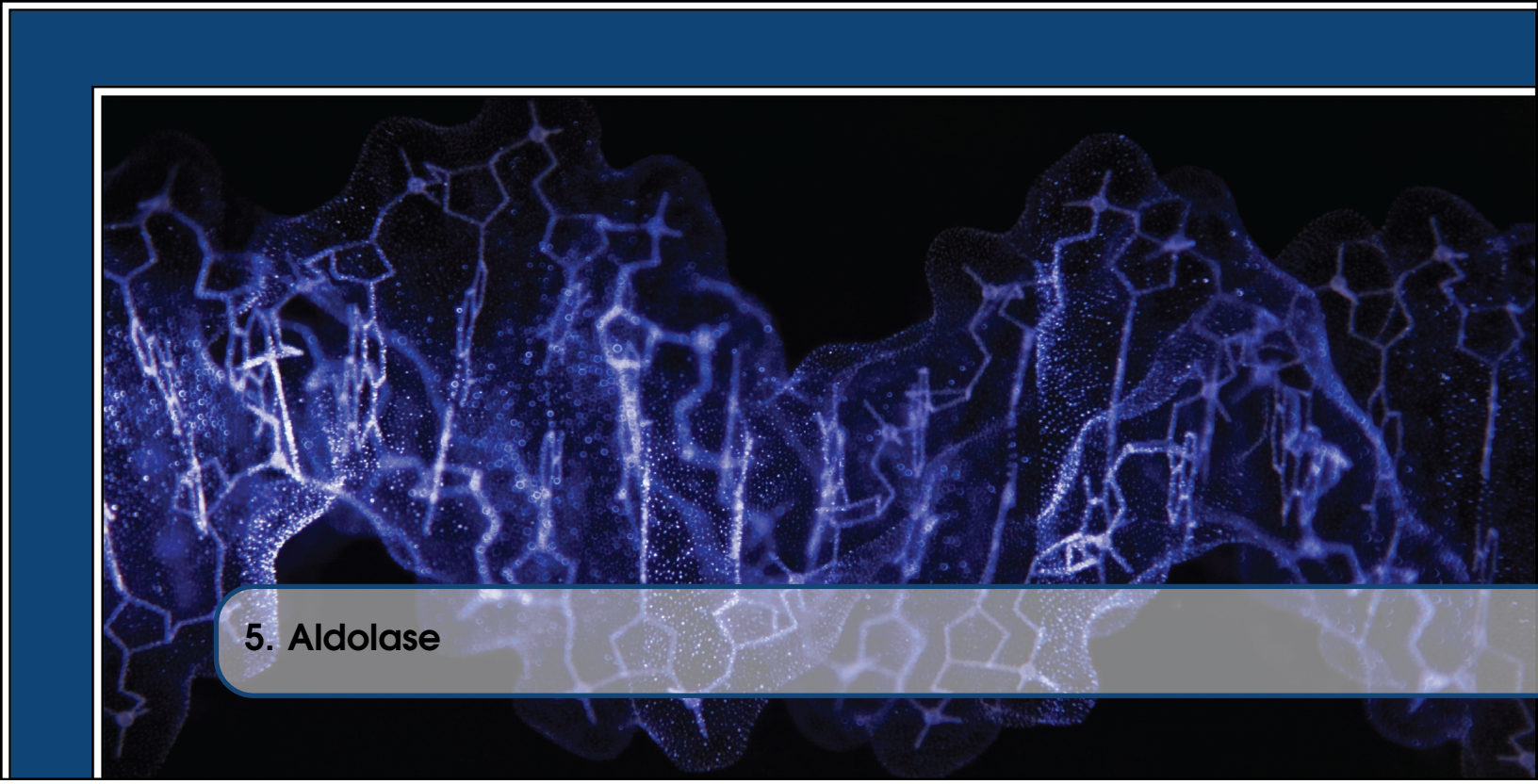

5. Aldolase

Aldolase is present in all animal and plant tissue, and in most microorganisms. Class I aldolase, found in animal and higher plant tissue, is characterized by the lack of requirement for a bivalent metal cofactor, and by the formation of ketimine Schiff base intermediate with the substrate dihyroxyacetone phosphate. Class II aldolase, found only in prokaryotes and lower eukaryotes, requires a bivalent metal cofactor (Heron and Caprioli 1975, London 1974, and Lebherz *et al*. 1973, Gefflaut *et al*. 1995). There are three types of Class I aldolases: Type A (the major form) is found in muscle; Type B in liver and kidney; and Type C (plus some A) in brain (Horecker *et al*. 1972). Fructose-biphosphate aldolase (ALD) catalyzes a key reaction in glycolysis and energy production:

D-Fructose-1,6-bisphosphate $\rightleftarrows$ dihydroxyacetone phosphate + D-glyceraldhyde-3-phosphate (5.1)

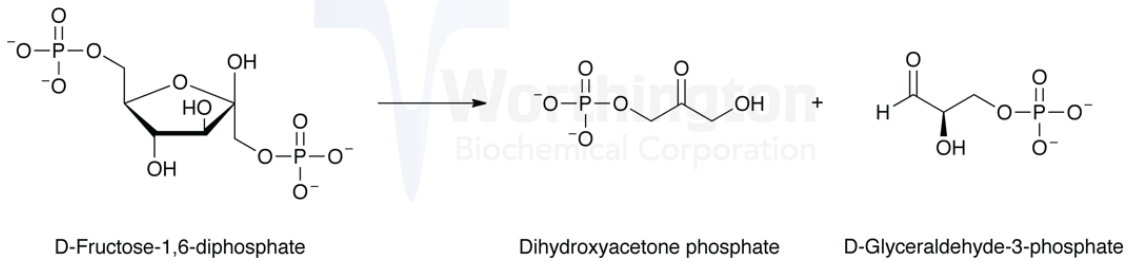

Figure 5.1: Enzymatic Reaction - Aldolase

History

In 1934, aldolase was found to be present in muscle and yeast by Meyerhof and Lohmann. Muscle aldolase was first separated from isomerase by Herbert *et al.* in 1940. Neuberg and Kobel first proposed the presence of aldolase in bacteria in 1934, which was later confirmed in *Escherichia coli* (Utter and Werkman 1941).

In the 1950s and 1960s the reaction mechanisms were investigated, and the classification scheme that divided aldolases into Class I and Class II was developed (Marsh and Lebherz 1992).

Work investigating the inhibitors began in the mid-1960s (Spolter *et al.* 1965), and early 1970s (Lehrer and Barker 1970, and Hinz *et al.* 1971). In 1969, the crystals of aldolase were studied in order to determine the tetrameric subunit structure (Eagles *et al.* 1969). The first low resolution crystal structure was determined in 1985 (Sygusch *et al.* 1985), and was refined in 1987 (Sygusch *et al.* 1987).

After some debate over the mechanism and the involvement of particular active side residues, Littlechild and Watson determined in 1993 that a cysteine residue was not present at or near the substrate binding site, despite initial studies concluding otherwise (Littlechild and Watson 1993). The first high resolution structure of rabbit aldolase was reported in 1997 (Blom and Sygusch 1997).

Today, work investigating aldolase inhibitors continues (Dax *et al.* 2005). Studies are also being conducted to better understand its utility in chemo-enzymatic syntheses (Bolt *et al.* 2008, and Calveras *et al.* 2009), its interactions with other cellular proteins (Forlemu *et al.* 2007), and its reaction intermediates (St.-Jean *et al.* 2005).

Molecular Characteristics

The primary structures of Class I eukaryotic enzymes are highly conserved (Kelly and Tolan 1986, and Freemont *et al.* 1988); however, there are no similarities between the sequences of Class I and Class II enzymes, as they have separate evolutionary origins (Marsh and Lebherz 1992). However, both Class I and Class II enzymes contain carboxy-terminal tyrosine residues that are required for maximal catalytic activity (Rutter 1961, and Morse and Horecker 1968). Prolonged contractile activity reduces the type A aldolase expression in mRNA by downregulating cytoplasmic glycolytic enzymes, and enhancing mitochondrial enzymatic activity (Williams *et al.* 1986).

Specificity

Aldolase is highly specific for its substrate, D-fructose.

Composition

The three isozymes (A, B, and C) found in mammals are composed of four identical subunits, each a polypeptide chain of 360 amino acids. The enzyme has a homotetrameric structure with subunits that are approximately 40 kDa (Marsh and Lebherz 1992). The structure of these subunits is a beta-barrel, surrounded by alpha-helices and connecting loops (Littlechild and Watson 1993). If activity of one of the four subunits of the homotetramer is knocked out, the entire complex is incapable of normal function, indicating a tight communication between active sites (Marsh and Lebherz 1992).

IUB

- 4.1.2.13

CAS

- 9024-52-6

Protein Accession Number

- P00883

CATH: Classification (v. 3.3.0)

Class:

- Alpha Beta

Architecture:

- Alpha-Beta Barrel

Topology:

- TIM Barrel

Molecular Weight

- 156.8 kDa

Optimal pH

- 6.9-8.8

Isoelectric Point

- 8.47

Extinction Coefficient

- 133,560 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280} = 8.52$

Active Residue

- Lysine (K229)
- Glutamate (E187)
- Plus other charged residues (functional assignment is complex due to the close proximity of the many charged amino acids found at the active site) (St.-Jean *et al.* 2005)

Inhibitors

- Heavy metals, especially Cu^{2+}, Zn^{2+}, and Ag^{+}
- Hydroxynaphthaldehyde phosphate derivatives (Dax *et al.* 2005)
- Phosphorylated aromatic compounds (Blonski *et al.* 1997)
- D-Erythrulose 1-phosphate (Ferroni *et al.* 1991)
- Phosphatidylserine liposomes (Kwiatkowska *et al.* 1994)
- Phosphates and adenine nucleotides (Callens *et al.* 1991)

Applications

- Chemo-enzymatic syntheses (Bolt *et al.* 2008 and Calveras *et al.* 2009)
- Structure-function studies (St.-Jean *et al.* 2005)
- Organic synthesis (Castillo *et al.* 2006, Concia *et al.* 2009)
- Quantification of D-fructose-1,6-diphosphate
- Metabolite determination in coupled reactions (Hiller *et al.* 2007)
- Molecular weight marking in columns and gels

Assay Information

Method

- Based on Boyer's modification of the hydrazine assay (Jagannathan et al. 1956) in which 3-phosphoglyceraldehyde reacts with hydrazine to form a hydrazone which absorbs at 240 nm. The advantages of the system are its simplicity and specificity. One unit is described as a change in absorbance of 1.00 per minute at 25°C and pH 7.5 under the specified conditions. Alternate methods for the assay of aldolase have been described by Rutter (1961). The coupled a-glycerophosphate dehydrogenase assay (Richards et al. 1961) has been used.

Reagents

- 0.0001 M EDTA, pH 7.5 containing 0.0035 M hydrazine sulfate
- Substrate: 0.012 M Fructose-1,6-bisphosphate pH 7.5

Enzyme

- Immediately before use, dilute in water to a concentration of 0.5 - 2.0 units per ml.

Procedure

Spectrophotometer Settings: Wavelength: 240 nm Temperature: 25°C

Pipette into cuvettes as follows:

Description	Blank	Test
Fructose-1,6-bisphosphate	1.0 ml	1.0 ml
Hydrazine sulfate	2.0 ml	2.0 ml
Distilled water	0.1 ml	—
Record ΔA_{240} for 10 minutes		
Add enzyme	—	0.1 ml
Record ΔA_{240} for 10 minutes		
Subtract $\frac{\Delta A_{240}}{\text{min}}$ of the blank from $\frac{\Delta A_{240}}{\text{min}}$ of the test		

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{240}}{\text{min}}}{\frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$

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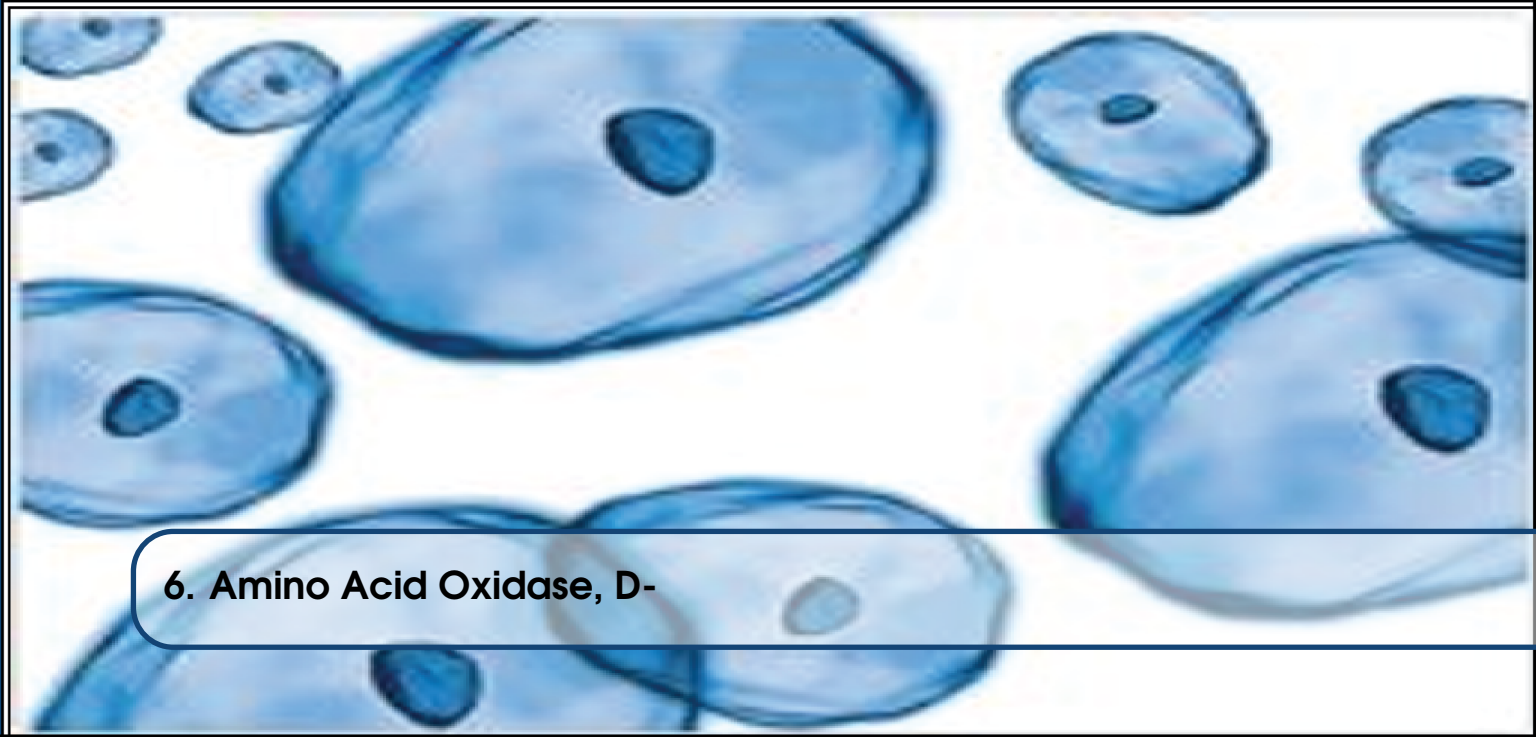

6. Amino Acid Oxidase, D-

D-amino acid oxidase (DAAO) is an oxidoreductase that oxidatively deaminates D-amino acids to the corresponding alpha-keto acid. DAAO is a flavoenzyme, containing 1 mole of FAD per monomer.

$$RCHNH_2COOH + O_2 + H_2O \rightarrow RCOCOOH + NH_3 + H_2O_2 \quad (6.1)$$

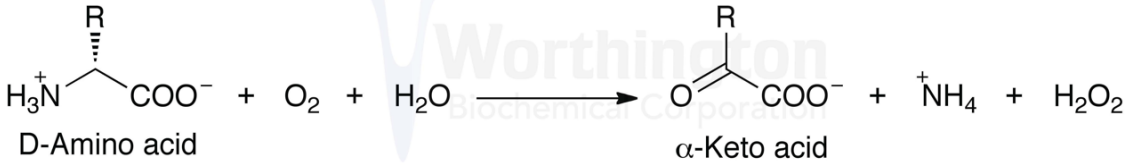

Figure 6.1: Enzymatic Reaction - Amino Acid Oxidase, D-

History

D-amino acid oxidase was the second flavoenzyme to be discovered. In 1935, Krebs discovered DAAO after he observed that porcine kidney homogenates deaminated amino acids of the "d-series... much more rapidly than the natural isomerides." In 1938, Warburg and Christian identified FAD as the enzyme's cofactor.

In the 1940s, Singer tested the role of sulfhydryl reagents as inhibitors (Singer 1948). In 1958, Kubo *et al.* developed a purification procedure for pkDAAO (porcine kidney D-amino acid oxidase). In 1961, Massey *et al.* improved this method, and it was improved again by Curti *et al.* (Curti *et al.* 1973). Curti *et al.* also first reported 5 sulfhydryl groups and 8 tryptophans per mole FAD. Tu and McCormick indicated that a tyrosine residue was probably involved in the active site, and Yagi *et al.* reported the monomer to be more active than the dimer (Tu and McCormick 1973, and Yagi *et al.* 1973b).

In the early 1980s, various inhibitors were investigated (Alston *et al.* 1983, and Carrea *et al.* 1983), and the amino acid sequence was preliminarily identified (Ronchi *et al.* 1982). Beginning in the mid-1980s, studies on DAAOs of microorganisms made it possible to carry out detailed biochemical studies (Pilone 2000). In the late 1980s, DAAO was expressed in *Escherichia coli* by Ciccarelli *et al.*, and molecular cloning and sequence analysis of cDNAs were performed by Fukui *et al.* (Fukui *et al.* 1987, and Ciccarelli *et al.* 1989).

In 1996, two groups independently reported on the 3D structure of pkDAAO, and Mettevi *et al.* provided strong support for a hydride mechanism (Mattevi *et al.* 1996, and Mizutani *et al.* 1996). Site directed mutagenesis studies (Harris *et al.* 1999, Molla *et al.* 2000, and Boselli *et al.* 2002) and the high resolution structure of yeast *Rhodotorula gracilis* support this hypothesis (Umhau *et al.* 2000). In 2006, Kawazoe *et al.* published the 3D structure of human DAAO.

Current research on DAAO is focused on its use as a biocatalyst (Truppo *et al.* 2009) and as a component of biosensors (van Staden *et al.* 2010, and Inaba *et al.* 2003).

Molecular Characteristics

The gene encoding mammalian DAAO is present in a single copy in the genome. A 1041 bp open reading frame encodes all 347 amino acids of the enzyme. This indicates posttranslational processing by proteolytic enzymes does not occur (Fukui 1987).

The primary structure of porcine D-amino acid oxidase was determined by Ronchi *et al.* (Ronchi *et al.* 1982), and the gene was cloned by Momoi *et al.* (Momoi *et al.* 1988). There are six regions of the primary structure that are highly conserved in DAAOs of various sources (Faotto *et al.* 1995). Regions I contains the consensus sequence GXGXXG, and both regions I and III have been found to be involved in coenzyme binding (Wierenga *et al.* 1983). Regions II, IV, and V contain the active site residues. The Ser-Lys/His-Leu terminal sequence is the peroxisomal targeting signal sequence (Subramani 1993, and Pilone 2000)

Mammalian DAAOs show 63% identity, and the three known DAAOs of microorganisms (*Rhodotorula gracilis*, *T. variabilis*, and *Fusarium solanii*) show a 18% identity. 30% identity is observed between yeast and mammalian DAAOs (Pilone 2000).

Specificity

The D-isomers of proline, methionine, isoleucine, alanine, valine and phenylalanine are good substrates (Scannone *et al.* 1964, and Dixon and Kleppe 1965b). The enzyme is reported to act on L-proline (Wellner and Scannone 1964) and D-lactate (Yagi and Ozawa 1964b). The best substrate for pkDAOO is

D-proline, and DAAOs exhibit very poor or no activity toward D-aspartate (Tishkov and Khoronenkova 2005).

The substrate-binding domains in various species' primary structures do not show high homology. This may reflect the wide variation in specificities observed for DAAOs from different origins (Tishkov and Khoronenkova 2005).

Composition

The active pkDAAO holoenzyme is a monomer of 347 amino acids that can undergo dimerization. The monomer has been found to be more active than the dimer, and contains 1 mol FAD noncovalently bound per monomer. All DAAOs characterized as of 2000 contain noncovalently bound FAD as their prosthetic group (Pilone 2000).

IUB

- 1.4.3.3

CAS

- 9000-88-8

Protein Accession Number

- P00371

CATH: Classification (v. 3.3.0)

Class:
- Alpha Beta

Architecture:
- 2-Layer Sandwich and 3-Layer (aba) Sandwich

Topology:
- D-Amino Acid Oxidase; Chain A, domain 2 and Rossmann fold

Molecular Weight

- 78.7 kDa

Optimal pH

- Dependent on the substrate: approximately 9 for D-alanine (Dixon and Kleppe 1965c)

Isoelectric Point

- 7.0, 7.2 (Tishkov and Khoronenkova 2005)

Extinction Coefficient

- 75,420 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 19.17

Active Residue

- Tyrosine (Y224)
- Aspartic acid (D228)
- Arginine (R283) (Pilone 2000)

Inhibitors

- 2-hydroxy acids, 2-oxo acids, and 2-oxobutyrate (Dixon 1965b)
- Metabolites and drugs (Hamilton and Buckthal 1982)
- Adenosine 5'-monophosphate and aniline (Yagi *et al.* 1972c)
- Benzoate (Pollegioni *et al.* 2007)
- Sodium benzoate (Nguyen *et al.* 2009)

Applications

- Keto acid preparation
- Oxidation reduction studies
- Separation of L-amino acids from racemic mixtures
- FAD determination
- D-alanine determination
- Biosensors (Inaba *et al.* 2003)

Assay Information

Method

- The reaction velocity is determined in a peroxidase coupled system by measuring the increase in A_{436} resulting from the oxidation of D-alanine. One unit oxidizes one micromole of D-alanine per min. at 37°C and pH 8.3 under the specified conditions.

Reagents

- 0.02 M Sodium pyrophosphate, pH 8.3
- 0.05 M D-Alanine in 0.02 M sodium pyrophosphate pH 8.3 (Substrate) and containing 0.0065% o-dianisidine
- 0.02 M Sodium pyrophosphate, pH 8.3 with 10 $\frac{\text{mg}}{\text{ml}}$ peroxidase (Worthington code: HPOD) in reagent grade water

Enzyme

- Dissolve purified enzyme at a concentration of 0.05-0.2 $\frac{\text{mg}}{\text{ml}}$ in 0.02 M sodium pyrophosphate buffer, pH 8.3. Dissolve crude enzyme at 1-5 $\frac{\text{mg}}{\text{ml}}$ in buffer.

Procedure

Spectrophotometer Settings: Wavelength: 436 nm Temperature: 37°C

Pipette 2.8 ml of pyrophosphate/alanine/dianisidine mixture into cuvette. Add 0.1 ml of 1% peroxidase and allow temperature to equilibrate. Record blank rate for 5-10 minutes if present. Add 0.1 ml of appropriately diluted enzyme to yield a rate of 0.015-0.05 $\frac{\Delta A}{min}$. Record rate for 10 minutes. Calculate rate from linear portion of curve. Subtract blank rate if present.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{436}}{\text{min}} \text{ x } 3.0 \text{ x dilution}}{8.1 \text{ x } 0.1 \text{ x } \frac{\text{mg}}{\text{ml}}}$

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7. Amino Acid Oxidase, L-

L-amino acid oxidase (LAAO) catalyzes the oxidative deamination of a number of L-amino acids, predominantly hydrophobic and aromatic L-amino acids. LAAO represents approximately 30% of the total venom of some snake species (Takatsuka *et al.* 2001).

$$RCHNH_2COOH + O_2 + H_2O \rightarrow RCOCOOH + NH_3 + H_2O_2 \quad (7.1)$$

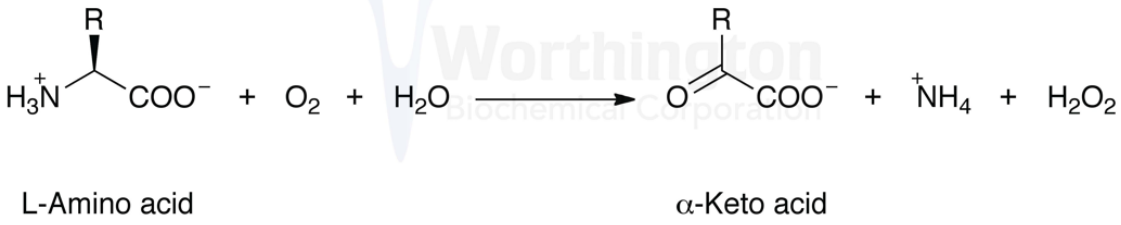

Figure 7.1: Enzymatic Reaction - Amino Acid Oxidase, L-

History

LAAO was first discovered by Zeller and Maritz (Zeller and Maritz 1944, 1945). It was found to occur in almost all snake venoms and designated a flavoprotein in 1948 (Zeller 1948).

LAAO was first prepared in crystalline form in 1958 by Wellner and Meister. In 1960, Wellner and Meister studied properties of the enzyme including prosthetic groups, electrophoretic fractions, stability, and pH dependence. In this study, they also isolated LAAO of Agkistrodon piscivorus piscivorus to compare it to that of C. adamanteus. Soon after, the enzyme mechanism was studied and further details of the mechanism were elucidated (Wellner and Meister 1961, and Massey and Curti 1967). The kinetics of the oxidase reaction were also investigated (Radd 1964, and Zeller *et al*. 1965).

In the 1970s vinylglycine was determined to be a suicide substrate/inactivator of LAAO (Marcotte and Walsh 1976), and the effect of pH and competitive inhibitors was also studied (de Kok and Veeger 1968). LAAO of C. adamanteus was the first LAAO found to act as a bactericidal agent, a property that is still being studied today (Skarnes 1970, and Zuliani *et al*. 2009). The gene sequence was determined in 1998 by Raibekas and Massey.

Recent work has involved using LAAOs to study apoptotic and cytotoxic effects (St*á*beli *et al*. 2007), and LAAO is also being studied for its use as an anti-parasitic agent (Sant'Ana *et al*. 2008).

Molecular Characteristics

LAAOs are widely distributed, being found in bacteria, fungi, green algae, plants, and snake venom. LAAOs of snake venom show a high degree of sequence homology, with conservation of at least 13 of the 24 N-terminal amino acids that are believed to be involved in substrate binding. (Zuliani *et al*. 2009). The N-terminal sequence contains a highly conserved beta-alpha-beta-fold domain involved in FAD binding (Du *et al*. 2002).

Specificity

LAAO is specific for L-isomers. Substrates are the L-isomers of leucine, isoleucine, norleucine, alpha-amino butyric acid, phenylalanine, tyrosine, tryptophan norvaline, methionine, histidine, and citrulline. Histidine and tyrosine cannot be determined in an L,D-mixture (Malmstadt and Hadjiioannov 1963). Methylene blue may be used as an electron acceptor. L-serine, threonine, aspartic acid, glutaric acid, lysine, and ornithine are deaminated only to a limited extent.

LAAO is believed to contain three hydrophobic substrate binding sites, designated a, b, and c. Subsite a accommodates one methylene carbon, b two, and c three. An amino binding subsite is designated d (Tan *et al*. 1991). This model is used to explain why amino acids with branching at the second carbon are unable to accommodate into subsite a, and are oxidized slowly or not at all (Zuliani *et al*. 2009).

Composition

LAAOs are FAD-dependent enzymes and are usually homodimeric, binding glycoproteins with molecular masses of 110-150 kDA. Important residues identified in C. rhodostoma LAAO include Glu63, Arg71, and Glu457, which interact with the FAD molecule. The dimethylbenzene ring cofactor is surrounded by Ile374, Trp420, and Ile430; Lys326 coordinates a water molecule (Pawelek *et al*. 2000). These residues have been found to be conserved in the majority of snake venom LAAOs (Pawelek *et al*. 2000, and Frana *et al*. 2007).

IUB

- 1.4.3.2

CAS

- 9000-89-9

Protein Accession Number

- O93364

CATH: Classification (v. 3.3.0)

Class:

- Alpha Beta; Alpha Beta; Mainly Alpha

Architecture:

- Alpha-Beta Complex; 3-Layer(bba) Sandwich; Orthogonal Bundle

Topology:

- Polyamine Oxidase (Chain A, domain 2); FAD/NAD(P)-binding domain; Guanine Nucleotide Dissociation Inhibitor (domain 1)

Molecular Weight

- 113.3 kDa

Optimal pH

- Approximately 7.5 for leucine (Wellner and Meister 1960)

Isoelectric Point

- 6.32

Extinction Coefficient

- 124,900 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{275}$ = 17.9

Inhibitors

- Cyanide
- Ammonium and benzoic acid (Meister and Wellner 1963)
- Aromatic carboxylates (de Kok and Veegar 1968)
- Benzoic acid derivatives (de Kok and Veeger 1968)

Applications

- Purification and determination of certain amino acids (Nicholson and Kim 1975)
- Preparation of alpha-keto acid (Nicholson and Kim 1975)
- Assaying peptidase activity (Nicholson and Kim 1975, and Donlon and Fottrell 1971)
- Oxidation reactions
- Catalyst in supercritical CO_2 (Findrik *et al.* 2005)

Assay Information

Method

- The reaction velocity is determined in a peroxidase coupled system by measuring the increase in A_{436} resulting from the oxidation of L-leucine. One unit oxidizes one micromole of L-leucine per minute at 25°C and pH 7.6 under the specified conditions.

Reagents

- 0.2 M Triethanolamine buffer pH 7.6 containing 0.1% L-leucine and 0.0065% o-dianisidine
- 1.0% Peroxidase: Dissolve Worthington Peroxidase (HPOD) at 10 $\frac{\text{mg}}{\text{ml}}$ in water.

Enzyme

- Dilute enzyme in reagent grade water to 0.05-0.2 $\frac{\text{units}}{\text{ml}}$.

Procedure

Spectrophotometer Settings: Wavelength: 436 nm Temperature: 25°C

Pipette into cuvettes as follows:

- 10 $\frac{\text{mg}}{\text{ml}}$ peroxidase - 0.01 ml
- 0.2 M triethanolamine-leucine-o-dianisidine mixture - 2.9 ml

Incubate in spectrophotometer at 25°C for 4-5 minutes to achieve temperature equilibration and record blank rate, if any. Add 0.1 ml of appropriately diluted enzyme and record increase in absorbance at 436 nm for 4-5 minutes. Calculate $\frac{\Delta A_{436}}{\text{min}}$ from the initial linear portion of the slope. Subtract blank rate if present.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{436}}{\text{min}} \text{ x } 3.0 \text{ x dilution}}{8.1 \text{ x } 0.1 \text{ x } \frac{\text{mg}}{\text{ml}}}$$

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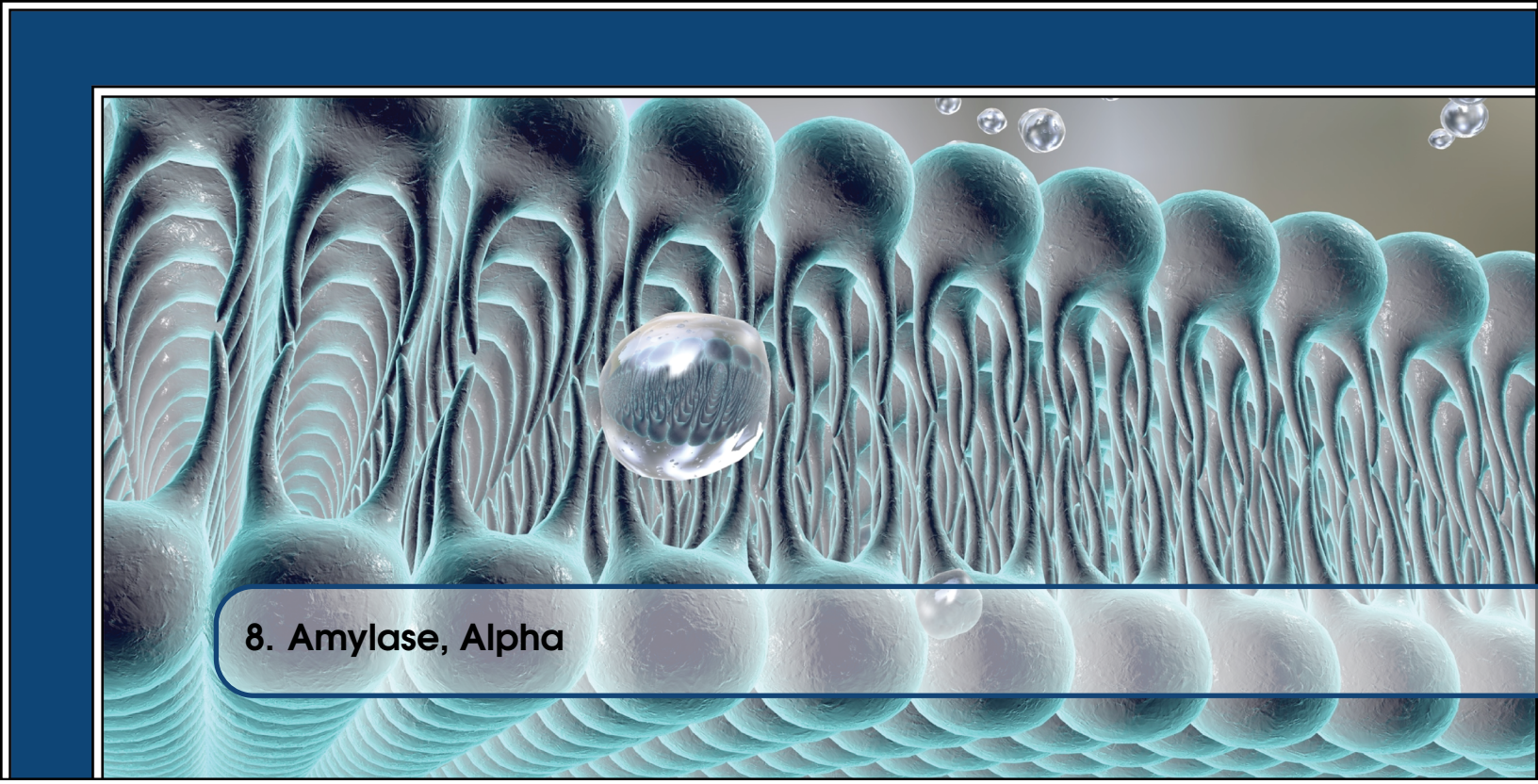

8. Amylase, Alpha

Porcine pancreatic alpha-amylase (PPA) acts upon large linear carbohydrate polymers at internal bonds. The hydrolytic products have alpha-configuration. alpha-Amylase activity is present in all living organisms; however, the enzymes vary remarkably even from tissue to tissue within a single species.

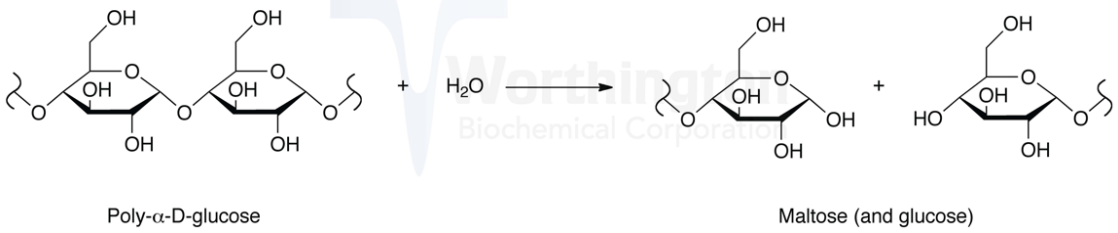

Figure 8.1: Enzymatic Reaction - Amylase, Alpha

History

The alpha-amylases were named by Kuhn in 1925, because the hydrolysis products are in the alpha configuration. In 1930, Ohlsson discovered another amylase, which yielded a beta-mannose. He named it beta-amylase. In 1947, the enzyme was first crystallized from pig pancreas by Meyer *et al.*, and Danielsson determined its molecular weight. In 1952, Caldwell *et al.* reported on an improved purification method.

Inhibitors of alpha-amylase were reported on as early as 1975 (Marshall and Lauda 1975). The first crystal structure of alpha-amylase was from *Aspergillus oryzae* and was determined in 1979 (Matsuura

et al. 1979). This 6 resolution structure was improved to a 3 resolution the following year (Matsuura *et al*. 1980).

In the 1980s, the amino acid sequences of the two forms (PPAI and PPAII) were determined (Kluh *et al*. 1981, and Pasero *et al*. 1986). The three dimensional crystal structures of each form were determined in the 1990s and found to be effectively identical (Qian *et al*. 1993, and Gilles *et al*. 1996). The complete cDNA sequence was determined by Darnis *et al*. in 1999.

Recent work with alpha-amylase has included isolating and studying the effects of inhibitors from natural sources (Narita and Inouye 2009, and Suda *et al*. 2011). Researchers have also studied the effects of bound carbohydrates on enzyme stability (Gopal *et al*. 2008) and analyzed the binding of inhibitors with the aim of designing novel inhibitors (Najafian *et al*. 2011).

Molecular Characteristics

Animal alpha-amylases are encoded by two loci, amy1 (salivary) and amy2 (pancreatic). The full length amy cDNA contains 1565 bp. It contains a 15 amino acid signal peptide and a 1536-nt open reading frame (ORF). A 29-nt 3' non-coding region is followed by a short poly(A) tail.

Animal alpha-amylases contain five conserved regions including four at or around the (alpha/beta)8-barrel and a short sequence near the C-terminus of domain B (Janecek 1993, Svensson 1994, and Janecek and Balaz 1995). Human and porcine pancreatic alpha-amylases are 87.1% identical, with only 64 amino acid substitutions. Mouse and rat alpha-amylase share a 85.5% identity with that of porcine. Amino acids 304-310 are conserved among mammalian alpha-amylases and are believed to be involved in the enzymatic mechanism (Darnis *et al*. 1999).

Specificity

alpha-Amylase catalyzes the hydrolysis of internal alpha-1,4-glucan links in polysaccharides containing 3 or more alpha-1,4-linked D-glucose units, yielding a mixture of maltose and glucose. See also Takeshita and Hehre (1975).

Composition

The enzyme is a glycoprotein (Beaupoil-Abadie *et al*. 1973). Its single polypeptide chain of about 475 residues has two free thiol groups, four disulfide bridges, and contains a tightly bound Ca^{2+} (Granger *et al*. 1975, and Steer *et al*. 1974). It exists in two forms (PPAI and PPAII), which have identical enzymatic properties and molecular mass of 55.4 kDa, differing only in electrophoretic mobility and pI (Cozzone *et al*. 1970b, Marchis-Mouren and Pasero 1967, and Darnis *et al*. 1999).

The structures of both forms of PPA consist of a major domain A (residues 1-99 and 170-404) organized as an (alpha/beta)8-barrel that contains a loop (domain B). Following the C-terminal domain C (residues 405-496) is a ten-beta-stranded Greek key motif (Darnis *et al*. 1999).

IUB

- 3.2.1.1

CAS

- 9000-90-2

Protein Accession Number

- P00690

CATH: Classification (v. 3.3.0)

Class:

- Alpha Beta; Mainly Alpha

Architecture:

- Alpha-Beta Barrel; Sandwich

Topology:

- TIM Barrel; Immunoglobulin-like

Molecular Weight

- 51.0-54.0 kDa (Cozzone *et al.* 1970a)
- 55.4 kDa (SDS-PAGE) (Alkazaz *et al.* 1996)

Optimal pH

- 7

Isoelectric Point

- PPAI: 7.5 (Ajandouz *et al.* 1995)
- PPAII: 6.4 (Ajandouz *et al.* 1995)

Extinction Coefficient

- 133,870 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280} = 26$

Inhibitors

- Phenolic compounds (Funke and Melzig 2005)
- Plant extracts (Karthic *et al.* 2008, and Buonocore *et al.* 1985)
- Urea and other amide reagents (Toralballa and Eitingon 1967)

Applications

- Ethanol production
- Reduction of starch or glycogen molecules

Assay Information

Method

- That of (1951) wherein the reducing groups released from starch are measured by the reduction of 3,5-dinitrosalicylic acid. One unit releases from soluble starch one micromole of reducing groups (calculated as maltose) per minute at 25°C and pH 6.9 under the specified conditions.

Reagents

- 0.02 M Sodium phosphate buffer, pH 6.9 with 0.006 M sodium chloride
- 2 N Sodium hydroxide
- Dinitrosalicylic acid color reagent. Prepare by dissolving 1.0 g of 3,5-dinitrosalicylic acid in 50 ml of reagent grade water. Add slowly 30.0 g sodium potassium tartrate tetrahydrate. Add 20 ml of 2 N NaOH. Dilute to a final volume of 100 ml with reagent grade water. Protect from carbon dioxide and store no longer than 2 weeks.
- 1% Starch. Prepare by dissolving 1.0 g soluble starch, (Merck) in 100 ml 0.02 M sodium phosphate buffer, pH 6.9 with 0.006 M sodium chloride. Bring to a gentle boil to dissolve. Cool and bring volume to 100 ml, with water, if necessary. Incubate at 25°C for 4-5 minutes prior to assay.
- Maltose Stock Solution. Prepare by dissolving 180 mg maltose (MW 360.3) in 100 ml reagent grade water in a volumetric flask.

Enzyme

- Dilute to a concentration of 1-10 $\frac{\mu g}{ml}$. A minimum of three different concentrations in this range should be run.

Procedure

Spectrophotometer Settings: Wavelength: 540 nm Temperature: 25°C

Using the maltose stock solution prepare a maltose standard curve as follows: In numbered tubes, prepare 10 maltose dilutions ranging from 0.3 to 5.0 $\frac{\mu moles}{ml}$. Include two blank tubes with reagent grade water only. Into a series of corresponding numbered tubes pipette 1 ml of each dilution of maltose. Add 1 ml of dinitrosalicylic acid color reagent. Incubate in boiling water bath for 5 minutes and cool to room temperature. Add 10 ml distilled water to each tube and mix well. Read A_{540} versus micromoles maltose.

Enzyme assay: Pipette 0.5 ml of respective enzyme dilutions into a series of numbered test tubes. Include a blank with 0.5 ml reagent grade water. Incubate tubes at 25°C for 3-4 minutes to achieve temperature equilibration. At timed intervals, add 0.5 ml starch solution (at 25°C). Incubate exactly 3 minutes and at timed intervals add 1 ml dinitrosalicylic acid color reagent to each tube. Incubate all tubes in a boiling water bath for 5 minutes. Cool to room temperature and add 10 ml reagent grade water. Mix well and read A_{540} versus blank. Determine micromoles maltose released from standard curve.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\text{micromoles maltose released}}{\text{mg enzyme in reaction mixture x 3 min}}$

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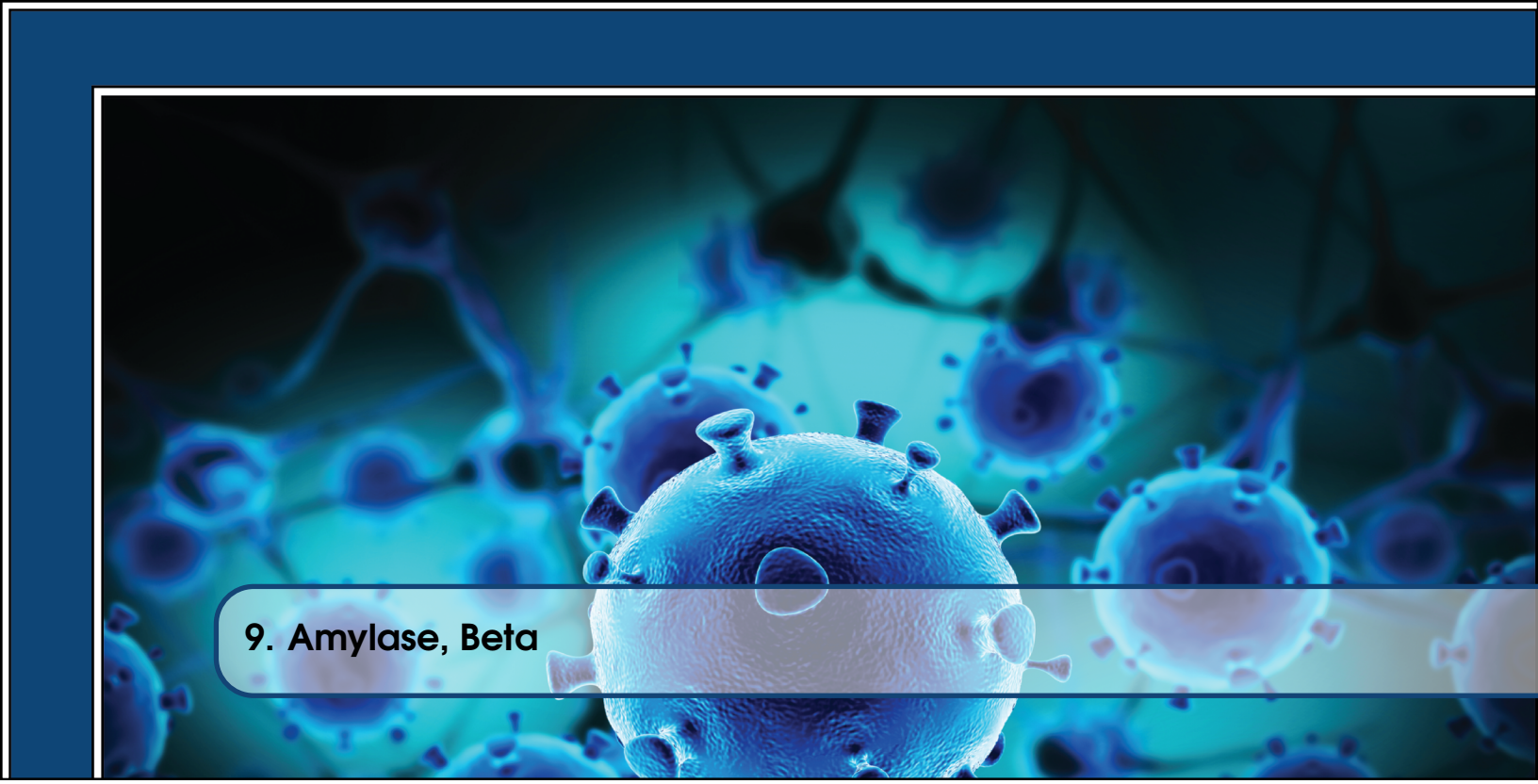

9. Amylase, Beta

β-Amylase is an exoenzyme that releases successive maltose units from the nonreducing end of a polysaccharide chain by hydrolysis of α-1,4-glucan linkages. The shortest normal saccharide attacked is maltotetraose (Myrback and Neumuller 1950). Since it is unable to bypass branch linkages in branched polysaccharides such as glycogen or amylopectin, the hydrolysis is incomplete and a macromolecular limit dextrin remains.

β-Amylase is found primarily in the seeds of higher plants and sweet potatoes. It yields a single product: maltose. Marshal and Whelan (1973) report on the removal of any contaminating β-glucosidase. The tuberous root of sweet potato is unusually rich in the enzyme, accounting for approximately 5% of the total soluble proteins. In contrast, other tuberous roots only contain trace amounts of β-amylase activity (Li and Oba 1985, and Yoshida and Nakamura 1991).

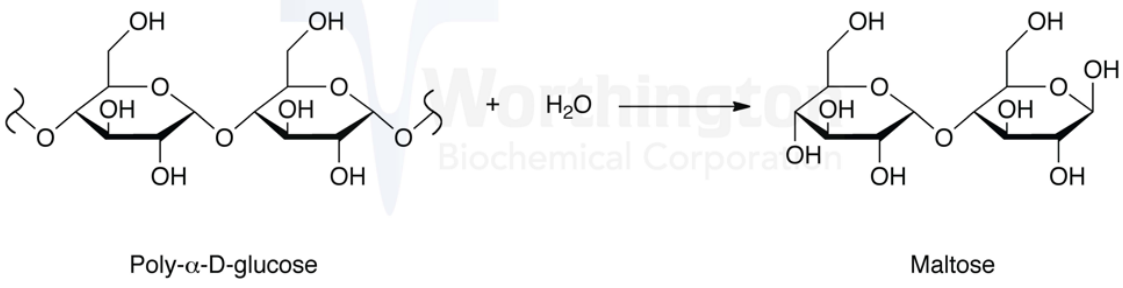

Figure 9.1: Enzymatic Reaction - Amylase, Beta

History

The α-amylases were named by Kuhn in 1925, because the hydrolysis products are in the alpha configuration. In 1930, Ohlsson discovered another amylase, which yielded a β-mannose and named it β-amylase.

β-amylase was first purified in the 1940s (Schoch 1942, and Haworth *et al.* 1946). Hopkins *et al.* first began to study the kinetics of β-amylase in 1948.

In the 1960s and 1970s, techniques in the purification of sweet potato β-amylase improved (Nakayama and Amagase 1963, Takeda and Hizukuri 1969, and Hegde *et al.* 1979). The enzyme was successfully immobilized on agarose and sepharose gels in the late 1970s (Caldwell *et al.* 1976a, b).

In 1991, β-amylase was successfully cloned and expressed in *Escherichia coli* (Yoshida *et al.* 1991). The complete amino acid sequence and preliminary crystal structure of the tetrameric enzyme were determined in 1993 (Toda *et al.* 1993, and Cudney and McPherson 1993). The crystal structure was refined in 1995 (Cheong *et al.* 1995). In 1996, Pujadas *et al.* studied the evolution of β-amylases determining the enzymes to be an example of parsimonious divergence illustrated by signature structural motifs.

In 2001, a simple purification method using affinity precipitation was developed (Teotia *et al.* 2001). The effects of guanidine hydrochloride and increased pressure on the activity of the enzyme were studied in 2002 (Tanaka *et al.* 2002).

The reaction of catalase occurs in two steps. A molecule of hydrogen peroxide oxidizes the heme to an oxyferryl species. A porphyrin cation radical is generated when one oxidation equivalent is removed from iron and one from the poryphyrin ring. A second hydrogen peroxide molecule acts as a reducing agent to regenerate the resting state enzyme, producing a molecule of oxygen and water (Switala and Loewen 2002).

Molecular Characteristics

The gene that encodes for β-amylase is amyB. A 1404 bp open reading frame encodes for the 499 amino acid precursor to the β-amylase subunit. After methionine processing, the mature form of the protein contains 498 residues (Yoshida and Nakamura 1991). The gene contains seven exons and six introns, and a TATA box sequence is present 26 to 30 bp upstream of the multiple transcription sites. The upstream region of amyB contains sequences to which several known plant nuclear factors bind; regulation of the amyB gene is thought to be controlled by one or more of the common cis-regulatory elements (Yoshida *et al.* 1992). Subunits of sweet potato β-amylase and seed β-amylase from barley and soybean share approximately 68% amino acid identity (Yoshida and Nakamura 1991).

Composition

Most β-amylases are monomeric enzymes (Thoma *et al.* 1971). However, that of sweet potato is tetrameric consisting of four identical subunits (Thoma *et al.* 1963). Each subunit consists of a large $(\alpha/\beta)_8$-barrel region also seen in a-amylase. A smaller, globular region is formed by long loops extending from β-strands. Between these two regions is a cleft believed to open and contain the

conserved Glu187. Cys96 is located at the entrance of the $(\alpha/\beta)_8$-barrel region and is important to inactivation by sulfhydryl reagents (Cheong *et al.* 1995).

IUB

- 3.2.1.2

CAS

- 9000-91-3

Protein Accession Number

- P10537

CATH: Classification (v. 3.3.0)

Class:
- Alpha Beta

Architecture:
- Alpha-Beta Barrel

Topology:
- TIM Barrel

Molecular Weight

- 223.8 kDa

Optimal pH

- 4.0-5.0 (French 1960)
- 5.5 (Ohba *et al.* 1979)

Isoelectric Point

- 5.17

Extinction Coefficient

- 388,640 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 17.7 (Takeda and Hizukuri 1969)

Active Residue

- Glutamate (G187 and G382)

Inhibitors

- Heavy metal ions
- PCMB and PMSF
- Iodoacetamide
- Ascorbate (Rowe and Weill 1962)
- Cyclohexaamylose
- Sulfhydryl reagents

Applications

- Starch and glycogen structural studies
- Fermentation in brewing and distilling industry
- Saccharification of liquefied starch: production of high maltose syrups and high conversion syrups

Assay Information

Method

- That of Bernfeld (1955) wherein the rate at which maltose is released from starch is measured by its ability to reduce 3,5-dinitrosalicylic acid. One unit releases one micromole of β-maltose per min at 25°C and pH 4.8 under the specified conditions.

Reagents

- 0.016 M Sodium acetate, pH 4.8
- 2 N Sodium hydroxide
- Dinitrosalicylic acid color reagent. Prepare by dissolving 1.0 g of 3,5-dinitrosalicylic acid in 50 ml of reagent grade water. Add slowly 30.0 g sodium potassium tartrate tetrahydrate. Add 20 ml of 2 N NaOH. Dilute to a final volume of 100 ml with reagent grade water. Protect from carbon dioxide and store no longer than 2 weeks.
- 1% Starch: Prepare by dissolving 1.0 gram of soluble starch (Merck) in 100 ml of 0.016 M sodium acetate buffer pH 4.8. Bring to a gentle boil to dissolve. Cool and, if necessary, dilute to 100 ml with reagent grade water. Incubate at 25°C for 4-5 minutes prior to assay.
- Maltose stock solution, 5 micromoles/ml. Prepare by dissolving 180 mg maltose (MW 360.3) in 100 ml reagent grade water. Incubate at 25°C for 4-5 minutes prior to assay.

Enzyme

- Dilute to a concentration of 1-10 $\frac{\mu g}{ml}$. A minimum of three different concentrations in this range should be run.

Procedure

Using the maltose stock solution prepare a maltose standard curve as follows: In numbered tubes, prepare 10 maltose dilutions ranging from 0.3 to 5.0 $\frac{\mu moles}{ml}$. Include two blank tubes with reagent grade water only. Into a series of corresponding numbered tubes pipette 1 ml of each dilution of maltose. Add 1 ml of dinitrosalicylic acid color reagent. Incubate in boiling water bath for 5 minutes and cool

to room temperature. Add 10 ml distilled water to each tube and mix well. Read A_{540} and plot A_{540} versus micromoles maltose.

Enzyme assay: Pipette 0.5 ml of respective enzyme dilutions into a series of numbered test tubes. Include a blank with 0.5 ml reagent grade water. Incubate tubes at 25°C for 3-4 minutes to achieve temperature equilibration. At timed intervals, add 0.5 ml starch solution (at 25°C). Incubate exactly 3 minutes and at timed intervals add 1 ml dinitrosalicylic acid color reagent to each tube. Incubate all tubes in a boiling water bath for 5 minutes. Cool to room temperature and add 10 ml reagent grade water. Mix well and read A_{540} versus blank. Determine micromoles maltose released from standard curve.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\text{micromoles maltose released}}{\text{mg enzyme in reaction mixture x 3 min}}$$

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10. Arginase

The enzyme participates in the Krebs-Henseleit urea cycle. It is most highly concentrated in mammalian liver, and is also present in abundance in mammary glands, where the urea cycle is not present. There are two distinct types of arginase: Type I (liver-type) and Type II (mitchondrial). Arginase is a hydrolase present in many tissues and organs that catalyzes the following reaction:

$$\text{Arginine} + H_2O \rightarrow \text{Ornithine} + \text{Urea} \tag{10.1}$$

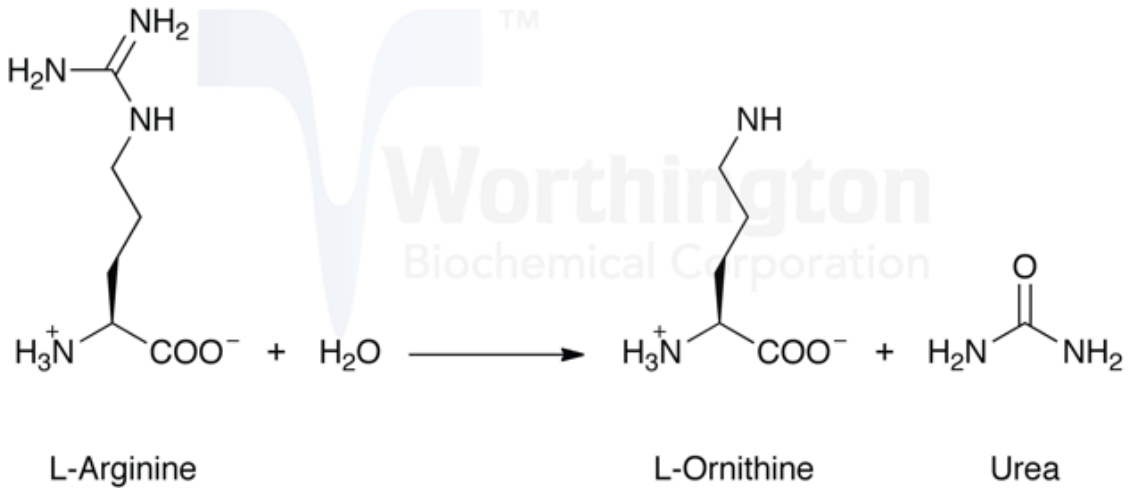

Figure 10.1: Enzymatic Reaction - Arginase

History

In 1928, Krebs and Henseleit conducted a series of experiments using liver slices and manometric assays to show that in the presence of arginase, ornithine produced urea (Jenkinson *et al*. 1996). Crude preparations of arginase were reported as early as 1931 (Salaskin and Solowjew 1931, and Waldschmidt-Leitz *et al*. 1931). Hellerman and Perkins first showed activation by bivalent metal ions including cobalt, nickel, manganese, and iron (Hellerman and Perkins 1935). In 1940 an improved purification developed by Richards and Hellerman showed that the activity could be restored to pH-inactivated arginase by Mn^{2+} and (to a lesser extent) Fe^{2+} (Richards and Hellerman 1940).

In 1956, the "partial" purification was further improved by Robbins and Shields. They demonstrated that the activity of arginase was dependent upon manganese, and found the optimal pH to be 9.2 (which has since been adjusted to 9.4) (Robbins and Shields 1956, and Xie *et al*. 2004).

Much work was done investigating inhibitors in the late 1970s and early 1980s (Rosenfeld *et al*. 1975, Bedino 1977, and Pace and Landers 1981). Bedino studied the effect of the product/inhibitor ornithine, and proposed an allosteric model for the regulation of the enzyme's activity (Bedino 1977).

Recent research has investigated the roles of arginases in vascular disease, pulmonary disease, infectious disease, and cancer (Zimmerman and Rothenberg 2006, Maarsingh *et al*. 2008, Santhanam *et al*. 2008, and Morris 2009). Varying levels of arginase have been found in the reproductive system of cattle (Razmi *et al*. 2005), as well as in the immune system of mice and humans (Munder 2009).

Molecular Characteristics

Arginase I and arginase II are each encoded by a different gene. Arginase I is located in the cytoplasm and expressed in the liver as part of the urea cycle. Arginase II is a mitochondrial enzyme, and is expressed primarily in the kidney (Romero *et al*. 2008). Based on sequence analysis, arginase is probably a primordial enzyme that was present in the universal common ancestor (Ouzounis and Krypides 1994). The arg1 gene is conserved in several species including human, dog, mouse, rat, zebrafish, and *S. cerevisiae*. The gene is induced in the late fetal period and is upregulated by high nutritional protein uptake (Morris *et al*. 1989). This is mediated by glucocorticoids and glucagon (Morris *et al*. 1987).

A cis element is present around -90 to -51 relative to the start site and is overlapped by two protein binding regions. These regions have been identified as footprint areas A and B. A is more upstream and binds a CCAAT/enhancer binding protein (C/EBP) related factor, while B is recognized by two other mutually exclusive factors (Takiguchi and Mori 1991, and Chowdhury *et al*. 1996). An enhancer region is also present 11 kb downstream from the start site (Gotoh *et al*. 1994). Hepatocyte nuclear factor-4 (HNF-4) has been shown to repress the C/EBP stimulated arginase promoter activity (Chowdhury *et al*. 1996).

Specificity

Arginase is highly specific for its substrate. Class I and Class II enzymes contain carboxy-terminal tyrosine residues required for maximal catalytic activity. Arginase has the highest level of specific activity of the urea cycle enzymes in the liver (Jenkinson *et al*. 1996).

Composition

Arginase is a homotrimer. Each subunit contains an active site, and the two essential manganese ions are bridged by oxygens and separated by approximately 3.3 . The mechanism is proposed to be a nucleophilic attack by the metal bridging hydroxide at the guanidinium carbon of the arginine substrate (Ash 2004).

IUB

- 3.5.3.1

CAS

- 9000-96-8

Protein Accession Number

- Q2KJ64

CATH: Classification (v. 3.3.0)

Class:

- Alpha Beta

Architecture:

- 3-Layer(aba) Sandwich

Topology:

- Arginase; Chain A

Molecular Weight

- 105.0 kDa

Optimal pH

- 9.4 (Xie *et al*. 2004)

Isoelectric Point

- 5.9 (Harell and Sokolovsky 1972)

Extinction Coefficient

- 74,340 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 7.08

Active Residue

- Histidine (H101, H126)
- Aspartate (D124, D128, D232, D234) (Residues coordinate bound manganese)

Activators

- Mn^{2+}, Mg^{2+}, Ca^{2+}, Ni^{2+}, and Co^{2+} (Dabir *et al.* 2005)

Inhibitors

- Indyl amino acid derivatives (Hrabak *et al.* 2008)
- EDTA (Esch *et al.* 1998)
- L-Proline and branched-chain amino acids (Dabir *et al.* 2006)
- Adenosine, inosine, uric acid (Rosenfeld *et al.* 1975)
- Possibly adenine (Rosenfeld *et al.* 1975, disputed by Pace and Landers 1981)

Applications

- Preparation of L-Ornithine
- Determination of L-Arginine in plasma and urine

Assay Information

Method

- A number of assay procedures have been used (Greenberg 1960). More recently, Geyer and Dabich (1971) have reported on an assay for arginase in tissue homogenates and Nishibe and Makino (1971) an automated method for erythrocyte enzyme. Hirsch-Kolb and Greenberg (1970) describe a microassay. The method used by this laboratory is based upon the colorimetric determination of released urea nitrogen, using 2,3-butanedione reagent. One unit releases one micromole of urea per minute at 37°C and pH 9.5 under the specified conditions.

Reagents

- 0.05 M Maleic acid, pH 7.0 with 0.05 M manganous sulfate
- 0.713 M L-arginine, pH 9.5
- Reagent grade water, pH 9.5 (20 mg NaOH per liter)
- 0.075%, 2,3-Butanedione in buffered arsenic-sulfuric acid

Enzyme

- Incubate a one $\frac{\text{mg}}{\text{ml}}$ solution of the enzyme in maleic-manganous sulfate buffer at 37°C for 4 hours. Following activation, dilute to 1-2 $\frac{\mu\text{g}}{\text{ml}}$ in reagent grade water, pH 9.5.

Procedure

Pipette the following into screw capped tubes:

Description	Blank Tube	Tube # 1	Tube # 2
Water, pH 9.5	0.3 ml	0.1 ml	0.2 ml
Diluted, activated enzyme	—	0.2 ml	0.1 ml
Incubate in 37°C water bath for 5 minutes to achieve temperature equilibration. At timed intervals start reaction by adding:			
Arginine	0.2 ml	0.2 ml	0.2 ml
Incubate for 30 minutes at 37°C. Stop reaction at timed intervals by adding:			
B.U.N. Reagent	4.0 ml	4.0 ml	4.0 ml
Cap tubes and develop color by boiling in water bath for 12 minutes. Chill tubes for 3 minutes in an ice bath. Read the color at 490 nm against blank.			

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\text{micromoles urea released}}{\text{30 x mg enzyme in reaction mixture}}$

Determine the micromoles of urea released from a standard urea curve in the range of 0.1-1.0 micromoles urea.

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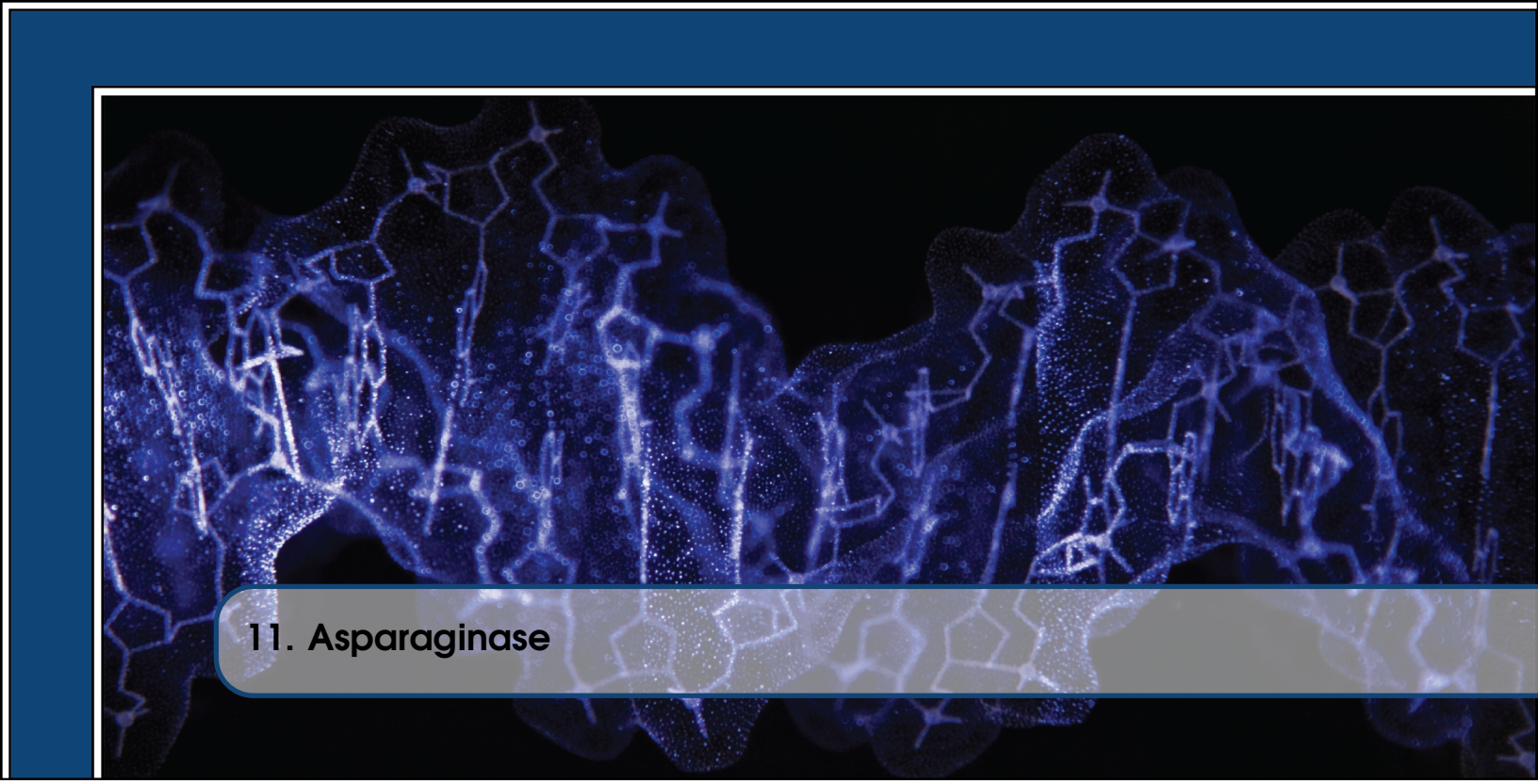

11. Asparaginase

Asparaginase catalyzes the hydrolysis of L-asparagine to produce L-aspartic acid and ammonia. Bacterial type L-asparaginases are classified into subtypes I and II, which is defined by their intra- or extracellular localization (Michalska and Jaskolski 2006). Type I (cytosolic) has a lower affinity for L-asparagine, while type II (periplasmic) has a high substrate affinity. Plant type L-asparaginases differ structurally and have a different evolutionary origin than bacterial L-asparaginases (Michalska *et al*. 2006)

$$HOOCCHNH_2CH_2CONH_2 + H_2O \rightarrow HOOCCHNH_2CH_2COOH + NH_3 \quad (11.1)$$

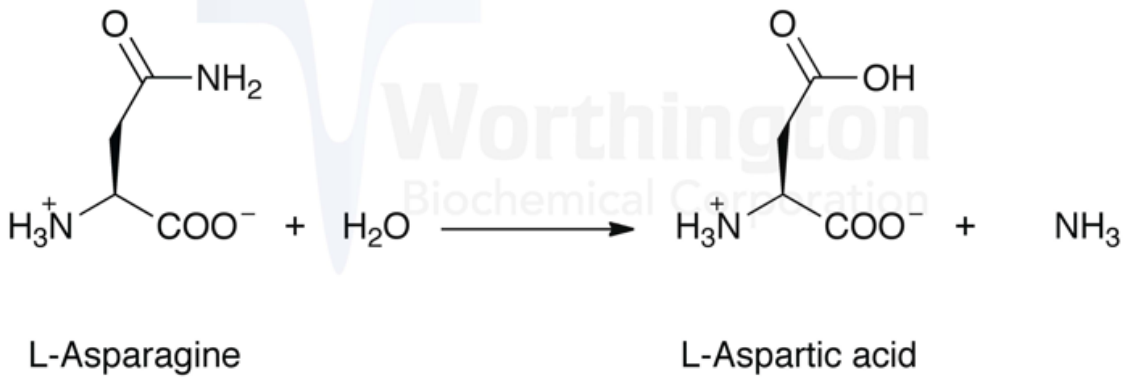

Figure 11.1: Enzymatic Reaction - Asparaginase

History

In 1904, Lang became the first to detect asparaginase activity in beef tissues. F*ü*rth and Friedmann confirmed these results in 1910. F*ü*rth and Friedmann believed asparaginase activity was found at the same level in all animal tissues, which was later refuted by Clementi (Clementi 1922) whose experiments concluded the enzyme was active in practically all tissues of herbivores, only in the liver of omnivorous animals, and absent in the organs of carnivorous animals, amphibians, and reptiles (Michalska and Jaskolski 2006).

In 1953, Kidd reported anti-tumor properties of guinea pig serum, which was later attributed to asparaginase activity (Broome 1961). In 1963, Mashburn and Wriston found that the *Escherichia coli* enzyme had anti-tumor activity. Work proceeded in several laboratories leading to clinical trials, making *Escherichia coli* asparaginase II the first antileukemic L-asparaginase to be used clinically (Roberts *et al*. 1966, Old *et al*. 1967, Boyse *et al*. 1967, Hill *et al*. 1967, Clarkson *et al*. 1970, and Oettgen *et al*. 1970). It was soon discovered that in addition to the secreted EcAII, *Escherichia coli* (and other bacteria) also produce cytosolic asparaginase, asparaginase I (EcAI), which has a lower affinity for L-asparagine and is not effective against cancer (Willis and Woolfolk 1974).

Preliminary efforts to determine the primary structure were carried out in 1974, and the sequence was confirmed in 1990 (Maita *et al*. 1974, Maita and Matsuda 1980, and Bonthron 1990). After crystallographic studies by Epp *et al*. in 1971, a partial model of the EcAII crystal structure was published in 1988 by Ammon *et al*.. Although not completely accurate, this structure was used as a stepping stone by Swain *et al*. who elucidated the true structure in 1993. Many crystal structures of L-asparaginase from a wide variety of organisms have been determined (Lubkowski *et al*. 2003).

Current research with L-asparaginase continues to focus on its oncological importance, and the specific transcription factors EcAII targets (Suto *et al*. 2010). Structure-function relationships of L-asparaginase from a variety of sources are also currently under investigation (Labrou and Papageorgiou 2010, and Bansal *et al*. 2010).

Molecular Characteristics

The promoter of the gene encoding L-asparaginase II, ansB, is coactivated by cAMP receptor protein (CRP) and the FNR protein (Scott *et al*. 1995). CRP is activated in response to carbon sources, and the FNR protein, the fnr gene product, in response to anaerobiosis. The FNR site is located 41.5 bp upstream of the major start site, and 28 bp upstream of the FNR site is the CRP regulation site (Jennings and Beacham 1993).

Specificity

EcAII exhibits a broad pH activity profile and higher substrate affinity than EcAI. The reaction consists of two steps. In the first step, the nucleophilic threonine attacks the carbonyl of the amide substrate to generate an acyl-enzyme intermediate. A molecule of ammonia is released. In the second step, water attacks the acyl-enzyme intermediate to produce L-aspartate (Michalska and Jaskolski 2006).

Composition

EcAII is a tetrameric protein composed of four identical subunits, each subunit contains 326 amino acid residues. The two threonine residues present at the active site are required for activity (see PDB 3ECA). Debate still continues over which threonine residue supplies the nucleophile. Most believe that it is the N-terminal threonine. The C-terminal threonine is part of a triad that resembles the typical SHD catalytic triad of serine proteases (Carter and Wells 1988).

IUB

- 3.5.1.1

CAS

- 9015-98-3

Protein Accession Number

- P00805

CATH: Classification (v. 3.3.0)

Class:
- Alpha Beta

Architecture:
- 3-Layer(aba) Sandwich

Topology:
- Rossmann Fold

Molecular Weight

- 138.4

Optimal pH

- 8.6 (Zhang *et al*. 2004)
- 8.0 (Wriston 1971)

Isoelectric Point

- 5.67

Extinction Coefficient

- 85,160 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{278} = 7.1$

Active Residue

- Threonine (T34, T111)
- Aspartate (D112)
- Lysine (K184)

Activators

- Anaerobic conditions (Cedar and Schwartz 1967)
- Amino acids (not specifically asparagine) (Cedar and Schwartz 1968)

Inhibitors

- 5-diazo-4-oco-L-norvaline (DONV) (Wriston and Yellin 1973)
- Dimethylsulfoxide (Wriston 1971)
- Glutaraldehyde (Zhang *et al*. 2004)
- L-Asparagine and L-aspartate (Wriston and Yellin 1973)
- NH_4^+ (Wriston 1971)
- Trypsin (Zhang *et al*. 2004)

Applications

- Treatment of acute lymphoplastic leukemia (Zhang *et al*. 2004)
- L-asparagine concentration determination (Gentili *et al*. 1994)

Assay Information

An assay method using a cationic glass electrode has been reported by Ferguson *et al*. (1974). Jayaram *et al*. (1974) report on a spectrophotometric assay and compare it with four other methods. Cooney *et al*. (1972) report on a colorimetric technique. See also Pajdak and Pajdak (1974 and 1972) Frohwein *et al*. (1971) and Kojima and Wacker (1969). The assay used at Worthington follows:

Method

- Essentially that of Mashburn and Wriston (1963) where the rate of hydrolysis of asparagine is determined by measuring released ammonia. One unit releases one micromole of ammonia per minute at 37°C and pH 8.6 under the specified conditions.

Reagents

- 0.05 M Tris · HCl buffer pH 8.6
- 0.01 M L-asparagine in 0.05 M Tris · HCl buffer, pH 8.6
- 1.5 M Trichloroacetic acid
- Nessler's Reagent
- Ammonia Standard - Dissolve 1.179 g ammonium sulfate to a final volume of 100 ml. Dilute 1.4 ml of this solution to 100 ml to give 1 μmole NH_3 per ml.

Enzyme

- Immediately prior to use, prepare several enzyme samples ranging from 0.1-1.0 $\frac{\text{mg}}{\text{ml}}$ in 0.2 M Tris · HCl pH 8.6.

Procedure

For each different enzyme dilution pipette into test tubes as follows:

Description	Test	Blank
0.05 M Tris · HCl pH 8.6	0.2 ml	0.2 ml
0.01 M L-asparagine	1.7 ml	1.7 ml
1.5 M TCA	—	0.1 ml

Incubate at 37°C for 5-6 minutes to achieve temperature equilibration. At zero time and at timed intervals add 0.1 ml diluted enzyme to Test and Blank tubes. Incubate at 37°C for exactly 10 minutes and stop reaction by adding 0.1 ml of 1.5 M TCA to Test tubes only. Clarify by centrifugation and add 0.5 ml of clear supernatant to 7.0 ml reagent grade water. Add 1.0 ml of Nessler's Reagent and incubate at room temperature for 10 minutes. Read A_{480} of Test tube versus the respective Blank. Determine micromoles of ammonia released from an ammonium sulfate standard curve. Standard is run in parallel by adding 0.5 ml of 1 μmole solution to 7.0 ml water and 1 ml of 1 μmole solution to 6.5 ml reagent grade water and adding 1 ml Nessler's Reagent to both.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\text{micromoles ammonia released}}{\text{10 minutes x mg enzyme in reaction}}$

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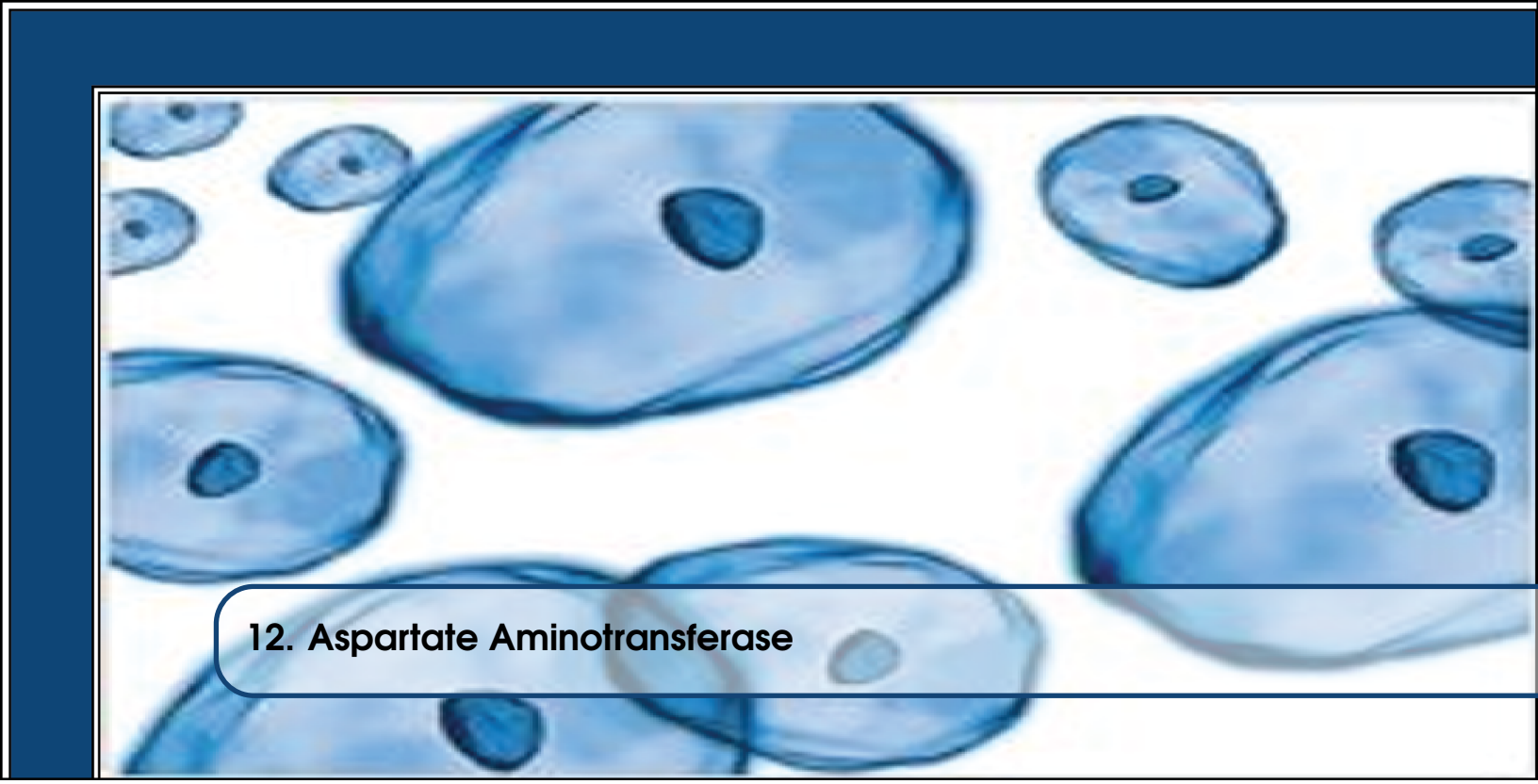

12. Aspartate Aminotransferase

Aspartate aminotransferase (AspAT) is a key enzyme of amino acid metabolism that catalyzes the reversible transfer of the amine group from L-aspartate to 2-oxoglutarate. In mammalian tissues the enzyme is especially concentrated in heart and liver tissue. The enzyme most extensively studied is that from pig heart. The usual preparations contain only the cytosolic enzyme (cAspAT).

$$\text{L-aspartate} + \text{2-oxoglutarate} \rightleftarrows \text{oxaloacetate} + \text{L-glutamate} \tag{12.1}$$

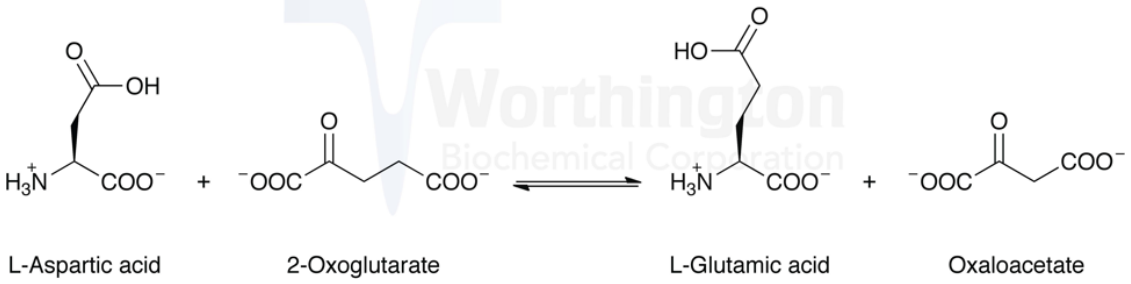

Figure 12.1: Enzymatic Reaction - Aspartate Aminotransferase

History

Transamination, the reversible transfer of the NH2 group from L-glutamate to pyruvate or other oxo acids together with an electron pair and a proton, was first demonstrated in 1937 by Braunstein and

Kritzmann.

In the 1950s, the enzyme's use in diagnostic testing for liver disease was introduced (Rej 1989). The enzyme has remained a primary diagnostic tool for liver disease for over half a century (Jeong *et al.* 2003).

In 1960, Fleischer *et al.* first reported on the separation of the two isozymes from dog heart and the differences in their substrate Kms and rate dependence on pH. In 1961, Boyd determined that the electrophoretically slow migrating enzyme was associated with the mitochondrial fraction (Martinez-Carrion *et al.* 1965). Research through the 1960s led to the purification of the two isozymes from a variety of tissues (Boyd 1966). In 1964, Wada and Morino purified the enzyme from porcine heart. Heart AspAT provided a unique system for studying isozyme structure and function because all forms contain a pyridoxal phosphate prosthetic group that can act as an indicator of active site events (Jenkins and D'Ari 1966b, and Martinez-Carrion and Tiemeir 1967).

The three dimensional structure of the porcine cytosolic enzyme was elucidated by Arnone *et al.* (Arnone *et al.* 1977 and 1985). In 1989, Nagashima *et al.* cloned and expressed porcine cAspAT in *Escherichia coli*.

Recent work has investigated cAspAT's role in fatty acid homeostasis (Tordjman *et al.* 2007) and protection against ischemic injury (Southwell *et al.* 2002). Work has also investigated the reduced activity of the enzyme as a result of crush syndrome (Desai and Desai 2008).

Molecular Characteristics

The gene encoding aspartyl aminotransferase (got1) is located on chromosome 14 in Sus scrofa. Nine exons encode the mature enzyme. The gene is conserved in human, chimpanzee, dog, cow, mouse, rat, chicken, zebrafish, fruit fly, mosquito, *C. elegans*, *S. pombe*, *S. cerevisiae*, *K. lactis*, *E. gossypii*, *M. grisea*, *Neurospora crassa*, *A. thaliana*, and rice (Gene ID: 396967).

Specificity

Aspartate aminotransferase has the highest reactivity toward L-aspartate and L-glutamate. In addition, the enzyme acts upon a number of aromatic amino acids. Non-aromatic amino acids are considerably poorer substrates (See tables in Shrawder and Martinez-Carron 1972, and Braunstein 1973, page 435).

Composition

Aspartate aminotransferase exists as two isozymes, one mitochondrial (mAspAT) and the other from the cytosol (cAspAT). Though differing markedly in primary structure, chemical and physical properties, both catalyze the same reaction with subtly different catalytic steps (Stankewicz *et al.* 1971, Wada *et al.* 1971, Watanabe and Wada 1971, and Martinez-Carrion *et al.* 1970).

cAspAT consists of two identical 412 residue subunits. Each subunit contains a large and small domain with the active site being located between the two domains. The pyridoxal phosphate cofactor is located in the large domain, covalently linked by an aldimine bond (Schiff base bond) to Lys258 (Rhee *et al.* 1997).

IUB

- 2.6.1.1

CAS

- 9000-97-9

Protein Accession Number

- P00503

CATH: Classification (v. 3.3.0)

Class:

- Alpha Beta

Architecture:

- Alpha-Beta Complex; 3-Layer(aba) Sandwich

Topology:

- Aspartate Aminotransferase (domain 1); Aspartate Aminotransferase (domain 2)

Molecular Weight

- Monomer: 47.0 kDa (Polyanovsky *et al.* 1972)
- Dimer: 93.1 kDa (Ovchinnikov *et al.* 1973)
- Dimer: 94.0 (Polyanovsky *et al.* 1972)

Optimal pH

- 8

Isoelectric Point

- 6.83

Extinction Coefficient

- 134,340 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 14.0 (Polyanovsky *et al.* 1972)

Active Residue

- Lysine (K258)

Activators

- Pyridoxal or pyridoxamine phosphate activate undenatured enzyme (Jenkins *et al.* 1959)
- In solution the enzyme may be stabilized with a-ketoglutarate and EDTA in maleate or succinate buffers (Jenkins *et al.* 1959).

Inhibitors

- 2-Oxoglutaconate, 2-oxoglutaconic acid dimethyl ester, and N-ethylmaleimide (Kato *et al.* 1996)
- N-5'-Phosphopyridoxyl L-aspartate (Izard *et al.* 1990)
- p-Mercuribenzoate (Stankewicz *et al.* 1971)
- L-a-Methylaspartic acid (Melander 1975)
- 2-Amino-3-butenoic acid (Rando 1974)
- Maleate, succinate, glutarate, and adipate (Jenkins *et al.* 1959)

Applications

- Clinical tests for diagnosis of liver and heart disorders
- Synthesis of unnatural L-amino acids from alpha-keto acids

Assay Information

Method

- Aspartate aminotransferase may be assayed spectrophotometrically in a coupled reaction with malate dehydrogenase in the presence of NADH (Karmen 1955; Amador and Wacker 1962). One unit oxidizes one micromole of NADH per minute at 25°C and pH 7.4 under the specified conditions.

Reagents

- Prepare reagent mixture containing:
- L-aspartate - 134.0 mM
- 2-oxoglutarate - 6.64 mM
- NADH - 0.24 mM
- Lactate dehydrogenase - 5 $\frac{\text{units}}{\text{ml}}$
- Malate dehydrogenase - 1.25 $\frac{\text{units}}{\text{ml}}$
- Sodium phosphate buffer, pH 7.4 - 50 mM

Enzyme

- Dissolve enzyme at a concentration of one $\frac{\text{mg}}{\text{ml}}$ in 0.1 M potassium phosphate pH 7.4. Immediately prior to use, dilute further in this buffer to a concentration of 0.05 - 0.25 $\frac{\text{units}}{\text{ml}}$.

Procedure

Spectrophotometer Settings: Wavelength: 340 nm Temperature: 25°C

Pipette 2.9 ml of the reagent mixture into cuvette and place in spectrophotometer. Incubate for 3 - 4 minutes to reach temperature equilibrium and establish blank rate, if any. At zero time, add 0.1 ml of

apropriately diluted enzyme and record the decrease in A_{340} for 4 - 5 minutes. Calculate $\frac{\Delta A_{340}}{min}$ from the initial linear portion of the curve.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{340}}{\text{min}}}{\frac{6.2 \text{ x mg enzyme}}{\text{ml reaction mixture}}}$$

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13. Avidin

Avidin is a glycoprotein consisting of four identical subunits. It is a minor constituent of the egg white of reptiles, amphibians, and birds. In the chicken, it is synthesized in the oviduct in response to progesterone and in several tissues in connection with inflammation and tissue damage (Elo *et al.* 1979, and Gope *et al.* 1987). It binds the vitamin biotin with high affinity and is therefore thought to be a defense protein against biotin-requiring microbes (Wallén *et al.* 1995). Avidin exhibits the highest known affinity in nature between a ligand and a protein (Livnah *et al.* 1993).

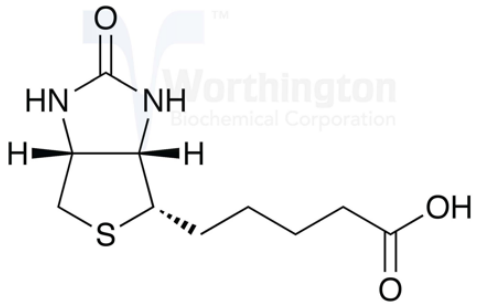

Figure 13.1: Enzymatic Reaction - Avidin

History

In 1916, Bateman first investigated the toxic effect of uncooked egg whites in animals. In 1927, Boas showed that "egg white injury" was caused by a protein in albumin. It was determined that this protein binds biotin forming a "non-digestible" complex that cannot be absorbed from the intestine of animals

or from the surrounding medium by microorganisms (Boas 1924, Pennington *et al.* 1942, Woolley and Longsworth 1942, and Hertz 1946). Eakin *et al.* first isolated the protein and named it avidin (Eakin *et al.* 1940a, b, and 1941).

In 1963, Green determined the dissociation constant of the avidin-biotin complex (10-15 M) indicating extreme stability of the complex *in vitro* (Green 1963a, b). Through the late 1970s and into the early 1980s, the complex formation of the avidin-biotin complex was a useful tool in biochemistry and immunocytochemistry (Guesdon *et al.* 1979, Bayer and Wilchek 1980 and Hsu *et al.* 1981). The sequence of chicken avidin was determined by DeLange and Huang in 1971. The cDNA was cloned and sequenced by Gope *et al.* in 1987, and the gene (avd) was cloned and sequenced by Wallén *et al.* in 1995.

Recent work with avidin has found its use in a variety of cancer treatments (Lesch *et al.* 2010). Both avidin and its derivatives continue to be used in the isolation of biotinylated proteins (Bolivar *et al.* 2008), and it has been incorporated into assays to detect levels of biotin in urine and serum (Zarogiannis *et al.* 2007).

Molecular Characteristics

The protein contains a 24 residue signal peptide. The avidin gene (avd) consists of four exons split by three introns (Wallén *et al.* 1995). Although avidin shares almost identical secondary, tertiary, and quaternary structure with streptavidin, the two proteins show only 30% sequence identity and are thought to be evolutionarily unrelated (Laitinen *et al.* 2006).

Composition

Avidin is a basic homotetrameric glycoprotein (Green 1964, and DeLange and Huang 1971). The carbohydrate moiety, a polysaccharide composed of four glucosamines and five mannoses, is attached to Asn-17 of each subunit (DeLange and Huang 1971). Each of the identical subunits binds one biotin molecule, and tryptophan is involved in non-covalent binding. Each subunit is folded into an eight-stranded anti-parallel beta-barrel, displaying up and down topology. The biotin binding site is located in the core of the barrel and is built by residues of the barrel itself and the loop of an adjacent subunit (Nardone *et al.* 1998). Although avidin shares almost identical secondary, tertiary, and quaternary structure with another biotin-binding protein, streptavidin, streptavidin is not glycosylated and is less susceptible to aggregation. Avidin has a higher affinity for unconjugated biotin than streptavidin, but a lower affinity for conjugated biotin (Laitinen *et al.* 2006).

The best preparations of avidin bind 15.1 μg of D-biotin per milligram of protein, corresponding to 4 moles of biotin bound per mole of protein. The dissociation constant for biotin is approximately 10-15 M, being about 103-106 times higher than that of a typical antigen-antibody interaction (Green 1963a, and Nardone *et al.* 1998). The biotin-avidin complex is dependent upon media ionic strength, but relatively stable over a wide pH range and to heat.

It is necessary to effect a reversible or irreversible denaturation to free biotin. Guanidinium chloride (6-8 M) at pH 1.5 and autoclaving at 120°C (15 minutes) are examples of sufficient dissociation conditions (Green 1970a, b).

CAS

- 1405-69-2

Protein Accession Number

- P02701

CATH: Classification (v. 3.3.0)

Class:
- Mainly Beta

Architecture:
- Beta Barrel

Topology:
- Lipocalin

Molecular Weight

- 54.7 kDa

Optimal pH

- Relatively stable over a wide pH range

Isoelectric Point

- 10 (Nardone *et al*. 1998)

Extinction Coefficient

- $E_{282}^{1\%}$ = 15.5 (Melamed and Green 1963)

Applications

- Biotin function studies
- Study of enzymes in which biotin is a coenzyme
- Avidin or avidin subunits used for affinity purification (Berger and Wood 1975, and Green and Toms 1973)
- *in vivo* for the targeting of solid tumors

Assay Information

The biotin-binding capacity of avidin is determined using the spectrophotometric method of Green (1963). One unit will bind one microgram of d-biotin at pH 8.9.

Reagents

- 0.2 M Ammonium carbonate, pH 8.9
- 0.001 M d-biotin (24.4 mg/100 ml) in ammonium carbonate buffer, pH 6.9

Enzyme

- Dissolve at 0.2 $\frac{\text{mg}}{\text{ml}}$ in 0.2 M ammonium carbonate buffer, pH 8.9

Procedure

Spectrophotometer Settings: Wavelength: 233 nm

Add 3 ml of stock avidin to control and test cuvette. Add 10 μl of d-biotin to test cuvette and record ΔA_{233}. Continue adding 10 μl biotin at a time to the test cuvette until no further increase in A_{233} occurs after three successive biotin additions. Plot the number of aliquots of biotin used versus A_{233}. Determine the equivalence point by drawing a straight line through the linear absorbance portion of the curve and by drawing a line through the portion where no further increase in A_{233} is observed. The intersection of these lines is the equivalence point. Determine the number of aliquots represented by the equivalence point.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\text{\# of aliquots at equivalence point x volume of aliquot (in ml) x biotin concentration (in } \frac{\mu\text{g}}{\text{ml}})}{\text{mg of avidin in 3 ml}}$$

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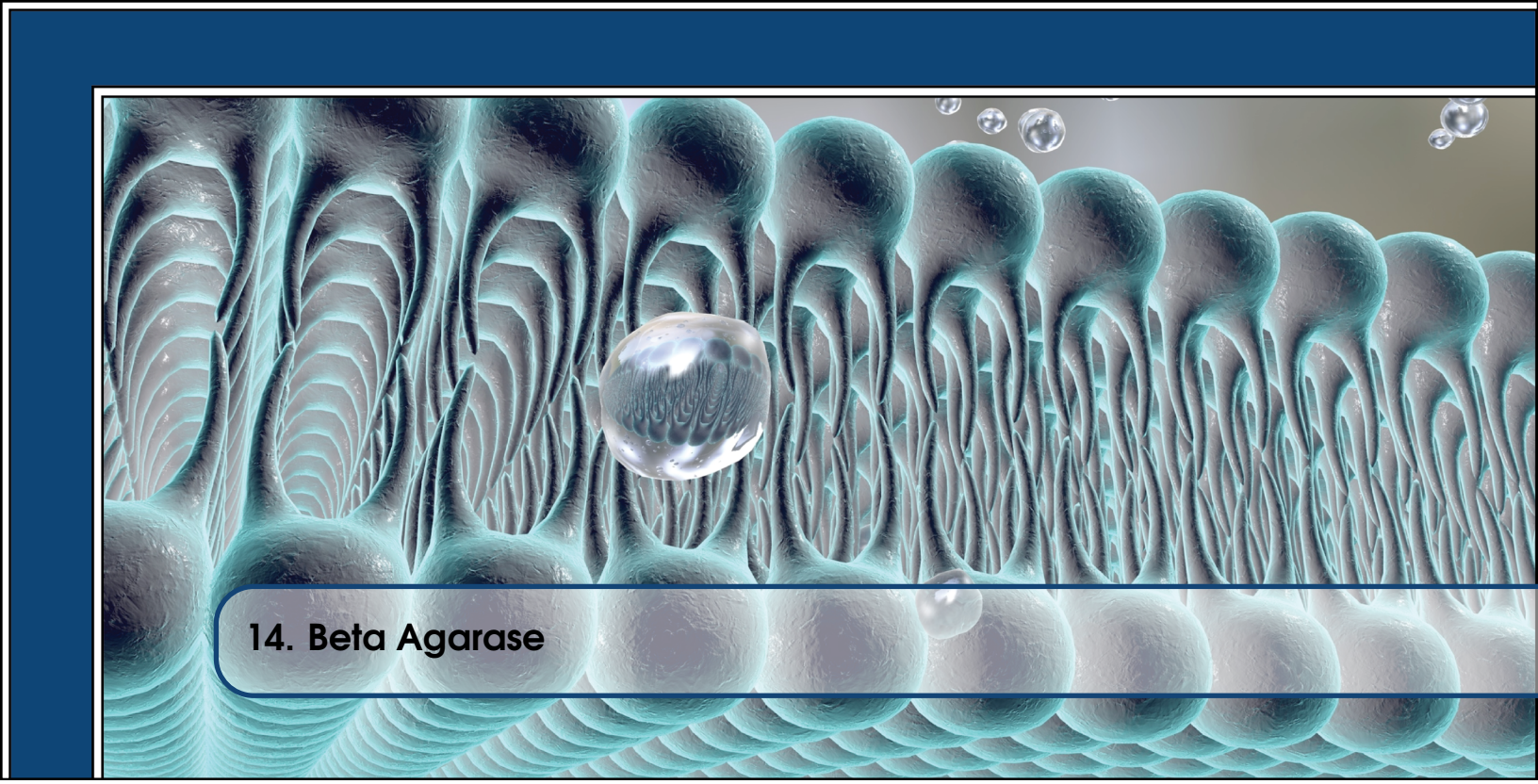

14. Beta Agarase

Agarose 3-glycanohydrolase

Agarases are glycoside hydrolases that catalyze the hydrolysis of agar. Agarases are primarily found in marine organisms (Fu and Kim 2010), with microorganisms being the primary sources for agarase production (Oh *et al.* 2010). Agarases are classified into two groups based on their mode of action. α-Agarases hydrolyze α-1,3-linkages to produce agarooligosaccharides of a series related to agarobiose, while β-agarases hydrolize β-1,4-linkages producing neoagarooligosaccharides of a series related to neoagarobiose (Fu and Kim 2010, and Oh *et al.* 2010).

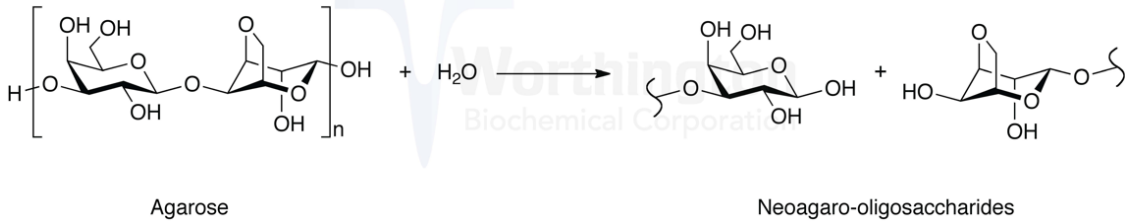

Figure 14.1: Enzymatic Reaction - Beta Agarase

History

Yaphe and colleagues first described the agarose-degrading system of Pseudomonas atlantica (Yaphe 1957, Yaphe 1966, Day and Yaphe 1975, and Groleau and Yaphe 1977). This enzyme was designated

β-Agarase II. The purification and characterization of the related β-Agarase I (32 kDa) was reported by Morrice *et al.* in 1983. The two enzymes differ significantly in that β-Agarase I is membrane-bound, while β-Agarase II is found extracellularly (Morrice *et al.* 1983a and b). That formerly manufactured by Worthington Biochemical was β-agarase II.

Belas *et al.* cloned β-agarase II from Pseudomonas atlantica gene in 1988, and the nucleotide sequence was determined in 1989 (Belas *et al.* 1988, and Belas *et al.* 1989). Worthington Biochemical first manufactured β-agarase II in the early 1990s.

Recent research has involved the identification of novel β-agarase sequences from agar-degrading bacterial genomes (Lee *et al.* 2015a and b) and the characterization of newly discovered agarases (Chi *et al.* 2015, and Hsu *et al.* 2015). In addition, the crystal structures of β-agarase enzymes continue to be elucidated (Takagi *et al.* 2014, and Pluvinage *et al.* 2013).

Molecular Characteristics

The gene encoding β-agarase (agrA) has a primary structure consisting of an ORF of 1,515 nucleotides. The primary translation product consists of 504 amino acids (Belas 1989). Three regions upstream to the start codon that are responsible for ribosomal binding and promoter sites of the gene sequence have been determined based on homology with *Escherichia coli* (Belas *et al.* 1989).

Specificity

β-Agarase is unique in its ability to break down the agarose polysaccharide core composed of repeating 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-alpha-L-galactopyranose into neoagarobiose oligosaccharides.

Composition

Based on catalytic domain similarities, β-Agarase II of Pseudoalteromonas atlantica has been designated part of the glycosyl hydrolase 86 (GH86) family. Other β-agarases belong to glycosyl hydrolase families 16 and 50, with all three groups containing a conserved glycoside hydrolase region responsible for catalysis (Fu and Kim 2010).

The NH2 terminus of the protein may function as a signal peptide in the export of extracellular β-agarase. Signal peptidase is believed to cleave between resides 22 and 23 of the primary agrA product, yielding a mature protein of 482 residues (Belas *et al.* 1989).

IUB

- 3.2.1.81

CAS

- 37288-57-6

Protein Accession Number

- P13734

CATH: Classification (v. 3.3.0)

Class:

- Mainly beta

Architecture:

- Sandwich

Topology:

- Jelly Rolls

Molecular Weight

- 55.1 kDa

Optimal pH

- 6.5-7.5 (Morrice *et al.* 1983b)

Isoelectric Point

- 5.9 (Oh *et al.* 2010)
- 5.95 (Theoretical)

Extinction Coefficient

- 97,250 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 17.66

Active Residue

- Glutamic Acid (E177 and E299)

Applications

- Food, cosmetic and medical industry (Kobayashi *et al.* 1997)
- Protoplast isolation from seaweeds (Araki *et al.* 1998)
- Production of agar-derived oligosaccharides
- DNA recovery from agarose gel: As a result of this enzymatic degradation the viscosity and gelling ability of the native low melting point agarose is eliminated liberating the nucleic acids. Since the oligosaccharides produced are alcohol soluble, intact DNA can be recovered in a simple and efficient manner by alcohol precipitation.

Assay Information

The Worthington enzyme can be 2-4 times higher in activity than preparations offered by other suppliers. One Worthington unit will hydrolyze 500 milligrams of molten 1% LMP agarose per hour at 40 - 42°C.

Reagents

- 0.1 M Sodium acetate, pH 5.0, containing 0.001 M EDTA
- 1% LMP Agarose, dissolved in 0.08 M Tris·borate buffer containing 0.001M EDTA, pH 8.3

Procedure

To a series of tubes in 40 - 42°C water bath add 0.5 ml (500 mg gel) 1% LMP agarose gel.
Add 0.25 ml of buffer to each tube.
Prepare dilutions of the enzyme in 0.01% BSA/water to 1:5, 1:10, 1:20, 1:30, 1:40, and 1:50.
Add 10 μl of each dilution to a labelled tube. Add 10 μl of 0.01% BSA enzyme diluent to a tube and label as BLANK.
Incubate one hour at 40 - 42°C.
Remove from water bath and chill in ice bath for 5 minutes.
Inspect tubes for the last dilution having complete digestion. Complete digestion is indicated by liquefaction of the agarose. Use this tube to select the dilution factor and in the calculation of activity.

Calculation

- $\frac{\text{Units}}{\text{ml}} = 100 \text{ x dilution}$
- $\frac{\text{Units}}{\text{mgP}} = \frac{\frac{\text{Units}}{\text{ml}}}{\frac{\text{mgP}}{\text{ml}}}$

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15. Carbonic Anhydrase

Carbonic anhydrase (CA) catalyzes the reversible hydration and dehydration reactions of CO_2/H_2CO_3. CAs are widespread in nature, being found in animals, plants, and certain bacteria. Sixteen isozymes have been identified and characterized in mammals. Since erythrocyte CA is relatively easy to obtain for experimental purposes, it is the most widely studied.

$$CO_2 + H_2O \rightarrow H_2CO_3 \tag{15.1}$$

History

CA was first discovered in vertebrate erythrocytes (Brinkman *et al.* 1932). It was inferred that a catalyst was present in blood that could dehydrate bicarbonate and allow it to escape as CO_2 (Stadie and O'Brien 1933). CA was soon isolated from erythrocytes by Meldrum and Roughton in 1933. Upon the discovery in 1940 that zinc is an intrinsic cofactor, CA became the first recognized metalloenzyme (Keilin and Mann 1940). It was also deduced that sulfanilamide is an inhibitor of the enzyme, which led to pharmacological investigations and the eventual important discovery of CA inhibitors as treatment for glaucoma (Mann and Keilin 1940, Maren 1995, and Khalifah 2003).

It took over twenty years for CA to gain the interest of the research community, and it did so because of the simple substrates upon which it acts. It became known as one of the most efficient acid-base catalysts (Eigen and Hammes 1963). Interest in the enzyme was also gained when Lindskog replaced the zinc ion with cobalt and found the activity of the enzyme was unaffected (Lindskog and Malström 1962). This led to many studies on the oxidation of the metals and theories that the activity of the enzyme is related to ionization of nearby groups (Whitney *et al.* 1967, Appleton and Sarkar 1974, and Shinar and Navon 1974).

Human erythrocytes were used in studies of the 1970s to isolate and obtain the first amino acid sequences of CA-I (Andersson *et al.* 1972, and Lin and Deutsch 1973) and CA-II (Henderson *et al.* 1973, and Lin and Deutsch 1974). John Edsall performed extensive studies on the kinetics of human erythrocyte CA, and also discovered the first competitive inhibitor, imidazole (Edsall 1968, and Khalifah 2003).

Current research on CA continues to investigate the mechanism of inhibitor binding (Safarian *et al.* 2007) and CA's role in inhibiting ectopic cardiovascular calcification (Rajachar *et al.* 2009).

Molecular Characteristics

Each isozyme from erythrocytes (CA-I and CA-II) is composed of a single chain peptide of 259 or 260 amino acid residues. The low activity form (CA-I) contains 260 residues, while the high activity form (CA-II) contains 259 residues. Erythrocyte high and low activity forms within a given species usually show greater than 50% sequence identity; for example, equine CA-I and CA-II forms only show 55% identity (Wendorff *et al.*1985). In contrast, the same forms from different species show much greater homology; human CA-II and bovine CA-II show 77% sequence homology (Tashian *et al.* 1980, Engberg *et al.* 1985, Alam *et al.* 2003). The isozymes are encoded by separate genes but given the great deal of homology, especially in the active center, they appear to have a common evolutionary history. Ubiquitin, a 76 residue protein with some enzymatic properties of CAs, has a distinct sequence homology to CA (Deutsch 1987).

Specificity

Blood CO_2 transport and excretion is largely dependent on the rapid catalysis of the CO_2 reactions within the erythrocyte by CA (Tufts *et al.* 2003). Bovine CA reversibly hydrates alkyl pyruvates and it exhibits hydratase activity toward a wide variety of substrates (Pocker *et al.* 1974, and Wells *et al.* 1975).

Composition

Sixteen CA isozymes have been described so far in mammals. Erythrocyte CAs, CA-I and CA-II, are most well known. CA-I, CA-II, CA-III, CA-VII, and CA-XIII are cytosolic. CA-IV, CA-IX, CA-XII, CA-XIV, and CA-XV are membrane bound. CA-VI is secreted in saliva. CA-VA and CA-VB are mitochondrial. There are also three acatalytic forms referred to as CA-related proteins (CARPs): CARP-VIII, CARP-X, and CARP-XI (Coban *et al.* 2009).

The zinc metal is always bound to histidines 93, 95, and 118 (mature chain numbering). A hydrogen bonded network, linked to the zinc-bonded water molecule and these histidines either directly or indirectly, includes 28-Ser, 91-Glu, 105-Glu, 106-His, 116-His, 193-Tyr, 198-Thr, 208-Trp, and 223-Asn. These residues have been found to be highly conserved (Lindskog 1982, and Lindskog *et al.* 1984). Bovine and human CA I and II contain a unique C-terminal knot structure, which has been shown to be important in enzymatic and mechanical properties (Alam *et al.* 2002).

IUB

- 4.2.1.1

CAS

- 9001-03-0

Protein Accession Number

- P00921

CATH: Classification (v. 3.3.0)

Class:
- Alpha Beta

Architecture:
- Roll

Topology:
- Carbonic Anhydrase II

Molecular Weight

- 29.0 kDa (Theoretical)
- 30 kDa (Lindskog *et al*. 1971)

Optimal pH

- 7.0-7.5 (Demir *et al*. 2000, and Tasgin *et al*. 2009)

Isoelectric Point

- 6.40 (Theoretical)

Extinction Coefficient

- 50,070 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 19.0 (Nyman and Lindskog 1964)

Active Residue

- Histidine (H63)
- Asparagine (N66)
- Lysine (K126)

Activators

- HPO_4^{2-} (Rowlett *et al*. 1991)
- SO_3^{2-} (Rowlett *et al*. 1991)

Inhibitors

- Monovalent anions (Lindskog *et al*. 1971, and Ward and Cull 1972)
- Sulfonates and sulfonamides (Pocker and Watamori 1973, and Binford *et al*. 1974)
- Imidazole (Edsall 1968)

Applications

- CO_2 determination in blood
- Elimination of CO_2 in reagents for acidity testing
- Carboxy group transfers
- Reduction reactions

Assay Information

Method

- The electrometric method of Wilbur and Anderson (1948) in which the time required (in seconds) for a saturated CO_2 solution to lower the pH of 0.012 M Tris · HCl buffer from 8.3 to 6.3 at 0°C is determined. The time without enzyme is recorded at T_0; with enzyme, T.
- A unit of activity $= \frac{2 \text{ x } (T_0 - T)}{T}$

Reagents

- 0.02 M Tris · HCl buffer, pH 8.0. Store in an ice bath at0 - 4°C before and during use.
- Carbon dioxide saturated water. Bubble CO_2 gas through 200 ml ice cold water for 30 minutes prior to assay. During saturation process, store water at0 - 4°C in an ice bath.

Enzyme

- Dissolve lyophilized powder at a concentration of 0.1 $\frac{\text{mg}}{\text{ml}}$ in ice cold water. Store in ice bath prior to use. IMMEDIATELY prior to use dilute suspensions or lyophilized materials to a concentration of approximately 0.01 $\frac{\text{mg}}{\text{ml}}$ in ice cold water.

Procedure

Blank Determination: Add 6.0 ml of chilled 0.02 M Tris · HCl buffer, pH 8.0 to a 15-20 ml beaker. Maintain temperature at0 - 4°C and record pH.
Withdraw in a 5 ml syringe, 4 ml of chilled CO_2 saturated water and add to Tris buffer. Immediately start a stop watch and record the time required for the pH to drop from 8.3 to 6.3. Record this time as T_0.
Enzyme Determination: Add 6.0 ml of chilled 0.02 M Tris · HCl buffer, pH 8.0 to a 20 ml beaker. Maintain temperature at0 - 4°C and record pH. Add 0.1 ml of freshly diluted enzyme. Quickly add 4 ml of CO_2 saturated water and record the time required for the pH to drop from 8.3 to 6.3. Record this time as T.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{2 \text{ x } (T_0 - T)}{T \text{ x mg enzyme in reaction mixture}}$

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16. Carboxypeptidase A

Carboxypeptidase A (CPDA) is a pancreatic metalloexopeptidase that hydrolyzes the peptide bond adjacent to the C-terminal end of a polypeptide chain.

$$\text{R-CONH-CHR'COOH} + H_2O \rightarrow \text{R-COOH} + H_2\text{NCHR'COOH} \quad (16.1)$$

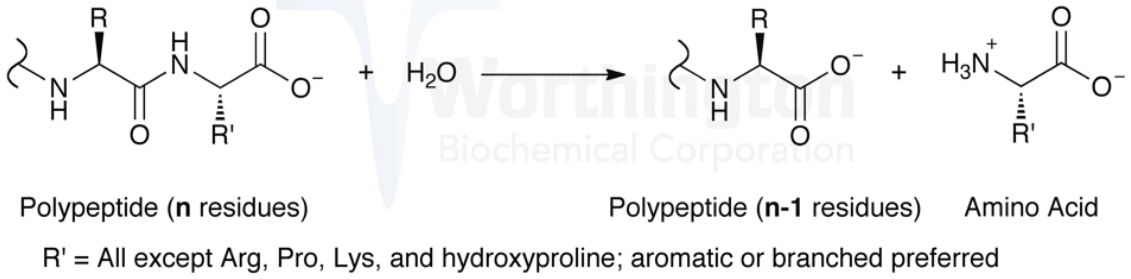

Figure 16.1: Enzymatic Reaction - Carboxypeptidase A

History

CPDA was first isolated by Waldschmidt-Leitz and Purr in 1929 and first crystallized by Anson in 1937 (Hartsuck and Lipscomb 1971, and Auld 2004). CPDA was the first metalloprotease and second zinc enzyme to be identified (Vallee and Neurath 1954, and Auld 2004).

In 1953 Thompson preliminarily determined the N-terminal amino acid sequence, establishing that CPDA was a single polypeptide chain. Through x-ray diffraction studies Reeke *et al.* resolved the structure of CPDA to 2.0 resolution and determined CPDA is composed of 307 amino acid residues (Reeke *et al.* 1967). Folk and Schirmer isolated porcine CPDA, and Folk established the relationships between the two forms of the enzyme (Folk and Schirmer 1963, and Folk 1963).

CPDA was one of the first enzymes to have its complex kinetic profile extensively studied (Slobin and Carpenter 1966, Whitaker 1966, Auld and Vallee 1970, Auld and Vallee 1971, Hass and Neurath 1971, Naik and Horton 1973, and Canonici and Behnke 1974). In 1969, Bradshaw *et al.* determined the amino acid sequence of bovine CPDA.

The mechanism of activation of CPDA was used as a model to investigate the role of zymogens in biological systems (Neurath 1984), and in the 1990s the gene of bovine pancreatic proCPDA was identified (Le Huërou *et al.* 1991, and Goo *et al.* 1995).

Recent studies have investigated binding of inhibitors through kinetic studies (Chong and Auld 2007) and the generation of an enzyme-inhibitor crystal structure (Arolas 2005). CPDA has also been used for the development of C-terminal affinity tags (Austin *et al.* 2010).

Molecular Characteristics

When Bradshaw *et al.* determined the complete amino acid sequence they confirmed that bovine CPDA exists in two allotypic forms (Bradshaw 1969b, and Goo *et al.* 1995). One form of the enzyme contains Ile179, Ala228, and Val305, while the other contains Val179, Glu228, and Leu305 (Petra *et al.* 1969). In 1995, Goo *et al.* isolated from bovine pancreas the major CPDA that contains Ile179, Ala228, and Val305. The nucleotide sequence showed an open reading frame encoding a 16 amino acid signal peptide, a 94 amino acid activation peptide, and a 309 amino acid mature CPDA (Goo *et al.* 1995).

The amino acid sequences of the family to which CPDA belongs to (M14) are between 60 and 80% conserved. Conserved residues believed to be important in specificity and catalysis include Glu270, Arg145, Arg127, Tyr248, and Tyr198 (Auld 2004). In 2009 Zimin *et al.* assembled the entire Bos taurus genome.

Specificity

C-terminal L-amino acids with aromatic or branched sidechains are preferentially cleaved (Libscomb 1970), and ester bonds of peptides with a free C-terminal carboxyl group can also cleaved by CPDA. Acylated -amino and -hydroxy carboxylic acids are also substrates (Auld 2004).

Composition

CPDA is secreted as a proenzyme, with a 94 amino acid segment that is cleaved by trypsin during activation. The active enzyme is composed of 309 amino acid residues (Guasch *et al.* 1992). Only one form of CPDA has been found in cattle, while two forms have been found to exist in humans and rats. The monomeric form of the proenzyme is found in most species; however, it also can be found as a binary or ternary complex. When it occurs as a binary complex, it is complexed with either

chymotrypsinogen C or proproteinase E, and when it occurs as a ternary complex it is complexed with both (Gomis-Rüth *et al*. 1995, and Vendrell *et al*. 2000). The activation segment lies at the center of the three subunits.

Arg145 is believed to be the site that interacts with the substrate's free alpha-carboxyl group, and Glu270 is the principle catalytic moiety (Hartsuck and Lipscomb 1971, and Rees *et al*. 1983).

IUB

- 3.4.17.1

CAS

- 11075-17-5

Protein Accession Number

- P00730

CATH: Classification (v. 3.3.0)

Class:
- Alpha Beta

Architecture:
- 3-Layer(aba) Sandwich

Topology:
- Aminopeptidase

Molecular Weight

- 35.3 (Bradshaw *et al*. 1969b)
- 34.8 (Theoretical)

Optimal pH

- 7-9, depending on the substrate

Isoelectric Point

- 6.08

Extinction Coefficient

- 65,670 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{278}$ = 19.4

Active Residue

- Arginine (R127 and R145)
- Glutamate (E270) (Auld 2004)

Activators

- The zinc component is essential for activity

Inhibitors

- Cysteine, sulfides, and cyanide, but not diisopropylphosphofluoridate (DFP) or phenylmethanesulfonyl fluoride (PMSF)
- Metal ions and anions (Geoghegan *et al*. 1983b)
- Strongly inhibited by the chelating agent 1,10-phenanthroline
- Ochratoxin A is a competitive inhibitor (Pitout and Nel 1969)
- Phosphonates (Kaplan and Bartlett 1991)
- Latexin (Normant *et al*. 1995)

Applications

- Resolution of racemic amino acids
- Sequence analysis
- Hydrolysis/condensation of amide bonds
- Downstream processing of fusion proteins that may precipitate at high salt concentrationes (White and White 1997)

Assay Information

Method

- The determination of reaction velocity is based upon the method of Folk and Schirmer (1963). The rate of hydrolysis of hippuryl-L-phenylalanine is determined by measuring the increase in absorbance at 254 nm. One unit hydrolyzes one micromole of hippuryl-L-phenylalanine per minute at pH 7.5 and 25°C under the specified conditions.

Reagents

- 0.025 M Tris · HCl buffer containing 0.5 M sodium chloride, pH 7.5
- 0.001 M Hippuryl-L-phenylalanine in 0.025 M Tris · HCl, pH 7.5 with 0.5 M sodium chloride
- 10% Lithium chloride

Enzyme

- Dissolve in 10% lithium chloride to a concentration of 1-3 units per ml. The enzyme crystals are not readily soluble in the diluent. Do not use solution for assay until the solution has cleared.
- Read A_{278} in cold 10% lithium chloride.

Procedure

Spectrophotometer Settings: Wavelength: 254 nm Temperature: 25°C

Pipette 2.0 ml of substrate into each cuvette and incubate in spectrophotometer at 25°C for 3-4 minutes to reach temperature equilibration and establish blank rate, if any. Add 0.1 ml of diluted enzyme and record increase in A_{254} for 3-5 minutes. Determine $\frac{\Delta A_{254}}{\text{min}}$ from the initial linear portion of the curve.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{254}}{\text{min}}}{\frac{0.36 \text{ x mg enzyme}}{\text{ml reaction mixture}}}$

where 0.36 is the extinction coefficient of hippuric acid formed during the reaction

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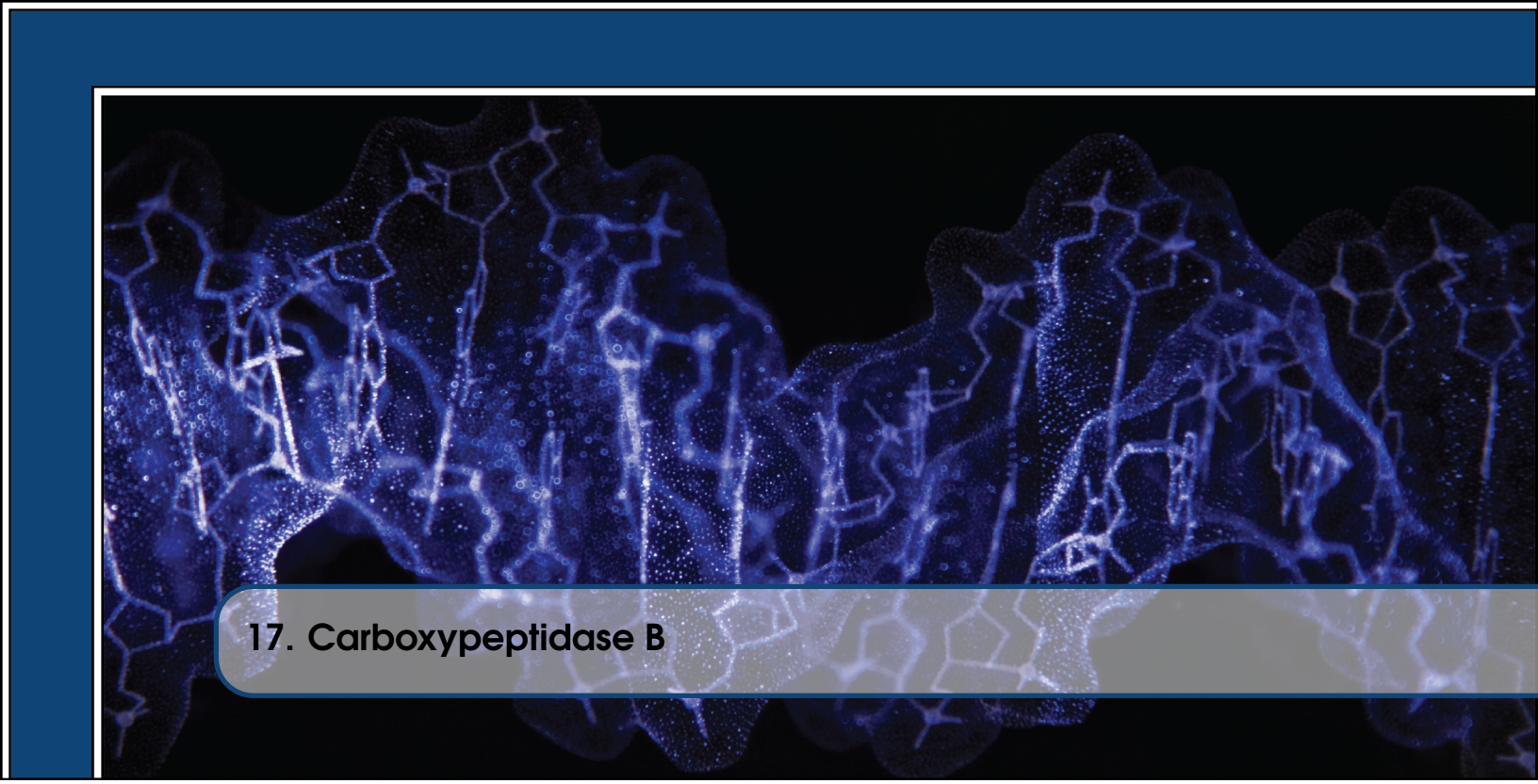

17. Carboxypeptidase B

Carboxypeptidases are secreted as zymogens by the acinar cells of the pancreas. The zymogens are activated by trypsin in the small intestine. Carboxypeptidase B (CPDB) is a metallocarboxypeptidase that catalyzes the hydrolysis of the basic amino acids, lysine, arginine, and ornithine from the C-terminal position of polypeptides:

Peptidyl-L-lysine (-L-basic amino acid) + H_2 → peptide + L-lysine (L-basic amino acid) (17.1)

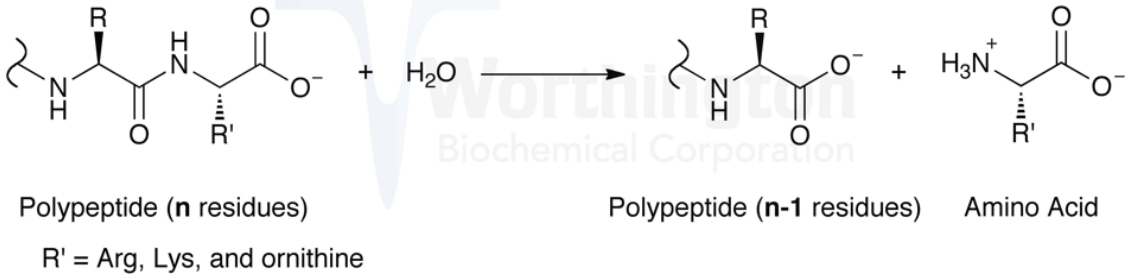

Figure 17.1: Enzymatic Reaction - Carboxypeptidase B

History

In 1931, Waldschmidt-Leitz *et al.* demonstrated that excretions from porcine pancreas contained an enzyme that catalyzed the release of arginine from proteins (Folk 1971). They termed the enzyme

"protaminase". In 1933, Calvery used egg albumin to show that it too could be susceptible to this enzyme, suggesting that lysine and histidine could also be released. Throughout the 1930s and 1940s, it was believed that this enzyme was identical to chymotrypsin (Sumner and Somers 1947, and Northrop *et al*. 1948).

In 1940, the work of Hoffmann and Bergmann demonstrated lysine was only slowly released from substrate in the presence of carboxypeptidase A (CPDA). The work of Folk in 1956 showed lysine was rapidly released in the presence of commercially available pancreas powder (Folk 1971). These two insights led to experiments that confirmed the presence of two separate carboxypeptidase enzymes (Folk 1956, and Folk and Gladner 1958). The carboxypeptidase B name was given by Folk *et al*. in 1960 in order to officially distinguish it from CPDA.

In 1975, Titani *et al*. determined the amino acid sequence. In 1976, Schmid and Herriott resolved the structure to a 2.8 resolution, which was later refined by Coll *et al*. in 1991. The globular domain of pancreatic porcine pro-CPDB pro-segment was derived through NMR spectroscopy by Vendrell *et al*. in 1990.

Recent work has included obtaining a higher resolution crystal structure of the proform of CPDB (Fern*á*ndez *et al*. 2010), as well as resolving the crystal structure of potent thiol inhibitors (Adler *et al*. 2005).

Molecular Characteristics

Clauser *et al*. studied the rat preprocarboxypeptidase B gene, and found that it is organized very similarly to the bovine CPB1 gene (Clauser *et al*.1988). The CPB1 gene is conserved in many eukaryotes and has been studied in human, chimpanzee, dog, mouse, rat, chicken, zebrafish, and fruit fly (Avil*é*s and Vendrell 2004). The mature form of rat CPDB is homologous to bovine CPDB (77% identical), and the amino acids involved in catalysis or ligand binding show no variance. The coding region of preprocarboxypeptidase B consists of 11 exons (Clauser *et al*. 1988). Comparisons of rat CPDB, rat CPDA, and rat CPDA2 genes have shown that the number, position, and sequence composition of the exons are conserved, despite large differences in intervening sequences (Gardell *et al*. 1988, and Clauser *et al*. 1988). Through cDNA and mRNA sequencing, CPDB mRNA has been determined to be 1385 nucleotides, excluding the poly(A) tail. The rat CPDB mRNA is 100 nucleotides longer the CPDA mRNA. This difference is primarily due to a larger 3' untranslated region (UTR) in the CPDB mRNA (Han *et al*. 1986).

Specificity

CPDB is activated by trypsin. CPDB is highly specific for lysine and arginine, but shows preference for arginine (Tan and Eaton 1995). It can also act (at a slower rate) on valine, leucine, isoleucine, asparagine, glycine, and glutamine (Villegas *et al*. 1995, Nishihira *et al*. 1995). The differences in specificities between carboxypeptidase A (CPDA) and CPDB can be attributed to the residues Ser205, Gly241, and Asp253 in CPDB as compared to Gly207, Ile243, and Ile255 in CPDA (Coll *et al*. 1991).

Composition

The major form of CPDB is found in the monomeric state. CPDB contains 1 atom of zinc per mole of protein. The residues coordinating the zinc residue are conserved and include two histidines, a glutamic acid, and a water (Avilés and Vendrell 2004).

IUB

- 3.4.17.2

CAS

- 9025-24-5

Protein Accession Number

- P09955

CATH: Classification (v. 3.3.0)

Class:

- Alpha Beta

Architecture:

- 3-Layer(aba) Sandwich

Topology:

- Aminopeptidase

Molecular Weight

- 34.3 kDa (Folk *et al.* 1960)
- 34.7 kDa (Theoretical)

Optimal pH

- 9.0 (Wolff *et al.* 1962)

Isoelectric Point

- 4.6-5.2 (Lipperheide and Otto 1986)

Extinction Coefficient

- 77,080 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 21.4 (Folk 1971)

Active Residue

- Glutamic acid (E378)
- Tyrosine (Y356)

Inhibitors

- Competitively inhibited by arginine, lysine, and ornithine (Wolff *et al.* 1962)
- Metal chelating agents such as 1,10-phenanthroline and EDTA
- Heavy metals
- Not inhibited by diisopropylfluorophosphate (DFP) or phenylmethylsulfonyl fluoride (PMSF)
- Potato carboxypeptidase inhibitor (Hass *et al.* 1981)
- Leech carboxypeptidase inhibitor (Reverter *et al.* 1998)

Applications

- Sequence analysis by successive cleavage of C-terminal basic amino acids
- Serum marker for acute pancreatitis diagnosis (Avilés and Vendrell 2004)
- Cytochrome C stepwise digestion (Südi *et al.* 1989)
- Human insulin production (Ladisch and Kohlmann 1992)

Assay Information

Method

- Activity is measured by the spectrophotometric method of Folk et al. (1960) where the reaction velocity is determined by an increase in absorbance at 254 nm resulting from the hydrolysis of hippuryl-L-arginine. One unit causes the hydrolysis of one micromole of hippuryl-L-arginine per minute at 25°C and pH 7.65 under the specified conditions.

Reagents

- 0.025 M Tris · HCl buffer, pH 7.65 containing 0.1 M sodium chloride
- 0.001 M Hippuryl-L-arginine in 0.025 M Tris · HCl pH 7.65 containing 0.1 M sodium chloride

Enzyme

- Dilute stock solution with reagent grade water to a concentration of 1-5 $\frac{\text{units}}{\text{ml}}$.

Procedure

Spectrophotometer Settings: Wavelength: 254 nm Temperature: 25°C

Pipette 2.9 ml of substrate into cuvette and incubate in spectrophotometer at 25°C for 3-4 minutes to reach temperature equilibration and establish blank rate, if any. Add 0.1 ml of diluted enzyme and record increase in A_{254} for 3-4 minutes. Determine $\frac{\Delta A_{254}}{\text{min}}$ from the initial linear portion of the curve.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{254}}{\text{min}}}{\frac{0.349 \text{ x mg enzyme}}{\text{ml reaction mixture}}}$

where 0.349 is the extinction coefficient of hippuric acid formed during the reaction

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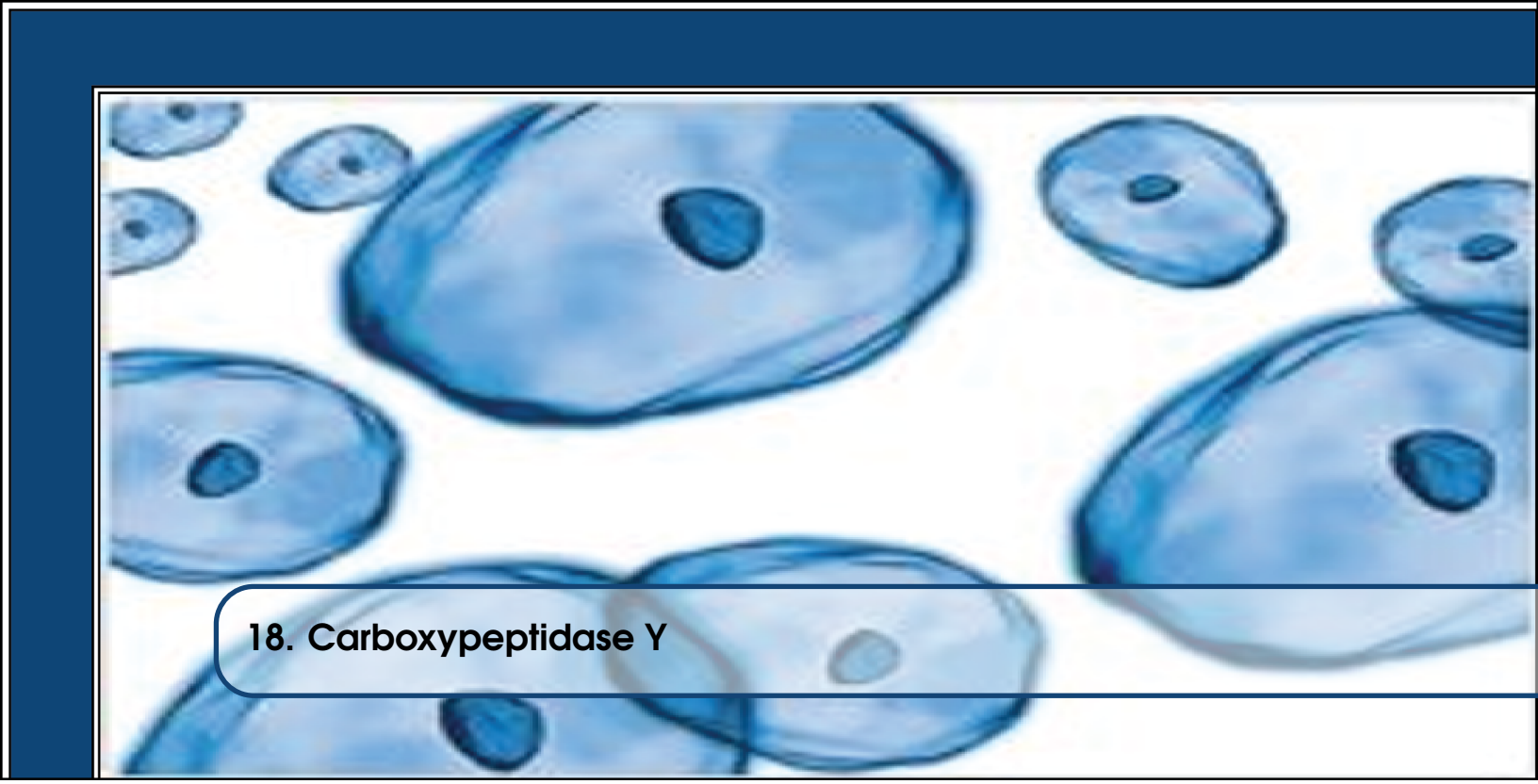

18. Carboxypeptidase Y

Carboxypeptidase Y (CPDY) is a glycoprotein exopeptidase of the acid and serine class.

$$\text{Peptidyl-L-amino acid} + H_2O \rightarrow \text{peptide} + \text{L-amino acid} \qquad (18.1)$$

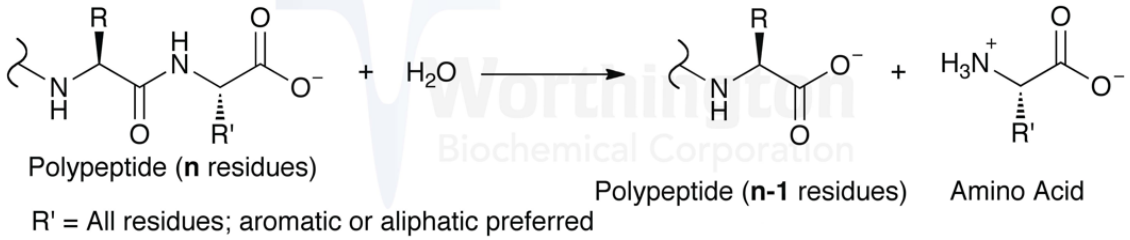

Figure 18.1: Enzymatic Reaction - Carboxypeptidase Y

History

CPDY was originally referred to as proteinase C by Hata *et al*. in 1967. In 1970, Hayashi *et al*. found that CPDY released C-terminal amino acids from peptides and determined it is inactivated by DFP at the active site serine residue, thus classifying it as a serine peptidase (Hayashi *et al*. 1970, and Mortensen *et al*. 2004).

Wolf and Fink (1975) first identified the structural gene for CPDY, which was later confirmed by Hemmings *et al.* (1981). In the 1980s, extensive work was done to better understand the signal sequence of CPDY and it was determined that the protein can be translocated without its amino-terminal sequence (Hemmings *et al.* 1981 and Blachly-Dyson and Stevens 1987). Throughout the 1990s, the carbohydrate contents of CPDY were studied (Ballou *et al.* 1990), and in 1994 the crystal structure was solved to a 2.8 resolution by Endrizzi *et al.*

Recent work has focused on transport of the nascent protein (Gharakhanian *et al.* 2011, Mukaiyama *et al.* 2010, and Burston *et al.* 2008) and inhibitory proteins (Gombault *et al.* 2009).

Molecular Characteristics

The mature chain of CPDY contains 421 amino acid residues. The gene, prc1, has been cloned and sequenced and encodes a prepro form of the enzyme (Stevens *et al.* 1986, Blachly-Dyson and Stevens 1987, Valls *et al.* 1987, and Mortensen *et al.* 2004).

Specificity

CPDY has broad amino acid specificity. It shows preference for hydrophobic amino acids in the P1' position of the substrate. CPDY is also able to catalyze aminolysis, the reverse reaction of hydrolysis (Remington and Breddam 1994). For details on the random order bi-bi mechanism CPDY employs, see Mortensen *et al.* 1994.

Composition

Unlike carboxypeptidases A and B, CPDY contains no metal ion. It is glycosylated at four positions, and contains 15% mannose. Some of the carbohydrate chains are phosphorylated (Trimble and Maley 1977, Hasilik and Tanner 1978, Hashimoto *et al.* 1981, Winther *et al.* 1991, and Mortensen *et al.* 2004). Each molecule of CPDY contains 4-5 diesterfied phosphates (Hashimoto *et al.* 1981).

The CPDY structure consists of fourteen a-helices, eleven strands of mixed β-sheets, five disulfide bridges, and one free cysteine residue (Endrizzi*et al.* 1994).

IUB

- 3.4.16.5

CAS

- 9046-67-7

Protein Accession Number

- P00729

CATH: Classification (v. 3.3.0)

Class:

- Alpha Beta; Mainly Alpha

Architecture:

- 3-Layer(aba) Sandwich; Orthogonal Bundle

Topology:

- Rossmann Fold; Helix Hairpins

Notes:

- CPDY contains two domains

Molecular Weight

- 64 kDa (Hayashi *et al.* 1973, and Mortensen *et al.* 2004)

Optimal pH

- 4.5-6.0

Isoelectric Point

- 3.6 (Hayashi *et al.* 1973)

Extinction Coefficient

- 88,940 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 15.0 (Hayashi *et al.* 1973, and Kuhn *et al.* 1973)

Active Residue

- Serine (S146)
- Aspartate (D338)
- Histidine (H397)

Inhibitors

- Diisopropyl fluorophosphate (DFP)
- PMSF
- APCK
- 4-Hydroxymercuribenzoate
- Aprotinin
- Sensitive to thiol-blocking reagents
- A high affinity inhibitor encoded by the TFSI gene has been characterized and shows homology to a family of lipid binding proteins (Bruun *et al.* 1998)

Applications

- C-terminal sequencing (Hayashi 1977)
- C-terminal modification/labeling of peptides and proteins (Remington and Breddam 1994)

Assay Information

Method

- The assay measures the rate of leucine liberated during the enzymatic hydrolysis of benzyloxycarbonyl-L-phenylalanyl-L-leucine under the specified conditions. One unit hydrolyzes 1μmoleof benzyloxycarbonyl-L-phenylalanyl-L-leucine per minute at 25°C, pH 6.5 under the conditions specified.

Reagents

- Substrate: 1 mM Benzyloxycarbonyl-L-phenylalanyl-L-leucine in 50 mM sodium phosphate, 0.15 M Sodium chloride, pH 6.5. Note: 0.5 ml of DMSO (dimethyl solfoxide) is used to dissolve the benzyloxycarbonyl-L-phenylalanyl-L-leucine before mixing with the buffer.
- 0.050 M sodium phosphate, 0.15 M Sodium chloride, pH 6.5
- 4% Ninhydrin in Methyl Cellosolve®
- 0.2 M Sodium citrate, 7.1 mM stannous chloride, pH 5.0
- Ninhydrin-citric acid mixture. Prepare by mixing 50 ml each of 4% ninhydrin in methyl cellosolve and 0.2 M sodium citrate (pH 5.0) - 7.1 mM stannous chloride. Stir for 15 minutes.
- 50% (V/V) n-Propanol
- 0.05 M L-leucine

Standard Curve

Leucine Standard Curve

0.05 M $NaPO_4$, 0.15 M NaCl (pH 6.5)	0.050 M L-Leucine	Concentratation (uM L-leucine)
4.90 ml	200 μl	0.392
5.0 ml	100 μl	0.196
5.05 ml	50 μl	0.098
5.08 ml	20 μl	0.039
5.10 ml	0 μl	0

Dilute each of the above 1:5 with $NaPO_4$ buffer.

Enzyme

- Allow enzyme to sit at room temperature for 2-3 hours before assaying. This equilibration stabilizes activity. Prepare a 1 $\frac{\text{mg}}{\text{ml}}$ solution of the enzyme, using reagent grade water. Dilute to concentrations of 5, 10, 15 and 20 $\frac{\mu\text{g}}{\text{ml}}$ by using 5, 10, 15 and 20 μl of the 1 $\frac{\text{mg}}{\text{ml}}$ solution, and taking to a final volume of 1 ml with reagent grade water.

Procedure

Pipette 1.0 ml of the substrate solution into test tubes labeled 5, 10, 15, 20 and BLANK. Pipette 1.0 ml of the 1:5 L-leucine into test tubes labeled 0.392, 0.196, 0.098, 0.039, and LEUCINE BLANK. Pre-incubate the five test tubes containing the substrate solutions for 10 minutes at 25°C. Start the enzyme reaction by pipetting 50 µl of the four enzyme samples into their respective test tubes. Add 50 µl of reagent grade water to the tube labeled BLANK. Allow to react at 25°C for 10 minutes. Add 1.0 ml of the ninhydrin reagent to each of the 10 test tubes. Place all tubes in a boiling water bath for 15 minutes. Remove tubes from bath and cool to below 30°C. Add 5.0 ml of the 50% propanol solution to each of the test tubes and mix well. Read the optical density of all tubes at 570 nm.

Calculation

- $\frac{\text{Units}}{\text{ml}} = \frac{\text{optical density - blank}}{\text{slope of standard curve x 10 min x 0.05}}$
- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\text{units}}{\text{ml}}}{\frac{\text{mg}}{\text{ml}}\ \text{sample}}$

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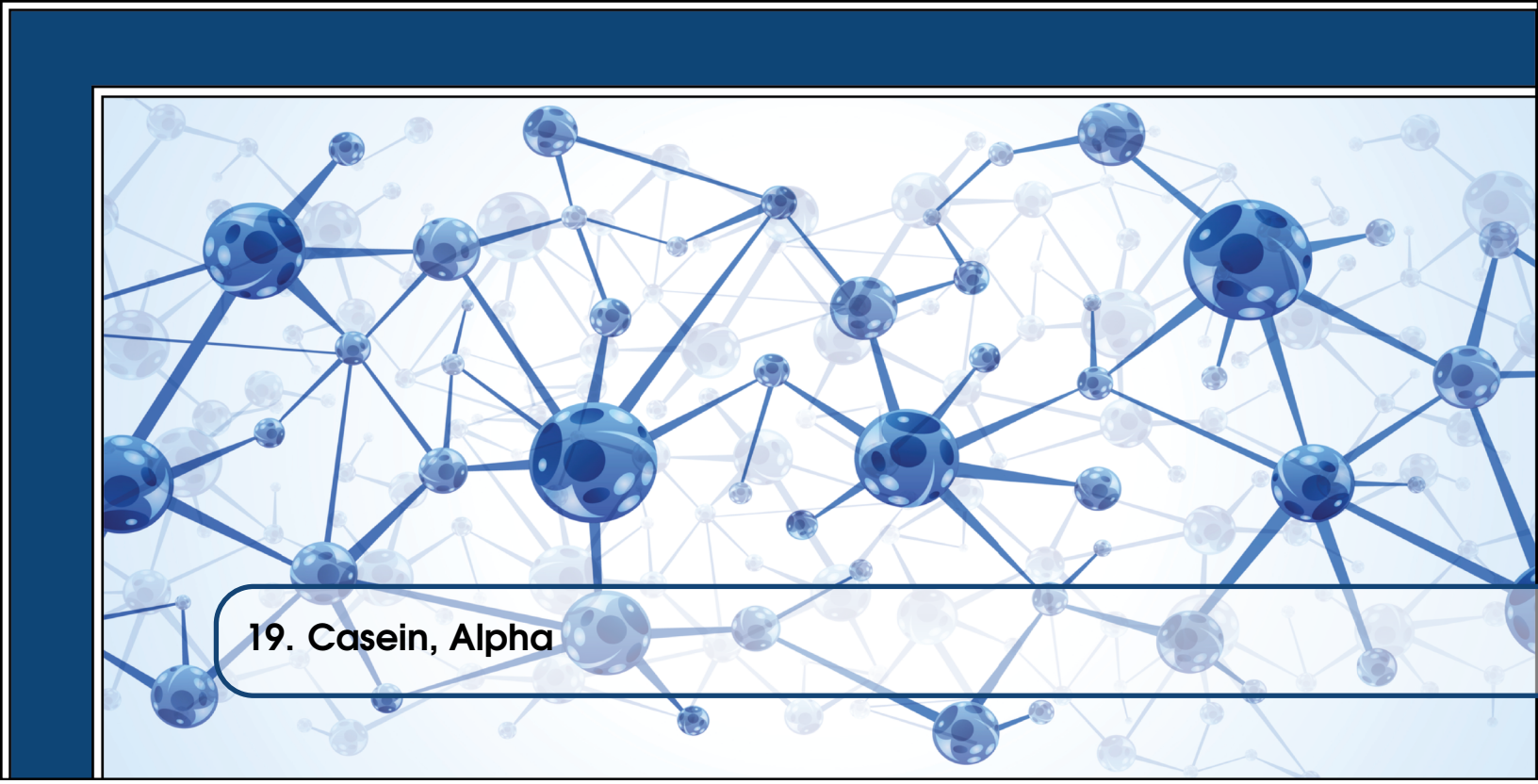

19. Casein, Alpha

The caseins are a group of proteins whose hydrophobicity and relatively high charge differentiate them from most other proteins. alpha$_{s1}$-Casein is the major protein fraction of bovine milk. A phosphoprotein, it exists as an equilibrium of soluble and complex colloidal aggregates (micelles). The casein is solubilized by dialyzing skim milk against phosphate buffer or by dilution. Bovine caseins include alpha$_{s1}$, alpha$_{s2}$, beta, and kappa-casein. The following information pertains to primarily bovine alpha$_{s1}$-casein.

History

In 1925 Linderstrom-Lang and Kodama first demonstrated the caseins to be composed of two discrete fractions instead of a homogeneous substance as they were initially regarded. In 1939 Mellander used zone electrophoresis to demonstrate three components he designated alpha-, beta-, and gamma-caesin.

Beginning in the 1950s, several models for the structure of casein association colloids (micelles) were proposed (Waugh and von Hippel 1956, Hill and Wake 1969, Bingham *et al*. 1972, Creamer *et al*. 1973, and Swaisgood 2003). In 1961, Wake and Baldwin refined the electrophoresis and found even more components. The tight linkage of alpha$_{s1}$-casein and beta-casein genes was first demonstrated in 1964 (Grosclaude *et al*. 1964), and the order of the three casein loci was first proposed in 1973 (Grosclaude *et al*. 1973).

As molecular biology techniques became available in the 1970s and 1980s, it was determined that the heterogeneity is in fact due to effects of post-translational processing, genetic polymorphisms, and alternative splicing of the gene product. Bovine caseins were confirmed as alpha$_{s1}$, alpha$_{s2}$, beta, and kappa-casein (Ng-Kwai *et al*. 1992, and Ginger and Grigor 1999).

Recently, investigators have studied the effects of selective serotonin reuptake inhibitor (SSRI) administration on inhibition of milk protein gene expression *in vitro* and *in vivo* and the consequent reduction of milk yield (Hernandez *et al*. 2011). Researchers have also characterized the caseins of goat milk to evaluate its suitability as an alternative for subjects allergic to cow's milk (Ballabio *et al*. 2011).

Molecular Characteristics

The gene that encodes alpha$_{s1}$-casein (csn1s1) is located on chromosome 6 in Bos taurus (GenBank accession: 282208). The as1-casein amino acid sequences of various species including rabbit (Devinoy *et al*. 1988), guinea-pig (Hall *et al*. 1984), mouse (Henninghausen and Sippel 1982), and human (Johnsen *et al*. 1995) have been found to contain a highly conserved 15 residue signal sequence but divergent mature chain sequences. The C-terminal region of bovine and rat contain only 29% identity, while bovine and pig share 48% identity. The alpha$_{s2}$-Caseins and beta-caseins also contain the highly conserved 15 residue signal sequence and divergent mature chain sequences (Ginger and Grigor 1999). kappa-Casein contains a 21 residue signal sequence (Bonsing *et al*. 1988).

Composition

alphas1-Casein is the most abundant protein of bovine milk. It is highly phosphorylated and exists as a major and minor form. The major form contains 8 bound phosphates and the minor 9 bound phosphates (Eigel *et al*. 1984). Phosphorylation occurs at Ser/Thr-X-Y motifs (Mercier 1981).

alpha$_{s2}$-Casein is also highly phosphorylated. That of bovine milk consists of four isoforms that contain between 10 and 13 phosphates (Brignon *et al*. 1977, and Eigel *et al*. 1984).

Bovine beta-casein exists in only one form, which contains 5 phosphates per mole. The beta-caseins of other species including human (Greenberg and Groves 1979), goat (Richardson and Mercier 1979), and possum (Ginger *et al*. 1999) contain multiple forms. Bovine milk has also been found to contain fragments of minor peptides including gamma$_1$, gamma$_2$, and gamma$_3$-caseins. The proteose peptone components 5 and 8-fast were initially found to be soluble in acid conditions and thought to be whey proteins. However, they were eventually determined to be products of the partial proteolysis of beta-casein by plasmin (Gordon *et al*. 1972, Groves *et al*. 1973, Eigel 1977, and Eigel 1981).

kappa-Casein is the only casein soluble in the presence of calcium ions. It also has the smallest amount of phosphate, with phosphorylation sites being present only in the C-terminal region. kappa-Casein is the only casein to contain carbohydrate moieties (Jollés *et al*. 1978).

Except for short alpha-helical regions, caseins have little secondary or tertiary structure (Huppertz *et al*. 2006). The caseins of milk exist in the form of colloidal complexes called micelles. The micelles contain an amorphous micellar calcium phosphate core, surrounded by a phosphopeptide (casein) shell (Swaisgood 2003).

CAS

- 9000-71-9

Protein Accession Number

- P02662

Molecular Weight

- 27 kDa (McKenzie 1967)
- 23.6 kDa (Mercier *et al*. 1971)

Isoelectric Point

- 4.91

Extinction Coefficient

- $E_{280}^{1\%}$ = 10.1 (Thompson and Kiddy 1964)

Applications

- Casein allergenicity studies
- Marker peptides for the detection of milk allergen in food samples
- Substrate in protease activity assays (beta-casein) (Mandl *et al*. 1953)

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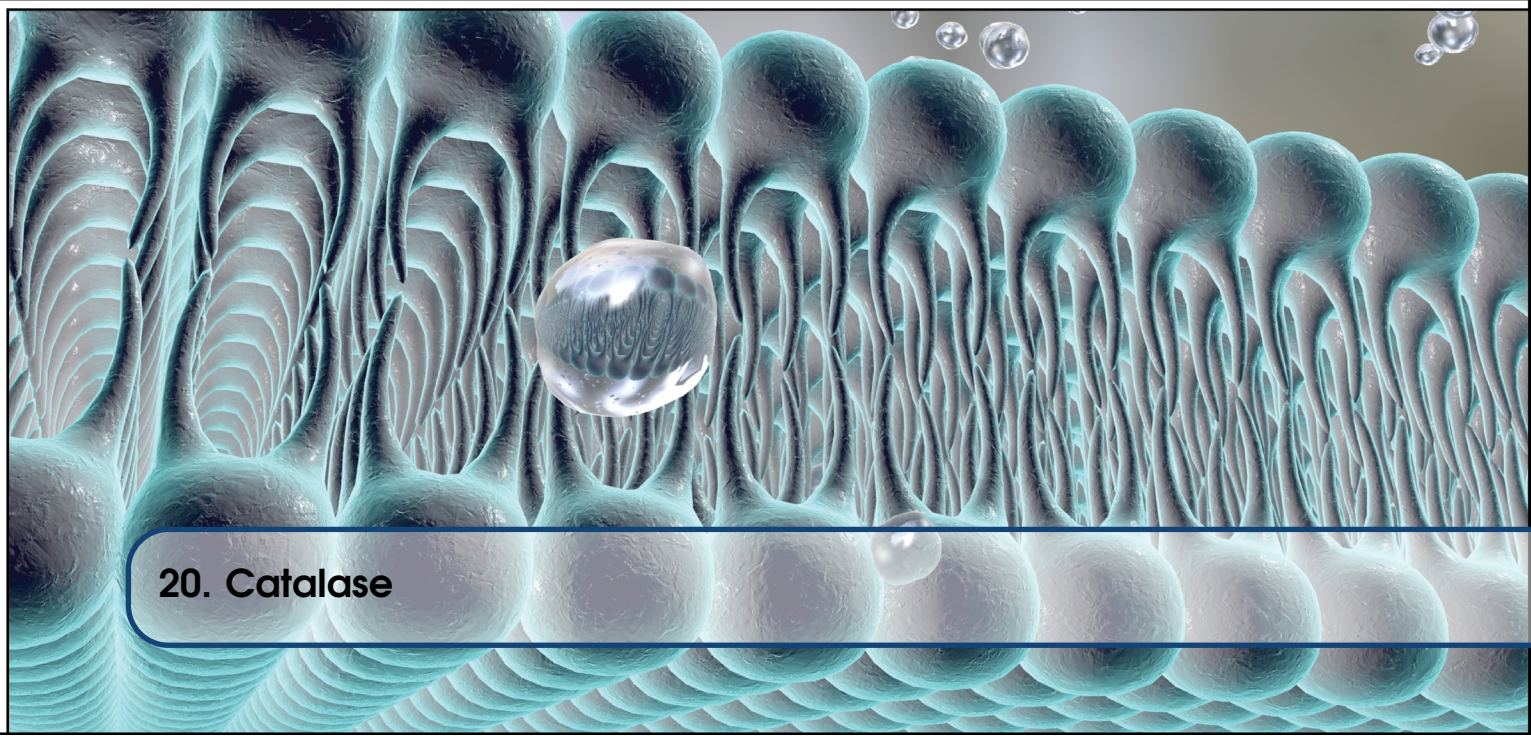

20. Catalase

Catalase is an enzyme responsible for the degradation of hydrogen peroxide. It is a protective enzyme present in nearly all animal cells.

$$2H_2O_2 \rightarrow 2H_2O + O_2 \qquad (20.1)$$

History

Bovine liver catalase was one of the first enzymes to be isolated to a high state of purity and the first iron-containing enzyme to be isolated (Sumner and Dounce 1937). The reaction mechanism was initially proposed to be a free radical mechanism by Oppenheimer and Stern in 1939. Throughout the next few decades, catalysis was determined to occur at the iron atom of the porphyrin (Warburg 1949, Keilin 1966, and Chance 1951). A more convenient method of preparing crystalline catalase from bovine liver was developed in 1952 by Tauber and Petit, and X-ray structure studies of the heme region of myoglobin examined the heme-containing active site (Stryer *et al*. 1964).

In the 1970s, X-ray and NMR studies provided further insight into the structure of the protein and the active site of hydroperoxidases in general (Larsson *et al*. 1970, and Hershberg and Chance 1975). In the 1980s, crystal structures of bovine liver catalases were published (Murthy *et al*. 1981, and Fita and Rossmann 1985).

In the 1990s, the inhibition of catalase by nitric oxide and the relation of this effect to nitric oxide cytotoxicity was investigated (Brown 1995). A study was conducted to compare the heme structures of catalases from various sources by Andersson *et al*. in 1995, and the unfolding and refolding of bovine liver catalase at various pHs and salt conditions was investigated by Prajapati *et al*. in 1998.

Recently, catalase has been investigated as a possible agent to support methods of intracellular drug delivery (Siwale *et al*. 2009). Catalase has also been incorporated into an assay for cholesterol quantification (Robinet *et al*. 2010) and a biosensor for alcohol determination (Hnaien *et al*. 2010).

Molecular Characteristics

The gene that encodes catalase, CAT, is located on chromosome 15 in Bos taurus. The gene is conserved in human, chimpanzee, dog, mouse, rat, chicken, zebrafish, fruit fly, mosquito, *C. elegans*, *S. pombe*, *S. cerevisiae*, *K. lactis*, *E. gossypii*, *Neurospora crassa*, *A. thaliana*, and rice (Gene ID: 531682).

Specificity

The reaction of catalase occurs in two steps. A molecule of hydrogen peroxide oxidizes the heme to an oxyferryl species. A porphyrin cation radical is generated when one oxidation equivalent is removed from iron and one from the poryphyrin ring. A second hydrogen peroxide molecule acts as a reducing agent to regenerate the resting state enzyme, producing a molecule of oxygen and water (Switala and Loewen 2002).

Composition

Catalase is a tetramer consisting of four identical, tetrahedrally arranged subunits. Each 60 kDa subunit contains a heme group and NADPH in its active center (Scibior and Czeczot 2006).

IUB

- 1.11.1.6

CAS

- 9001-05-2

Protein Accession Number

- P00432

Molecular Weight

- 232 kDa (Schroeder *et al*. 1969)
- 240 kDa (Herskovits 1969)
- Monomer: 57.5 (Schroeder *et al*. 1969)

Optimal pH

- 7.0 (Maehly and Chance 1954)

Isoelectric Point

- 5.4 (Samejima *et al*. 1962)

Extinction Coefficient

- 246,000 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{276}$ = 12.9 (Herskovits 1969)

Active Residue

- Histidine (H74)
- Asparagine (N147)

Activators

- Sodium arsenate (Kertulis-Tartar *et al.* 2009)

Inhibitors

- Cyanide, azide, hydroxylamine, aminotriazole, and mercaptoethanol (Switala and Loewen 2002)
- Ascorbate alone as well as with Cu^{2+} (Orr 1967a, b)
- Freezing and lyophilization cause inactivation (Tanford and Lovrien 1962, and Deisseroth and Dounce 1967)
- Inactivated by sunlight under aerobic conditions (Mitchell and Anderson 1965)
- Peroxide (Altomare *et al.* 1974)
- Nitro and nitroso compounds (Titov *et al.* 2008)

Applications

- Commercially wherever hydrogen peroxide is used as a germicide (Chu *et al.* 1975)
- Milk preservative with peroxidase (Collins 1971)
- Increases synthesis and stability of diacetyl in cultured milk (Collins 1971)
- Free radical research (Lardinois 1995)
- Deodorization (White and White 1997)
- Decomposition of residual hydrogen peroxide after bleaching woven and knitted cotton fabrics before drying (White and White 1997)
- Cysteamine determination (White and White 1997)
- Gluconic and glycolic acid production (Seip *et al.* 1994, and Godjevargova *et al.* 2004)
- Cleaning silicon and semiconductor plates (White and White 1997)

Assay Information

There are numerous assays for catalase. Gregory and Fridovich (1974) report on a sensitive activity stain for catalase applicable to a polyacrylamide gel electrophoretogram, Haining and Legan (1972) describe a polarographic assay utilizable in tissue homogenates, and Kroll *et al.* (1989) discuss a rapid method for estimating the bacterial content of foods. The subject has been reviewed by Maehly and Chance (1954) and Chance and Maehly (1955). The assay used at Worthington follows:

Method

- Essentially that described by Beers and Sizer (1952) in which the disappearance of peroxide is followed spectrophotometrically at 240 nm. One Unit decomposes one micromole of H_2O_2 per minute at 25°C and pH 7.0 under the specified conditions.

Reagents

- 0.05 M Potassium phosphate, pH 7.0
- 0.059 M Hydrogen peroxide (30%) in 0.05 M potassium phosphate, pH 7.0

Enzyme

- Immediately prior to use dilute the enzyme in 0.05 M phosphate buffer, pH 7.0 to obtain a rate of 0.03-0.07 $\frac{\Delta A}{min}$.

Procedure

Spectrophotometer Settings: Wavelength: 240 nm Temperature: 25°C

Pipette into each cuvette as follows:

- Reagent Grade Water - 1.9 ml
- 0.059 M Hydrogen peroxide - 1.0 ml

Incubate in spectrophotometer for 4-5 minutes to achieve temperature equilibration and to establish blank rate if any. Add 0.1 ml of diluted enzyme and record decrease in absorbance at 240 nm for 2-3 minutes. Calculate $\frac{\Delta A_{240}}{min}$ from the initial (45 second) linear portion of the curve.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{240}}{\text{min}} \text{ x } 1000}{\frac{43.6 \text{ x mg enzyme}}{\text{ml reaction mixture}}}$$

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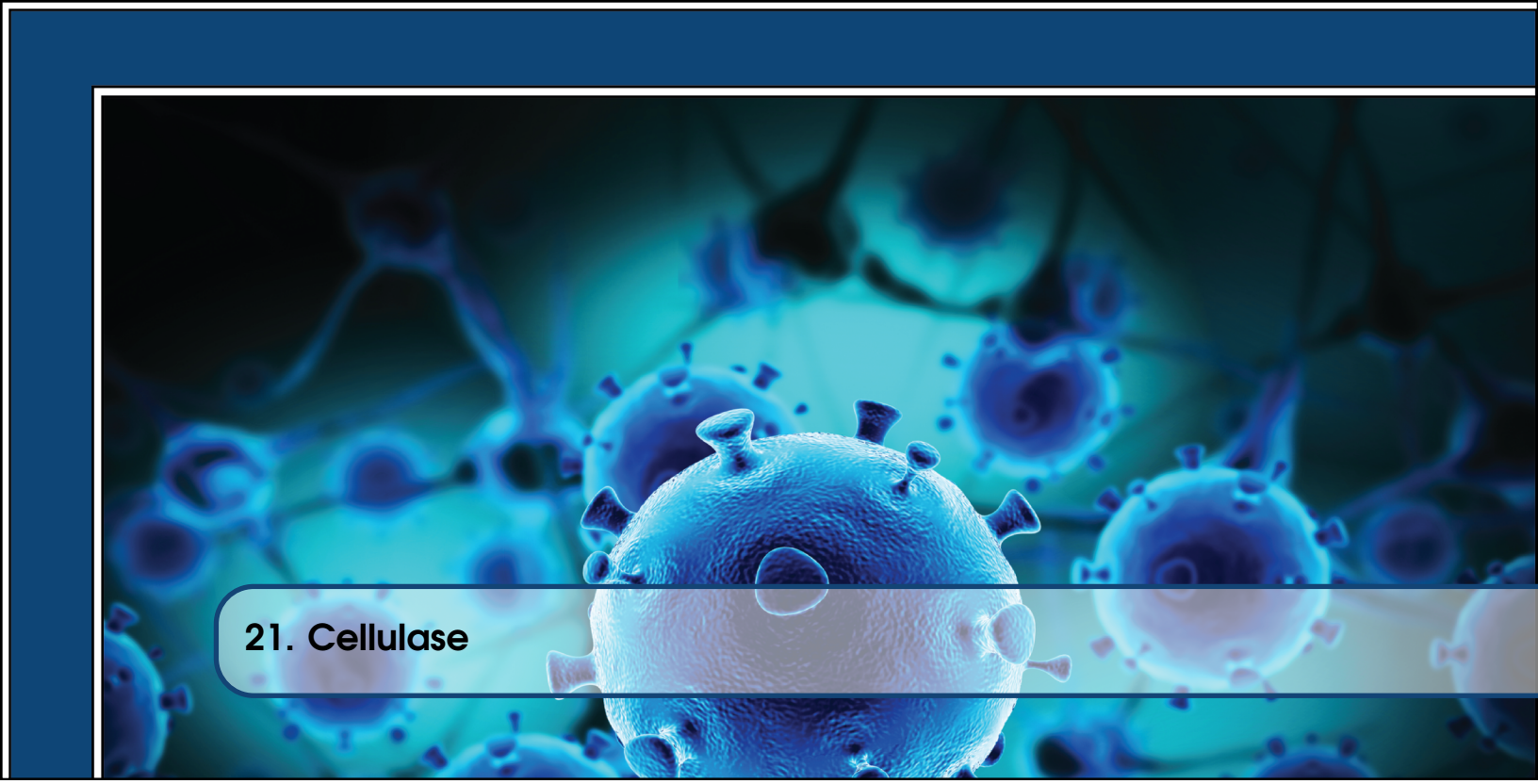

21. Cellulase

1,4-(1,3;1,4)-β-D-Glucan-4-glucanohydrolase

Cellulase refers to a group of enzymes which, acting together, hydrolyze cellulose.

Cellulose is a linear polysaccharide of glucose residues connected by β-1,4 linkages. Like chitin it is not cross-linked. Native crystalline cellulose is insoluble and occurs as fibers of densely packed, hydrogen bonded, anhydroglucose chains of 15 to 10,000 glucose units. Its density and complexity make it very resistant to hydrolysis without preliminary chemical or mechanical degradation or swelling. In nature cellulose is usually associated with other polysaccharides such as xylan or lignin. It is the skeletal basis of plant cell walls. Cellulose is the most abundant organic source of food, fuel and chemicals (Spano *et al*. 1975). However, its usefulness is dependent upon its hydrolysis to glucose. Acid and high temperature degradation are unsatisfactory in that the resulting sugars are decomposed; enzymatic degradation (cellulase) is the most effective means of degrading cellulose into useful components. Although cellulases are distributed throughout the biosphere, they are most prevalent in fungal and microbial sources.

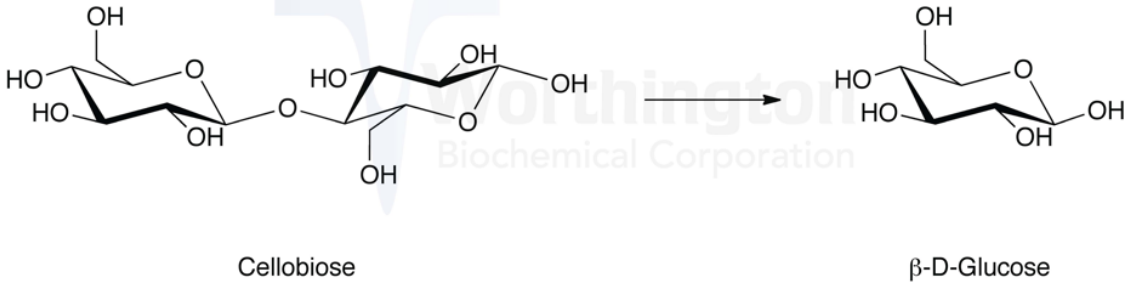

Figure 21.1: Cellobiase

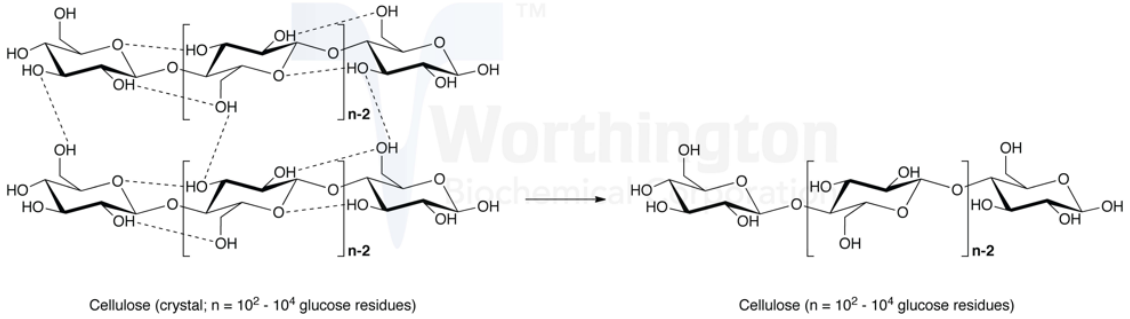

Figure 21.2: Endcellulase

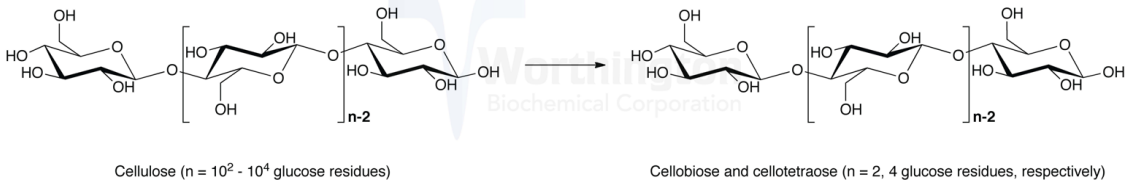

Figure 21.3: Exocellulase

History

Reese *et al*. (1950) first proposed the enzyme mechanisms involved in the degradation of cellulose. At least two steps are involved: first, a prehydrolytic step wherein anhydroglucose chains are swollen or hydrated and secondly, hydrolytic cleavage of the now susceptible polymers either randomly or endwise.

Reese and Levinson made an extensive comparative study of the action of cellulolytic organisms showing many cellulolytic organisms degrade modified cellulose, but often do not show corresponding activity on native cellulose (Reese and Levinson 1952). Mandels and Reese first described the cellulase complex from *Trichoderma viride*, later named *Trichoderma reesei*, from which Worthington cellulase is produced (Mandels and Reese 1957).

The original Worthington assay was developed in our laboratory in 1957 and used carboxymethylcellulose, unaware that a test using this substrate had been described by Levinson and Reese in 1950 (Levinson and Reese 1950). Today's Worthington assay uses the rate of glucose formation, a technique first described in 1960 (Meyers *et al*. 1960).

The cellulase complex of *Trichoderma reesei* has been most thoroughly studied. It is complete in that it can convert native cellulose as well as derived celluloses to glucose (King and Nessal 1969). Howell and Stuck (1975) have described the complex and indicate it to be remarkably resistant to inhibitors except carbohydrates, particularly cellobiose and excess cellulose. Okada (1975) obtained a number of active fractions from *Trichoderma reesei* differing in carbohydrate content and specificity but otherwise identical.

Berghem and Pettersson (1973) reported on the *Trichoderma reesei* enzyme involved in the first step of cellolose degratation, a β-1,4-glucan cellobiohydrolase which acts upon crystalline cellulose. Cellobiose is the principle product. The enzyme was further characterized by Berghem, Pettersson and Axio-Fredriksson (1975). They indicated a molecular weight of 42,000 based on amino acid and carbohydrate analysis. The enzyme contained 9.2% carbohydrate covalently bound. The pH optimum is about 4.8 and the reaction rate can be enhanced by addition of cellobiase and additional endo-glucanase. The identification of the genes for the other cellulase and hemicellulase enzymes of *Trichoderma reesei* has been completed, and the characterization of many of these enzymes has also been achieved (Kubicek 2011).

Recently researchers have been investigating the genomes of mutant species to correlate cellulase activity with specific gene sequences (Le Crom *et al*. 2009, and Vitikainen *et al*. 2010). Current research has explored the use of cellulase in the production of biofuels and biorefinery products (Kubicek 2012).

The Cullulase Complex from *Trichoderma reesei*:

The *Trichoderma reesei* complex is a true cellulase in the most rigid sense, being able to convert crystalline, amorphous, and chemically derived celluloses quantitatively to glucose. It has been established that: a) the system is multi-enzymatic; b) that at least three enzyme components are both physically and enzymatically distinct; and c) that all three components play essential roles in the overall process of converting cellulose to glucose (King and Vessal 1969).

Molecular Characteristics

The *Trichoderma reesei* genome contains 9143 genes, with a surpsingly small number of cellulase and hemicellulase genes as compared to other Trichoderma species. *Trichoderma reesei* contains a total of 200 glycosyl-hydrolase (GH) encoding genes, several of which share homology with bacteria suggesting they may have been obtained by horizontal gene transfer (Kubicek 2013). 41% of the GH and other carbohydrate active genes were reported to occur in 25 discreet clusters (Martinez *et al*. 2008). Several of these clusters also contain genes encoding non-ribosomal peptide and polyketide synthases suggesting these genes are important for the ability of Trichoderma to compete in its natural environment (Kubicek 2013). Production of cellulases is induced by the presence of cellulose, lactose, or sophorse; the enzyme complexes are not formed constitutively. Expression is controlled by a variety of positive and negative transcription factors (Kubicek *et al*. 2009).

Composition

Cellobiohydrolase I (Cel7A) and cellobiohydrolase II (Cel6A) are the predominant cellulase proteins in all major commercial preparations. The endoglucanases and hemicellulases represent less than 10% of the extracellular extract (Chundawat *et al*. 2011).

The cellulase complex from *Trichoderma reesei* contains endoglucanases, cellobiohydrolases, and beta-glucosidases. Information concerning the known sequences of these indivudual enzymes have been organized in the following table. Each individual enzyme functions as a monomer.

IUB

- 3.2.1.4

CAS

- 9012-54-8

Protein Accession Number

- Endoglucanase I: P07981
- Endoglucanase II: P07982
- Endoglucanase IV: O14405
- Endoglucanase V: P43317
- Endoglucanase VII: Q7Z9M7
- beta-1,4-glucanase: O00095
- Cellobiohydrolase I: P62694
- Cellobiohydrolase II: P07987
- beta-Glucosidase I: AAA18473
- beta-Glucosidase II: O93785

CATH: Classification (v. 3.3.0)

Class:

- Endoglucanase I: Mainly beta
- Endoglucanase II: Alpha Beta
- beta-1,4-glucanase: Mainly beta
- Cellobiohydrolase I: Mainly beta
- beta-Glucosidase I and II: Alpha Beta

Architecture:

- Endoglucanase I: Distorted sandwich
- Endoglucanase II: Alpha-Beta Barrel
- beta-1,4-glucanase: Sandwich
- Cellobiohydrolase I: Distorted Sandwich
- beta-Glucosidase I and II: Alpha-Beta Barrel

Topology:

- Endoglucanase I: 1,4-Beta-D-Glucan Cellobiohydrolase
- Endoglucanase II: TIM Barrel
- beta-1,4-glucanase: Jelly Rolls
- Cellobiohydrolase I: 1,4-Beta-D-Glucan Cellobiohydrolase I; Chain A
- beta-Glucosidase I and II: TIM Barrel

Molecular Weight

- Endoglucanase I: 46.0 kDa
- Endoglucanase II: 42.2 kDa
- Endoglucanase IV: 33.4 kDa
- Endoglucanase V: 22.8 kDa
- Endoglucanase VII: 25.1 kDa
- beta-1,4-glucanase: 23.5 kDa
- Cellobiohydrolase I: 52.2 kDa
- Cellobiohydrolase II: 47.2 kDa
- beta-Glucosidase I: 75.3 kDa
- beta-Glucosidase II: 52.1 kDa

Optimal pH

- Varies with the substrate in the range 4.2 - 5.2

Isoelectric Point

- 4.5-7.2

Activators

- Nonionic detergents like Triton X-100 (White and White 1997)

Inhibitors

- Carbohydrates, particularly cellobiose and excess cellulose (Howell and Stuck 1975)

Applications

- Digestive tablets
- Removal or softening of cellulose in food preparation (Toyama 1963)
- Protoplast preparation from plants (Cocking 1960)
- Various manufacturing processes (White and White 1997)

Assay Information

Method

- Cellulase activity is determined by its effect on microcrystalline cellulose with respect to glucose formation. Released glucose is determined in a hexokinase/glucose-6-phosphate dehydrogenase system at 340 nm. One unit of activity releases 0.01 mg glucose per hour from micro-crystalline cellulose at 37°C and pH 5.0 under the specified conditions.

Reagents

- 0.05 M Acetic acid, pH 5.0
- Microcrystalline cellulose (Avicel, F.M.C. or equivalent))

Glucose determination reagent (Can be replaced with a glucose assay utilizing a standard curve)

- ATP - 0.77 $\frac{\mu\text{mol}}{\text{ml}}$
- Hexokinase - 1.5 $\frac{\text{units}}{\text{ml}}$
- NAD - 0.91 $\frac{\mu\text{mol}}{\text{ml}}$
- Glucose-6-phosphate dehydrogenase - $\geq$1.9 $\frac{\text{units}}{\text{ml}}$
- Tris $\cdot$ HCl buffer pH 7.6 $\pm$0.2 - 0.1 M

Enzyme

- Dissolve enzyme in reagent grade water at a concentration of 1-0.1 $\frac{\text{mg}}{\text{ml}}$.

Procedure

Spectrophotometer Settings: Wavelength: 340 nm Temperature: 25°C

Measure into clean dry test tubes as follows:

Description	Test	Blank
Microcrystalline cellulose	200 mg	200 mg
0.05 M Acetic acid	4.0 ml	4.0 ml
Reagent grade water	—	1.0 ml
Enzyme dilution	1.0 ml	—

Incubate with stirring or shaking for 2 hours at 37°C. Remove tubes to an ice bath and allow sediment to settle. Clarify by centrifugation. Store in an ice bath.

Place 3.0 ml glucose reagent in a cuvette and incubate in spectrophotometer set at 340 nm and 25°C to achieve temperature equilibration. Record the A_{340} of the glucose reagent in the cuvette. Add 0.1 ml of the supernatant from each reaction tube and record increase in A_{340} until no further change occurs in

3-5 minutes. Record final A_{340}.

Calculation

- $\Delta A_{340} = \underset{\text{Final}}{A_{340}} - \underset{\text{Initial}}{A_{340}}$
- $\frac{\text{Units}}{\text{mg}} = \frac{(\underset{\text{Sample}}{\Delta A_{340}} - \underset{\text{Blank}}{\Delta A_{340}}) \times 3.1 \times 180 \times 5}{6.22 \times 10^3 \times 0.1 \times 2 \times 0.01 \times \text{mg enzyme in mixture}}$

Notes

- Worthington purified cellulases contain less than 0.02 units per mg dry weight lipase activity. One unit of lipase activity releases one micromole of fatty acid per minute from emulsified olive oil at 25°C and pH 8.0.
- Nuclease activity is evaluated by incubating 1 μg and 10 μg cellulase with 1 μg ΦX174DNA in 0.5 ml at 37°C for 16 hours under optimum conditions. Degradation of the DNA (evidence of exonuclease) and conversion of the DNA to the Rf2 or linear form (evidence of endonuclease) are monitored by agarose electrophoresis. CEL cellulase exhibits no evidence of nuclease contamination at the 1 μg level, and only a trace of endonuclease contamination at a 10 μg level. CELF grade shows slight endonuclease contamination at the 1 μg level, and partial exonuclease contamination at a 10 μg level.
- One mg of purified cellulase exhibits less tryptic-like proteolytic activity on a casein-agar radial diffusion plate than 0.01 μg purified trypsin after 16 hours at 25°C.

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22. Cholesterol Esterase

Cholesterol esterase catalyzes the hydrolysis of sterol esters into their component sterols and fatty acids. The enzyme is found primarily in the pancreas, but has been detected in other tissues as well. Bile salts, such as cholate and its conjugates, are required to stabilize the enzyme in its native polymeric form and to protect it from proteolytic hydrolysis in the intestine (Vahouny and Treadwell 1968). Cholesterol esterase finds clinical applications in the determination of serum cholesterol (Allain *et al*. 1974).

Sterol ester $\rightleftarrows$ sterol + fatty acid (22.1)

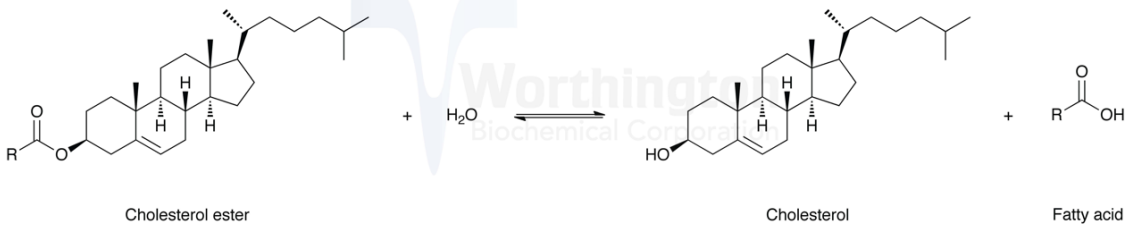

Figure 22.1: Enzymatic Reaction - Cholesterol Esterase

History

Throughout the early 1900s the enzymatically catalyzed synthesis and hydrolysis of cholesterol esters in the presence of certain tissues was observed and described (Kondo 1910, Schultz 1912, Cytronberg 1912, Gardner and Lander 1913, Mueller 1916, Porter 1916, Nomura 1924, Shope 1928, Nedswedski

1935, Klein 1939, Sperry 1935, and Sperry and Stoyanoff 1937).

In 1949 and 1950, two papers were published as part of an extensive study on cholesterol esterase. This research described a method for crude enzyme preparation that focused on the importance of stable emulsions of cholesterol and cholesterol esters for activity (Yamamoto *et al.* 1949, and Swell and Treadwell 1950). Throughout the 1950s, the cholesterol esterase of serum and various other tissues was studied (Korzenovsky 1960). In 1957, Hernandez and Chaikoff studied the properties of pancreatic cholesterol esterase specifically and devised a rapid turbidimetric method for enzymatic activity estimation (Hernandez and Chaikoff 1957).

In the 1960s, Vahouny *et al.* studied methods for protecting cholesterol esterase from proteolytic inactivation (Vahouny *et al.* 1964). This study coupled with the known correlation between cardiovascular disease and high serum cholesterol (Keys 1980, and Goldstein and Brown 2003) led to the development of total serum cholesterol determination methods using cholesterol esterase (Allain *et al.* 1974).

In 1989, Kyger *et al.* sequenced a cDNA clone of bovine pancreatic cholesterol esterase. In 1994, the landmark "4S" study showed for the first time that lowering LDL levels through the use of statins could prevent heart attacks and prolong life (Goldstein and Brown 2003). With such treatment options available and the need for testing total cholesterol in serum rising dramatically, cholesterol esterase has become one of the most widely used enzymes in clinical laboratories (MacLachlan 2000).

Molecular Characteristics

The gene that encodes cholesterol esterase in pigs (lipA) is located on chromosome 14 (GENE ID: 100142668). The LIPA gene is conserved in human, chimpanzee, dog, cow, mouse, rat, chicken, zebrafish, fruit fly, mosquito, *C. elegans*, *S. pombe*, *S. cerevisiae*, *E. gossypii*, *M. grisea*, N. cassa, *A. thaliana*, and rice.

Specificity

Cholesterol esterase is synthesized in the acinar cells of the pancreas, stored in zymogen granules, and secreted as a component of pancreatic juice into the lumen of the small intestine (Labow *et al.* 1983). Cholesterol esterase hydrolyzes a wide range of ester substrates including cholesteryl esters, acylglycerides, phospholipids (Brockerhoff and Jensen 1974), retinyl esters (Fredrikzon *et al.* 1978), vitamin esters, and phenyl esters (Rudd and Brockman 1984). The enzyme has also been found to have amidase activity (Hui *et al.* 1993). The enzyme is useful as a biocatalyst because of its ability to catalyze transacylation reactions in a water-limited environment (Gallo *et al.* 1977, Kazlauskas 1989, and Feaster *et al.* 1996).

Composition

Cholesterol esterase is a glycoprotein that in the presence of salts aggregates to a hexamer (Hyun *et al.* 1971). Cholesterol esterase belongs to the alpha/beta-hydrolase fold family (Ollis *et al.* 1992, and Cygler *et al.* 1993). Most members of this family are esterases and share secondary and tertiary features. Nearly all use a serine esterase catalytic mechanism, which resembles that of the serine proteases (Kraut 1977).

IUB

- 3.1.1.13

CAS

- 9026-00-0

Protein Accession Number

- NP_001116606

Molecular Weight

- Monomer: 65 kDa (Hyun *et al.* 1971)
- Monomer: 43.3 kDa (Theoretical)
- Hexamer: 400 kDa (Hyun *et al.* 1971)

Optimal pH

- For esterification, 6.1-6.2 (Vahouny and Treadwell 1968)
- For hydrolysis, 6.6-7.0 (Vahouny and Treadwell 1968)

Isoelectric Point

- 7.91

Extinction Coefficient

- 86,680 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 20.01

Active Residue

- Serine (S194)
- Histidine (H435)
- Aspartate (D320)

Activators

- Bile salts
- Cholate (Vahouny *et al.* 1964)
- Glycocholate (Vahouny *et al.* 1964)
- Taurocholate (Vahouny *et al.* 1964)

Inhibitors

- PMSF and p-chloromercuribenzoate (Hyun *et al.* 1971, and Vahouny *et al.* 1964)
- Diisopropyl fluorophosphate (Momsen and Brockman 1976)
- Hg^{2+}, Ag^{+}, and ionic detergents
- Aryl carbamates (Feaster *et al.* 1996)

Applications

- Determination of cholesterol in serum and plasma, with cholesterol oxidase or peroxidase
- Synthesis of optically active alcohols and carboxylic acids (via ester hydrolysis, esterification, or transesterification)

Assay Information

Method

- The initial rate of hydrolysis of cholesterol ester is determined by following the increase in absorbance at 505 nm. One unit hydrolyzes 1 μmole of cholesterol ester per minute at 37°C and pH 7.0 under the specified conditions.

Reagents

- 0.1 M Phosphate buffer, pH 7.0
- 0.7 mM 4-aminoantipyrine in 0.1 M Phosphate buffer, pH 7.0. Discard after 8 hours.
- 5.0 mM Phenol in 0.1 M Phosphate buffer, pH 7.0. Discard after 8 hours. Use only fresh, white phenol crystals.
- 5% Triton X-100. Store at 4°C.
- Sodium Cholate: Dissolve 3.51 g Sodium cholate in 10.0 ml reagent grade water. Discard after 8 hours.
- Cholesterol Oxidase: Dissolve 28.5 units cholesterol oxidase/ml in reagent grade water. Discard after 8 hours.
- Peroxidase: 8200 $\frac{\text{units}}{\text{ml}}$ in reagent grade water. Discard after 8 hours.
- Cholesteryl Acetate. Dissolve 220 mg Cholesteryl Acetate (Eastman) in 50.0 ml Isopropanol (ACS Grade). Stir vigorously to dissolve. Store at 4°C.

Enzyme

- Dissolve one $\frac{\text{mg}}{\text{ml}}$ in 0.1 M Phosphate buffer and then prepare further dilutions (usually 2-3x) to achieve a rate of approximately $\frac{0.045}{\text{min}}$.

Procedure

Spectrophotometer Settings: Wavelength: 505 nm Temperature: 37°C

Prepare master batch, sufficient for 5 tests, by pipetting into a beaker:

- 0.7 mM 4-aminoantipyrine - 7.0 ml
- 5.0 mM Phenol - 7.0 ml
- Sodium Cholate - 0.5 ml
- 5% Triton X-100 - 0.5 ml

- Cholesterol Oxidase - 0.25 ml
- Peroxidase - 0.05 ml

Mix gently. Stored at 4°C, master batch is stable for 8 hours.

Pipette into test and blank cuvettes 3.0 ml master batch. Add 0.10 ml cholesterol acetate. Mixture should be used within 1 hour. Equilibrate temperature to37°C. Add to test cuvettes, 0.1 ml enzyme dilution and 0.1 ml reagent grade water to the blank. If the blank rate exceeds 0.005 $\frac{\Delta A_{505}}{min}$, reincubate for 5 more minutes. Determine ΔA_{505} of test and blank cuvettes using the linear portion of the first 5 minutes of the reaction. Subtract blank rates, if any.

Calculation

- $\frac{\text{Units}}{\text{mg}}$ dry weight = $\frac{\frac{\Delta A_{505}}{min} \text{ x } 3.2 \text{ x dilution}}{0.1 \text{ x } 6.89}$

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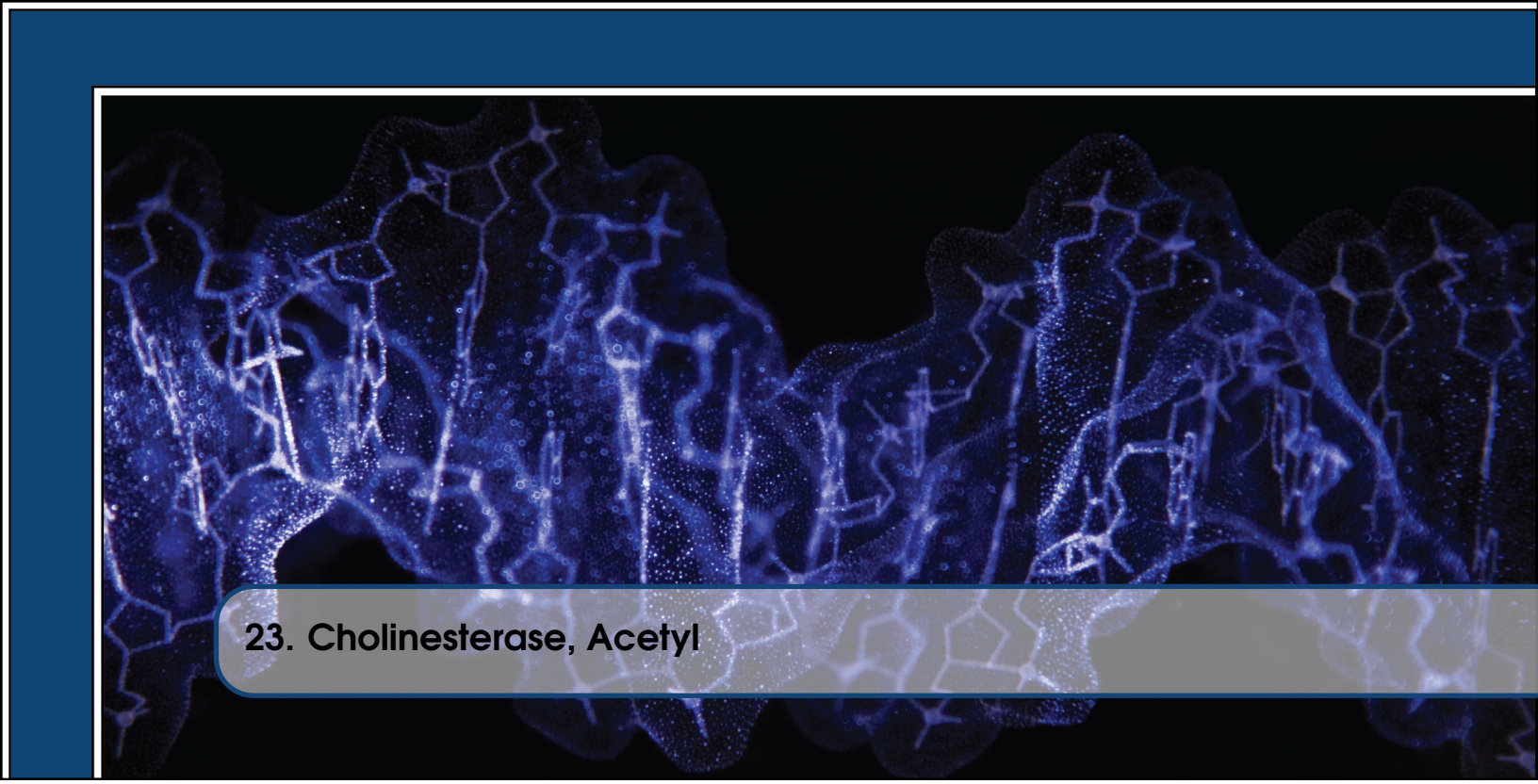

23. Cholinesterase, Acetyl

Acetylcholine acetylhydrolase

The enzyme is bound to cellular membranes of excitable tissue (synaptic junction, endoplasmic reticulum, etc.) and is believed to be associated with nerve impulse conduction (Politoff *et al* ; 1975, Friedenberg and Seligman 1972, Nachmansohn 1970). AChE is also found in red blood cells. A second cholinesterase found in blood serum hydrolyzes butyrylcholine 4 times faster than acetylcholine. They are two distinct enzymes. See monograph on butyrylcholinesterase (E.C.3.1.1.8). See also, Chatonnet and Lockridge (1989). Enzymatic and immunochemical properties of AChE from electric eel have been reported by Gurari *et al*. (1974) and Leuzinger (1971). See also Sadar and Laidler (1975), Jain *et al*. (1973), Robaire and Kato (1973), Dudai *et al*. (1972), Rosenberry and Bernhard (1972), and Froede and Wilson (1971).

Other reported sources of AChE are: mouse brain (Adamson *et al*. 1975); houseflies (Devonshire 1975); venom (Kumar and Elliott 1973); pig brain (McIntosh and Plummer 1973); human erythrocytes (Sihotang 1974; Chajilani, *et al*. 1989; Paniker *et al*. 1973); rat liver (Wheeler *et al*. 1972); and mollusc (Bevelaqua *et al*. 1975). See also Krupka and Hellenbrand (1974) and Ngo and Laidler (1975a and b). Goodson *et al*. (1973) report on immobilized AChE. Acetylcholinesterase (AChE) catalyzes the hydrolysis of acylcholinesters with a relative specificity for acetylcholine:

$$\text{Acetylcholine} + H_2O \rightarrow \text{acetate} + \text{choline} \tag{23.1}$$

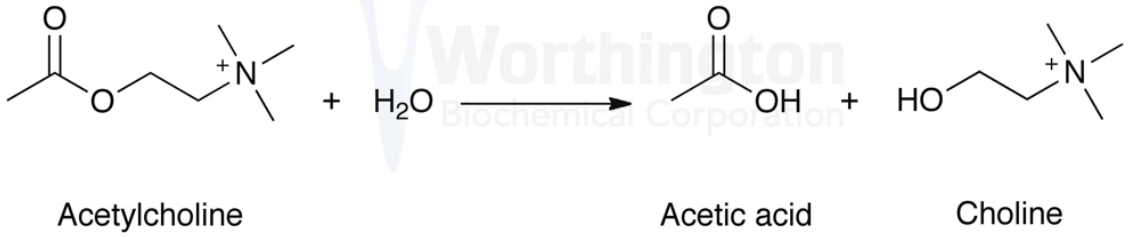

Figure 23.1: Enzymatic Reaction - Cholinesterase, Acetyl

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Electric Eel

Specificity

At substrate concentrations of 1 x 10^{-3}M and constant enzyme level (approximately optimal for acetylcholine), relative rates of hydrolysis for several esters are: acetylcholine, 100; propionylcholine, 96; butyrylcholine, negligible; and triacetin, 11 (Nachmansohn 1959). See also Moore and Hess (1975).

Composition

Levinson and Ellory (1974) indicate that the enzyme in its natural state is a monomer of approximately 75,000; in solution, aggregates are present. Rosenberry *et al.* (1974) report subunits to have a molecular weight of 70,000 and one active site. See also Chen *et al.* (1974) and Berman (1973). Dudai *et al.* (1973) have also reported on molecular structure.

There is evidence that eel AChE is a glycoprotein (Bon and Rieger 1975; Powell *et al.* 1973). Mooser and Sigman (1974 and 1972) report on a non-catalytic ligand binding-site remote from the active site. See also: Cocolas *et al.* (1974); Fuchs *et al.* (1974); Hochachka (1974); Marquis and Webb (1974); Roskowski (1974); Kato *et al.* (1972); Mooser *et al.* (1972); Rieger *et al.* (1972); Rosenberry (1975) and Rosenberry *et al.* (1972).

IUB

- 3.1.1.7

Molecular Weight

- 260,000 (Leuzinger *et al.* 1969)

Optimal pH

- 7

Isoelectric Point

- 5.35

Extinction Coefficient

- $E_{280}^{1\%}$ = 16.1 (Leuzinger *et al*. 1968)

Activators

- 0.02 M Mg^{2+} is stimulatory in purified preparations.

Inhibitors

- The classical inhibitors of AChE are organophosphate compounds (Bartels and Nachmansohn 1969 and Ashani *et al*. 1972) Most carbamates inhibit it (Hetnarski and O'Brian 1975). Light-sensitive inhibitors have been studied by Deal *et al*. (1969). See also: Maheshwari *et al*. (1975); Reiner *et al*. (1975); Millner *et al*. (1974); Stanley *et al*. (1974); deJong and vanDijk (1972); Allen and Abeles (1989). Moss *et al*. (1974) report on its reaction with puromycin.

Stability/Storage

- The enzyme is stable as a lyophilized powder for months at -20°C and in solution at pH 7.0 for several days at 4°C.
- Reports on methods of assay include the following: Schnitzerling and Nolan (1975); Stoops and Bender (1975); Augustinsson and Eriksson (1974); Lewis and Eldefrawi (1974); Smith (1974); Ellin *et al*. (1972); Vete *et al*. (1972); and vanHooidonk *et al*. (1972). See also comments by Ellin (1972).

Assay Information

Method

- The rate of acid production resulting from the hydrolysis of acetylcholine is measured titrimetrically. One unit hydrolyzes one micromole of acetylcholine per minute at 25°C and pH 7.0 under the specified conditions.

Reagents

- 0.02 M Potassium phosphate, pH 7.0
- 0.005 M Acetylcholine
- 0.01% Gelatin, containing 0.2 M sodium chloride and 0.04 M magnesium chloride
- 0.01-0.02 N Sodium hydroxide, standardized

Enzyme

- Dissolve at 1 $\frac{\text{mg}}{\text{ml}}$ in 0.02 M potassium phosphate, pH 7.0. Dilutions are made in 0.02 M potassium phosphate buffer, pH 7.0 to obtain a response between 0.05-0.2 $\frac{\text{ml}}{\text{min}}$. immediately before use.

Procedure

The titration can be carried out with either an automatic titrator or a laboratory pH meter. The reaction vessel must be maintained at 25°C.

Pipette the following into a 25 ml beaker:

- 0.005 M Acetylcholine - 7.5 ml
- 0.01% Gelatin-salt mixture - 7.5 ml

At zero time, add 0.1-1.0 ml of appropriately diluted enzyme and adjust the pH to 7.0.

After a constant rate is achieved, record the volume of base required to maintain the pH at 7.00 for 5-6 minutes.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\text{ml base added}}{\text{min}} \text{ x base normality x } 1000}{\text{mg enzyme in reaction mixture}}$$

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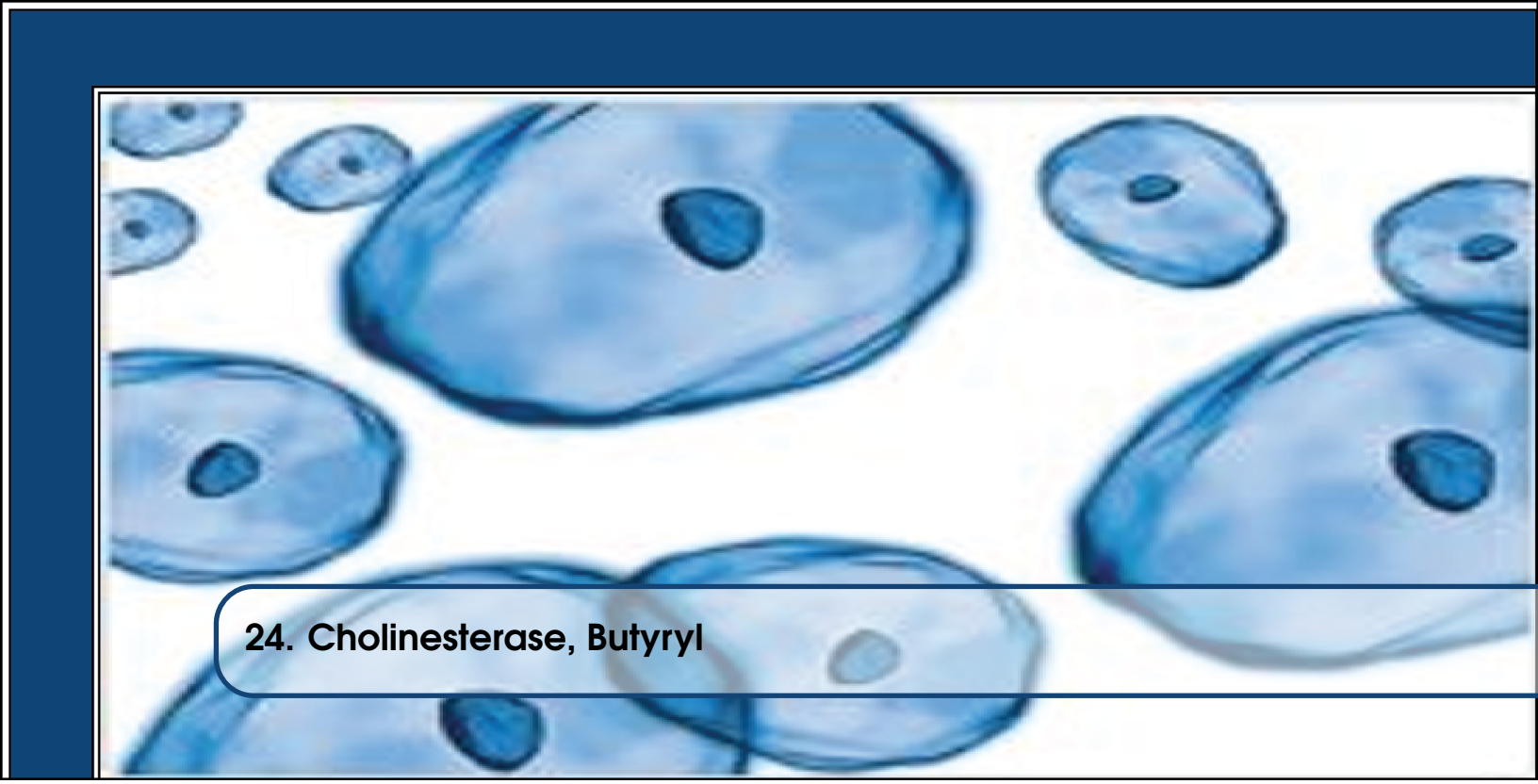

24. Cholinesterase, Butyryl

Acylcholine acylhydrolase

The enzyme is found in mammalian blood plasma, liver, pancreas, intestinal mucosa and the white matter of the central nervous system. It is sometimes referred to as serum cholinesterase as opposed to red cell cholinesterase (AChE). It hydrolyzes butyrylcholine 4 times more rapidly than acetylcholine. ChE does not hydrolyze D-β-methyl acetylcholine whereas AChE does. Both are inhibited by 10^{-5}M physostigmine. Because the enzyme is so markedly inhibited by organophosphate compounds used as insecticides and neurotoxins, it is widely used in monitoring systems. An immobilized cholinesterase detection device has been described by Goodson *et al.* (1973). Butyryl cholinesterase, (ButChE), catalyzes the hydrolysis of a number of choline esters:

$$\text{Acetylcholine} + H_2O \rightarrow \text{choline} + \text{acid} \qquad (24.1)$$

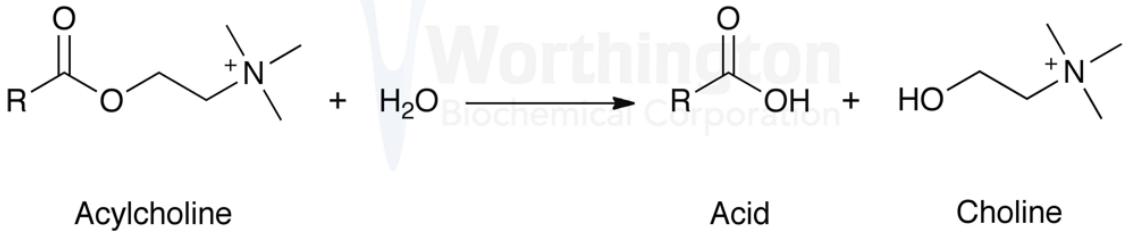

Figure 24.1: Enzymatic Reaction - Cholinesterase, Butyryl

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Horse Serum

Specificity

The enzyme is more active with butyryl and propionyl choline than with acetyl choline. See also Main *et al*. (1974) for some comparative activities. Non-choline esters (e.g., procaine, morphine esters, atropine and cocaine) are susceptible to the action of ButChE (Augustinsson 1960).

Composition

Lee and Harpst (1973) indicate ButChE to be a tetrameric structure with equally sized subunits of 110,000 daltons. It is a glycoprotein. Main *et al*. (1974) have determined the amino acid and carbohydrate composition, based upon a particle weight of 101,000. See also Chiu *et al*. (1972) and Pavlic (1972).

IUB

- 3.1.1.8

CAS

- 9001-08-5

Molecular Weight

- 440,000 (Lee and Harpst 1973; see also Main *et al*. 1974)

Optimal pH

- 6.0-8.0 (Augustinsson 1960)

Extinction Coefficient

- $E^{1\%}_{280}$ = 13.6 (Main *et al.* 1974)

Activators

- Ca^{2+} and Mg^{2+} (Augustinsson 1960)

Inhibitors

- Numerous organophosphate esters, the carbamate derivatives and quaternary ammonium salts (Augustinsson 1960). See also Kamaric (1975), Koelle *et al.* (1974), Millner *et al.* (1974), Stanley *et al.* (1974), Ashani *et al.* (1972), Post (1971).

Assay Information

A number of assays have been developed for BChE, including Grubic *et al.* 1975, Augustinsson and Ericksson 1974, Smith 1974, Brownson and Watts 1973, Dietz *et al.* 1973, Ellin 1972, Baum and Ward 1971, Garry 1971, Garry *et al.* 1971, and Hanss and Rey 1971 who report on a conductivity method. The following assay is used at Worthington.

Method

- The reaction velocity is determined by a titrimetric measurement of the acid released during the hydrolysis of acetylcholine. One Unit causes hydrolysis of one micromole of acetylcholine per minute at 25°C and pH 7.4, under the specified conditions.

Reagents

- 0.02 M Tris · HCl buffer, pH 7.4
- 0.2 M Magnesium chloride
- 2.2 M Acetylcholine chloride
- Titrant: 0.01-0.02 N NaOH, standardized

Enzyme

- Dissolve at one $\frac{mg}{ml}$ in reagent grade water.

Procedure

The titration can be measured with either an automatic titrator or with a laboratory pH meter. The reaction vessel should be maintained at 25°C. Pipette the following into a titration vessel at 25°C.

- Reagent Grade Water - 7.0 ml
- 0.2 M Magnesium chloride - 3.0 ml
- 0.02 M Tris · HCl buffer, pH 7.4 - 3.0 ml
- Enzyme - 0.1 to 0.5 ml

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\text{ml base added}}{\text{min}} \text{ x normality x } 1000}{\text{mg enzyme in reaction mixture}}$

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25. Chymotrypsin

Chymotrypsin is a serine endopeptidase produced by the acinar cells of the pancreas. Chymotrypsin becomes activated after proteolysis of chymotrypsinogen by trypsin. While trypsin hydrolyzes at lysine and arginine, chymotrypsin selectively cleaves peptide bonds formed by aromatic residues (tyrosine, phenylalanine, and tryptophan) (Hedstrom *et al.* 1992). Two predominant forms of chymotrypsin, A and B, are found in equal amounts in cattle pancreas. They are very similar proteins (80% identical), but have significantly different proteolytic characteristics (Hartley 1964, Meloun *et al.* 1966, Smillie *et al.* 1968, and Gráf *et al.* 2004). The information below pertains primarily to the A form of chymotrypsinogen and chymotrypsin.

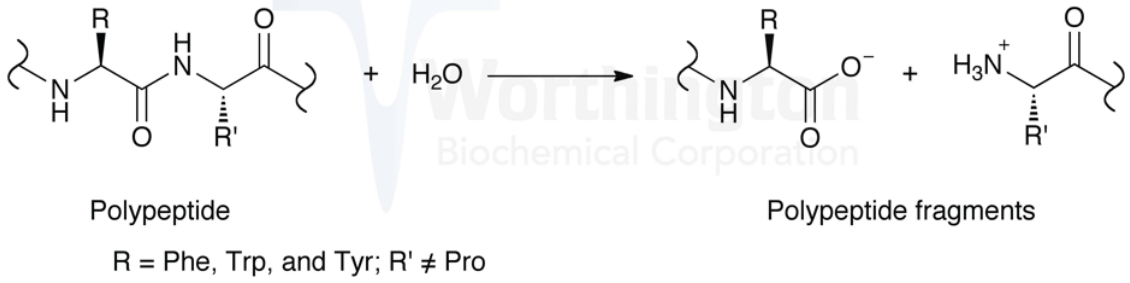

Figure 25.1: Enzymatic Reaction - Chymotrypsin

History

In the early 1900s, Vernon proposed that pancreatic preparations could give rise to an intrinsic activator of its own enzymes (Vernon 1901). Vernon's milk-clotting experiments determined there were at least two enzymes present and that one was more stable than the other (Vernon 1902). However, this idea was not widely accepted until 1934 when Kunitz and Northrop confirmed the presence of an enzyme in addition to trypsin, naming it chymotrypsin. They were able to crystallize chymotrypsin, as well as the inactive precursor, chymotrypsinogen (Kunitz and Northrop 1934). In 1938, Kunitz isolated different active forms of chymotrypsin, designating them as alpha, beta, and gamma (Kunitz 1938).

In the early 1940s Fruton and Bergmann further studied the specificity of chymotrypsin, reporting on several new substrates (Fruton and Bergmann 1942). Jacobsen soon identified additional forms of chymotrypsin, designating them as delta and pi (Jacobsen 1947). In 1948, Schwert further characterized the molecular weights of chymotrypsin and chymotrypsinogen.

In 1954, the first evidence for the three-step mechanism of chymotrypsin hydrolyzing amide and ester substrates was reported on by Hartley and Kilby, who hypothesized the presence of an acyl enzyme intermediate, which was later proven to be true (Henderson 1970). In 1955, Laskowski obtained a second crystalline chymotrypsinogen, naming it chymotrypsinogen B. In 1964 Hartley determined the amino acid sequence of chymotrypsin A, which was later refined by Meloun *et al.* in 1966. In 1968, Smillie *et al.* determined the amino acid sequence of chymotrypsin B, which revealed 80% sequence identity with chymotrypsin A. Throughout the 1970s and 1980s research was done to better understand the mechanism of action, and identify the differences in amino acid sequences between trypsin and chymotrypsin (Steitz *et al.* 1969, Cohen *et al.* 1981, Asbóth and Polgár 1983, and Gráf *et al.* 1988).

In the 1990s, chymotrypsin was purified from other sources including Atlantic cod (Ásgeirsson and Bjarnason 1991), and camel (Al-Ajlan and Bailey 1997). Work also begun on investigating inhibitors (Baek *et al.* 1990), and Frigerio *et al.* elucidated the crystal structure of bovine chymotrypsin to a 2.0 resolution (Frigerio *et al.* 1992).

Recent research has investigated the folding and denaturation of chymotrypsin over a range of concentrations (Ghaouar *et al.* 2010), chymotrypsin's interaction with nanoparticle substrates (You *et al.* 2006, and Jordan *et al.* 2009), and increasing chymotrypsin stability by conjugating to Polyethylene Glycol (PEG) molecules (Castellanos *et al.* 2005, and Rodríguez-Martínez *et al.* 2009).

Molecular Characteristics

Chymotrypsin A and B share 80% sequence identity (Hartley 1964, Meloun *et al.* 1966, Smillie *et al.* 1968, and Gráf *et al.* 2004). The amino acids of the catalytic triad (H57, D102, and S195) are highly conserved in the sequences of the peptidases of family S1 (Gráf *et al.* 2004). The serine at position 214 is also highly conserved in the family and has been proposed as the fourth member of the catalytic triad (Ohara *et al.* 1989, and McGrath *et al.* 1992).

Specificity

Chymotrypsin is activated through cleavage of the bond between arginine and isoleucine (R15 and I16) by trypsin, causing structural modifications and formation of the substrate binding site (Sears 2010).

Chymotrypsin differs from trypsin in that trypsin cleaves peptides at arginine and lysine residues, while chymotrypsin prefers large hydrophobic residues (Hedstrom *et al*. 1992). Chymotrypsin preferentially catalyzes the hydrolysis of peptide bonds involving L-isomers of tyrosine, phenylalanine, and tryptophan. It also readily acts upon amides and esters of susceptible amino acids. Chymotrypsin's specificity for large hydrophobic residues can be explained by a hydrophobic S1 binding pocked formed by residues 189 through 195, 214 through 220, and 225 through 228 (Cohen *et al*. 1981).

Although the structure of trypsin and chymotrypsin's S1 site show only one difference (at position 189), site-directed mutagenesis of trypsin and chymotrypsin have failed to interchange specificities, suggesting the mechanism by which trypsin and chymotrypsin achieve substrate specific catalysis is not fully understood (Steitz *et al*. 1969, and Gráf *et al*. 1988).

Composition

The three amino acid residues of the catalytic triad (H57, D102, and S195) are essential for peptide bond cleavage and are stabilized by hydrogen bonds (Sears 2010, and Gráf *et al*. 2004). G193 and S195 make up the oxyanion hole and interact with the carbonyl group of the scissile peptide bond, orienting it to form the tetrahedral intermediate (Rühlmann *et al*. 1973, Huber and Bode 1978, and Gráf *et al*. 2004).

IUB

- 3.4.21.1

CAS

- 9004-07-3

Protein Accession Number

- P00766

CATH: Classification (v. 3.3.0)

Class:

- Mainly Beta

Architecture:

- Beta Barrel

Topology:

- Trypsin-like Serine Protease

Molecular Weight

- 25.6 kDa (Wilcox 1970)

Optimal pH

- 7.8-8.0 (Rick 1974)

Isoelectric Point

- 8.52 (Chymotrypsinogen, Theoretical)
- 8.33 (Chymotrypsin, Theoretical)

Extinction Coefficient

- 51,840 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 20.57 (Chymotrypsin, Theoretical)

Active Residue

- Histidine (H57)
- Aspartate (D102)
- Serine (S195)

Activators

- Cetyltributylammonium bromide (Spreti *et al*. 2008)
- Dodecyltrimethylammonium bromide (Abuin *et al*. 2005)
- Hexadecyltrimethylammonium bromide (Celej *et al*. 2004)
- Tetrabutylammonium bromide (Spreti *et al*. 2001)

Inhibitors

- Hydroxymethylpyrroles (Abell and Nabbs 2001)
- Boronic acids (Smoum *et al*. 2003)
- Courmarin derivatives (Pochet *et al*. 2000)
- Peptidyl aldehydes (Lesner *et al*. 2009)
- Peptides from natural sources (Telang *et al*. 2009, Roussel *et al*. 2001, and Chopin *et al*. 2000)
- Peptides containing an unnatural amino acid (Legowska *et al*. 2009, and Wysocka *et al*. 2008)

Applications

- Sequence analysis
- Peptide synthesis
- Peptide mapping
- Peptide fingerprinting

Assay Information

Method

- The reaction velocity is determined according to Hummel (1959) by measuring an increase in absorbance at 256 nm resulting from the hydrolysis of benzoyl-L-tyrosine ethyl ester. One unit

hydrolyzes one micromole of benzoyl-L-tyrosine ethyl ester (BTEE) per minute at pH 7.8 and 25°C under the specified conditions.
- 45 BTEE units = 10,000 optical density units = 1,330 N.F. (ATEE) units.

Reagents

- 0.08 M Tris · HCl buffer, pH 7.8 containing 0.1 M calcium chloride
- 0.00107 M Benzoyl-L-tyrosine ethyl ester (BTEE) in 50% w/w methanol (63 ml absolute methanol added to 50 ml reagent grade water)
- 0.001 N HCl

Enzyme

- Dissolve enzyme at one $\frac{\text{mg}}{\text{ml}}$ in 0.001 N HCl. Dilute in 0.001 N HCl to 10-30 $\frac{\mu\text{g}}{\text{ml}}$ for assay.

Procedure

Spectrophotometer Settings: Wavelength: 256 nm Temperature: 25°C

Pipette into cuvettes as follows:
- 0.08 M Tris · HCl buffer, pH 7.8 with 0.1 M $CaCl_2$ - 1.5 ml
- 0.00107 M BTEE - 1.4 ml

Incubate in spectrophotometer at 25°C for 4-5 minutes to achieve temperature equilibrium and record blank rate, if any. Add 0.1 ml of appropriately diluted enzyme and record increase in absorbance at 256 nm for 4-5 minutes. Calculate $\frac{\Delta A_{265}}{\text{min}}$ from the initial linear portion of the curve.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{256}}{\text{min}} \text{ x } 1000}{964 \text{ x } \frac{\text{mg}}{\text{ml}} \text{ in the reaction mixture}}$$

where 964 is the extinction coefficient of BTEE at 256 nm

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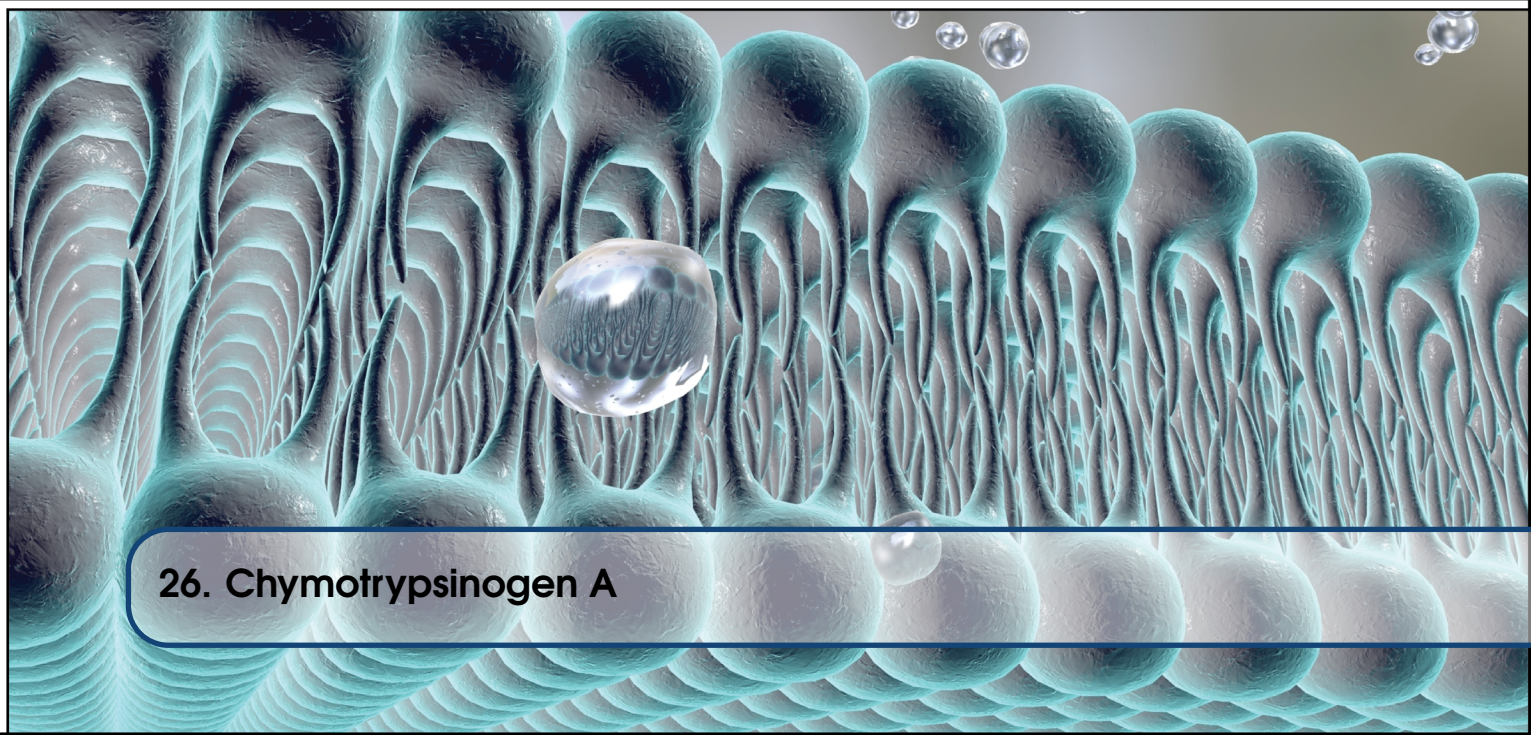

26. Chymotrypsinogen A

Chymotrypsin is a serine endopeptidase produced by the acinar cells of the pancreas. Chymotrypsin becomes activated after proteolysis of chymotrypsinogen by trypsin. While trypsin hydrolyzes at lysine and arginine, chymotrypsin selectively cleaves peptide bonds formed by aromatic residues (tyrosine, phenylalanine, and tryptophan) (Hedstrom *et al.* 1992). Two predominant forms of chymotrypsin, A and B, are found in equal amounts in cattle pancreas. They are very similar proteins (80% identical), but have significantly different proteolytic characteristics (Hartley 1964, Meloun *et al.* 1966, Smillie *et al.* 1968, and Gráf *et al.* 2004). The information below pertains primarily to the A form of chymotrypsinogen and chymotrypsin.

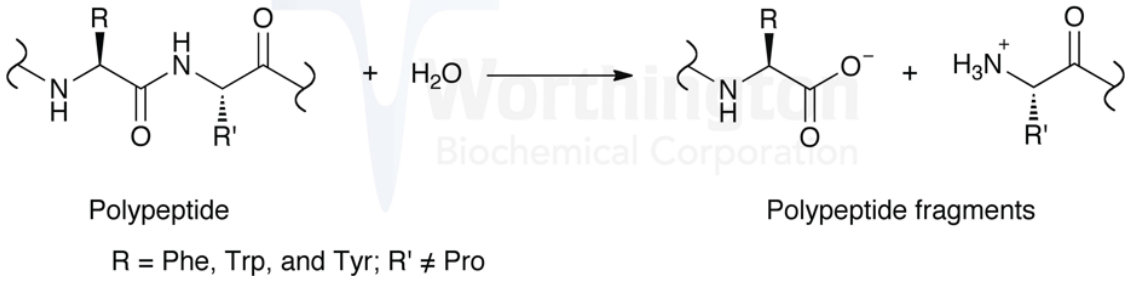

Figure 26.1: Enzymatic Reaction - Chymotrypsinogen A

History

In the early 1900s, Vernon proposed that pancreatic preparations could give rise to an intrinsic activator of its own enzymes (Vernon 1901). Vernon's milk-clotting experiments determined there were at least two enzymes present and that one was more stable than the other (Vernon 1902). However, this idea was not widely accepted until 1934 when Kunitz and Northrop confirmed the presence of an enzyme in addition to trypsin, naming it chymotrypsin. They were able to crystallize chymotrypsin, as well as the inactive precursor, chymotrypsinogen (Kunitz and Northrop 1934). In 1938, Kunitz isolated different active forms of chymotrypsin, designating them as alpha, beta, and gamma (Kunitz 1938).

In the early 1940s Fruton and Bergmann further studied the specificity of chymotrypsin, reporting on several new substrates (Fruton and Bergmann 1942). Jacobsen soon identified additional forms of chymotrypsin, designating them as delta and pi (Jacobsen 1947). In 1948, Schwert further characterized the molecular weights of chymotrypsin and chymotrypsinogen.

In 1954, the first evidence for the three-step mechanism of chymotrypsin hydrolyzing amide and ester substrates was reported on by Hartley and Kilby, who hypothesized the presence of an acyl enzyme intermediate, which was later proven to be true (Henderson 1970). In 1955, Laskowski obtained a second crystalline chymotrypsinogen, naming it chymotrypsinogen B. In 1964 Hartley determined the amino acid sequence of chymotrypsin A, which was later refined by Meloun *et al.* in 1966. In 1968, Smillie *et al.* determined the amino acid sequence of chymotrypsin B, which revealed 80% sequence identity with chymotrypsin A. Throughout the 1970s and 1980s research was done to better understand the mechanism of action, and identify the differences in amino acid sequences between trypsin and chymotrypsin (Steitz *et al.* 1969, Cohen *et al.*1981, Asbóth and Polgár 1983, and Gráf *et al.* 1988).

In the 1990s, chymotrypsin was purified from other sources including Atlantic cod (*á*sgeirsson and Bjarnason 1991), and camel (Al-Ajlan and Bailey 1997). Work also begun on investigating inhibitors (Baek *et al.* 1990), and Frigerio *et al.* elucidated the crystal structure of bovine chymotrypsin to a 2.0 resolution (Frigerio *et al.* 1992).

Recent research has investigated the folding and denaturation of chymotrypsin over a range of concentrations (Ghaouar *et al.* 2010), chymotrypsin's interaction with nanoparticle substrates (You *et al.* 2006, and Jordan *et al.* 2009), and increasing chymotrypsin stability by conjugating to Polyethylene Glycol (PEG) molecules (Castellanos *et al.* 2005, and Rodríguez-Martínez *et al.* 2009).

Molecular Characteristics

Chymotrypsin A and B share 80% sequence identity (Hartley 1964, Meloun *et al.* 1966, Smillie *et al.* 1968, and Gráf *et al.* 2004). The amino acids of the catalytic triad (H57, D102, and S195) are highly conserved in the sequences of the peptidases of family S1 (Gráf *et al.* 2004). The serine at position 214 is also highly conserved in the family and has been proposed as the fourth member of the catalytic triad (Ohara *et al.* 1989, and McGrath *et al.* 1992).

Specificity

Chymotrypsin is activated through cleavage of the bond between arginine and isoleucine (R15 and I16) by trypsin, causing structural modifications and formation of the substrate binding site (Sears 2010).

Chymotrypsin differs from trypsin in that trypsin cleaves peptides at arginine and lysine residues, while chymotrypsin prefers large hydrophobic residues (Hedstrom *et al*. 1992). Chymotrypsin preferentially catalyzes the hydrolysis of peptide bonds involving L-isomers of tyrosine, phenylalanine, and tryptophan. It also readily acts upon amides and esters of susceptible amino acids. Chymotrypsin's specificity for large hydrophobic residues can be explained by a hydrophobic S1 binding pocked formed by residues 189 through 195, 214 through 220, and 225 through 228 (Cohen *et al*. 1981).

Although the structure of trypsin and chymotrypsin's S1 site show only one difference (at position 189), site-directed mutagenesis of trypsin and chymotrypsin have failed to interchange specificities, suggesting the mechanism by which trypsin and chymotrypsin achieve substrate specific catalysis is not fully understood (Steitz *et al*. 1969, and Gráf *et al*. 1988).

Composition

The three amino acid residues of the catalytic triad (H57, D102, and S195) are essential for peptide bond cleavage and are stabilized by hydrogen bonds (Sears 2010, and Gráf *et al*. 2004). G193 and S195 make up the oxyanion hole and interact with the carbonyl group of the scissile peptide bond, orienting it to form the tetrahedral intermediate (Rühlmann *et al*. 1973, Huber and Bode 1978, and Gráf *et al*. 2004).

CAS

- 9035-75-0

Protein Accession Number

- P00766

CATH: Classification (v. 3.3.0)

Class:

- Mainly Beta

Architecture:

- Beta Barrel

Topology:

- Trypsin-like Serine Protease

Molecular Weight

- 25.6 kDa (Wilcox 1970)

Optimal pH

- 7.8-8.0 (Rick 1974)

Isoelectric Point

- 8.52 (Chymotrypsinogen, Theoretical)
- 8.33 (Chymotrypsin, Theoretical)

Extinction Coefficient

- 51,840 cm-1 M-1
- $E^{1\%}_{280}$ = 20.19 (Chymotrypsinogen, Theoretical)

Active Residue

- Histidine (H57)
- Aspartate (D102)
- Serine (S195)

Activators

- Cetyltributylammonium bromide (Spreti *et al*. 2008)
- Dodecyltrimethylammonium bromide (Abuin *et al*. 2005)
- Hexadecyltrimethylammonium bromide (Celej *et al*. 2004)
- Tetrabutylammonium bromide (Spreti *et al*. 2001)

Inhibitors

- Hydroxymethylpyrroles (Abell and Nabbs 2001)
- Boronic acids (Smoum *et al*. 2003)
- Courmarin derivatives (Pochet *et al*. 2000)
- Peptidyl aldehydes (Lesner *et al*. 2009)
- Peptides from natural sources (Telang *et al*. 2009, Roussel *et al*. 2001, and Chopin *et al*. 2000)
- Peptides containing an unnatural amino acid (Legowska *et al*. 2009, and Wysocka *et al*. 2008)

Applications

- Sequence analysis
- Peptide synthesis
- Peptide mapping
- Peptide fingerprinting

Assay Information

Method

- Chymotrypsinogen is assayed first for intrinsic activity, then activated and re-assayed. The activity will be determined in the chymotrypsin assay as described above.

Reagents

- 1 N H_2SO_4
- 0.5 M Sodium potassium phosphate buffer, pH 7.6
- Trypsin solution: Dissolve Worthington trypsin (Code: TRL), at a concentration of 1 $\frac{mg}{ml}$ in 0.001 N HCl. Prepare immediately before use.

Procedure

Intrinsic chymotrypsin activity: Dissolve enzyme at a concentration of 10 $\frac{mg}{ml}$ in 0.001 N HCl. Assay for chymotrypsin as described above. Calculate $\frac{units}{mg}$ protein.

Chymotrypsinogen activation: Dissolve 100 mg enzyme in 5 ml reagent grade water. Adjust pH to 3.0 with 1 N H_2SO_4. Add 2.5 ml of 0.5 M phosphate buffer pH 7.6 and readjust pH to 7.6 if necessary. Add 1 ml (1 mg) trypsin and dilute to a final volume of 10 ml with reagent grade water. A 1:500 dilution will probably be required. Assay immediately using the chymotrypsin assay as described above. Repeat the assay at 10 minute intervals until the activity begins to decline. As activation continues, dilution may increase. Calculate $\frac{units}{mg}$ chymotrypsin activity.

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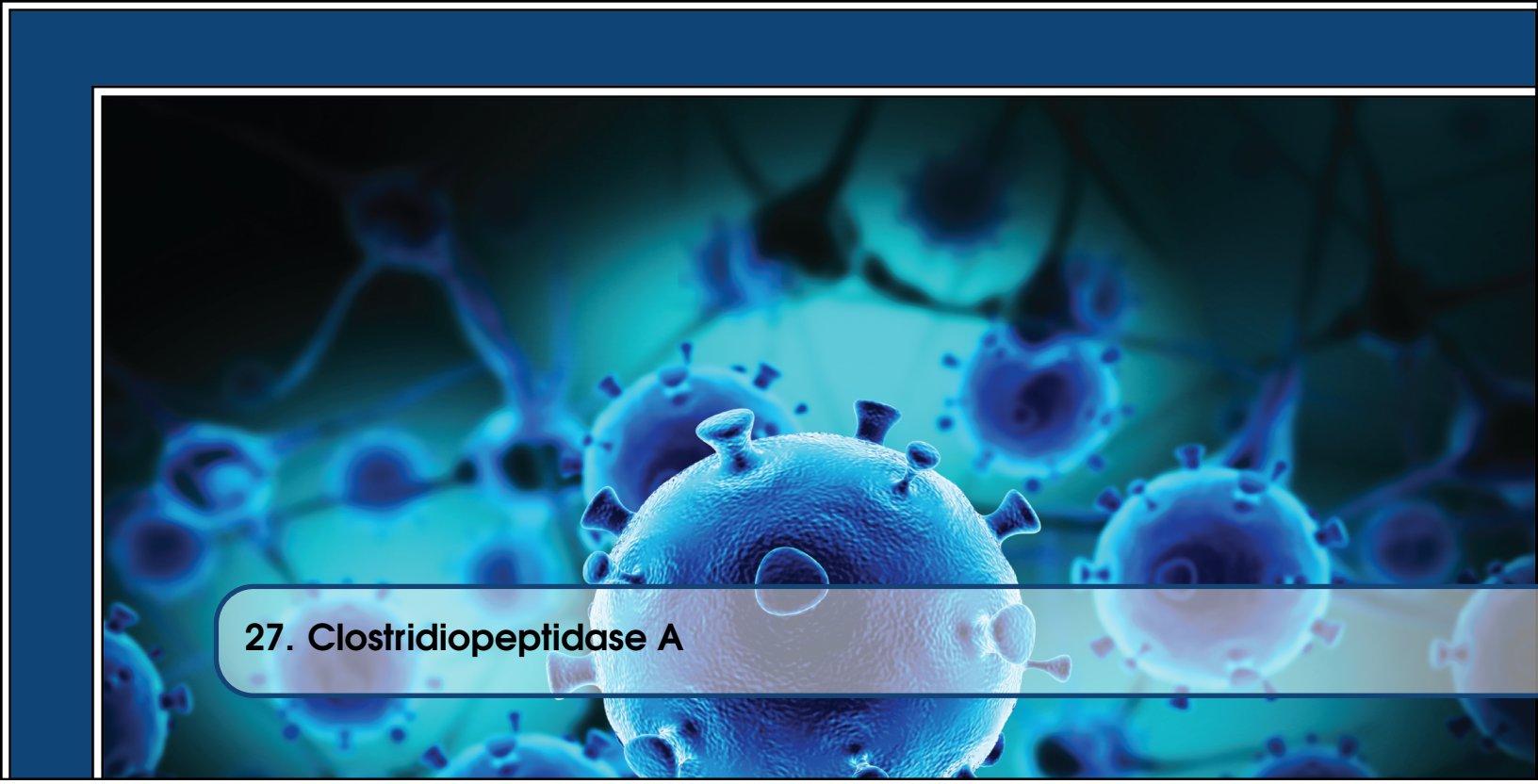

27. Clostridiopeptidase A

Clostridium histolyticum produces two distinct types of collagenase: Collagenase 1 is very active on native collagen and synthetic substrates but with negligible activity on gelatin and azocoll, and collagenase 2 is active on gelatin but less active on synthetic substrates. Clostridiopeptidase A is a collagenase 1. It degrades the helical regions on native collagen preferentially at the Y-Gly bond in the sequence Pro-Y-Gly-Pro where Y is most frequently a neutral amino acid. This cleavage yields products susceptible to further peptidase digestion. Activity is measured by the specific assay of W*ü*nsch and Heidrich. CLOSA is suitable for more critical experiments requiring only partial collagen degradation.

Worthington's clostridiopeptidase A was a highly purified form of collagenase that we no longer manufacture. For additional information please see our collagenase manual entry.

1 unit releases 1 micromole of p-phenylazobenzyloxycarbonyl-L-prolyl-L-leucine per minute at 25°C, pH 7.1

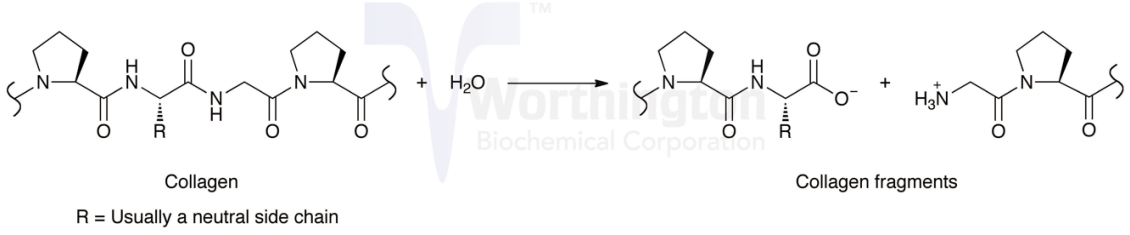

Figure 27.1: Enzymatic Reaction - Clostridiopeptidase A

Stability/Storage

- Stable for years at 2 - 8°C

Assay Information

Method

- The assay is based on the enzyme-specific splitting of a Pz-Pro-Leu-Gly-Pro-Arg substrate between leucine and glycine (W*ü*nch and Heidrich, 1963). This results in a colored lipophilic fragment and a non-colored tripeptide. The change in absorbance of the fragment at 320 nm per unit time is a measure of enzyme activity.

Reagents

- 0.1 M Tris · HCl, pH 7.1
- 0.1 M Calcium chloride
- 25 mM Citric acid, pH 3.5
- Ethyl acetate
- Anhydrous sodium sulfate
- Substrate: Pz-Pro-Leu-Gly-Pro-Arg (molecular weight 812.93). Dissolve to 5 $\frac{mg}{ml}$ in methanol. Dilute to 1 $\frac{mg}{ml}$ with Tris buffer.
- Standard: Pz-Pro-Leu (molecular weight 466.54). Dissolve to 5 $\frac{mg}{ml}$ in methanol. Dilute to 1 $\frac{mg}{ml}$ with Tris buffer.

Enzyme

- Stock solution: Dissolve at 1 $\frac{mg}{ml}$ in reagent grade water. Dilute stock 1:20 and 1:50 for assay.

Procedure

Determine the standard curve.

Description	0 μg of std	50 μg of std	100 μg of std	200 μg of std	400 μg of std	600 μg of std	800 μg of std
Standard	0 ml	0.05 ml	0.1 ml	0.2 ml	0.4 ml	0.6 ml	0.8 ml
Tris buffer	1.1 ml	1.05 ml	1.0 ml	0.9 ml	0.7 ml	0.5 ml	0.3 ml
Calcium Chloride	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml

Mix well and transfer 0.5 ml to labeled tubes containing 1 ml citric acid and 5 ml ethyl acetate. Vortex for 15 seconds. All the phases separate at room temperature. Transfer the organic phase to tubes containing 350 mg sodium sulfate. Shake gently to allow ethyl acetate to dry. Read the A_{320} of the dried ethyl acetate vs. air.

Determine the net A_{320} (test - blank) and plot A_{320} / $\geq$ g standard from the slope of the curve. Determine the calibration factor as follows:

Factor = $\frac{1}{\frac{A_{320}}{\mu g} \text{ x } \frac{466\ \mu g}{\mu mol}}$

Enzyme Assay: Pipette 0.2 ml calcium chloride and 1.0 ml substrate into a series of numbered tubes. Incubate in a water bath at 25°C to achieve temperature equilibration. At timed intervals, add 0.1 ml

enzyme dilution to the respective tubes. Include 2 tubes with 0.1 ml water as blanks. Incubate exactly 15 minutes at 25°C and at timed intervals withdraw 0.5 ml and transfer to tubes containing 1 ml citric acid and 5 ml ethyl acetate. Proceed as with standard curve, reading A_{320} of dried ethyl acetate vs. air. Determine net A_{320}.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\text{Net } A_{320} \text{ x dilution x Factor}}{0.1 \text{ x } 15 \text{ x } \frac{\text{mg}}{\text{ml}} \text{ in stock}}$$

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28. Clostripain (Endoproteinase-Arg-C)

Clostripain is a cysteine-activated protease found, along with collagenase and other proteases, in culture filtrates of Clostridium histolyticum. It is unique in its specificity for the carboxyl peptide bond of arginine and its dependence on thiol and calcium ions.

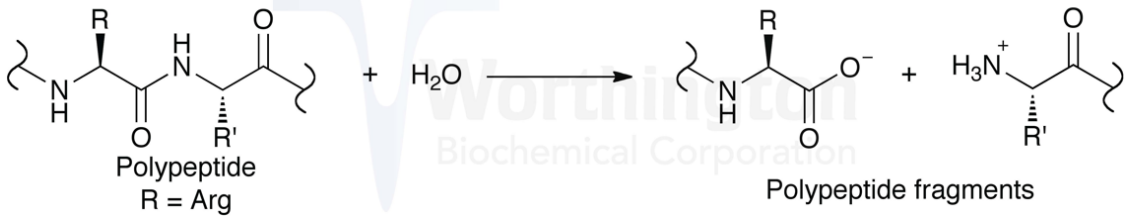

Figure 28.1: Enzymatic Reaction - Clostripain (Endoproteinase-Arg-C)

History

The bacteria from which clostripain is purified first gained attention during World War I because of its severe consequences to the wounded (Mitchell and Harrington 1971). Cl. hisolyticum is only one of the organisms with consequential pathogenic properties, but its proteolytic activity in cell-free culture filtrates gained attention as far back as 1917 (Weinberg and Ségun 1917, and Mitchell and Harrington 1971).

In 1931, digestion of horse tendons was described by Weinberg and Randin. A year later they identified

an exotoxin, which they termed "ferment fibrinolytique", as the cause of digestion (Weinberg and Randin 1932). It was later found that this digestion was caused by a variety of proteolytic enzymes, including a cysteine-activated proteinase, clostripain (Kochalaty and Weil 1938, and Maschmann 1938).

In 1948, Kochalaty and Krejci first successfully isolated clostripain in relatively pure form (Mitchell and Harrington 1968). Ogle and Tytell refined the purification technique in 1953 and first reported on its specificity (Ogle and Tytell 1953).

When the narrow substrate specificity of clostripain became of interest, confusion existed in the literature regarding the identity of clostripain (Mitchell and Harrington 1971). Prior to its description as clostripain by Labouesse and Gros in 1960, and later Mitchell and Harrington in 1968, it was referred to as g-protease (Bard and McClung 1948, and Oakley and Warrack 1950), amidase-esterase (Nordwig and Strauch 1963), and clostridiopeptidase B (Mitchell and Harrington 1968).

Recent work with clostripain has included cell isolation and its use as a model target of protease inhibitors for the treatment of clostridial infections (Wang *et al*. 2004, and Gusman *et al*. 2001).

Molecular Characteristics

Both the heavy and light chains are encoded by a single gene with a 1581 nucleotide open reading frame (ORF). Upon expression of the gene, the entire ORF (the signal region, proregion, and 9 amino acid peptide linker) is transcribed. Postranslational processing produces the heterodimeric active enzyme (Dargatz *et al*. 1993).

Specificity

Clostripain selectively hydrolyzes arginyl bonds and lysyl bonds at a lower rate. It can also act as a transpeptidase with maximal activity at pH 7.6-9.0 (Anderson 1985, and Fortier and MacKenzie 1986).

Composition

Clostripain is a heterodimer. The mature chain is composed of 526 residues. The two chains are held together by strong noncovalent forces (Gilles *et al*. 1979, and Ullman and Bordusa 2004). The catalytic sulfhydryl residue of the active site is believed to be Cys41 (heavy chain residue). The precursor contains a 27 amino acid putative signal peptide, a 23 amino acid propeptide, a 131 amino acid light chain subunit, a 9 amino acid linker peptide, and a 336 amino acid heavy chain subunit (Ullman and Bordusa 2004).

IUB

- 3.4.22.8

CAS

- 9028-00-6

Protein Accession Number

- P09870

Molecular Weight

- 53.0 kDa (Theoretical)
- Light chain: 12.5 kDa, Heavy chain: 45 kDa (Gilles *et al.* 1979)

Optimal pH

- 7.4-7.8 (activity against a-benzoyl-arginine ethyl ester) (Mitchell and Harrington 1968)

Isoelectric Point

- 4.8-4.9 (Mitchell and Harrington 1971)

Extinction Coefficient

- 87,890 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 16.57

Active Residue

- Cysteine (C41, heavy chain)

Activators

- Sulfhydryl requirement: dithiothreitol, cysteine, or other reducing agents
- Calcium ion is essential
- Reducing agents

Inhibitors

- EDTA
- Oxidizing agents
- Sulfhydryl reagents (such as TLCK) (Porter *et al.* 1971)
- Co^{2+}, Cu^{2+}, Cd^{2+}, and heavy metal ions
- Citrate, borate and Tris anions partially inhibit

Applications

- Peptide mapping
- Sequence analysis
- Cell isolation (Wang *et al.* 2004)
- Hydrolysis/condensation of amide bonds
- Peptide synthesis (Meiwes *et al.* 1991)

Assay Information

Method

- The reaction velocity is measured as an increase in absorbance at 253 nm resulting from the hydrolysis of N-benzoyl-L-arginine ethyl ester. One unit hydrolyzes one micromole of BAEE per minute at 25°C and pH 7.6 under the conditions specified.

Reagents

- 0.075 M Sodium phosphate buffer, pH 7.6
- 7.5 mM Dithiothreitol (DTT)
- 0.75 mM N-Benzoyl-L-arginine ethyl ester (BAEE)
- 1.0 mM Calcium acetate containing 2.5 mM dithiothreitol (activation solution)

Enzyme

- Dissolve or dilute the enzyme at a concentration of 1 $\frac{\text{mg}}{\text{ml}}$ in water. Immediately prior to assay, dilute the enzyme further in 1.0 mM Calcium acetate containing 2.5 mM dithiothreitol to a concentration of 0.2-0.8 $\frac{\text{units}}{\text{ml}}$.

Procedure

Spectrophotometer Settings: Wavelength: 253 nm Temperature: 25°C

Pipette into each cuvette as follows:

- 0.075 M phosphate buffer, pH 7.6 - 1.0 ml
- 7.5 mM DTT - 1.0 ml
- 0.75 mM BAEE - 1.0 ml

Incubate in spectrophotometer for 3-5 minutes to achieve temperature equilibrium and establish blank rate, if any. At zero time, add 0.1 ml of appropriately diluted enzyme and record A_{253} for 4-5 minutes. Determine $\frac{\Delta A_{253}}{\text{min}}$ from the linear portion of the curve. Note: The reaction appears to be most linear with respect to enzyme concentration when $\frac{\Delta A_{253}}{\text{min}}$ is between 0.007 and 0.030.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{253}}{\text{min}} \text{ x } 1000}{1150 \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$$

where 1150 is the extinction coefficient of BAEE at 253 nm

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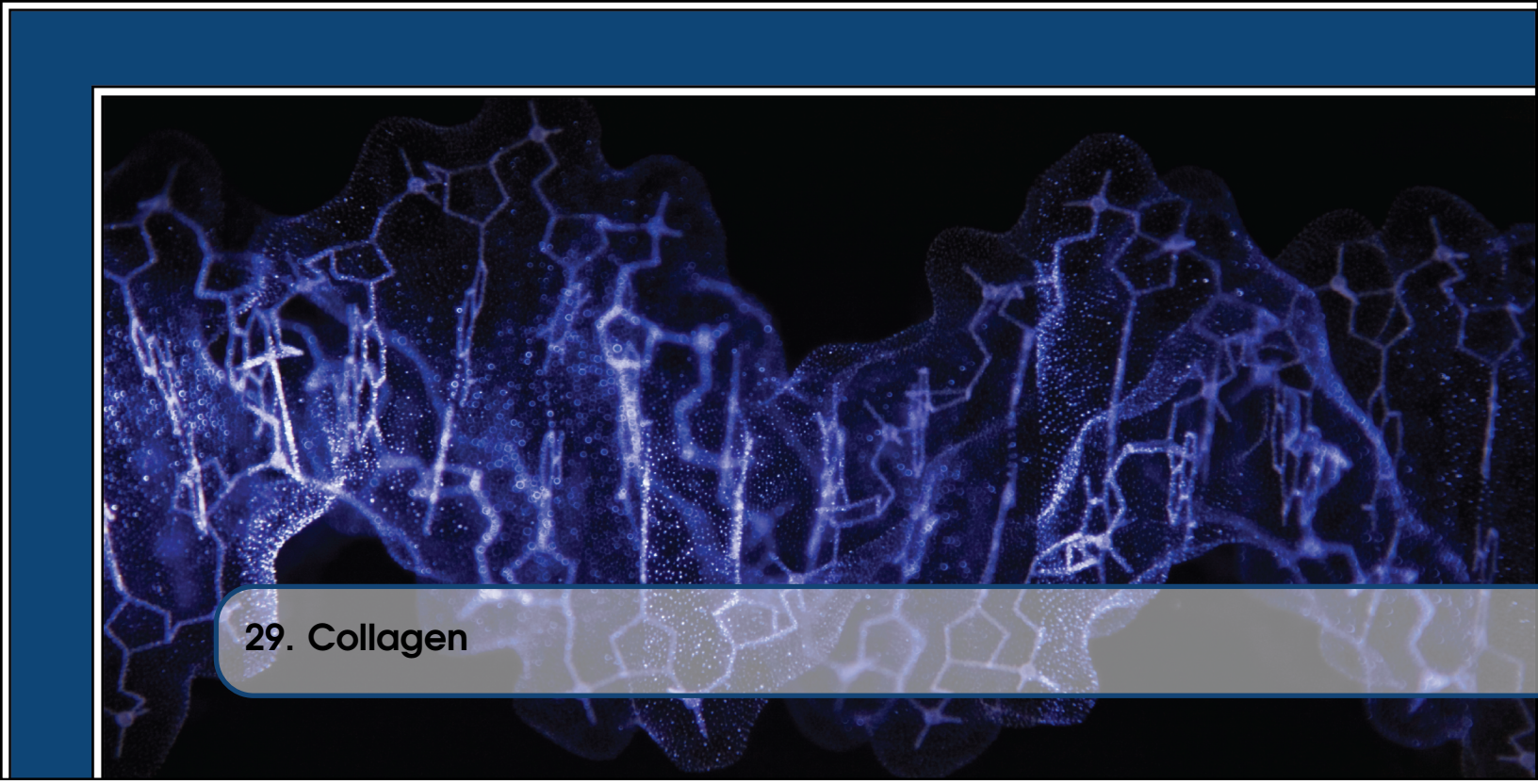

29. Collagen

Collagen is an inert, rigid protein found predominantly in skin, ligaments, bones and teeth. Its most distinctive attribute, essential to a transmitter of mechanical force, is inelasticity. Its fundamental structural unit is tropo-collagen, a molecular rod about 2600 in length and 15 in diameter and 300,000 molecular weight. In tendons these macromolecules, grouped as collagen fibrils, run parallel to the axis, in skin the fibrils are interlaced and branched. Collagen has been reviewed by Gallop and Seifter (1963). See also the monograph on collagenase and review by Bornstein and Sage (1980).

Collagen fibers with limited crosslinkages (i.e. unaged) will dissolve to some extent in dilute acid or concentrated neutral salt solutions. Natural tendon (aged) collagen is insoluble in aqueous solutions.

Dissolved calf skin collagen in 0.075 M sodium citrate buffer, pH 4.3-4.5 (approx. 6 mg collagen/ml) can be repeatedly transformed into a stable gel on titration to pH 7.0 with 0.5 M sodium carbonate and by warming to 37°C. Such gels, cast as membranes or solids, are of interest; as for example, their use as support of immobilized enzymes. See Venkatasubramanian *et al*. (1974) and Wang and Vieth (1973).

Soluble collagen is also of importance in platelet aggregation assays (Swann *et al*., 1974; Mustard *et al*., 1973; Packman and Guccione, 1973; Puett and Cunningham, 1973; Jamieson *et al*., 1971; and Nakanishi *et al*., 1971). Worthington soluble calf skin collagen has been found to be suitable for this assay. It may be used directly or diluted with 0.9% saline.

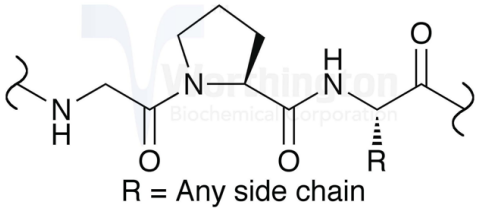

Figure 29.1: Composition: Predominant repeating sequences -Gly-Pro-X-

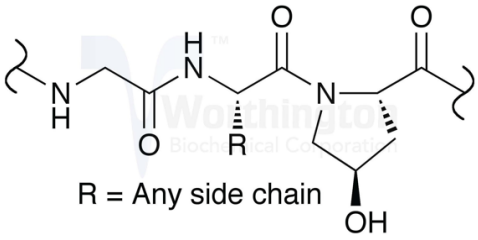

Figure 29.2: Composition: Predominant repeating sequences -Gly-Y-Hyp-

CAS

- 9007-34-5

Protein Accession Number

- P02453 (alpha-1(I) chain) & P02465 (alpha-2(I) chain)

Stability/Storage

- Bovine achilles tendon collagen is stable. Soluble calf skin collagen is stable for 3 - 6 months at 2 - 8°C

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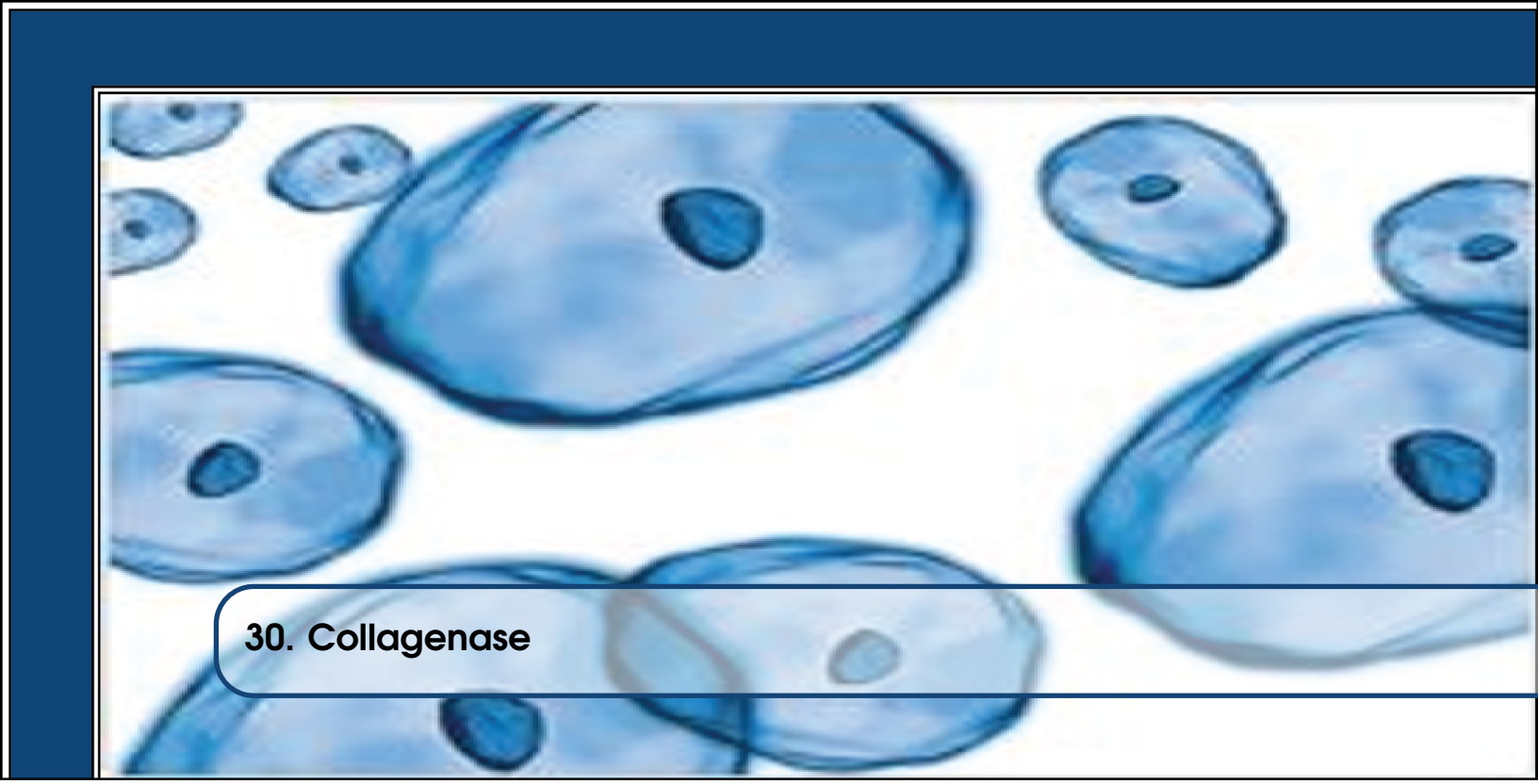

30. Collagenase

Collagenases are endopeptidases that digest native collagen in the triple helix region. Collagens are the major fibrous component of animal extracellular connective tissue. Bacterial collagenases differ from vertebrate collagenases in that they exhibit broader substrate specificity (Peterkofsky 1982, Birkedal-Hansen 1987). Unlike animal collagenases that split collagen in its native triple-helical conformation (Woolley *et al.* 1975, Gross *et al.* 1974), bacterial collagenase is unique because it can degrade both water-insoluble native collagens and water-soluble denatured ones. It can attack almost all collagen types, and is able to make multiple cleavages within triple helical regions (Mookhtiar and Van Wart 1992).

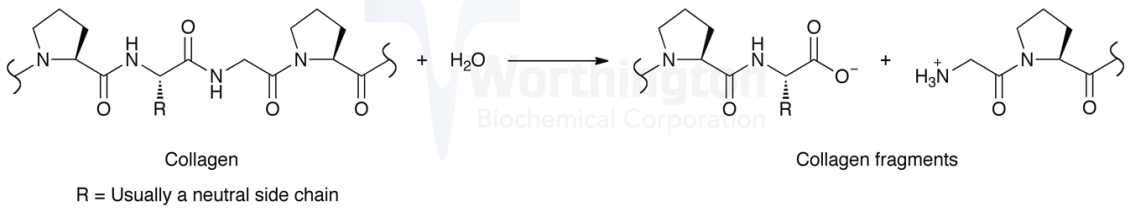

Figure 30.1: Enzymatic Reaction - Collagenase

History

Much of what is presently known about the characteristics of clostridial collagenase comes from the pioneering studies in the 1950s by Mandl, Seifter, Harper and their associates, and the later classification work of Van Wart and Bond (Mandl *et al.* 1953, Mandl *et al.* 1958, Seifter *et al.* 1959, Harper *et al.*

1965, Bond and Van Wart 1984).

In 1959, the first commercially available collagenase isolated from *Clostridium histolyticum* was offered by Worthington. At that time, only one preparation of crude enzyme was offered.

After collagenases from *Clostridium histolyticum* were first prepared by Mandl *et al*. (1953), studies from the late 1950s to the mid 1980s found that several separable collagenases exist and these fractions' specificities and stabilities were partially characterized. (Grant and Alburn 1959, Mandl *et al*. 1964, Yoshida and Noda 1965, Kono 1968, Seifter and Harper 1970, Harper and Kang 1970, Lwebuga-Mukusa *et al*. 1976, Bond and Van Wart 1984). The molecular weights of these seven collagenases were found to range from 68 kDa to 130 kDa, and were classified as either Class I or Class II, based on a variety of properties (Bond and Van Wart 1984). These classes were found to differ with respect to their activities, stabilities, and amino acid composition, but they share many similarities.

Until 1962, most interest in collagenases centered on clostridial collagenases. In that year, Gross and Lapiere obtained evidence for a collagenase in bullfrog tadpole tissue culture media (Gross 1962, Harper 1980), which was the discovery of the first vertebrate collagenase. Subsequent to this discovery, a large number of collagenases were found in marine life, other bacteria (Schoellmann and Fisher 1966, Welton and Woods 1973 and 1975, Keil *et al*. 1975), amphibians, and mammals.

Further studies of collagenases from human and other mammalian sources were reported on, and continue to be actively studied in order to better understand the pathology and treatment of human diseases. Of particular interest is the relationship between collagenase and rheumatoid arthritis (Abe and Nagai, 1973, Kruze and Wojtecka 1972, Bauer *et al*. 1971, Evanson *et al*. 1968), metastasis (Harris *et al*. 1972, McCroskery *et al*. 1973 and 1975, Alaho *et al*. 2005), wound debriding (Hoover 1959), herniated disc treatment (Sussman 1968), angiogenesis, tissue repair, and cirrhosis.

Molecular Characteristics

Collagenase is produced by two separate and distinct genes in *Clostridium histolyticum*. Both genes have been cloned and sequenced (Yoshihara 1994). The colG gene codes for type I collagenase, a 936 amino acid peptide. The colH gene codes for type II collagenase, a 1021 amino acid peptide. These genes share 72% identity, and the proteins share 43% identity. Both gene products can be present as two or more isoforms differing in molecular weight. Crude collagenase mixtures can therefore contain six to eight different molecular weight species ranging from 68 to 130 kDa. Substrate specificity studies have demonstrated that the colG gene prefers natural substrates such as intact collagen, compared to the colH gene product. Conversely, the colH gene product preferentially acts on short synthetic substrates (FALGPA) relative to the colG gene product (Eckhard *et al*. 2009 and Matsushita 1999).

Worthington Collagenases:

Type AFA: Derived from cultures grown in medium completely devoid of animal based components and designed for bioprocessing applications where introduction of potential animal derived pathogens must be prevented. Levels of secondary proteases are similar to Types 1 and 2.

Type 1: Contains average amounts of assayed activities (collagenase, caseinase, clostripain, and tryptic

activities). It is generally recommended for fat, adrenal, and liver cells.

Type 2: Contains greater clostripain activity. It is generally used for heart, bone, muscle, thyroid, cartilage, and liver cells.

Type 3: Contains low proteolytic activity. It is usually used for mammary and fetal cells.

Type 4: Contains low tryptic activity. It is commonly used for islets and other applications where receptor integrity is crucial.

CLSPA: Chromatographically purified collagenase, which is specifically purified to reduce the caseinase activity and provide high specific activity. It is typically used for pancreatic and parotoid acini isolations and collagen structural analysis.

Crude preparations contain not only several collagenases but also a sulfhydryl protease, clostripain (Mitchell 1968), a trypsin-like enzyme (Peterkofsky and Diegelmann 1971, Sparrow and McQuade 1973), and an aminopeptidase (Kessler and Yaron 1973). Sugasawara and Harper (1984) and Bond and Van Wart (1984) report on purification of the collagenases of Cl. histolyticum.

Specificity

The hyper-reactive sites where the Class I and Class II enzymes initially attack all three collagen types were identified by French *et al*. (1992). The cleavage sites are all at Yaa-Gly bonds in the repeating Gly-X-Y collagen sequence (Barrett 1998). Clostridial collagenases' ability to digest native, triple-helical types I, II, and III collagens into a mixture of small peptides is its primary distinguishing factor. This is accomplished by making multiple scissions in the triple helix. Digestion is completed by hydrolyzing those fragments into a mixture of small peptides. Conversely, vertebrate collagenases initiate collagenolysis by making a single scission across all three alpha chains after which attack on those alpha chains is very limited. Gelatinases and other proteases then carry out collagenolysis only after denaturing of the triple helix (Barrett 1998).

Composition

Clostridium collagenases represent unusually large metalloproteases, a family of proteases that shares a zinc-containing motif at the center of the active site (Gonzales and Robert-Baudouy 1996).

IUB

- 3.4.24.3

CAS

- 9001-12-1

Protein Accession Number

- Q9X721 (ColG, Theoretical)

- Q46085 (ColH, Theoretical)

CATH: Classification (v. 3.3.0)

Class:
- Mainly beta

Architecture:
- Sandwich

Topology:
- Jelly rolls

Molecular Weight

- 68-130 kDa

Optimal pH

- 6.3-8.5 (Soru and Zaharia 1972)

Isoelectric Point

- 5.63 (ColG, Theoretical)
- 5.58 (ColH, Theoretical)

Extinction Coefficient

- 159,970 $\frac{l}{cm}\frac{1}{M}$ (ColG, Theoretical)
- 159,130 $\frac{l}{cm}\frac{l}{M}$ (ColH, Theoretical)
- $E^{1\%}_{280}$ = 13.20 (ColG, Theoretical)
- $E^{1\%}_{280}$ = 13.40 (ColH, Theoretical)

Activators

- Ca^{2+}
- Zn^{2+}

Inhibitors

- EDTA, EGTA
- Cysteine, histidine
- DTT
- 2-mercaptoethanol
- o-phenanthroline
- Hg^{2+}, Pb^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}
- Not inhibited by DFP or serum (White and White 1997)

- Collagenase is also inhibited by α2-macroglobulin, a large plasma glycoprotein (Werb *et al.* 1974). Nagase *et al.* (1983) and Stricklin and Welgus (1983) report on natural collagenase inhibitors. The human skin enzyme is inhibited by human serum (Eisen *et al.* 1970), but granulocyte collagenase is not (Lazarus *et al.* 1968). Human serum contains α2-macroglobulin and α1-antitrypsin that may inhibit certain collagenases as well as a third inhibitor reported by Woolley *et al.* (1975). It has also been shown that collagenase A is photo-inactivated in the presence of methylene blue (Takahashi and Seifter 1970 and Karakiulakis 1991).

Applications

- Isolation of adipocytes, hepatocytes, and cells from lung, epithelium and adrenal tissue
- Isolation of cardiomyocytes and cells from bone, cartilage, muscle, thyroid, and endothelium
- Isolation of mammary and various other soft tissues
- Isolation of human and porcine pancreatic islet cells (Kin 2007)
- Treatment of tissues with crude collagenase, with its mixture of proteolytic activities, provides gentle, selective digestion of the intercellular matrix with little damage to cells or loss of viability
- AFA collagenase is suitable for applications needing to avoid introduction of animal derived pathogens into bioprocessing procedures
- NOTE: For collagen structural and biosynthetic studies researchers generally use more highly purified collagenase preparations free of other proteolytic activities.

Assay Information

Assay methods utilizing labelled collagen have been reported by Gisslow and McBride (1975), Robertson *et al.* (1972) and Sakamoto *et al.* (1972). Since true collagenase attacks the helical region of the molecule, change in optical rotary dispersion reflects collagen degradation (Keil *et al.* 1975). Worthington collagenase products CLS and CLSPA are assayed as described below:

Method

- A modification of the procedure of Mandl et al. (1953). Collagenase is incubated for 5 hours with native collagen. The extent of collagen breakdown is determined using the Moore and Stein (1948) colorimetric ninhydrin method. Amino acids liberated are expressed as micromoles leucine per milligram collagenase. One unit equals one micromole of L-leucine equivalents from collagen in 5 hours at 37°C and pH 7.5 under the specified conditions.

Reagents

- 0.05 M TES [tris(hydroxymethyl)-methyl-2-aminoethane sulfonate] buffer with 0.36 mM calcium chloride, pH 7.5
- 4% Ninhydrin in methyl cellosolve with 7.1 mM stannous chloride
- 0.2 M Sodium citrate, pH 5.0
- Ninhydrin-citric acid mixture: Prepare by mixing 50 ml of the 4% ninhydrin in methyl cellosolve containing 7.1 mM stannous chloride with 50 ml of 0.2 M citrate pH 5.0. Allow mixture to stir for 5 minutes.
- 50% n-Propanol
- Substrate: Worthington bovine achilles tendon collagen (Code: CL) and vitamin free casein

- 50% (w/v) Trichloroacetic acid

Enzyme

- Dissolve enzyme at a concentration of 1 $\frac{mg}{ml}$ in 0.05 M TES with 0.36 mM calcium chloride, pH 7.5. Dilutions run are 1/10 and 1/20 in the above buffer.

Procedure

Weigh 25 mg of Worthington bovine collagen into each of four test tubes. Include at least two tubes to serve as blanks which will contain no enzyme. Add 5.0 ml of 0.05 M TES buffer to the tubes and incubate at 37°C for 15 minutes. Start the reaction by adding 0.1 ml of enzyme dilution to appropriate tubes.

After 5 hours, stop the collagenase reaction by transferring 0.2 ml of solution (leaving behind the collagen) to test tubes containing 1.0 ml of ninhydrin-citric acid mixture. Include an enzyme blank (collagen incubated with 0.1 ml TES buffer in place of enzyme). Heat for 20 minutes in a boiling water bath. After cooling, dilute with 5 ml of 50% n-propanol. Let stand for 15 minutes and read absorbance at 600 nm. From an L-leucine standard curve determine micromoles amino acid equivalent to leucine liberated.

Non-specific protease activity (i.e. caseinase activity) is determined using the above assay and substituting 25 milligrams vitamin free casein for collagen. The reaction is stopped after 5 hours by the addition of 0.5 ml of 50% trichloroacetic acid. After centrifugation, 0.2 ml of the supernatant is transferred to 1.0 ml of ninhydrin and treated as above. Caseinase activity is calculated as collagenase activity.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\text{micromoles L - leucine equivalents liberated}}{\text{mg enzyme in digestion mixture}}$
- $\text{Factor} = \frac{1}{\frac{A_{320nm}}{\mu g} \text{ x } 466 \frac{\mu g}{\mu mol}}$
- $\frac{\text{Units}}{\text{mg}} = \frac{\text{Net } A_{320} \text{ x dilution x Factor}}{0.1 \text{ x } 15 \text{ x } \frac{mg}{ml} \text{ in stock}}$

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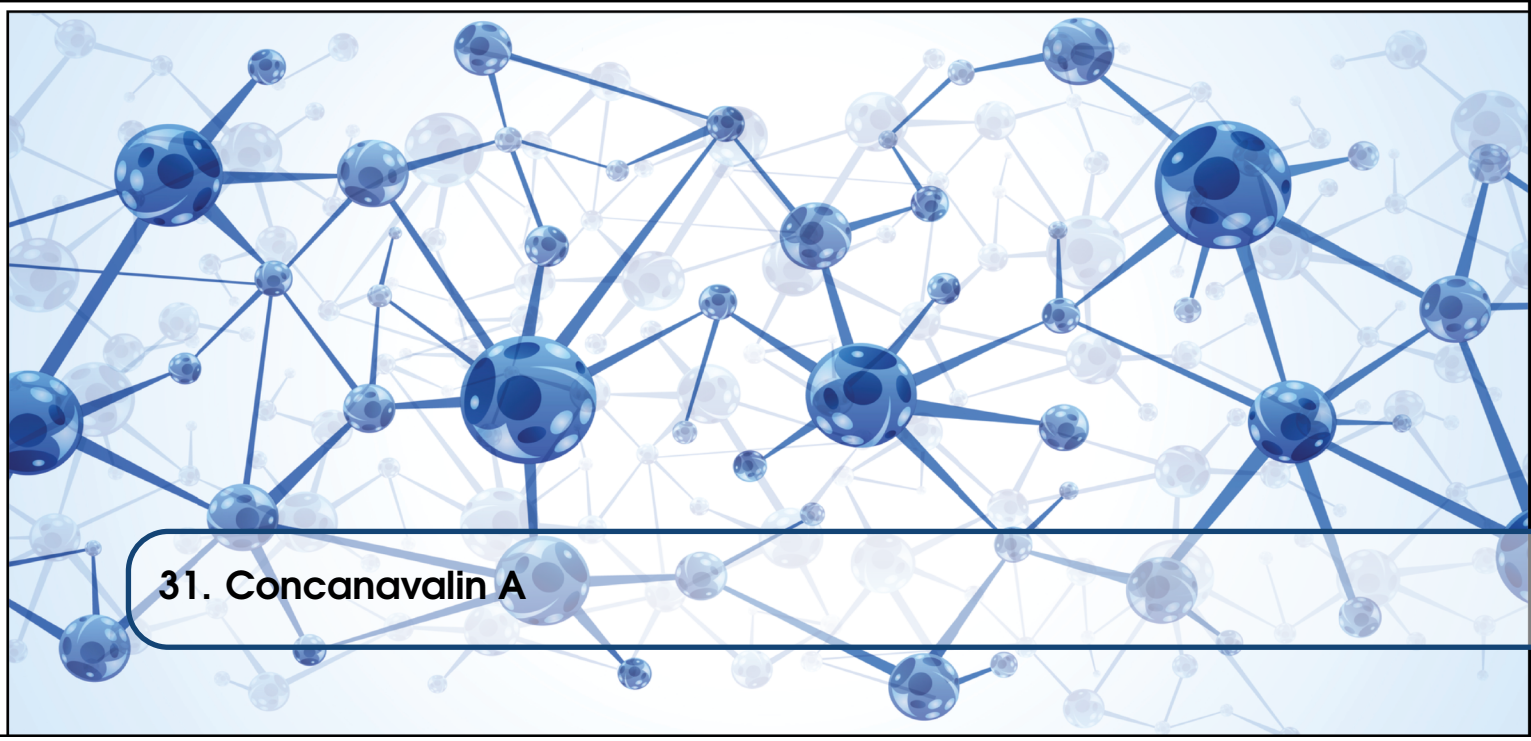

31. Concanavalin A

First crystallized by Sumner and Howell (1936), Concanavalin A (ConA) has proven to be an interesting and useful lectin. Lectins are proteins that react with specific terminal sugar residues and are useful probes in studying carbohydrates of cell surfaces. See review by Sharon and Lis (1972). The properties of ConA have been extensively dealt with in the published proceedings of an International Symposium on ConA (Concanavalin A, T.K. Chowdhury and A.K. Weiss, eds., Plenum Press, New York, 1975).

Characteristics of Concanavalin A from Jack Bean:

The molecular structure of ConA has been reported by Becker *et al.* (1976), Becker *et al.* (1975), Cunningham *et al.* (1975), Reeke *et al.* (1975) and Wang *et al.* (1975). It is composed of identical subunits of 237 amino acid residues (M.W.: 26,000) with no covalently bound carbohydrate or other prosthetic group. Olson and Liener (1967) report no cystine residues. See also Edmondson *et al.* (1971). According to McKenzie *et al.* (1972), at pH 4.5-5.6 (I=0.1), ConA exists as a single dimer (M.W.: 53,000). Above pH 7 it is predominantly tetrameric (Wang *et al.* 1975). Its optimal activity is near pH 7.

ConA binds two metal ions per monomer: a transition metal, nominally Mn^{2+} (See Becker *et al.* 1975) at site S1 and Ca^{2+} at S2. Both must be present for saccharide binding. (Grimaldi and Sykes 1975; Jack *et al.* 1971; Poretz and Goldstein 1970; Greer *et al.* 1970; Agrawal *et al.* 1968; Goldstein *et al.* 1965). See also Barber and Carver (1975) and Barber *et al.* (1975). The transitional metal site has been reported on by Sherry *et al.* (1975).

ConA reacts with non-reducing α-D-glucose and α-D-mannose - it is the ring form that participates in the reaction. Smith and Goldstein (1967) report on the stereochemical requirements of the active sites. α-methyl-D-glucopyranoside acts as a competitive inhibitor. Goldstein *et al.* (1965) report on simple sugar inhibition and indicate unmodified OH groups at C-3, C-4 and C-6 are essential for binding.

Magnetic resonance studies have been reported by Barber *et al*. (1975) and Grimaldi and Sykes (1975). Luminescent properties are similar to other tyrosine and tryptophane containing proteins (Miller and Nwokedi 1975). Beppu *et al*. (1975) report on labelled ConA.

As indicated above, the usefulness of ConA lies in its specific binding action with certain carbohydrate-containing receptors. It agglutinates red blood cells and complexes with blood group substances (Clark and Denborough 1971) and immunoglobulin glycopeptides (Kornfeld and Ferris 1975) and carcinoembryonic antigens (Brattain *et al*. 1975; Boenisch and Norgaard-Pedersen 1975). See also Pitlick (1975). Harmony and Cordes (1975) report on its interaction with human plasma low density lipoprotein.

ConA exhibits mitogenic activity with lymphocytes (Ruscetti and Chervenick 1975; Novogrodsky and Katchalski 1971; Perlmann *et al*. 1970). Bessler and Lipps (1976) report its effect on protozoan nucleus.

Cancer cells are readily aggregated by ConA; normal cells are not (Shoham *et al*. 1970; Inbar and Sachs 1969). See also Ben-Bassat and Goldblum (1975) and Kolata (1975). Moscona (1971) reports that embryonic cells are also aggregatable. Normal cells react after proteolytic treatment. Nicholson (1972) suggests that trypsinization causes clustering of the membrane ConA sites. On the other hand, Burger and Noonan (1970) report that ConA treated with trypsin can restore growth patterns of transformed fibroblasts to normal.

Studies of ConA with particular cell types include locust muscle fibers, Mathers and Usherwood (1976); lymphocytes, Ben-Bassat and Goldblum (1975), Beyer and Bowers (1975), DePetris (1975), Pommier *et al*. (1975); fibroblasts, Huet and Bernadac (1975); adipocytes, Katzen and Soderman (1975); rat liver plasma membrane components, Berzins and Blomberg (1975). Sutou and Shindo (1975) report that ConA induces endoreduplication in mammalian cells and Kubota and Kanatani (1975) indicate that it induces oocyte maturation-inducing substance in starfish follicle cells. ConA reaction with *Escherichia coli* has been reported by Picken and Beachman (1975); that with Dictyostelium discoideum by Weeks (1975); and that with B. substilis by Birdsell and Doyle (1973). Brunson and Watson (1975) report on activity with lipopolysaccharide.

Immobilized specific lectins are useful for purifying glycoprotein (Avrameas and Guilbert 1971; Allan *et al*. 1972; and Anderson and Lee-Own 1974) and removing contaminants, (Edelman 1975). Wood and Sarinana (1975) report on use of ConA in studying nerve glycoproteins.

CAS

- 11028-71-0

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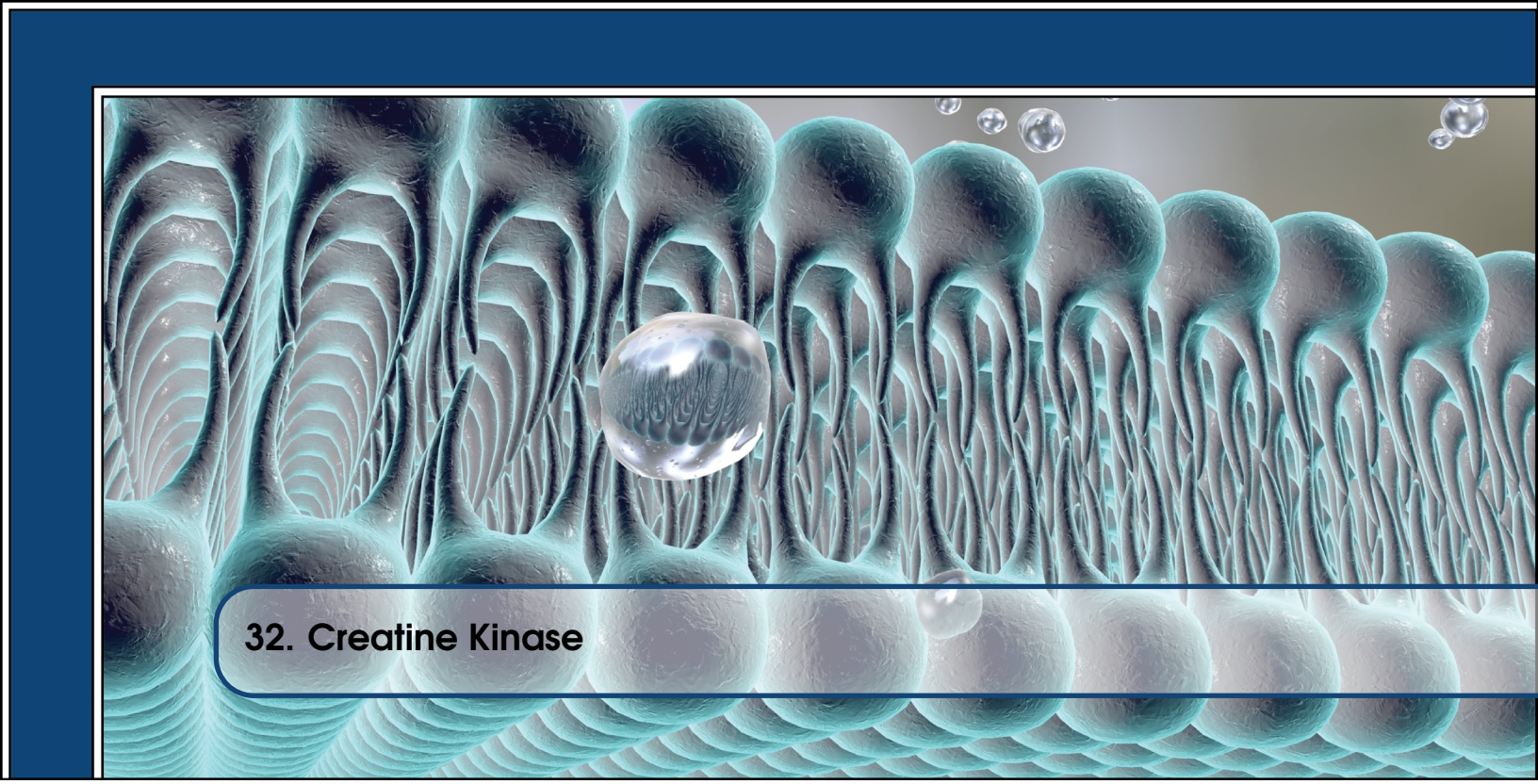

32. Creatine Kinase

ATP: Creatine N-phosphotransferase

Creatine kinase (CK) is widely distributed and seems to be primarily concerned with ATP regeneration. According to Watts (1973) CK may represent 10-20% (w/v) of muscle cytoplasmic protein.

The enzyme is dimeric and exists as three isozymes: MM (muscle), MB (hybrid), and BB (brain). Names indicate major tissue of origin. Since the MB isozyme has its highest concentration in heart muscle, its level in the serum has diagnostic value.

Most investigational work has been on the soluble enzyme from rabbit muscle. (There is also an insoluble CK associated with mitochondria). Creatine kinase catalyzes the following reaction:

$$\text{ATP} + \text{creatine} \rightleftarrows \text{ADP} + \text{phosphocreatine} \qquad (32.1)$$

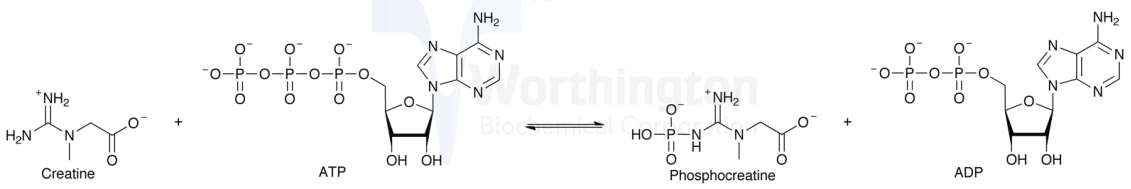

Figure 32.1: Enzymatic Reaction - Creatine Kinase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Rabbit Muscle

Specificity

Besides creatine (N-methylglycocyamine) only N-ethylglycocyamine and glycocyamine act as phosphoryl acceptors. Creatinine, D- and L-arginine, histidine, and taurocyamine are inactive (Kuby and Noltmann 1962).

Composition

The enzyme is dimeric, its identical subunits will combine with others from different species to form active hybrids (Dawson *et al*. 1967). The amino acid composition has been determined (Watts 1973). Active center studies have been reported by Borders and Riordan (1975); James and Cohn (1974) and Buechter *et al*. (1992). Smith and Kenyon (1974) question whether the "active" SH group of CK is, in fact, essential. See also McLaughlin (1974) and McLaughlin *et al*. (1972) and Roustan *et al*. (1973). O'Sullivan *et al*. (1972) report on ADP analog binding.

IUB

- 2.7.3.2

Molecular Weight

- 81,000 (Kuby and Noltmann 1962)

Extinction Coefficient

- $E_{280}^{1\%}$ = 8.76 (Kuby *et al*. 1954)

Inhibitors

- Various sulfhydryl reagents, chelating agents, some adenosine phosphate compounds, orthophosphate, pyro- and tripolyphosphate, adenosine, Cl^-, SO_4^{2-}, acetate (slight), and other compounds such as dibenamine, phenothiazone, and 3,5-dinitro-o-cresol. ADP strongly inhibits the forward reaction competitively with respect to ATP and noncompetitively with respect to creatine (Kuby and Noltmann 1962). Creatinine phosphate acts as competitive inhibitor with respect to phosphocreatine (Gercken and Dö̈ring 1974).

Stability/Storage

- If kept dry, the refrigerated lyophilized preparation is stable for 6-12 months.

Activity

- Engelborghs *et al*. (1975) have reported on initial product formation, and Gercken and D*ö*ring (1974) show that creatinine phosphate is not a substrate but is a competitive inhibitor to creatine phosphate. Various divalent cations such Mg^{2+}, Ca^{2+}, and Mn^{2+} activate the enzyme.

Stabilizers

- Reducing agents such as cysteine and thioglycolate.

Assay Information

Alternate assay methods are described by Dinovo *et al*. (1973).

Method

- Creatine kinase activity is determined in a coupled enzyme system utilizing pyruvate kinase (PK) and lactate dehydrogenase (LDH). The following procedure is essentially that described by Tanzer and Gilvarg (1959). One Unit is defined as the conversion of one micromole of creatine to creatine phosphate per minute at 25°C and pH 8.9 under the specified conditions.

Reagents

- Buffered Creatine: 0.40 M Glycine containing 53.2 mM creatine and 62 mM potassium carbonate. Adjust the pH to 8.9 with NaOH.
- Enzyme diluent: Freshly prepared 5 mM glycine pH 9.0

Reagent solution – the required amount of solution should be prepared containing:

- ATP - 8.5 mM
- NADH - 1.22 mM
- PEP - 2.0 mM
- LDH - 15.0 $\frac{\text{units}}{\text{ml}}$
- PK - 7.0 $\frac{\text{units}}{\text{ml}}$
- $MgSO_4$ - 28.0 mM
- Glutathione (reduced) - 26.0 mM
- pH adjusted to - 7.4

Enzyme

- Prepare $\frac{\text{mg}}{\text{ml}}$ in 5 mM glycine, pH 9.0.
- Dilute to 0.1-10 $\frac{\mu\text{g}}{\text{ml}}$ in glycine buffer.

Procedure

Spectrophotometer Settings: Wavelength: 340 nm Temperature: 25°C

Pipette into each cuvette as follows:

- Reagent Solution - 0.7 ml
- Buffered Creatine - 2.2 ml

Incubate in spectrophotometer at 25°C for 3-5 minutes to achieve temperature equilibrium and establish blank rate, if any. Add 0.1 ml diluted enzyme and record decrease in A_{340} for 5-8 minutes. An initial lag period may occur. Determine ΔA_{340} from linear portion of the curve.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{340}}{\text{min}}}{6.2 \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$$

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33. Cytochrome C Oxidase

Cytochrome c oxidase (EC 1.9.3.1), the terminal enzyme in the respiratory chain, catalyzes the four-electron reduction of molecular oxygen and couples this reaction to the generation of a proton gradient across the mitochondrial inner membrane.

The enzyme activity is associated with a protein lipid complex containing two dissimilar heme-copper catalytic centers (heme Aa + CuA and heme Aa3 + CuB) combined with a number of other subunits. Some of the subunits are synthesized in the mitochondria while others are synthesized in the cytosol and imported into the mitochondria. The roles of the various subunits and the question of which subunits are essential to activity have received much attention.

One Unit oxidizes one micromole of ferrocytochrome c per minute at 25°C, pH 6.8.

IUB

- 1.9.3.1

CAS

- 9001-16-5

Stability/Storage

- Stable as supplied for 12 months stored at -20°C. Dilutions at 0.05mg BCA protein per milliliter in 0.05M potassium phosphate, pH 6.8 containing 0.1% laurylmaltoside are stable for 24hr. stored at 2 - 8°C but significant losses occur at 25°C. The enzyme shows poor stability when diluted in water.

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34. Deoxyribonuclease I

Bovine pancreatic deoxyribonuclease is an endonuclease that preferentially splits phosphodiester linkages adjacent to a pyrimidine nucleotide, yielding 5'-phosphate terminated polynucleotides with a free hydroxyl group at the 3' position. DNase I is secreted by exocrine glands, and found most abundantly in the pancreas and parotid. It is also present in lower quantities in other tissues (Chen and Liao 2006, and Nadano *et al*. 1993).

DNase is known to be involved in apoptosis and has been proposed to play a role in the regulation of actin polymerization in cells. In addition to its use in molecular biology, DNase I has been used as a treatment for cystic fibrosis, and systemic lupus erythematosus (Chen and Liao 2006).

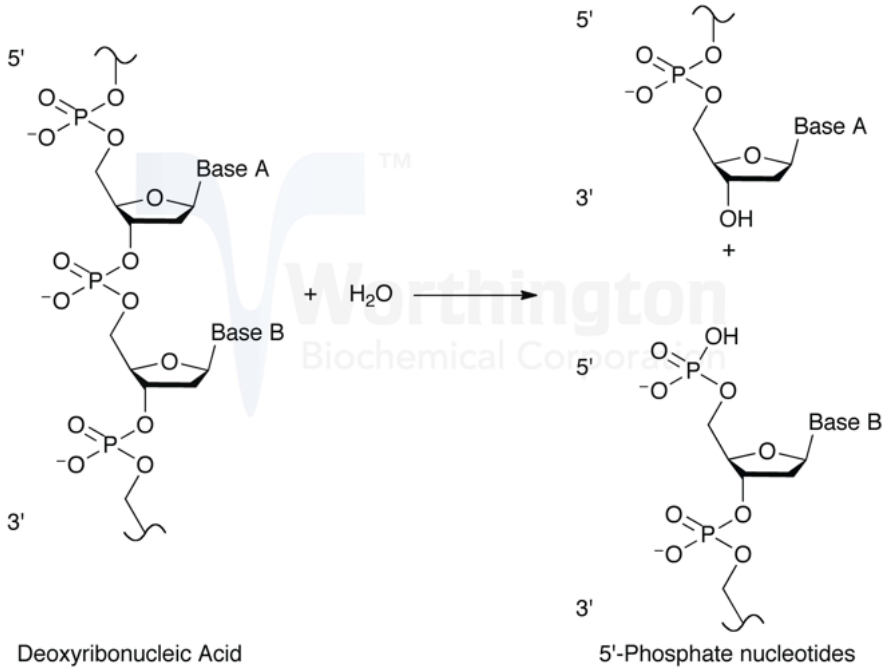

Figure 34.1: Enzymatic Reaction - Deoxyribonuclease I

History

In 1903, Araki liquefied gels of alpha-nucleic acid with extracts of liver, spleen, and thymus tissue (Araki 1903, and Kunitz 1950). Plenge also showed similar activities in microorganisms (Plenge 1903). In 1913, De la Blanchardire developed an assay for the liquification of thymus DNA gel, and showed it was not accompanied by the destruction of DNA bases (Kunitz 1950).

Over the next few decades various authors described enzymes that cleaved nucleotides with names such as nucleinase (Levene and Medigreceanu 1911), nucleogelase (Feulgen 1923), polynucleotidase (Levene and Dillon 1932), desoxyribonucleodepolymerase (Greenstein 1943), thymonucleodepolymerase (Laskowski 1946), and desoxyribonuclease (McCarty 1946). In 1950, Kunitz first crystallized and coined today's name, deoxyribonuclease.

In the early 1970s, Salnikow *et al*. found DNase I was present in four isoforms distinguishable by their sialic acid contents (Salnikow *et al*. 1970). Soon after, the polypeptide chain structure was elucidated (Salnikow *et al*. 1973b, Liao *et al*. 1973, and Catley 1973). The crystal structure was determined at a 2.0 resolution by Suck and Oefner in 1986 and later refined (Oefner and Suck 1986).

In 1990, the gene coding for Bovine pancreas DNase I (bpDNase I) was constructed from synthetic oligonucleotides, and then cloned and expressed in *Escherichia coli* (Worrall and Connolly 1990). In 1992, the X-ray structure of the bpDNaseI-d(GGTATACC)2 complex was obtained at a 2.3 resolution (Weston *et al*. 1992). In 1998, the bpDNaseI gene was cloned from bp-cDNA and expressed in *Escherichia coli* (Chen *et al*. 1998).

Today, researchers continue to investigate the residues involved in substrate binding and catalytic activity (Chen *et al*. 2008), novel inhibitors (Chen and Liao 2008), the specificity of DNAse I for specific DNA sequences (Heddi *et al*. 2010), and the conformational changes that occur in both DNAse I and DNA upon protein-DNA complexation (N'soukpo*é*-Kossi *et al*. 2008).

Molecular Characteristics

Similar to human, mouse, and rat, the bovine pancreas DNase I gene consists of nine exons, and only the last eight encode the protein (De Mar*í*a and Arruti 2003). The nascent protein is directed to the secretory pathway organelles by a 22 amino acid signal sequence, encoded by exon two. The primary active site residue, H166, is encoded by exon six (De Mar*í*a and Arruti 2003, and Kraehenbuhl *et al*. 1977). Although exon lengths are almost equivalent in bovine and other mammalian species, the intron lengths vary greatly. A TATA box sequence is located 35 bp upstream of exon I (De Mar*í*a and Arruti 2003). It has been proposed that multiple splicing events that affect the coding sequence may be a mechanism to downregulate DNase I expression (Liu *et al*. 1997).

Specificity

bpDNase I is not base nor sequence specific; however, it does not cleave randomly. It shows preference for cleavage at the 5' side of pyrimidines, and is particularly pronounced in alternative copolymers (Bernardi *et al*. 1975, and Lomonossoff *et al*. 1981). It has been shown that variations in the twist angle are recognized by DNase I (Dickerson and Drew 1981). The specificity of DNase I also depends on the divalent cations present. In the presence of Ca^{2+} and Mg^{2+}, it causes single strand breaks, and in the presence of Mn^{2+} double strand breaks have been reported (Junowicz and Spencer 1973, and Campbell and Jackson 1980).

Composition

bpDNase I exists as many isoforms, which differ both genetically and in sialic acid content (Liao 1974, Liao 1981, and Chang *et al*. 1994). The polypeptide chain is glycosylated at Asn-18 and because of the sialic acids of the carbohydrate side chain, shows charge heterogeneity (Chen and Liao 2006).

The enzyme is particularly stable due to an extended hydrophobic core, which is composed of two tightly-packed six-stranded beta-pleated sheets. These are surrounded by eight helices and several loop regions, which are stabilized by bound calcium atoms. The enzyme is further stabilized by intramolecular hydrogen bonds, salt bridges, and two disulfide bonds (Chen and Liao 2006).

IUB

- 3.1.21.1

CAS

- 9003-98-9

Protein Accession Number

- P00639

CATH: Classification (v. 3.3.0)

Class:
- Alpha Beta

Architecture:
- 4-Layer Sandwich

Topology:
- Deoxyribonuclease I; Chain A

Molecular Weight

- 29.1 kDa

Optimal pH

- 7.8

Isoelectric Point

- 5.08

Extinction Coefficient

- 36,750 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 11.1

Active Residue

- Tyrosine (Y87)
- Glutamate (E100)
- Histidine (H156)

Activators

- Bivalent metal ions (Junowicz and Spencer 1973b, Poulos and Price 1972, Price 1969, and Price 1975)

Inhibitors

- 2-mercaptoethanol (Laskowski 1971)
- 2-nitro-5-thiocyanobenzoic acid (Moore 1981)
- Actin (Mannherz *et al.* 2008, and Lazarides and Lindberg 1974)
- Alfatoxin B2a, G2, G2a, and M1 (non-competitive) (Schabort 1970)

- EGTA (Moore 1981) and EDTA (Junowicz and Spencer 1973)
- Sodium dodecyl sulfate (Liao 1975a)
- Calf spleen inhibitor protein (Laskowski 1971)
- Carbodiimide and cholesterol sulfate (Iwamori 2000)
- Iodoacetate (Moore 1981)

Applications

- DNA removal in primary cell isolation: decreases viscosity providing better yields
- DNA removal in bioprocessing applications
- Removing genomic DNA from RNA preparations prior to RT-PCR
- *in vitro* transcription
- Nick translation
- DNase footprinting
- Actin binding
- UV crosslinking of proteins to nucleic acids
- Radioactive labeling

Assay Information

Method

- That developed by Kunitz (1950) based upon the increased absorbance at 260 nm observed during the depolymerization of DNA by DNase. A unit causes an increase in absorbance at 260 nm of 0.001 per minute per ml when acting upon highly polymerized DNA at 25°C and pH 5.0 under the specified conditions. A standard enzyme preparation should be run in parallel with an unknown because standardization of DNA preparations and their degree of polymerization in solution is not possible.

Reagents

- 1.0 M Acetate buffer, pH 5.0
- 6.25 mM Magnesium sulfate in reagent grade water
- Worthington Standard DNase Vial (Code:DSV) containing a defined activity of approximately 2000 DNase units per vial.
- Worthington Highly Polymerized DNA (Code: DNA). Dissolve 10 mg DNA in 200 ml of 6.25 mM magnesium sulfate. Let stand overnight at room temperature. Add 25 ml of 1.0 M acetate buffer, pH 5.0 and dilute to a final volume of 250 ml with reagent grade water. (Substrate solution may be prepared in larger batches and stored for 2-3 weeks at0 - 4°C.)

Enzyme

- Note: Pancreatic deoxyribonuclease is unusually sensitive to physical denaturation by shaking. Mixing should be done by gentle inversion. Dissolve the standard vial in 1.0 ml of reagent grade water. Care must be taken when opening the vial that no lyophilized material is lost. This solution will contain the number of $\frac{\text{units}}{\text{ml}}$ as stated on the label. Dilute further to a concentration of 20-60 $\frac{\text{units}}{\text{ml}}$. All dilutions are made in reagent grade water.

- Sample to be assayed: Dissolve at a concentration of 1 $\frac{mg}{ml}$. Dilute further to a concentration of 20-60 $\frac{units}{ml}$ immediately before the assay.

Procedure

Spectrophotometer Settings: Wavelength: 260 nm Temperature: 25°C

Pipette 2.5 ml of substrate into cuvettes and incubate in spectrophotometer at 25°C for 3-4 minutes to establish blank rate if any, and to reach temperature equilibration. Add 0.5 ml of diluted standard and record A_{260} for 8 - 10 minutes. Calculate $\geq \frac{A_{260}}{min}$ from linear portion of curve following a brief lag. Note: The change in A_{260} for this assay is not generally linear from the initial time and is linear for only short periods. The most linear portion should be used in determining the activity. A rate of 0.008 - 0.018 $\geq \frac{Alpha}{min}$. is recommended.

Calculate the factor for the standard vial.

$$\text{Factor} = \frac{\text{activity of standard as stated on the label}}{\frac{\Delta A_{260}}{min} \text{ x dilution}}$$

Using the diluted sample to be tested, repeat the above procedure. Record the $\geq \frac{A_{260}}{min}$ from the linear portion of the curve.

Calculation

- $\frac{Units}{mg} = \frac{\Delta A_{260}}{min}$ x dilution x factor

Activity is compared to that of the standard vial. Deoxyribonuclease activity can also be conveniently measured in a radial diffusion assay system.

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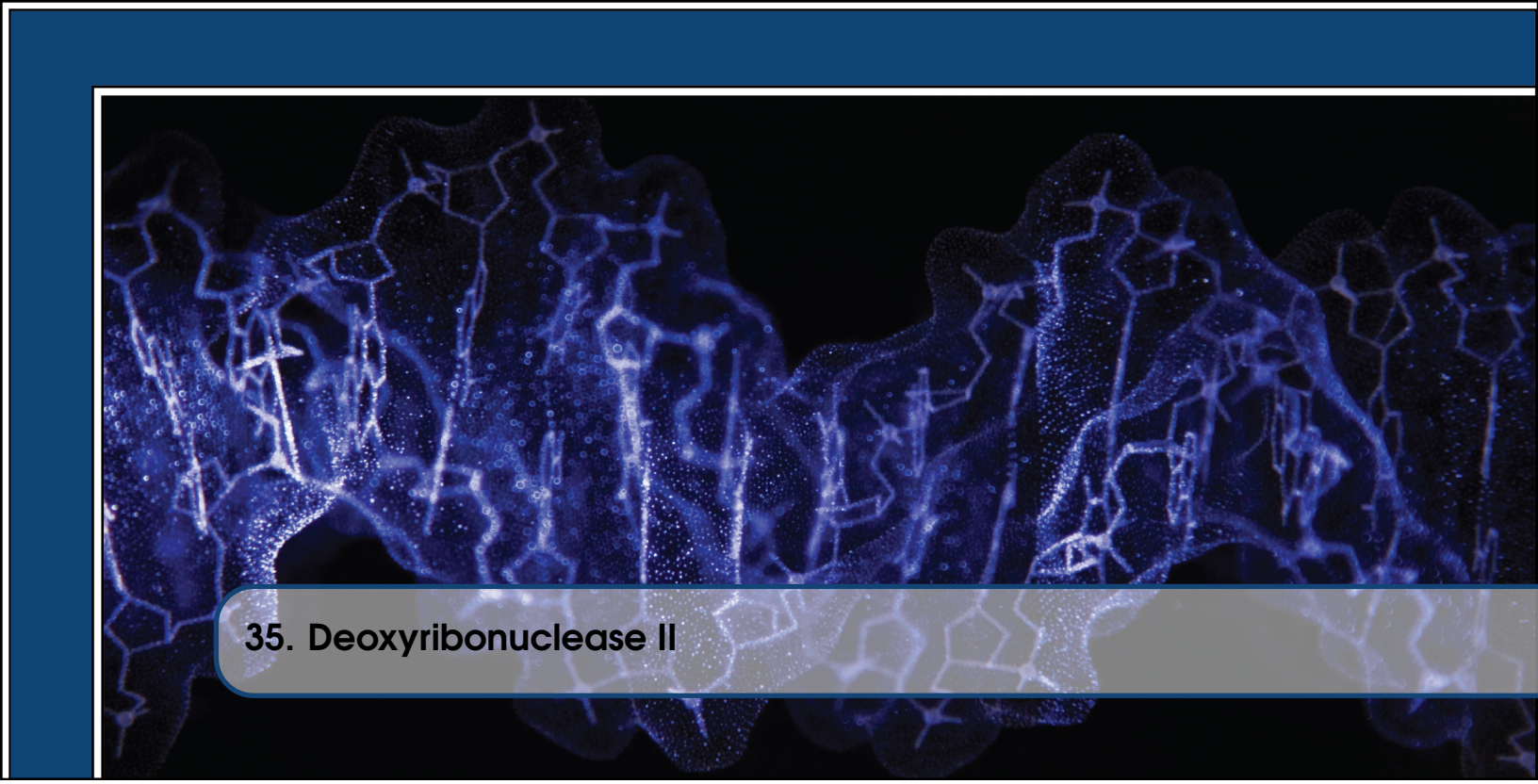

35. Deoxyribonuclease II

Porcine DNase II (pDNase II) is an acid endonuclease widely found in animal cells, mostly associated with lysosomes. It hydrolyzes the phosphodiester bonds of DNA under acidic conditions without the requirement for divalent metal ions and is important for DNA fragmentation and degradation.

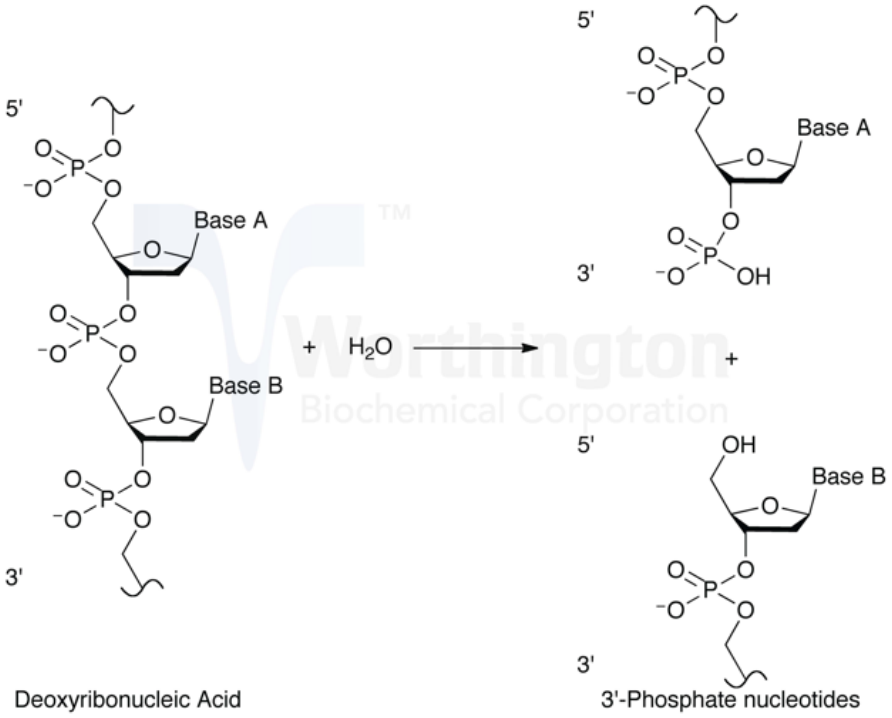

Figure 35.1: Enzymatic Reaction - Deoxyribonuclease II

History

In the late 1940s Catchside and Holmes first observed a mammalian DNase that was optimally active at an acidic pH (pH 4.5-5.5) and termed it "acid DNase" (Evans and Aguilera 2003).

Spleen tissue displays particularly high activity, so it was often referred to as "spleen acid DNase". However, in 1968 Cordonnier and Bernardi found that acid DNase was found to be widely distributed in animal cells, and it was soon suggested that the enzyme be called DNase II to better distinguish it from pancreatic DNase (DNase I) (Cunningham and Laskowski 1953, and Evans and Aguilera 2003).

In the 1970s, Bernardi extensively investigated the inhibitory effects of various compounds on DNase II, finding actinomycin D, N-bromosuccinimide, and divalent cations to be inhibitory (Bernardi 1971). In 1973, Oshima and Price predicted the involvement of a histidine side chain in DNase II catalysis, and in 1985 Liao demonstrated this to be true.

In 1998, Liao first identified the structure of DNase II as a heterodimer, however it was soon shown by Wang *et al*. to be a heterotrimer (Liao *et al*. 1998, Wang *et al*. 1998). Yasuda *et al*. first characterized the human DNase II gene, the first DNase gene to be identified (Yasuda *et al*. 1998). Soon after, Baker *et al*. reported on cloning of the human gene as well (Baker *et al*. 1998).

Recent research has aimed at better understanding the role of DNase II in apoptosis (Kitahara *et al*. 2010) and its relevance to autoimmunity (Ueki *et al*. 2010).

Molecular Characteristics

The genes encoding DNase II proteins have been described by Baker *et al*. 1998, Krieser and Eastman 1998, Shiokawa and Tanuma 1998, and Yasuda *et al*. 1998). The DNase2 gene is conserved in human, chimpanzee, dog, cow, mouse, rat, zebrafish, fruit fly, mosquito, and *C. elegans*. Porcine and human DNase II have been shown to share 73.4% identity (Shiokawa and Tanuma 1998).

The cDNA of pDNase II contains 1292 bases, with an open reading frame that encodes 364 amino acids, including six N-glycosylation sites (Shiokawa and Tanuma 1998).

To form the mature enzyme, a putative transmembrane peptide at the N-terminus is removed during the translation of the nascent protein. After protein folding, further processing within the lysosome removes a peptide at the C-terminus and two connecting peptides between the alpha1 and beta subunits and between beta and alpha2 (Wang *et al*. 1998). The resulting protein is a heterotrimer, as described in the Composition section.

Specificity

Through a nicking mechanism, DNase II generates 3'-phosphate groups by hydrolyzing the phosphodiester linkanges of DNA (Bernardi 1971, Harosh *et al*. 1991, and Baker *et al*. 1998).

A catalytic center, with a critical histidine residue, mediates the cleaveage (Oshima and Price 1973). Specifically, site-directed mutagenesis studies of histidine residues in pDNase II have suggested His115, His132, and His297 play crucial role in the enzyme's function, and show absolute evolutionary conservation (Cheng *et al*. 2006).

The mechanism of DNase II has been shown to occur in three phases. Initially, multiple single strand breaks are induced within the DNA backbone. During the middle phase, acid soluble nucleotides and oligonucletides are produced. A slow, non-linear hyperchromic shift occurs in the last phase (Bernardi 1971).

Composition

It was initially discovered that the mature DNase II enzyme was a non-covalently bound alpha/beta heterodimer. It was proposed that during the production of endogenous pDNase II, the initial 45 kDa translation product is targeted to the lysosome where proteases cleave the translation product to two fragments of 35 kDa and 10 kDa, forming the dimer (Liao *et al*. 1989). However, Wang *et al*. showed that the two polypeptides of the alpha subunit dissociate into alpha1 and alpha2 subunits after disulfide reduction, forming a heterotrimeric structure composed of alpha1, beta, and alpha2 subunits (Wang *et al*. 1998, and Huang *et al*. 2009).

IUB

- 3.1.22.1

CAS

- 9025-64-3

Protein Accession Number

- O62855

Molecular Weight

- 38.0 kDa (Bernardi *et al*. 1965)

Optimal pH

- 5.0 (although activity can be detected over a significant pH range) (Lyon and Aguilera 1997, Lyon *et al*. 2000)
- 4.5-5.0 at ionic strength 0.15 (Bernardi and Griffe 1964)

Isoelectric Point

- 8.10 (Theoretical)
- 10.2 (Bernardi *et al*. 1965)

Extinction Coefficient

- 82,750 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 12.1 (Bernardi *et al*. 1965)

Active Residue

- Histidine (H115, H132, H297) (Cheng *et al*. 2006)

Activators

- Cysteine (Bernardi 1971)
- EDTA (Bernardi 1971)

Inhibitors

- Actinomycin D (Bernardi 1971)
- Iodoacetate (Liao *et al*. 1989)
- N-Bromosuccinimide and H_2O_2 (Bernardi 1971)
- Divalent cations such as zinc and copper (Bernardi 1971)
- Sulfate (Bernardi and Griffe 1964)
- NaCl (above 250 mM) (Ikeda 2002)
- DNA (above 0.4 $\frac{\text{mg}}{\text{ml}}$), rRNA, tRNA (Bernardi 1971)
- Iodoacetamide (slightly inhibitory) (Bernardi 1971)

Applications

- DNA fragmentation
- Apoptosis research
- Molecular weight marker
- Control in antibody reactions

Assay Information

Method

- That developed by Kunitz (1950) based upon the increase in absorbance at 260 nm during the hydrolysis of DNA. The method has been adapted from that used for DNase I by reducing the pH to 4.6 and lowering the magnesium concentration to 0.001 M. One unit causes an increase in absorbance at 260 nm of 0.001 per minute per ml at 25°C and pH 4.6 under specified conditions. Bernardi (1971) discusses the relationship of absorbance units so defined to terminal phosphate formation, i.e., substrate bonds hydrolyzed.

Reagents

- 1.0 M Acetic acid, pH 4.6
- 0.02 M Magnesium sulfate

Substrate

- Dilute 12.5 ml of 0.02 M magnesium sulfate to a volume of 200 ml with reagent grade water. Add 10 mg Worthington calf thymus DNA (Code: DNA). Let stand overnight at room temperature. Add 25 ml of 1.0 M acetic acid, pH 4.6 and adjust the pH to 4.6. Dilute to a final volume of 250 ml with reagent grade water. Store at 2 - 8°C.

Enzyme

- Dissolve enzyme at one $\frac{\text{mg}}{\text{ml}}$ in reagent grade water. Dilute further in water immediately prior to use to a concentration of 50 - 150 $\frac{\text{units}}{\text{ml}}$.

Procedure

Spectrophotometer Settings: Wavelength: 260 nm Temperature: 25°C

Pipette 2.5 ml of substrate into cuvette and incubate in the spectrophotometer for 3 - 4 minutes to establish blank rate, if any, and to reach temperature equilibrium. Add 0.5 ml diluted enzyme. Record increase in A_{260} from linear portion of curve. Note: the change in A_{260} for this assay is not generally linear from the initial time and is linear for only short periods. The change in A_{260} should be determined from the portion which is most linear.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{260}}{\text{min}} \text{ x } 1000 \text{ x } 6}{\text{mg enzyme in reaction}}$

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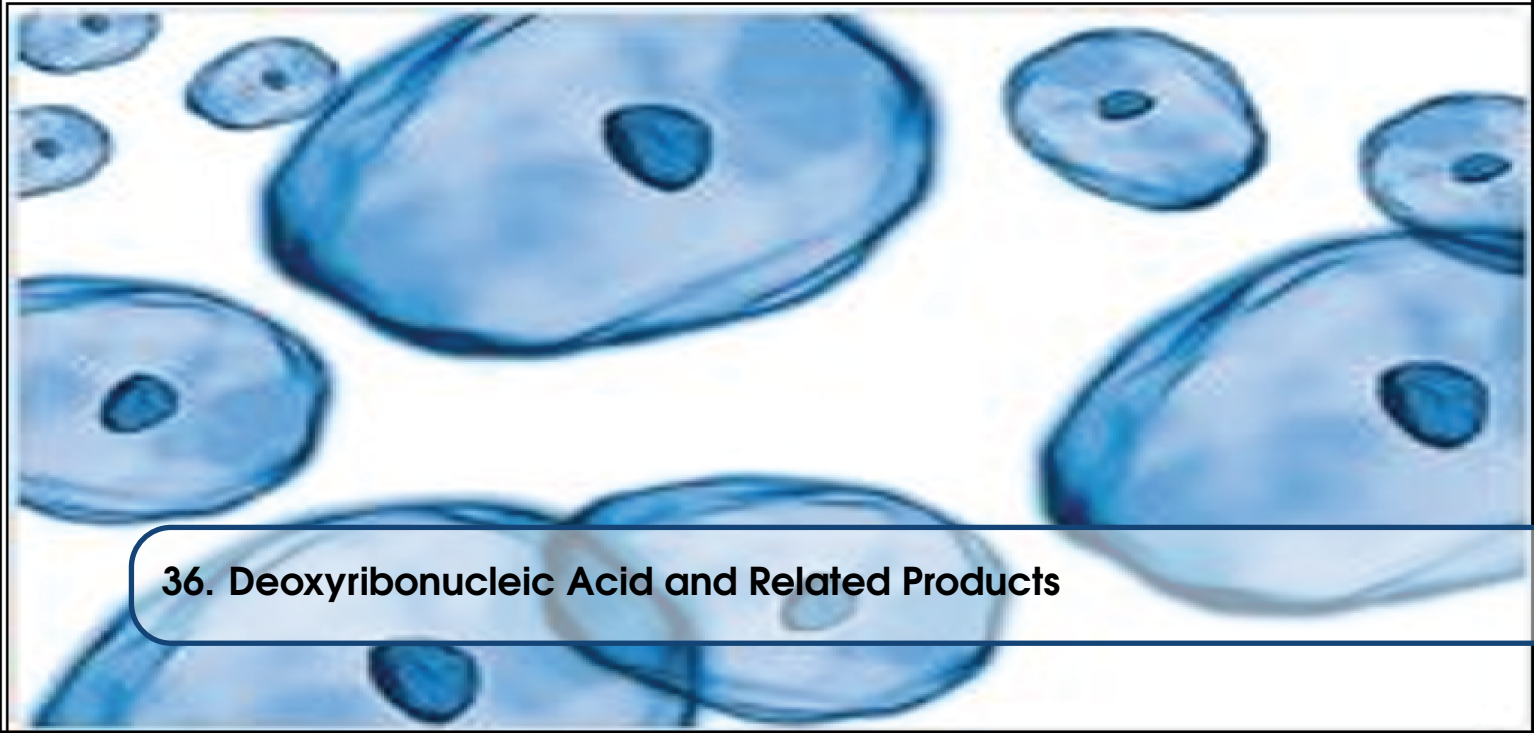

36. Deoxyribonucleic Acid and Related Products

Deoxyribonucleic acid (DNA) is a double stranded, helical nucleic acid molecule that consists of nucleotide monomers, each composed of a deoxyribose sugar, a phosphate group, and one of four nitrogenous bases: adenine, cytosine, guanine, and thymine.

Each chromosome contains one long molecule of DNA, with hundreds or thousands of genes arranged along its length. The DNA molecule is a double helix, with sugar-phosphate bonds on the outside, and base pairs on the inside. The bases pair as such: adenine (A) with thymine (T), and guanine (G) with cytosine (C). DNA replicates semiconservatively and requires an RNA primer. When the parental strands separate, each serves as the template for making a new, complementary strand. *Escherichia coli* DNA replication is semidiscontinuous; one strand is replicated continuously and the other is replicated discontinuously. This discontinuous replication forms Okazaki fragments. *Escherichia coli* proofreading is carried out by DNA polymerase III, which will only use a base-paired nucleotide as a primer, stalling DNA replication if a misincorporation is present. During this stall, 3' to 5' exonuclease of the DNA polymerase III holoenzyme can remove the misincorporated base. Mammalian cells have five polymerases: alpha, beta, delta, epsilon, and gamma. alpha and delta participate in replication of both DNA strands, and beta and epsilon function in DNA repair. gamma is believed to replicate mitochondrial DNA (Weaver 2008).

History

In 1869 Friedrich Miescher discovered a mixture of compounds in the nucleus of a cell. He termed this mixture nuclein (Miescher 1871). The major component of nuclein is DNA. As the chromosome theory of inheritance was gradually accepted, scientists agreed that chromosomes consisted of some sort of polymer, but did not understand whether it was DNA, RNA, or protein. Building upon experiments performed by Frederick Griffith where he transferred a genetic trait from one bacterium to another

(Griffith 1934), Avery, MacLeod, and McCarty demonstrated DNA to be the genetic material in 1944 (Avery *et al.* 1944).

In 1952, Hershey and Chase confirmed DNA as the genetic material with work on a T2 phage whose structure had recently been shown by electron microscopy. In 1953, James Watson and Francis Crick built models of the DNA molecule based on data gathered from x-ray diffraction experiments by Rosalind Franklin and Maurice Wilkins for which Watson, Crick, and Wilkins jointly received the Nobel Prize in 1962. Watson and Crick proposed DNA is a double helix and that the bases of each strand are on the inside of the helix, with the base of one strand pairing to the base of the other strand. The two strands being complementary allows DNA to replicate semiconservatively–one strand of the parental double helix is conserved in each daughter strand. In 1958, Meselson and Stahl proved DNA replication in bacteria is semiconservative.

In 1969, Hershey shared the Nobel Prize in Physiology or Medicine for his work on determining the properties of DNA. In the 1970s restriction enzymes were discovered. It became clear that the discovery of restriction enzymes and the new era of recombinant DNA technology was transforming biology. In 1973, Stanley Cohen, Herbert Boyer, and Paul Berg created the first genetically engineered organisms (Cohen *et al.* 1973, and Jackson *et al.* 1972). In 1978, Werner Arber, Daniel Nathans, and Hamilton Smith shared the Nobel Prize in Physiology or Medicine for the discovery of restriction enzymes.

The late 1970s and 1980s brought the principles and techniques of DNA testing. The first genetic test using DNA was restriction fragment length polymorphism (RFLP, pronounced "rif-lip") and required large blood samples (Jeffreys *et al.* 1987). Polymerase Chain Reaction (PCR) was developed in the 1980s by Kary Mullis (Mullis 1990). It became the standard test for DNA testing in the 1990s, enabling researchers to quickly produce millions of copies of a specific DNA sequence, bypassing the need for bacteria and large quantities of DNA (Chakraborty *et al.* 1992). In 1989, Francis Collins and Lap-Chee Tsui sequenced the first human gene containing the defective gene that causes cystic fibrosis (CFTR) (Riordan *et al.* 1989). In 2001, the Human Genome Project and Celera Genomics released the first draft of human genome. The thirteen-year Human Genome Project was successfully completed in 2003. DNA polymorphisms began to be widely used to reconstruct human evolutionary history, and greater understanding of the DNA damage sensing, signaling, and the interplay between protein phosphorylation and ubiquitin pathway has shown important implications for aging and cancer.

CAS

- 9007-49-2

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37. Dextranase

1,6-α-D-Glucan 6-glucanohydrolase

Dextranases catalyze the endohydrolysis of 1,6-alpha-glucosidic linkages in dextran.

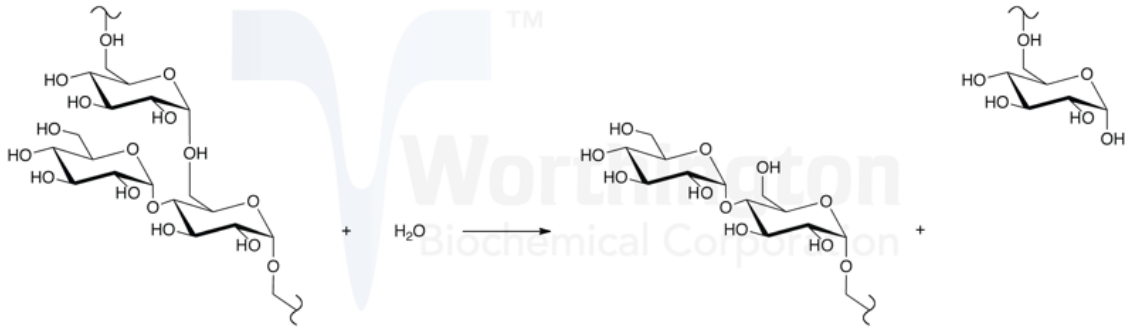

Figure 37.1: Enzymatic Reaction - Dextranase

History

The search for organisms producing large amounts of an enzyme to break down dextran began in the 19th century (van Tieghem 1878).Organisms producing trace amount of both intracellular and extracellular dextranase were isolated in the late 1940s (Ingelman 1948, and Hultin and Nordström 1949) but it was not until the 1950s that a Penicillium species producing large amounts of extracellular

dextranase was reported (Tsuchiya *et al.* 1952).

In the early 1970s, the first purifications and characterizations of *Penicillium dextranase* were being conducted (Baastad and Rolla 1970, Chaiet *et al.* 1970, and Fukumoto *et al.* 1971). In the later 1970s, research began to explore the use of dextranase for the treatment of tooth decay (Minah *et al.* 1972, Goldstein-Lifschitz and Bauer 1976, and Simonson *et al.* 1979).

In the 1990s, the *Penicillium dextranase* gene was cloned and expressed in Pichia pastoris (Garcia *et al.* 1996, and Roca *et al.* 1996). An early classification system for dextranase and other dextran-hydrolyzing enzymes was also developed using sequence-analysis software (Aoki and Sakano 1997).

The crystal structure of *Penicillium minioluteum* was elucidated by Larsson *et al.* in 2003. This study also importantly suggested the reaction mechanism proceeds by net inversion (rather than retention) of the anomeric carbon. Recent research has aimed to improve enzyme stability of fungal dextranase using site-directed mutagenesis (Chen *et al.* 2009).

Molecular Characteristics

Extracellular dextranase is encoded by the dex gene. Post-translational modification includes cleavage of the signal peptide and N-glycosylation. Amino acid sequence analyses have revealed 29% identity between *Penicillium minioluteum* and Arthrobacter sp. (Roca *et al.* 1996).

Specificity

Dextranase preferentially cleaves the alpha-1,6 linkages of dextran, releasing shorter isomaltosaccharides, with net inversion of the anomeric carbon configuration (Larsson *et al.* 2003, and Sugiura *et al.* 1973).

Composition

Dextranase enzymes belong to two glycoside hydrolase families, either 49 or 66, which do not share significant sequence similarity. Dextranases from Penicillium and Arthrobacter species are classified as family 49, while those of the Streptococcus species are found in family 66 (Coutinho and Henrissat 1999).

Penicillium dextranase and related enzymes contain two domains. The first domain resembles the immunoglobulin fold and consists of 200 amino acid residues forming 13 beta strands. A beta sandwich is formed by nine of those strands, with all but strands 5 and 13 being antiparallel. The second domain contains a right-handed parallel beta-helical fold containing 3 parallel beta sheets (Larsson *et al.* 2003). These two domains are connected by a large interface, which contains 29 amino acids completely conserved in the glycoside hydrolase family 49. Disulfide bridges are formed by four of the six cysteines present in the protein, none of which are conserved within the glycoside hydrolase family 49 (Larsson *et al.* 2003).

IUB

- 3.2.1.11

CAS

- 9025-70-1

Protein Accession Number

- CAB91097

CATH: Classification (v. 3.3.0)

Class:

- Mainly Beta

Architecture:

- Sandwich; 3 Solenoid

Topology:

- Dex49a from *Penicillium minioluteum* complex, domain 1; Pectate Lyase C-like

Molecular Weight

- 64.6 kDa

Optimal pH

- 5.0-7.0 (Chaiet *et al.* 1970)

Isoelectric Point

- 4.55 $\pm$ 0.05 (Chaiet *et al.* 1970)
- 3.88 (Raices *et al.* 1991)

Extinction Coefficient

- 119,350 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 20.0 (Chaiet *et al.* 1970)

Activators

- Co^{2+}, Mn^{2+}, Cu^{2+} (Sugiura *et al.* 1973)

Inhibitors

- Hg^{2+}, Ag2+
- N-bromosuccinimide
- I2 (Sugiura *et al.* 1973)

Applications

- Viscosity reduction in cell culture (Lindskog *et al.* 1987)
- Glycobiology
- Reduction in viscosity caused by dextran during sugar processing (Larsson *et al.* 2003)

Assay Information

Method

- A modification of that described by Janson and Porath (1966). One Unit of activity causes the release of one micromole isomaltose from dextran per minute at 37°C and pH 6.0 under the specified conditions.

Reagents

- 0.1 M Potassium phosphate, pH 6.0
- 2% Dextran. Dissolve 1.0 g dextran 500 in 30 ml 0.1 M potassium phosphate pH 6.0. under the specified conditions. Bring to a final volume of 50 ml.
- 2% Sodium hydroxide
- Dinitrosalicylic acid color reagent. Prepare by dissolving 1.0 g of 3,5-dinitrosalicylic acid, 200 mg phenol, 50 mg sodium sulfite, 20 g sodium potassium tartrate tetrahydrate in 60 ml of 2% NaOH. Q.S. to 100 ml with 2% NaOH. Protect from carbon dioxide and store no longer than 2 weeks.

Enzyme

- Dissolve enzyme at one $\frac{\text{mg}}{\text{ml}}$ in reagent grade water. Immediately prior to use, dilute further to 5-20 $\frac{\mu\text{g}}{\text{ml}}$.

Procedure

Into a series of numbered tubes pipette 1.9 ml dextran substrate. Include one tube to be used as a blank. Incubate in a 37°C water bath. At timed intervals, to each tube add 0.1 ml of an enzyme dilution. To the blank, add 0.1 ml of reagent grade water in place of the enzyme.
Incubate at 37°C for 30 minutes. Stop the reaction at timed intervals by removing one ml aliquots of enzyme-substrate mix to tubes containing one ml dinitrosalicylic acid reagent. Incubate for 15 minutes in a boiling water bath. Cool to room temperature, add 10 ml reagent grade water to each tube, mix and read A_{540}.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\text{micromoles maltose in aliquot x 2}}{\text{30 x mg enzyme in reaction mixture}}$

Determine micromoles of maltose in aliquot from a standard curve.

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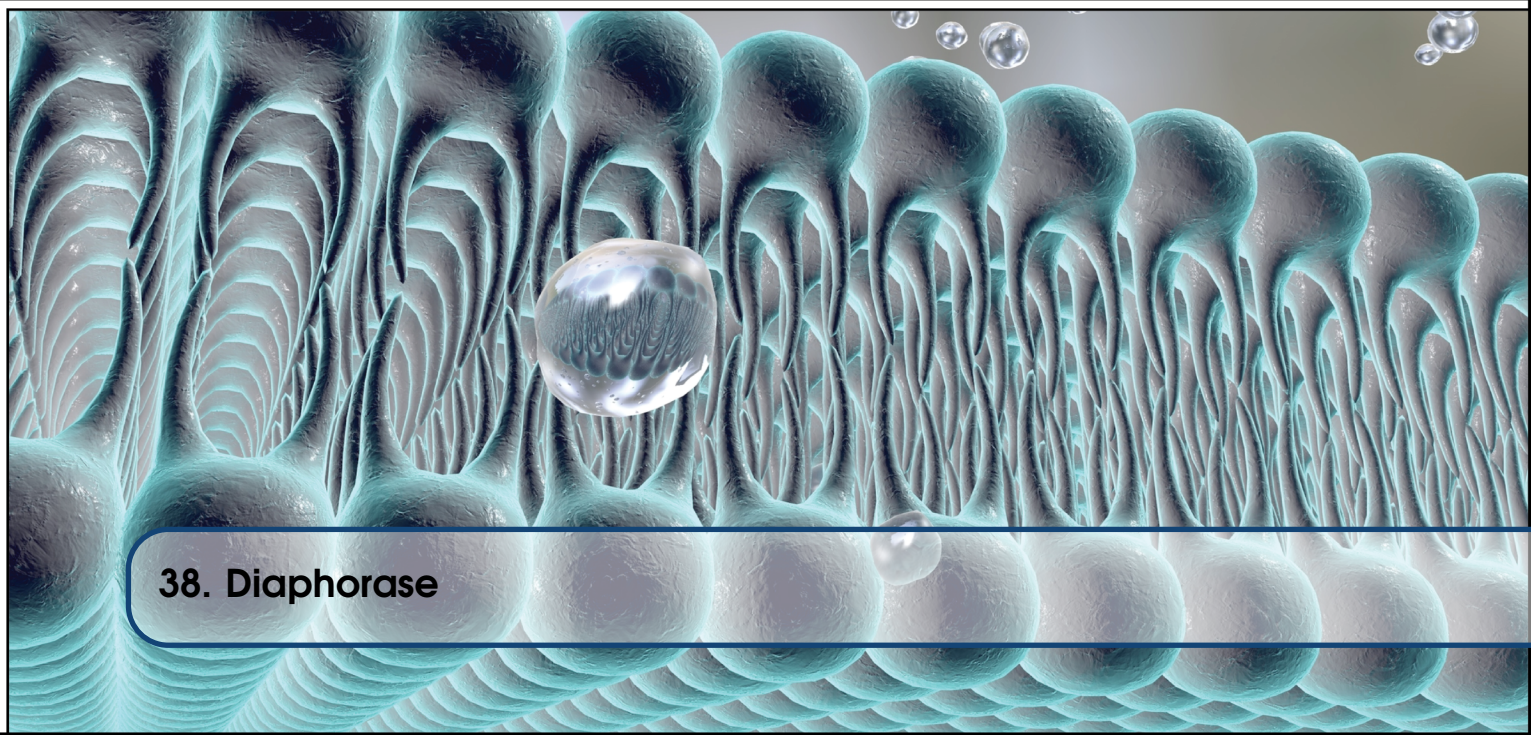

38. Diaphorase

NADPH: (Acceptor) Oxidoreductase

The diaphorases are a ubiquitous class of flavin-bound enzymes that catalyze the reduction of various dyes which act as hydrogen acceptors from the reduced form of di- and tri- phosphopyridine nucleotides, i.e., NADH, NADPH. The first such enzyme to be purified was that from heart muscle (Straub 1939). Almost twenty years later heart diaphorase was shown to be identical to lipoyl dehydrogenase (Massey 1958, 1963). Other diaphorases have been described and purified from various bacteria, plants and mammalian organs. Diaphorase activity of a partially purified extract of *Clostridium kluyveri* cells, originally described as a source of NADH and NADPH oxidase (Ciotti and Kaplan 1957), was observed in this laboratory and its use applied to the coupled, colorimetric determinations of dehydrogenases and ethanol. (Teller 1958). These methods, based on the decolorization of 2,6-dichlorophenolindophenol were improved by the substitution of a tetrazolium dye which becomes chromogenic on reduction. (Brower and Woodbridge 1970; Nachlas *et al*. 1960).

The enzyme from *Clostridium kluyveri* has been purified and characterized by Kaplan, Setlow, and Kaplan (1969), who used starting material equivalent to the present Worthington product.

Unless otherwise noted, the following data have been obtained from Kaplan *et al*. (1969).

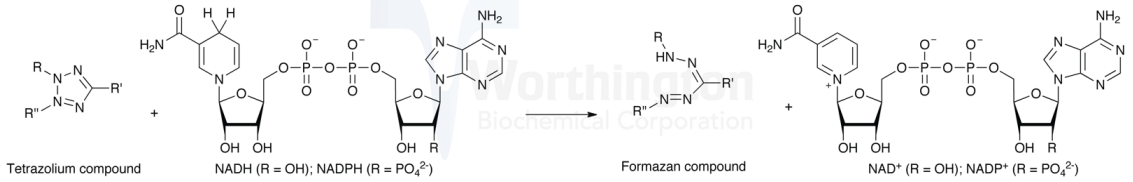

Figure 38.1: Enzymatic Reaction - Diaphorase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Clostridium kluyveri*

Specificity

Either NADH or NADPH may be used as reductants. However, no exchange of hydrogen between the coenzymes is catalyzed. Neither oxygen nor cytochrome C is reduced by *Clostridium kluyveri* diaphorase.

Composition

The enzyme contains one molecule of flavin mononucleotide per molecule. The amino acid composition has been determined.

IUB

- 1.6.99.1

CAS

- 9001-68-7

Protein Accession Number

- Q97E86 (*Clostridium acetobutyliucum*)

Molecular Weight

- 151 kDa (homotetramer)

Optimal pH

- 8.5. At pH 7.4 and 9.4 the activity is reduced 50%. Both NADH and NADPH share this optimum.

Activators

- We have found that excess flavin mononucleotide (FMN) appears to stimulate the reaction with dichlorophenol-indophenol slightly.

Inhibitors

- N-ethylmaleimide inactivates diaphorase at concentrations of under 5 mM.

Stability/Storage

- Though of reasonable stability, if held for prolonged periods, the dry enzyme should be stored in a freezer. Solutions, especially, should always be kept out of strong light.

Stabilizers

- NADH, NADPH and FMN protect the enzyme against denaturation by urea and guanidine.

Constants

- K_m for NADH was reported to be 9 x 10^{-5} and for NADPH it was much lower.

Assay Information

ASSAY for Product Code DIL

Method

- The oxidation of NADH is determined by measuring colorimetrically the reduction of 2,6-dichlorophenolindophenol. The assay is based on that described by Edelhoch et al. (1952) and Mahler et al. (1952) with modifications to provide greater linearity of reaction rate and proportionality of initial rate to enzyme concentration. One unit is defined as the amount of enzyme which reduces one micromole of 2,6-dichlorophenolindophenol per minute at 25°C and pH 8.5 under the specified conditions.

38.0.1 Reagents

- 0.2 M Tris · HCl buffer, pH 8.5
- 0.12 M Zinc chloride in 0.005 M HCl
- 0.006 M NADH. Prepare fresh daily.
- 0.0024 M Dichlorophenolindophenol (DCPIP) Prepare fresh daily.
- Enzyme diluent: 0.2 M Tris · HCl pH 8.5 containing 0.3 M potassium chloride, 0.025% flavin mononucleotide (FMN), and 0.025% bovine serum albumin. Prepare fresh daily and protect from strong light.

38.0.2 Enzyme

- Dissolve lyophilized powders at 10 $\frac{\text{mg}}{\text{ml}}$ in 0.2 M Tris · HCl pH 8.5. Immediately prior to use, dilute further in enzyme diluent to a concentration of 0.1-0.15 $\frac{\text{mg}}{\text{ml}}$.

Procedure

Spectrophotometer Settings: Wavelength: 600 nm Temperature: 25°C

Pipette into cuvettes as follows:

Description	Test	Blank
0.2 M Tris · HCl, pH 8.5	2.6 ml	2.6 ml
0.12 M ZnCl2	0.1 ml	0.1 ml
0.006 M NADH	0.1 ml	0.1 ml
0.0024 M 2,6-Dichlorophenolindophenol (DCPIP)	—	0.1 ml
Enzyme diluent	—	—

Incubate in spectrophotometer at 600 nm and determine A_{600} of blank cuvette for 8-10 minutes. Determine ΔA_{600} from initial linear portion of curve. To test cuvette add 0.1 ml of 0.0024 M DCPIP followed immediately by 0.1 ml of diluted enzyme. Record decrease in A_{600} for 3-4 minutes. Calculate ΔA_{600} from the initial linear portion of the curve. The reaction is linear for no more than 1-2 minutes.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{600}}{\text{min}}_{\text{(Test)}} - \frac{\Delta A_{600}}{\text{min}}_{\text{(Blank)}}}{19 \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$$

Assay Information

ASSAY for Product Code DILW

Method

- One unit equals a decrease in absorbance of 1.0 per minute at 25°C at pH 7.5 with 2,6-dichlorophenolindophenol as the chromogen.

Reagents

- 0.2 M Tris · HCl buffer, pH 7.5
- 0.006 M NADH. Prepare fresh daily.
- 0.0012 M Dichlorophenolindophenol (DCPIP) Prepare fresh daily.
- Stock Enzyme Solution
- Prepare a 10 $\frac{\text{mg}}{\text{ml}}$ solution of enzyme in 0.2 M Tris · HCl, pH 7.5.
- Dilute further immediately before use to give $\frac{\Delta A}{\text{min}}$ of 0.15-0.20.

Procedure

Spectrophotometer Settings: Wavelength: 600 nm Temperature: 25°C

Mix quickly and measure the decrease in absorbance at 600 nm for 2-3 minutes.

Determine the $\frac{\Delta A}{min}$. from the initial linear portion of the curve. (Use portion of curve from t=0 to t=1 minute; the rate is linear for 1/2 to 1 minute.)
Pipette into cuvettes as follows:

Description	Test	Blank
0.2 M Tris · HCl, pH 7.5	0.3 ml	0.3 ml
0.006 M NADH	0.1 ml	0.1 ml
Water	2.4 ml	2.5 ml
Mix thoroughly and incubate in spectrophotometer to achieve temperature equilibration and add simultaneously:		
Dye	0.1 ml	0.1 ml
Sample	0.1 ml	—

Calculation

- $\frac{\text{Units}}{\text{mg}}$ dry weight = $\frac{(\frac{\Delta A}{min}_{(\text{Test})} - \frac{\Delta A}{min}_{(\text{Blank})}) \text{ x Dilution}}{10 \frac{\text{mg}}{\text{ml}} \text{ x } 0.1}$

References

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39. DNA Ligase, T4

T4 DNA ligase is an ATP-dependent ligase that catalyzes a joining reaction between DNA molecules. By joining the 3'-hydroxy and 5'-phosphate termini to form a phosphodiester, DNA ligases are absolutely essential for DNA replication and repair in all organisms. The phage-encoded T4 DNA ligase is produced during infection of *Escherichia coli* by bacteriophage T4.

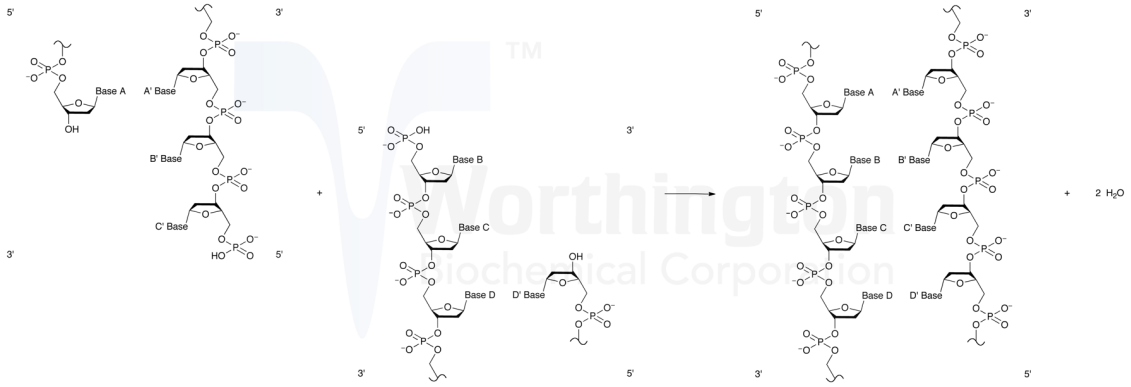

Figure 39.1: Enzymatic Reaction: Ligation

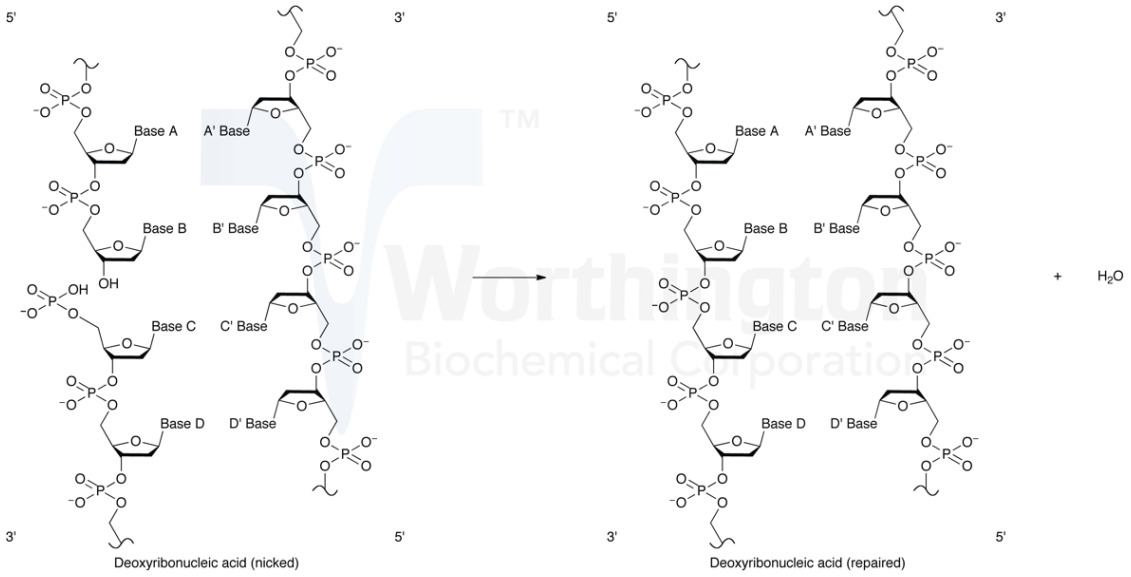

Figure 39.2: Enzymatic Reaction: Strand Repair

History

The search for T4 DNA ligase stemmed from two discoveries during the early 1960s. Meselson and Weigle, and Kellenberger *et al*. discovered that genetic recombination can occur by the breakage and rejoining of DNA molecules; and Bode and Kaiser discovered that a large fraction of linear DNA from bacteriophage l is rapidly converted to covalently closed duplex circles soon after infection of the host (Meselson and Weigle 1961, Kellenberger *et al*. 1961, Young and Sinsheimer 1964, and Bode and Kaiser 1965). The race to find the enzyme responsible for this DNA joining activity is evident from the independent and nearly concurrent discovery of DNA ligases by five different laboratories (Gellert 1967, Weiss and Richardson 1967, Olivera and Lehman 1967, Gefter *et al*. 1967, and Cozzarelli *et al*. 1967). In 1968, Okazaki *et al*. discovered another function of DNA ligase: DNA may be replicated discontinuously, and short segments can be joined into continuous strands that make up the chromosome (Lehman 1974).

Soon after, the widespread distribution in a variety of eukaryotic tissues including rabbit, rat, and lily microsporocytes was observed (Lindahl and Edelman 1968, Tsukada and Ichimura 1971, and Howell and Stern 1971). The gene encoding T4 DNA ligase was cloned by Wilson and Murray in 1979, and the primary structure and genetic organization of T4 DNA ligase was investigated by Armstrong *et al*. in 1983.

Ligases were critical reagents in molecular cloning development and many applications in DNA biotechnology. There has been a recent rejuvenation in ligase research upon the discovery that most organisms have multiple ligases functioning either in DNA replication or particular repair pathways (Tomkinson *et al*. 2006, Ellenberger and Tomkinson 2008, Shuman and Glickman 2007, and Shuman 2009).

Molecular Characteristics

T4 DNA ligase is a 487 residue protein. Bacteriophage T4 has a double stranded genome of approximately 169 kb, encoding 300 genes (Bullard and Bowater 2006). The genome of phage T4 is approximately four times the size of those of T7 and lambda, suggesting it undergoes less selective pressure to minimize the length of its DNA (Armstrong *et al.* 1983). T4 DNA ligase is encoded by gene 30 (gp30).

Specificity

DNA ligases repair nicks in double stranded DNA with 3' OH and 5' phosphate ends. Typically, bacterial ligases are NADH-dependent, while eukaryotic ligases are ATP-dependent; however, despite T4 DNA ligase's existence being confined within a prokaryote, it encodes an ATP-dependent ligase. T4 DNA ligase has a lower affinity for DNA than multidomain ligases, which could also explain its sensitivity to salt concentration. DNA ligation occurs in a three-step reaction. The first step is a nucleophilic attack on the alpha-phosphorus of ATP by the conserved lysine residue, releasing PPi and generating a covalent enzyme-adenylate intermediate in which AMP is linked to the terminal side-chain nitrogen of lysine. In the second step, a nucleophilic attack occurs on the phosphorus of the enzyme-adenylate by the 5'-phosphate-terminated DNA substrate, releasing lysine and forming DNA-adenylate. In the final step, DNA-adenylate is attacked by 3'-OH of another DNA strand, releasing AMP and joining the polynucleotides (Shuman 2009).

Composition

T4 DNA ligase is a single polypeptide. Despite extensive purification of T4 DNA ligase, attempts to crystallize the protein, both with and without cofactor, have been unsuccessful. A similar structure, that of T7 DNA Ligase, has been solved (Subramanya *et al.* 1996, Doherty *et al.* 1996, and Shuman and Schwer 1995). Alignment studies of DNA ligases indicate a highly conserved central DNA binding cleft, active site lysine residue, and nucleotide binding site (Subramanya *et al.* 1996).

IUB

- 6.5.1.1

CAS

- 9015-85-4

Protein Accession Number

- P00970

Molecular Weight

- 77 kDa (Sedimentation, Lehman 1974)
- 74 $\pm$ 3 kDa (SDS Page, Lehman 1974)
- 55.3 kDa (Theoretical)

Optimal pH

- 7.5-8.0

Isoelectric Point

- 6.14

Extinction Coefficient

- 54,770 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280} = 9.91$

Activators

- Requires ATP, Mg^{2+}, and sulfhydryl reagents (DTT, 2-mercaptoethanol)

Inhibitors

- NaCl (concentrations exceeding 200 mM)

Applications

- Joining DNA molecules with compatible cohesive termini, or blunt ended double stranded DNA to one another or to synthetic linkers
- Only T4 DNA ligase joins fragments with overlapping complementary single-stranded protrusions as well as blunt ends
- Unlike *Escherichia coli* DNA ligase, T4 enzyme joins duplex DNA molecules at blunt ends
- Repairs single-stranded nicks in duplex DNA
- Repairs nicks in duplex DNA, RNA, or DNA/RNA hybrids
- Ligase chain reaction (LCR)

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40. DNA Polymerase I

DNA polymerase I participates in the DNA replication of prokaryotes. DNA chain growth is in the 5' to 3' direction with addition at the 3' hydroxyl end. The new chain is base-paired with the template, and the new chain and template are antiparallel. DNA polymerase I is the most abundant polymerase and functions to fill gaps in DNA that arise during DNA replication, repair, and recombination.

$$(\text{dNMP})_n + \text{dNTP} \rightarrow (\text{dNMP})_{n+1} + \text{PPi} \tag{40.1}$$

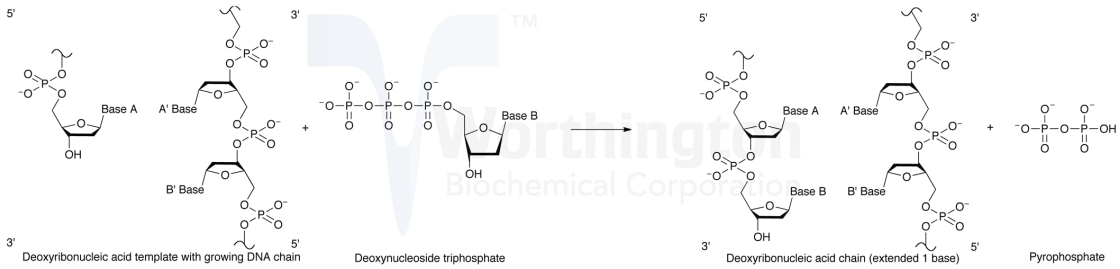

Figure 40.1: Enzymatic Reaction: Polymerization

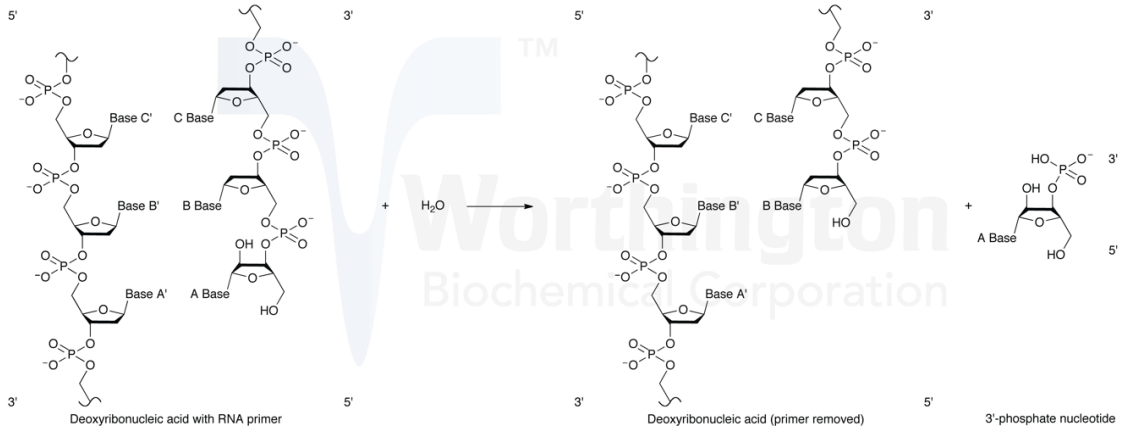

Figure 40.2: Enzymatic Reaction: Proofreading

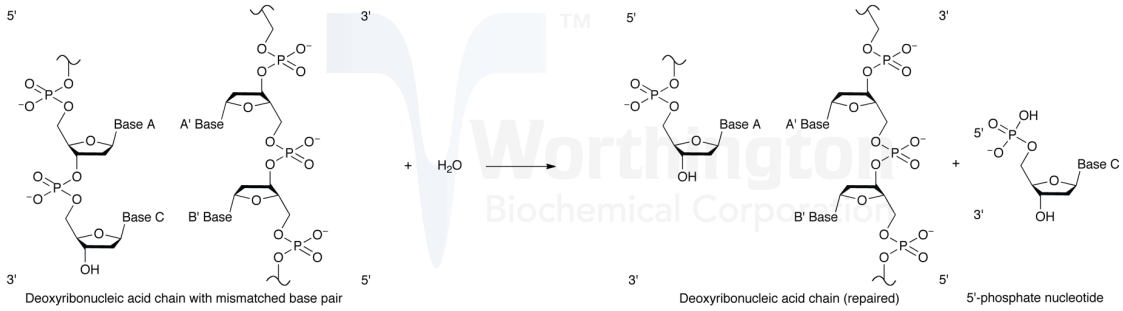

Figure 40.3: Enzymatic Reaction: Primer Removal

History

DNA polymerase I was discovered by Arthur Kornberg *et al.* in 1956. His initial results were first presented at the 1956 annual meeting of the Federation of American Societies for Experimental Biology (FASEB) in Atlantic City, New Jersey. Reviewers of his initial paper suggested that the authors refer to the product as 'polydeoxyribonucleotide' rather than 'DNA'; 'DNA' was only approved after an appeal to the editor-in-chief, John Edsall (Friedberg 2006). Two more papers were published in 1958 by Lehman *et al.* and Bessman *et al.*, which definitively established DNA polymerase was performing DNA replication. Kornberg was awarded the Nobel Prize in 1959 for his discovery of DNA polymerase I.

In 1969, Jovin *et al.* elucidated the amino acid composition (Jovin *et al.* 1969a, b). That same year, DeLucia and Cairns isolated an *Escherichia coli* strain with a mutation that affected the DNA polymerase and surprisingly found that the mutant synthesized DNA normally. This discovery cast doubts on the role of DNA polymerase in replication and led groups to search for other replication enzymes. At the same time, Klenow and colleagues showed that the treatment of DNA polymerase with the proteolytic enzyme subtilisin (type Carlsberg) resulted in an increase of polymerase activity and decrease of exonuclease activity. The resulting DNA polymerase was isolated and was named the "Klenow fragment" (Klenow and Henningsen 1970a, and Klenow and Overgaard-Hansen 1970).

In 1970, DNA polymerase II of *Escherichia coli* was isolated and characterized by Arthur Kornberg's son, Thomas Kornberg (Kornberg and Gefter 1970). DNA polymerase II was also independently reported on by Knippers and by Moses and Richardson in 1970 (Moses and Richardson 1970b). A year later, Thomas Kornberg and Gefter identified DNA polymerase III (Kornberg and Gefter 1971).

Recent work with DNA polymerase I has included investigating the molecular basis of substrate specificity through thermodynamic studies (Wowor *et al*. 2010) and single-molecule FRET experiments (Santoso *et al*. 2010). Hastings *et al*. have investigated the interactions of the five *Escherichia coli* DNA polymerases during cellular stress (Hastings *et al*. 2010), and Kukreti *et al*.'s studies have aimed to determine which residues are important for 3'-5' exonuclease activity (Kukreti *et al*. 2008).

Molecular Characteristics

The gene encoding DNA polymerase I (polA) contains approximately 3,000 base pairs and encodes approximately 1,000 amino acid residues in a simple polypeptide chain. Even organisms separated by a billion years of evolution (such as Deinococcus-Thermus genera and *Escherichia coli*) have approximately 35% amino acid identity and approximately 50% homology (Patel *et al*. 2001).

Specificity

DNA synthesis requires a primer strand with a free 3'-hydroxyl terminus annealed to a DNA template strand and the deoxynucleotide triphosphates form base pairs with the template. Addition is in the 5' to 3' direction with release of pyrophosphate. The enzyme is active with DNAs containing single stranded gaps and also with DNAs with single-strand breaks or nicks. Under some conditions, RNA-DNA hybrids and an RNA duplex may serve as template-primer (Setlow 1972).

The 5' to 3' exonuclease activity associated with DNA polymerase I degrades both single and double stranded DNA in the 5' to 3' direction, yielding 5'-mononucleotides. The 5' to 3' exonuclease activity is specific for double stranded DNA, yielding 5'-mononucleotides and oligonucleotides. DNA polymerase I can also excise mismatched regions in DNA (Setlow 1972).

The similar structure of DNA polymerases has indicated that most DNA polymerase enzymes use an identical two metal ion-catalyzed polymerase mechanism. One metal ion activates the primer's 3'-OH for attack on the a-phosphate of the dNTP. The other metal ion stabilizes the negative charge of the leaving oxygen and chelates the b- and g-phosphates (Steitz 1999).

The Klenow fragment is a proteolytic product of *Escherichia coli* DNA polymerase I that retains polymerization and 3' to 5' exonuclease activity, but has lost 5' to 3' exonuclease activity.

Composition

DNA polymerase I is the predominant polymerizing enzyme found in *Escherichia coli*. It contains a single disulfide bond and one sulfhydryl group (Jovin *et al*. 1969b). Five distinct DNA polymerases have been isolated from *Escherichia coli* and have been designated I, II, III, IV, and V. DNA polymerase I functions to fill DNA gaps that arise during DNA replication, repair, and recombination. DNA

polymerase II also functions in editing and proofreading mainly in the lagging strand (Kim *et al.* 1997, Wagner and Nohmi 2000). DNA polymerase III is the main replicative enzyme. DNA polymerase IV and V have large active sites that allow for more base misincorporation, and are therefore more error-prone. They also lack proofreading-exonuclease subunits to correct misincorporations (Nohmi 2006, and Hastings *et al.* 2010). DNA polymerase V is present at significant levels only in SOS-induced cells and over-expression restricts DNA synthesis (Marsh and Walker 1985).

The domain shape of all polymerases whose structures are known has been described as a "right hand" with "thumb", "palm", and "finger" domains (Kohlstaedt *et al.* 1992). The palm region is thought to catalyze the phosphoryl transfer, and the finger region is thought to interact with the incoming nucleoside triphosphate and the template base it is paired to. The thumb is believed to help in positioning the DNA and in translocation (Brautigam and Steitz 1998).

IUB

- 2.7.7.7

CAS

- 9012-90-2

Protein Accession Number

- P00582

Molecular Weight

- 109 kDa (Jovin *et al.* 1969a, b)
- Klenow fragment: 70 kDa (gel filtration, Klenow and Overgaard-Hansen 1970)

Optimal pH

- Maximum activity is obtained at pH 7.4 with potassium phosphate buffer for native DNA or poly dAT template-primer systems (Richardson *et al.* 1964)
- Klenow fragment: Maximal activities are obtained at 7.4 with phosphate buffer and at 8.4 with Tris · HCl buffer

Isoelectric Point

- 5.4

Extinction Coefficient

- 81,030 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 7.86, In 10 mM sodium bicarbonate, the $\frac{A_{280}}{A_{260}}$ ratio is 1.81 and the absorbance at 280 nm of a 1 $\frac{\text{mg}}{\text{ml}}$ solution is 0.85 (Jovin *et al.* 1969a)

Activators

- A divalent cation is required for activity
- Mg^{2+} at a concentration of 7 mM yields optimum activity under the conditions of the standard assay (Richardson *et al.* 1964)
- Mn^{2+} can partially fulfill the metal ion requirement
- Enzyme activity is also influenced by concentrations of monovalent cations such as K^+, Rb^+, Cs^+, and NH_4^+

Inhibitors

- Kanchanomycin, mitomycin, bleomycin, phleomycin, ananthramycin, plurmycin A (Tanaka *et al.* 1965), and neomycin (Lazarus and Kitron 1973)
- Actinomycin inhibits only when guanosine and cytosine nucleotides are present (Cohen and Yielding 1965)
- Dideoxynucleoside
- Arabinosyl nucleotide triphosphate
- Deoxyuridine-5'-triphosphate and analogues of uridine and deoxyuridine with 5'-hydroxy or amino substituents (Kornberg 1974)
- Chloroquine and some of its analogs (Cohen and Yilding 1965)

Applications

- High percentage incorporation of radioactivity for nick translation assays
- Standard reference material for the study of DNA polymerases
- Manufacturing of alternating copolymers such as poly d(A-T) and homopolymers such as poly dG-poly dC
- Klenow fragment: DNA sequencing (Sanger *et al.* 1977), fill-in of 5' overhangs and removal of 3' overhangs to form blunt ends (Sambrook 1989), and second strand synthesis in mutagenesis (Gubler 1987)

References

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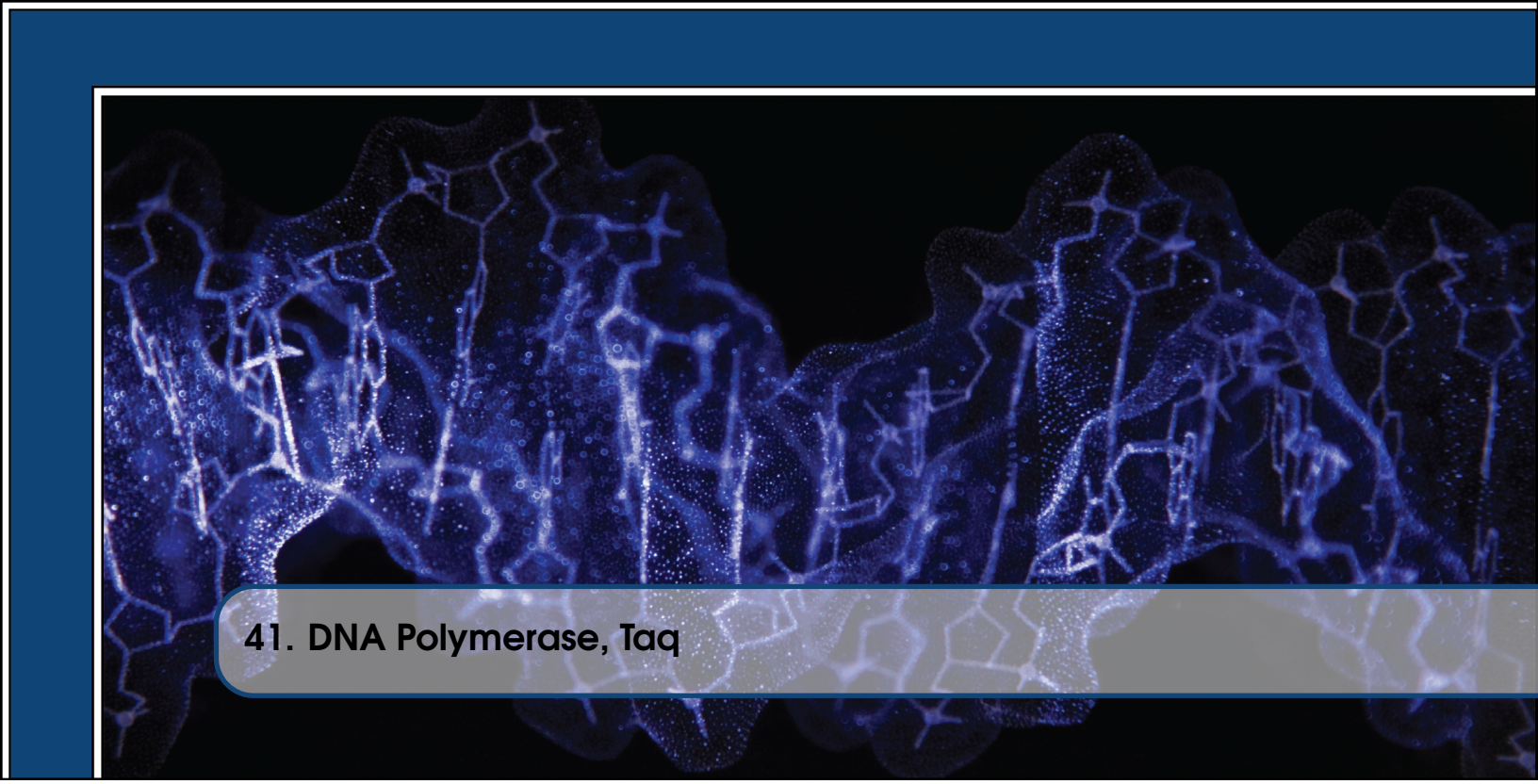

41. DNA Polymerase, Taq

Taq DNA polymerase is a DNA-dependent polymerase. Taq refers to Thermus aquaticus, the bacteria from which this thermostable polymerase was first isolated. The enzyme exhibits optimal activity at 75°C.

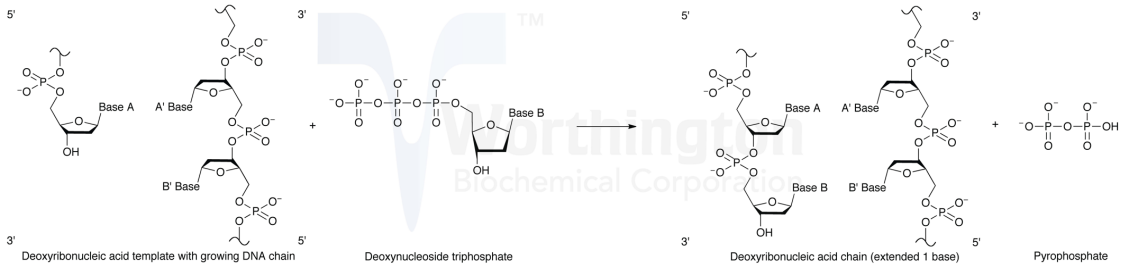

Figure 41.1: Enzymatic Reaction - DNA Polymerase, Taq

History

DNA polymerase I was the first enzyme thought to be involved in DNA synthesis and was the only DNA polymerase to be studied from the late 1950s to the early 1970s (Kornberg *et al.* 1956, Bessman *et al.* 1957, Buttin and Kornberg 1966, Englund *et al.* 1968, Deutsch and Kornberg 1969, Englund *et al.* 1969, and Kornberg *et al.* 1974). In 1969, DeLucia and Cairns isolated an *Escherichia coli* strain with a mutation that affected the DNA polymerase and surprisingly found that the mutant synthesized DNA normally. This discovery casted doubts on the role of DNA polymerase in replication and led groups to search for other replication enzymes.

DNA polymerase II and III of *Escherichia coli* were soon isolated and characterized (Kornberg and Gefter 1972, and Livingston and Richardson 1975). However, few attempts were made to isolate DNA polymerase from thermophiles (Stenesh and Roe 1972). In 1976, DNA polymerase from the thermophilic bacterium Thermus aquaticus was first isolated and purified, and its remarkable stability at high temperatures was described (Chien *et al*. 1976).

Soon after its purification, Taq DNA polymerase was found to be highly useful in the polymerase chain reaction (PCR) method of DNA amplification. Compared to *Escherichia coli* DNA polymerase I, Taq polymerase's high specificity of primer binding at elevated temperatures was found to give a higher yield of the desired product with less non-specific amplification product (Saiki *et al*. 1985, Mullis and Faloona 1987, and Lawyer *et al*. 1989). This feature paved the way for dramatic improvements to the PCR method. Since its optimal temperature is so high, Taq polymerase only needs to be added to a PCR reaction at the beginning, rather than before each round of amplification.

The crystal structure was determined by Kim *et al*. in 1995. Recent work with Taq polymerase has included developing inhibitor-resistant mutants of Taq polymerases for use in PCR assays (Baar *et al*. 2011) and investigating the molecular basis of substrate specificity through thermodynamic and structural studies (Obeid *et al*. 2010, and Wowor *et al*. 2010).

Molecular Characteristics

Taq polymerase displays little, if any, 3'to 5' exonuclease activity. It is possible that mutations, deletions, or insertions destroyed this activity; *Escherichia coli* polymerase I displays little sequence similarity in the region believed to be the 3' to 5' exonuclease region. Six amino acid residues believed to be essential for polymerase activity in DNA polymerase I include Met-512, Arg-682, Lys-758, Tyr-766, Arg-841, and His-881 (Lawyer *et al*. 1989). All residues except Met-512 are conserved in Taq polymerase.

Thermophilic organisms have been found to have high GC content. *Escherichia coli* polymerase has been shown to contain 52.0% GC conent, whereas the gene for Taq polymerase contains 67.9% (Lawyer *et al*. 1989).

Specificity

DNA synthesis proceeds in the 5' to 3' direction and requires the presence of dNTPs and primer template DNA. The similar structure of DNA polymerases has indicated that these enzymes use an identical two metal ion-catalyzed polymerase mechanism (Beese and Steitz 1991). One metal ion activates the primer's 3'-OH for attack on the a-phosphate of the dNTP. The other metal ion stabilizes the negative charge of the leaving oxygen and chelates the b- and g-phosphates (Steitz 1999).

The enzyme is thermostable and exhibits optimal activity at 75°C. It is stable at -20°C when undiluted. If needed, it is recommended to dilute the enzyme in storage buffer containing 0.1% nuclease-free BSA (Worthington Code:BSANF).

Composition

The enzyme consists of a single polypeptide chain. The domain shape of all polymerases whose structures are known has been described as a "right hand" with "thumb", "palm", and "finger" domains

(Kohlstaedt *et al*. 1992). The palm region is thought to catalyze the phosphoryl transfer, and the finger region is thought to interact with the incoming nucleoside triphosphate and the template base it is paired to. The thumb is believed to help in positioning the DNA and in translocation (Brautigam and Steitz 1998).

IUB

- 2.7.7.7

CAS

- 9012-90-2

Protein Accession Number

- P19821

Molecular Weight

- 94 kDa (gel filtration, Lawyer *et al*. 1993)

Optimal pH

- Active from 7.5-9.5, around 9 is optimal (20°C)

Isoelectric Point

- 6.03

Extinction Coefficient

- 110,380 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 11.75

Inhibitors

- Bromophenol blue (Wittwer and Garling 1991)
- Aptamer (Yakimovich *et al*. 2003)
- Pyranicin (Takahashi *et al*. 2008)
- Catalpol (Pungitore *et al*. 2004)
- KCl (greater than [75 mM])
- Urea, DMSO, DMF, formamide, and SDS (Wittwer and Garling 1991)

Applications

- Polymerase chain reaction (PCR)
- Primer dependent DNA labeling or DNA sequencing (particularly since the reaction can proceed at high temperature)

Assay Information

Method

- One unit incorporates 10 nanomoles of total deoxynucleotides into acid precipitable products with activated calf thymus DNA as primer-template, for 30 min. at 37°C, prepared according to Aposhian and Kornberg 1962.

Reagents

- 150 mM Tris · HCl, pH 8.5 with 5 mM ammonium sulfate, 24 mM $MgCl_2$, 30 mM 2-mercaptoethanol and 0.2 $\frac{mg}{ml}$ BSA
- Nucleoside Triphosphates: 30 μM dATP, dCTP, dGTP, dTTP spiked with [^{3}H]dTTP to 10^6 - 2 x 10^6.
- 0.5 $\frac{mg}{ml}$ activated calf thymus DNA
- 10% perchloric acid
- 1% perchloric acid
- Methyl Cellosolve® (ethylene glycol monomethyl ether)

Enzyme

- 0.05 - 0.2 units in 5 μl - 10μl Tris · HCl.

Procedure

To clean glass tubes, add 50 μl each of Tris · HCl and DNA. Add 0.1 ml deoxynucleoside triphosphates. Add enzyme 0.05 - 0.2 units in 5 μl - 10 μl volume (for dilution of the enzyme use Tris · HCl). Include one tube without enzyme as a blank. Incubate at 37°C for 30 minutes. Stop reaction by the addition of 1 ml of 10% cold perchloric acid. Filter by suction through cellulose acetate filters, pores 1 μm - 10 μm. Wash four times using 2 ml of 1% cold perchloric acid for each wash. Transfer filtrate to scintillation vials and add 2 ml Cellosolve® to dissolve filter. Add 10 ml scintillation cocktail and count.

Calculation

- $\frac{\text{units}}{\text{ml}} = \frac{\text{3.6 x (reaction CPM - blank CPM)}}{\text{total CPM x reaction volume in ml}}$
- $\frac{\text{units}}{\text{mgP}} = \frac{\frac{\text{units}}{\text{ml}}}{\frac{\text{MgP}}{\text{ml}}\text{ Lowry}}$

References

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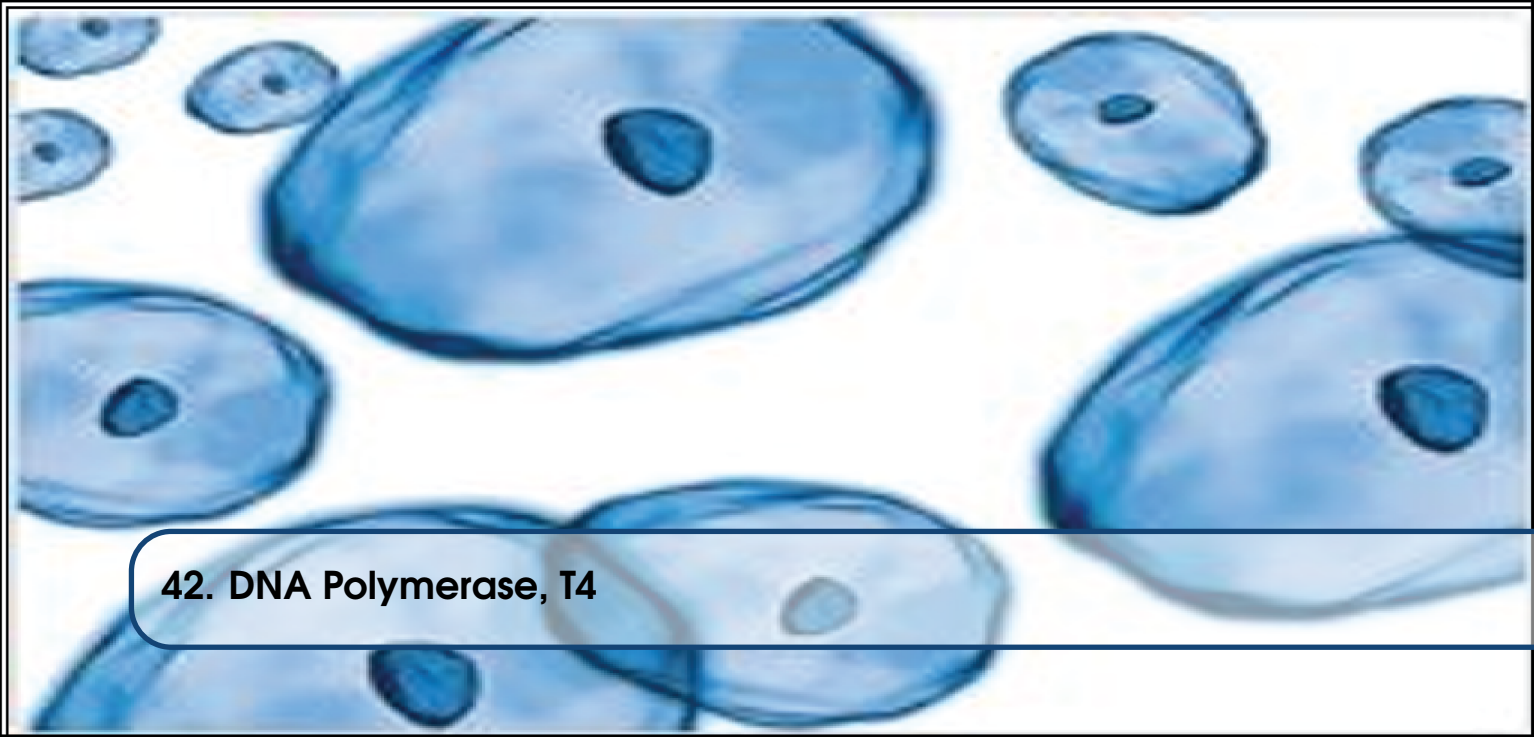

42. DNA Polymerase, T4

Bacteriophage T4 DNA polymerase is a DNA-directed 5’ to 3’ DNA polymerase. It is the product of gene 43 of the bacteriophage T4, and is therefore often referred to as T4 gp43 DNA Polymerase.

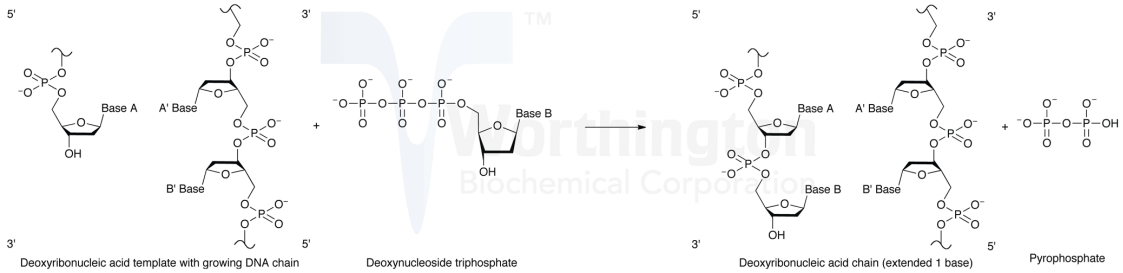

Figure 42.1: Enzymatic Reaction: Polymerization

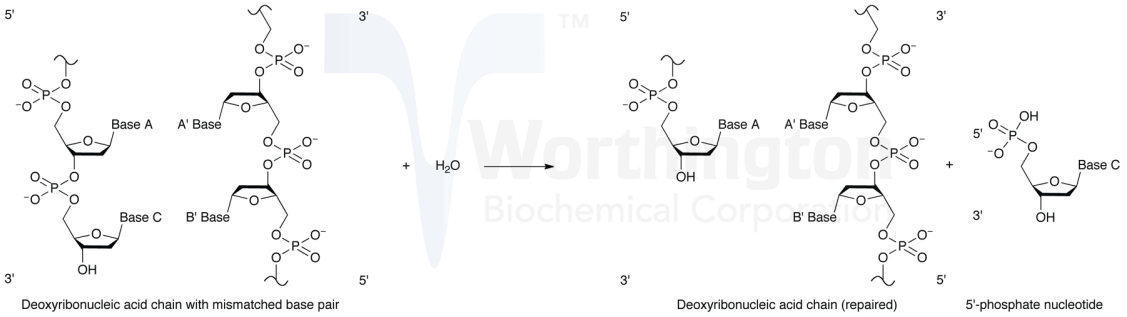

Figure 42.2: Enzymatic Reaction: Proofreading

History

Bacteriophages were used by Alfred Hershey and Martha Chase in early experiments to support the theory of DNA as the genetic material (Hershey and Chase 1952, and Hershey 1953).

Throughout the 1960s, the genetic studies on T4 mutants by Epstein, Edgar and their colleagues led to the construction of a T4 phage genetic map (Epstein *et al.* 1963, Edgar *et al.* 1964, and Edgar and Lielausis 1964). Additional experiments with T4 showed that replication fidelity was determined by nucleotide selection accuracy during polymerization, and by a balance between polymerization and 3' to 5' excision/proofreading (Goodman *et al.* 1993, and Nossal 1998). In 1965, de Waard *et al.* demonstrated that T4 DNA polymerase was encoded by gene 43. Soon after, Speyer *et al.* found that mutation frequencies in unlinked genes were increased after infection with phage and some polymerase mutations (Speyer *et al.* 1966). These findings led to the surprising discovery that some polymerase mutations could act as "antimutators" and decrease the frequency of other mutations (Drake and Allen 1968, and Drake *et al.* 1969).

The replication complex of bacteriophage T4 was one of the first systems to be successfully reconstituted *in vitro* (Morris *et al.* 1975, and Young *et al.* 1992). Throughout the 1970s, the laboratories of Bruce Alberts and Nancy Nossal were instrumental in identifying the seven T4 coded gene products as well as the single stranded DNA binding protein (Nossal 1979, and Nossal and Peterlin 1979).

T4 DNA polymerase found its use in molecular biology because, unlike other DNA polymerases, it does not have the ability to extend from a nick, which is important in site-directed mutagenesis (Henikoff 1990). T4 DNA polymerase's lack of 5' to 3' exonuclease activity also prevents digestion of annealed mutagenic oligonucleotides and reversion to parental sequence (Doetsch 1985).

Recent research on T4 DNA polymerase mutants has identified a new motif in the family of B DNA polymerases (Li *et al.* 2010). Nelson and Bencovic have also studied the activity of the enzyme during DNA replication after DNA lesions are introduced (Nelson and Benkovic 2010).

Molecular Characteristics

The T4 DNA polymerase is an 898 amino acid residue protein. It is related to the Pol B family, which includes eukaryotic polymerases a, d, and e. The structure of RB69 DNA polymerase (a 903 residue

protein) has been solved (Wang *et al.* 1997, and Franklin *et al.* 2001). Sequence alignment shows the two polymerases are 62% identical. The N-terminal domain of T4 DNA polymerase consists of residues 1-102 and 340-380. The C-terminal domain contains a PCNA interacting peptide (PIP box) motif at residues 883-903 which interacts with the sliding clamp protein (Mueser *et al.* 2010).

The bacteriophage T4 consists of an icosahedral head that contains the 166 kb genome and a tail segment that contains tail fibers responsible for the virus binding to the surface of *Escherichia coli*.

Specificity

The enzyme catalyzes the polymerization of deoxynucleotide triphosphates in a 5' to 3' direction. It possesses very active 3' to 5' exonuclease activity that is more active on single than double stranded DNA; T4 DNA polymerase has no 5' to 3' exonuclease activity. For polymerase activity the enzyme requires DNA with a 5' protruding end and a high concentration of dNTPs. In the presence of a high concentration of dNTPs the exonucleolytic activity is inhibited by the polymerase activity. The enzyme is not active with intact double stranded DNA as the template. For polymerase activity the enzyme requires a primed single strand of DNA, a duplex DNA with gaps, or a single stranded DNA with protruding 5' termini.

Bacteriophage T4 DNA polymerase is also capable of an exchange (replacement) reaction. In the presence of only one dNTP the 3' to 5' exonuclease will degrade double stranded DNA from the 3' hydroxyl terminus until a base is exposed that is complementary to the dNTP present. A continuous series of syntheses and exchange reactions will take place at that position.

Composition

The bacteriophage T4 encodes ten proteins known collectively as the replisome. These proteins are responsible for the replication of the phage genome and are divided into three activities: replicase, primosomal, and Okazaki repair. T4 DNA polymerase is part of the replicase, along with the gene 45 sliding clamp, the gene 44 and 62 encoded ATP-dependent clamp loader, and the gene 32 single stranded DNA binding protein (Frankllin *et al.* 2001, and Mueser *et al.* 2010). T4 DNA polymerase is active as a monomer, but it has been suggested that dimerization is necessary for coordination of leading and lagging strand synthesis (Salinas and Benkovic 2000).

IUB

- 2.7.7.7

CAS

- 9012-90-2

Protein Accession Number

- P04415

Molecular Weight

- 103.6 kDa (Spicer *et al.* 1988)

Optimal pH

- 8.0-9.0. At pH 7.5 and pH 9.7 the activity is only 50%

Isoelectric Point

- 5.93

Extinction Coefficient

- 130,120 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280} = 12.56$

Active Residue

- Asp 411, 621, 622, 684, and 686 (a cluster of aspartate residues)

Activators

- Requires Mg^{2+} and sulfhydryl reagents for activity

Inhibitors

- 4-Hydroxymercuribenzoate (Lehman 1974)
- Butylphenyl nucleotides (Khan *et al.* 1994)
- Aphidicolin (Khan *et al.* 1994)
- Pyrophosphate analogs (Khan *et al.* 1994)
- Pyranicin (Takahashi 2008)

Applications

- Filling or labeling recessed 3' termini created by digestion of DNAs with restriction enzymes (Richardson 1964)
- Radioactive labeling of the 3' termini of DNA (Goulian *et al.* 1968): The advantage is that DNA labeled by using T4 enzyme lacks the hairpin structures that can be produced during nick translation, and the labeled DNA can easily be converted into strand specific probes by cleavage with suitable restriction enzymes (Challberg and Englund 1980). The disadvantage is that this method does not produce a uniform distribution of label along the length of the DNA.
- Conversion of duplex DNA fragment ends to a double-ended structure suitable for blunt ended ligation in cloning
- Detection of stable DNA lesions (Doetsch 1985)

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43. Elastase

Elastase is a serine protease that also hydrolyzes amides and esters. It is produced in the pancreas as an inactive zymogen, and activated in the duodenum by trypsin. The following information applies to porcine elastase.

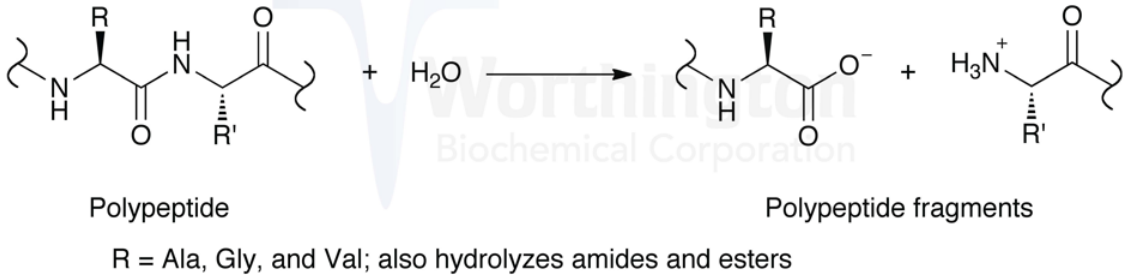

Figure 43.1: Enzymatic Reaction - Elastase

History

Eijkman is believed to be the first to have studied elastase as a product of bacteria (Eijkman 1904). However, it was not shown to differ from other proteolytic components of the pancreas, such as trypsin and chymotrypsin, until the Bal*ó* and Banga publication in 1949.

In 1968, Shotton and Hartley were the first to obtain crystalline porcine elastase. In the late 1960s and into the early 1970s, elastase's involvement in emphysema, atherosclerosis, and acute hemorrhagic

pancreatitis was investigated (Janoff and Sherer 1968, Janoff and Basch 1971, Talamo *et al.* 1971, Kaplan *et al.* 1973, and Bieth *et al.* 1974). In 1974, Ardelt discovered a second elastolytic proteinase, naming it pancreatic "elastase II" and the original "elastase I".

In the 1980s, the catalytic properties of elastase were studied (Ascenzi *et al.* 1983), and inhibitors were identified (Largman *et al.* 1980, Lestienne *et al.* 1981, Kettner and Shenvi 1984, Poncz *et al.* 1984, and Imperiali and Abeles 1986). Elastase I and II genetic information was studied into the 1990s (Ornitz *et al.* 1985, Stevenson *et al.* 1986, Swift *et al.* 1984, Tani *et al.* 1987, and Gestin *et al.* 1997).

Elastase has recently been used in investigations of the mechanical forces and enzyme activity in extracellular matrix breakdown (Jesudason *et al.* 2010). Its use in artificial organ development has also been studied (Tedder *et al.* 2010), and it is used as a model to investigate the catalytic activity of the serine proteases (Tamada *et al.* 2009).

Molecular Characteristics

Porcine pancreatic elastase is composed of a single peptide chain of 240 amino acids, and contains 4 disulfide bridges (Sawyer *et al.* 1973). It has a high degree of sequence identity with pancreatic elastases from other species, such as rat with whom it shares 86% identity (MacDonald *et al.* 1982). Elastase I and II genes share sequence similarity, especially in the 5' proximal flanking regions, which include the TATA box and a putative tissue-specific enhancer sequence (Ornitz *et al.* 1985, Stevenson *et al.* 1986, Swift *et al.* 1984, Tani *et al.* 1987, and Gestin *et al.* 1997).

Specificity

Porcine elastase I is specific for Ala-Ala and Ala-Gly bonds, while elastase II has a broad specificity for substrates with medium to large hydrophobic amino acids in the P1 position (Gertler *et al.* 1977, Del Mar *et al.* 1980, and Gestin *et al.* 1997). Porcine elastase is the most potent elastase, having a rate 20-fold higher than that of human leukocyte elastase (Bieth 1978, Bieth 1986, and Largman 1983).

Hydrolysis occurs in several steps. An adsorption complex between elastase and its substrate is formed, followed by nucleophilic attack (S214) to form an acyl-enzyme intermediate, and release of the first product (the C-terminal end of the substrate). The intermediate is hydrolyzed in a deacylation step, regenerating the active enzyme and releasing the second product (Bieth 1986).

Composition

The catalytic triad is formed by three hydrogen-bonded amino acid residues (H71, D119, and S214). The polypeptide chain is composed of two antiparallel beta-barrel domains, which form a crevice containing the catalytic triad, and a small proportion of alpha-helices (Bieth 2004).

IUB

- 3.4.21.36

CAS

- 9004-06-2

Protein Accession Number

- P00772

Molecular Weight

- 26.0 kDa (Bieth 2004)

Optimal pH

- Close to 7 (Bieth 1978)

Isoelectric Point

- 9.5 (Bieth 2004)

Extinction Coefficient

- 54,870 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280} = 21.18$

Active Residue

- Histidine (H71)
- Aspartic acid (D119)
- Serine (S214)

Inhibitors

- Competitively inhibited by derivatives of dipeptides of alanine, valine, leucine, and isoleucine
- Inhibitors from natural sources (e.g. serpins) (Tsunemi *et al.* 1996, Matern *et al.* 2003, and Dementiev *et al.* 2006)
- Modified natural inhibitors (Ay *et al.* 2003, and Hilpert *et al.* 2003)
- Sulfate-modified lipids (Ito *et al.* 1998)

Applications

- Tissue dissociation: Because elastin is found in highest concentrations in the elastic fibers of connective tissues, elastase is frequently used to dissociate tissues that contain extensive intercellular fiber networks. For this purpose, it is usually used with other enzymes such as collagenase, trypsin, and chymotrypsin.
- Membrane protein solubilization
- Protein sequence studies

Assay Information

Bieth *et al*. (1974) have described an excellent assay using succinyl-(L-alanine)3-p-nitroanilide. Other suggested assays have been described: Keller and Mandl (1971), Feinstein *et al*. (1973), and Bieth and Meyer (1973). Schumacher and Schill (1972) describe a radial diffusion assay. The assay used in this laboratory is as follows:

Method

- Derived from that of Feinstein et al. (1973) and using the more soluble substrate of Bieth et al. (1974). An increase in absorbance at 410 nm results from the hydrolysis of N-succinyl-L-Ala-L-Ala-L-Ala-p-nitroanilide (Suc Ala_3NA). One unit hydrolyzes one micromole of Suc Ala_3NA per minute at 25°C and pH 8.0 under the specified conditions.

Reagents

- 0.1 M Tris buffer, pH 8.0
- 0.0044 M Suc Ala_3NA substrate dissolved in Tris buffer. (2 $\frac{mg}{ml}$)

Enzyme

- Prepare a one $\frac{mg}{ml}$ solution in Tris buffer. Immediately before use dilute further to obtain a rate of 0.02-0.04 $\frac{\Delta A}{min}$.

Procedure

Spectrophotometer Settings: Wavelength: 410 nm Temperature: 25°C

Pipette into each cuvette as follows:

- Tris Buffer - 2.7 ml
- Enzyme - 0.1 ml

Mix and incubate in the spectrophotometer 4-5 minutes to achieve temperature equilibration. To test cuvette add 0.2 ml of substrate, mix, and record increase A_{410} for 3-5 minutes. Calculate $\frac{\Delta A}{min}$ from the linear portion of the curve.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{410}}{\text{min}}}{\frac{\text{8.8 x mg enzyme}}{\text{ml reaction mixture}}}$$

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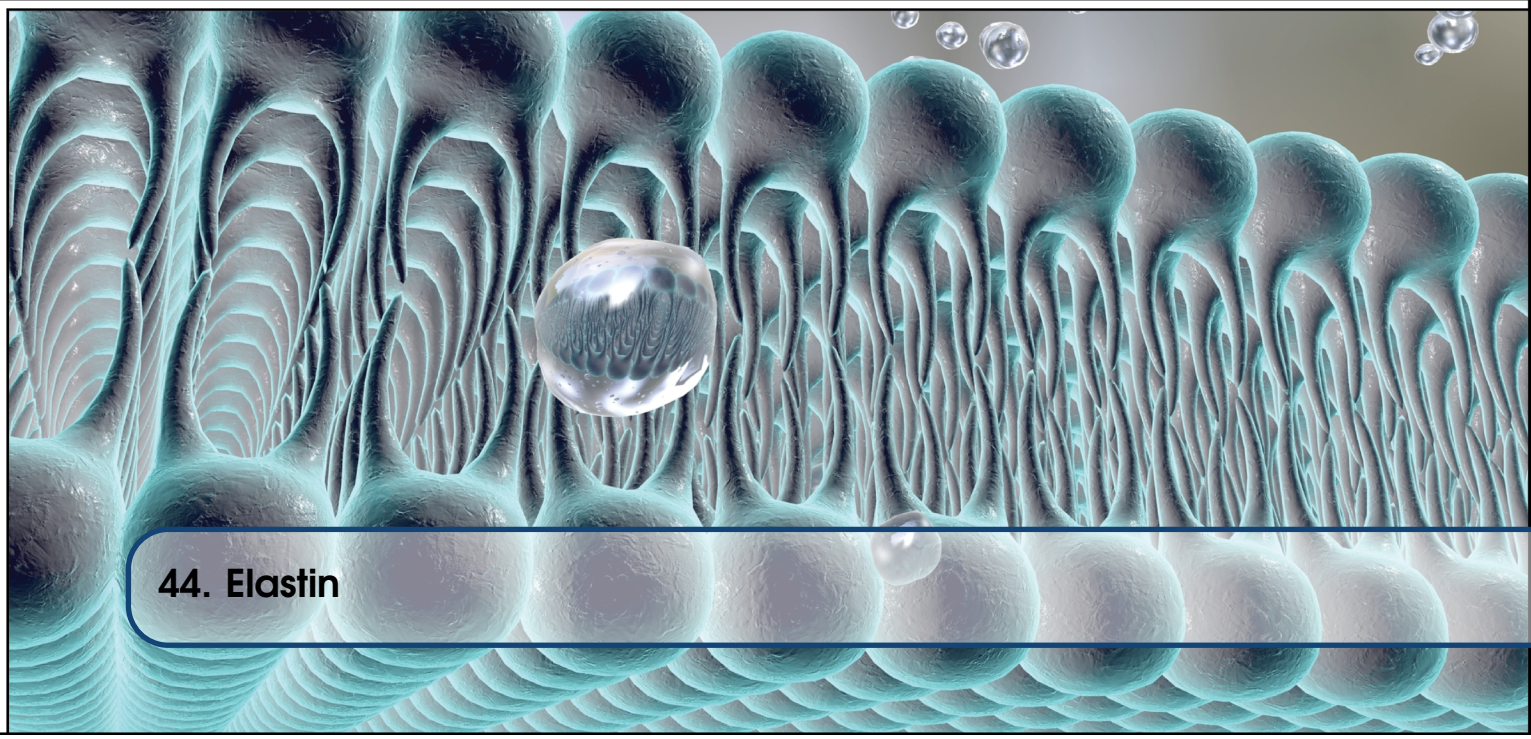

44. Elastin

Elastin is the insoluble, elastic protein of high tensile strength found in intercellular spaces of the connective tissues of large arteries, trachea, bronchi and ligaments. It has been reviewed by Seifter and Gallop (1966). Elastin consists of covalently cross-linked polypeptide chains "randomly kinked" (Sykes and Partridge 1972). Although it is characterized by non-polar amino acid residues it has an affinity for calcium ions. Urry (1971) has indicated that calcification of aortic elastin may be the start of chronic arteriosclerosis. See also Hornebeck and Partridge (1975), Abatangelo, *et al.* (1974), Urry *et al.* (1971). The polypeptide structural units are linked by two novel amino acids: desmosine and isodesmosine (Davis and Anwar 1970; Shimada *et al.* 1969; Thomas *et al.* 1963). The soluble, unlinked polypeptides (tropoelastin) are obtainable (Smith *et al.* 1968). According to Sykes and Partridge (1972) the cross-bonding involves oxidative deamination of lysine residues - a reaction inhibited by Cu^{2+} deficiency and lathyrism. See also: Foster *et al.* (1975), Narayanan *et al.* (1974), Sandberg *et al.* (1971 and 1969). Petruska and Sandberg (1968) report on the amino acid composition of soluble and insoluble elastin and indicate a minimum M.W. of 30,000; Smith *et al.* (1972) report it to be 74,000 using pig aorta. Robert *et al.* (1971) report a varying proportion of glycoprotein. See also Long *et al.* (1975), Lyerla and Torchia (1975), Castellani and Nolpin (1974), >Urry *et al.* (1974), Cox *et al.* (1973), Starcher *et al.* (1973), Beevers (1971), Grant *et al.* (1971). Gerber and Anwar (1975) report that bovine aortic and ligamentum nuchae elastins have identical sequences. See also Steven and Jackson (1968). Dorrington *et al.* (1975) and Gray *et al.* (1973) report on its elasticity. Another characteristic is fluorescence activated by ultraviolet light. See Thornhill (1975). Kakivaya and Hoeve (1975) indicate that the glasslike rigidity that occurs on heating is related to its H_2O content. Elastin is not hydrolyzed by trypsin, chymotrypsin or pepsin but it is acted upon by plant proteases: bromelin, ficin and papain (Seifter and Gallop 1966) and some from microbial sources (Suss *et al.* 1969); Morihara and Tsuzuki 1967; Morihara *et al.* 1965). Keller and Mandl (1971) report on the determination of elastolytic activity.

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45. Galactose Oxidase

Galactose oxidase (GAO) is a fungal secretory enzyme that catalyzes the oxidation of a range of primary alcohols to the corresponding aldehyde, with reduction of dioxygen to hydrogen peroxide.

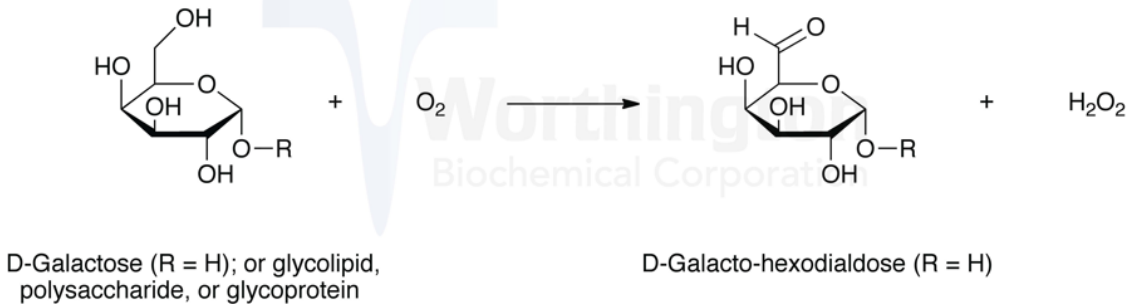

Figure 45.1: Enzymatic Reaction - Galactose Oxidase

History

GAO was discovered in the extracellular culture medium of the fungus *Polyporus circinatus* by Cooper *et al.* in 1959. A method to prepare the crystalline enzyme was developed (Avigad *et al.* 1962, Amaral *et al.* 1963, Kelly-Falcoz *et al.* 1965, and Amaral *et al.* 1966), and it was determined that the original sample was actually Dactylium dendroides and had been misnamed as *Polyporus circinatus* (Nobles and Madhosingh 1963). Purification procedures were later modified by Bauer *et al.* in 1967 and Hamilton *et al.* in 1973.

In the 1980s, the role of carbohydrate content in GAO's activity was studied (Kornfield and Kornfield 1985, and Medonca and Zancan 1988), and Whittaker did extensive studies on the unique active site of GAO (Whittaker *et al*. 1988, and Whittaker *et al*. 1989). Further understanding of the active site was gained through the gene cloning and sequence analysis by McPherson *et al*. (McPherson *et al*. 1992, and McPherson *et al*. 1993).

Recent work with GAO has included successfully cloning and expressing it in *Escherichia coli* and P. pastoris (Spadiut *et al*. 2010). An *Escherichia coli* expression system was also constructed to enable the screening of a library of mutant enzymes (Deacon and McPherson 2011).

Molecular Characteristics

The gaoA gene contains a long open reading frame from +324 to +2507, including the mature protein-coding sequence (+521 to +2507). It also contains a long untranslated upstream region and a putative pro-sequence with a monobasic cleavage site (McPherson *et al*. 1992).

Specificity

GAO has a wide substrate specificity, but remarkable stereospecificity, only oxidizing D-isomers of substrates (McPherson *et al*. 1992). GAO will oxidize galactose and some galactose derivatives in both free and polymeric form. Oxidation occurs at the C6 position.

Composition

GAO contains one Cu(II) atom yet catalyzes a two-electron transfer reaction (McPherson *et al*. 1992). The copper is bound by two tyrosines, and two histidines (Tyr272, Tyr495, His496, and His581). In a novel post-translational covalent modification, Tyr272 is linked by a thioether bond to cysteine (Cys228), suggesting the involvement of a tyrosine radical in the catalytic mechanism. Stabilization of the radical occurs because Tyr272 of the thioether bond is liganded to the copper, creating a stacking interaction with Trp290 (Whittaker *et al*. 1989, Ito *et al*. 1991, and Whittaker *et al*. 2005). The structure of the enzyme has revealed extensive beta-sheet secondary structure, consistent with the high stability of the enzyme (Kosman *et al*. 1974).

Most extracellular proteins of eukaryotes are modified by glycosylation during passage through the ER and golgi, leading to greater glycosylation of extracellular than intracellular forms of a protein. Unusually, the intracellular form of GAO is more highly glycosylated (9% carbohydrate) and exhibits greater stability than the extracellular form (2% carbohydrate) (Medonca and Zancan 1988). Additionally, most proteins are modified by O- and/or N-glycosylation while GAO is only modified only by O-glycosylation (Kornfield and Kornfield 1985, and McPherson *et al*. 1992)

IUB

- 1.1.3.9

CAS

- 9028-79-9

Protein Accession Number

- P0CS93

CATH: Classification (v. 3.3.0)

Class:
- Mainly Beta

Architecture:
- Sandwich, 7 Propellor

Topology:
- Jelly Rolls, Methylamind Dehydrogenase; Chain H, Immunoglobulin-like

Molecular Weight

- 68.5 kDa (calculated from translated DNA sequence and SDS-polyacrylamide gel electrophoresis, McPherson *et al.* 1992)
- 68.0 ± 3.0 kDa (determined from physical measurements, Cooper *et al.* 1959)

Optimal pH

- 7.0 (Cooper *et al.* 1959)

Isoelectric Point

- 7.75

Extinction Coefficient

- 122,480 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 17.87

Inhibitors

- Cyanide
- Diethyldithiocarbamate
- Azide
- Hydroxylamine
- EDTA
- Applications
- Quantitative determination of galactose in blood and other biological fluids (Frings and Pardue 1964, Hankin 1966, and Roth *et al.* 1965)
- Locating galactose histochemically (Roberts and Gupta 1965)
- Detecting and distinguishing glycoproteins (Itaya *et al.* 1975)

Assay Information

Method

- The reaction velocity is measured in a peroxidase/o-tolidine coupled system as an increase in A_{425} resulting from the oxidation of galactose. One unit results in a change in A_{425} of 1.0 per minute at 25°C and pH 6.0 under the defined conditions.

Reagents

- 0.1 M Potassium phosphate buffer, pH 6.0
- 0.5% o-tolidine. Note: o-tolidine has been reported to be carcinogenic. Handle with care.
- Peroxidase. Dissolve Worthington peroxidase (Code: HPOD) at a concentration of approximately 60 $\frac{\text{units}}{\text{ml}}$ in reagent grade water.
- 10% galactose. Allow to come to equilibrium of mutarotation by allowing to stand overnight.

Enzyme

- Dissolve at a concentration of 1 $\frac{\text{mg}}{\text{ml}}$ in reagent grade water. Dilute further for assay to a concentration of 0.2 - 0.5 $\frac{\text{units}}{\text{ml}}$.

Procedure

Spectrophotometer Settings: Wavelength: 425 nm Temperature: 25°C

Prepare tolidine-buffer mixture by adding 0.1 ml tolidine to 12 ml 0.1 M potassium phosphate buffer pH 6.0.

Pipette into each cuvette as follows:

- Tolidine-buffer solution - 1.7 ml
- 10% Galactose - 1.5 ml
- Peroxidase - 0.1 ml

Incubate in spectrophotometer at 25°C for 3 - 4 mintues to achieve temperature equilibration and establish blank rate, if any. Add 0.1 ml of appropriately diluted enzyme and record increase in $\frac{A_{425}}{\text{min}}$. from initial linear portion of the curve.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{425}}{\text{min}}}{\text{mg enzyme in reaction mixture}}$

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46. Galactosidase, Beta

β-D-Galactoside Galactohydrolase

β-Galactosidases are widespread, in microorganisms, animals and plants. That from the Escherichia coli strain K12 has been particularly studied at Anfinsen's laboratory in connection with genetic experiments on gene regulation of protein synthesis. Craven, Steers and Anfinsen 1965). The enzyme has been reviewed in detail by Wallenfels and Weil (1972). Lactase may be used as a reagent for determining lactose in blood and other biological fluids.

Another important application is in food processing. Of special interest is its use in the treatment of milk to meet the needs of the large percentage of the world population afflicted with lactose intolerance. Industrial applications necessitate the enzyme's immobilization on which considerable investigation has been reported (Byrne and Johnson 1975; Narinesingh *et al*. 1975; Paine and Carbonell 1975; Faulsitch *et al*. 1974; Bunting and Laidler 1972 , Lilly 1971, Khare and Gupta 1988, and Park and Hoffman 1990). Beta-galactosidase (Lactase) catalyzes the following hydrolysis:

$$\beta\text{-D-galactoside} + H_2O \rightarrow \text{galactose} + \text{alcohol} \qquad (46.1)$$

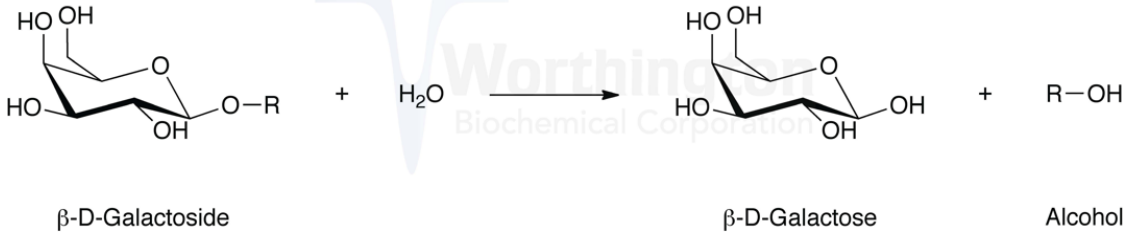

Figure 46.1: Enzymatic Reaction - Galactosidase, Beta

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Escherichia coli*

Specificity

A review of substrate requirements is offered by Wallenfels and Malhotra (1960).

Composition

The enzyme is tetrameric, being composed of four identical subunits of 135,000 daltons, each with an active site which may be independently active (Melcher and Messe 1973). The enzyme is readily fragmented into small peptides (Marinkovic *et al.* 1975). The amino acid analysis indicates approximately 1170 residues per subunit (Fowler and Zabin 1970). See also Langley *et al.* (1975) and Naider *et al.* (1972). Kaneshiro *et al.* (1975) report on an active dimer.

Contaxis and Reithel (1974) report a small molecule with high 260 nm absorbance which is bound to the enzyme in crude extracts as a stabilizer. On adding it to the pure enzyme, activity and heat stability are enhanced.

Bronskil and Wong (1971) suggest that the ribosomal enzyme may be an intermediate stage in the assembly of the quarternary structure.

IUB

- 3.2.1.23

CAS

- 9031-11-2

Molecular Weight

- 540,000 (Steers *et al.* 1965; Cohen and Mire 1971; Contaxis and Reithel 1971).

Optimal pH

- 6 - 8. See also Tenu *et al*. (1971 and 1972).

Extinction Coefficient

- $E^{1\%}_{280}$ = 20.9

Inhibitors

- In the absence of Mg^{2+} ions, β-mercaptoethanol causes dissociation (Shifrin *et al*. 1970. See also Case *et al*. (1973) and Loontiens *et al*. (1970).

Stability/Storage

- The enzyme is stable 4 - 6 months when stored at 5°C.

Activity

- The reaction probably involves a galactosyl-enzyme intermediate (van de Groen 1973). See also Hill and Huber (1971) and Sinnott and Souchard (1973). Hartl and Hall (1974) report a second β-galactosidase without lactase activity *in vivo* but with high ONPG (o-nitrophenyl, β-D-galactopyranoside) activity. Monovalent cations have a stimulatory effect on the enzyme (Becker and Evans 1969). The alcohols, methanol, ethanol, i-propanol, and n-propanol, at 5% concentration, all increase the rate of o-nitrophenyl, β-D-galactopyranoside cleavage (Shifrin and Hunn 1969). The enzyme is protected against heat-inactivation by 5-phosphorylribose 1-pyrophosphate in the presence of β-mercaptoethanol (Moses and Sharp 1970).

Assay Information

Method

- Essentally that of Craven et al. (1965). One unit causes the hydrolysis of one micromole of o-nitrophenyl-β-D-galactopyranoside per minute at 25°C and pH 7.5 under the specified conditions.

Reagents

- 0.3 M Sodium phosphate buffer, pH 7.5 with 0.003 M magnesium chloride
- Substrate diluent: 0.01 M Tris · HCl, pH 7.5 with 0.01 M magnesium chloride, 0.01 M mercaptoethanol and 0.01 M sodium chloride
- 0.10 M Sodium/potassium phosphate buffer, pH 7.0
- 1.0 M Mercaptoethanol
- 0.014 M o-Nitrophenyl-β-D-galactopyranoside (ONPG) in substrate diluent. Prepare fresh daily.

Enzyme

- Prepare a one $\frac{\text{mg}}{\text{ml}}$ stock solution in 0.10 M sodium/potassium phosphate buffer pH 7.0. Immediately prior to use, dilute further to 0.02 - 0.04 $\frac{\Delta A}{\text{min}}$. in enzyme diluent. The protein concentration of the chromatographically purified enzyme (Code: BGC) may be determined as follows:

- $\frac{\text{mg protein}}{\text{ml}} = A_{280} \times 0.478$
- Note: This enzyme is not stable when diluted. All dilutions should be made as quickly as possible and used immediately.

Procedure

Spectrophotometer Settings: Wavelength: 405 nm Temperature: 25°C

Pipette into cuvette as follows:

- 0.3 M sodium phosphate buffer - 1.0 ml
- 1.0 M mercaptoethanol - 0.3 ml
- 0.014 M ONPG - 0.5 ml
- Reagent grade water - 1.1 ml

Incubate in spectrophotometer at 25°C for 3 - 5 minutes to achieve temperature equilibration and establish blank rate, if any. Add 0.1 ml freshly diluted enzyme and record increase in A_{405} for 4 - 5 minutes. Calculate $\frac{\Delta A_{405}}{\text{min}}$ from initial linear rate.

Calculation

- $\frac{\text{Units}}{\text{mgP}} = \frac{\frac{\Delta A_{405}}{\text{min}}}{\frac{3.1 \times \text{mg enzyme}}{\text{ml reaction mixture}}}$

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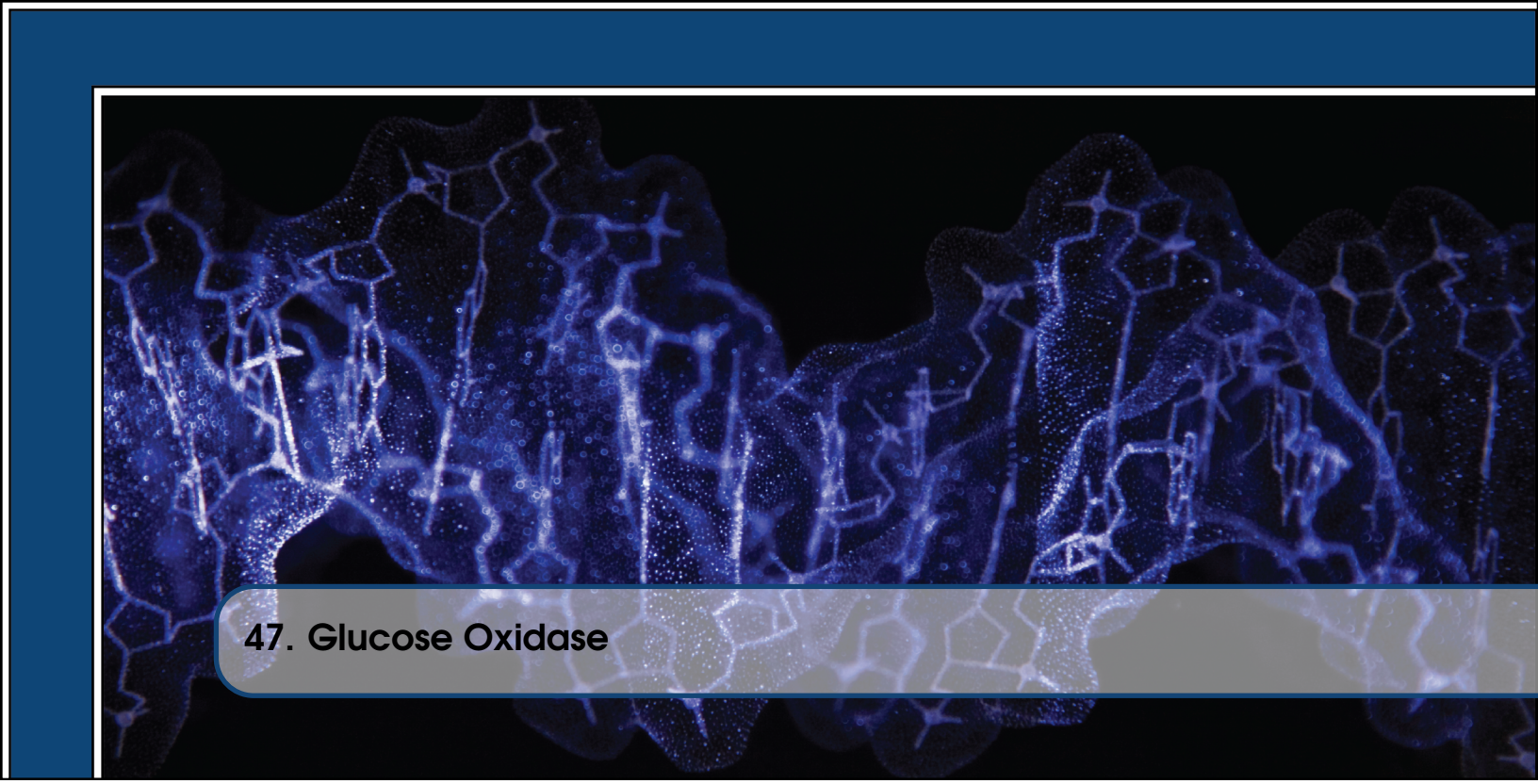

47. Glucose Oxidase

β-D-Glucose: Oxygen 1-Oxidoreductase

Bright and Porter (1975) have reviewed the kinetic behavior and redox states of the flavin coenzyme. Bentley (1963) has reviewed the general properties of the enzyme.

Since its discovery as an "antibiotic" (shown subsequently to be due to peroxide formation) there has been an interest in glucose oxidase, chiefly because of its utility in glucose estimation. Following Keston's report in 1956 of coupling the reaction to peroxidase and a chromogen, (qualitative) glucose "dip-sticks" became available for screening for urine glucose. Based on Teller's paper in the same year, Worthington offered the first quantitative enzymatic system for the colorimetric determination of glucose. For most clinical work the crude form of the *Aspergillus niger* enzyme has been satisfactory. However, it contains trace amount of polysaccharidases such as amylase, maltase, and sucrase which can contribute to falsely high glucose levels. The purified enzyme is free of these traces and is recommended for analytical use in the presence of di- or polysaccharides. Reports on glucose analytical systems employing the enzyme include: Williams *et al.* (1976), and Auses *et al.* (1975) wherein H_2O_2 produced reacts with Fe(CN) – in the presence of luminol to produce luminescence proportional to the initial glucose concentration. See also Lott and Turner (1975).

Considerable work has been done on immobilized glucose oxidase. Reports include the following: Greenfield *et al.* (1975), Greenfeld and Laurence (1975), Lahoda *et al.* (1975), Mell and Maloy (1975), Tran-Minh and Broun (1975), Kunz and Stastny (1974), Miwa and Okuda (1974), Weibel *et al.* (1973), Gestrelius *et al.* (1972), *et al.* (1972), Jemmali and Rodriquez-Kabana (1972), Okuda and Miwa (1971), Weibel and Bright (1971a), Hornby *et al.* (1970), Weetall and Hersh (1970). Glucose oxidase,

a flavoenzyme, catalyzes the following reaction:

$$\text{Enzyme-FADH}_2 + O_2 \rightarrow \text{Enzyme-FAD} + H_2O_2 \tag{47.1}$$

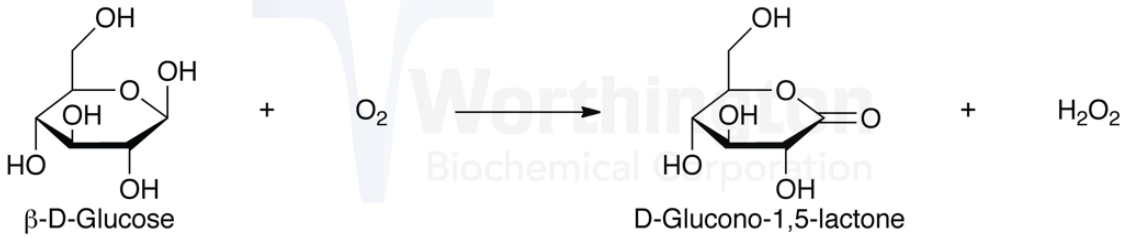

Figure 47.1: Enzymatic Reaction: Oxidation

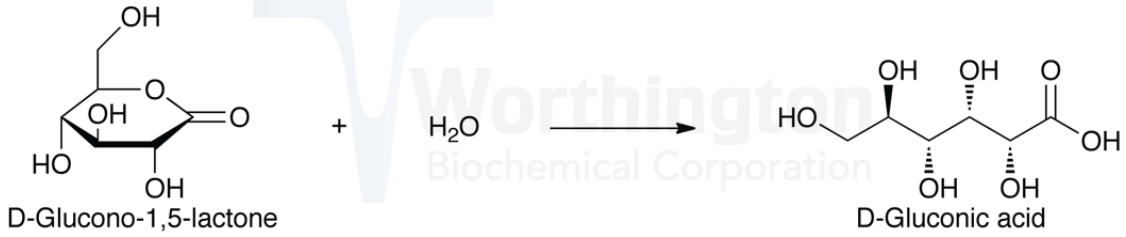

Figure 47.2: Enzymatic Reaction: Hydrolysis

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Aspergillus niger*

Specificity

The enzyme is highly specific for β-D-glucose. The α anomer is not acted upon. 2-deoxy-D-glucose, D-mannose and D-galactose exhibit low activities as substrate. (See Bentley 1966).

Composition

The enzyme consists of two identical polypeptide chain subunits (80,000 daltons) covalently linked by disulfide bonds (O'Malley and Weaver 1972). Each subunit contains one mole of Fe and one mole of FAD (flavin-adenine dinucleotide). Tsuge *et al*. (1975) report the molecule to be approximately 74% protein, 16% neutral sugar and 2% amino sugars. They indicate that the FAD is replaceable with FHD (flavin-hypoxanthine dinucleotide) without loss of activity.

IUB

- 1.1.3.4

Molecular Weight

- 160,000 (Tsuge *et al*. 1975)

Optimal pH

- 5.5 with broad range 4 - 7 (Bright and Appleby 1969). See also Weibel and Bright (1971b).

Inhibitors

- Ag^{+}, Hg^{2+}, Cu^{2+} (Nakamura and Ogura 1968). FAD binding is inhibited by several nucleotides (Swobada 1969). See also Rogers and Brandt (1971).

Stability/Storage

- Dry preparations are stable for years when stored cold. Solutions are reasonably stable under a variety of conditions.

Assay Information

Method

- The reaction velocity is determined by an increase in absorbance at 460 nm resulting from the oxidation of o-dianisidine through a peroxidase coupled system. One unit causes the oxidation of one micromole of o-dianisidine per minute at 25°C and pH 6.0 under the conditions specified.

Reagents

- 0.1 M Potassium phosphate buffer, pH 6.0
- 1% o-Dianisidine: Note: o-dianisidine has been reported to be carcinogenic in the solid form. Handle with care.
- Peroxidase: Dissolve peroxidase (Worthington product code HPOD) at a concentration of 200 $\frac{\mu g}{ml}$ in reagent grade water.
- 18% Glucose: Allow mutarotation to come to equilibrium by standing overnight at room temperature.
- Dianisidine-buffer mixture: Prepare by diluting 0.1 ml of 1% o-dianisidine in 12 ml of 0.1 M potassium phosphate buffer pH 6.0. Saturate with oxygen for 10 minutes within 30 minutes of use.

Enzyme

- Dissolve at one $\frac{mg}{ml}$ in reagent grade water. Dilute further to 0.02 - 0.06 $\frac{\Delta A}{min}$. in reagent grade water for assay.

Procedure

Spectrophotometer Settings: Wavelength: 460 nm Temperature: 25°C

Pipette into cuvette as follows:

- Dianisidine-buffer mixture, pH 6.0 (oxygenated) - 2.5 ml
- 18% Glucose - 0.3 ml
- Peroxidase - 0.1 ml

Incubate in spectrophotometer for 3 - 5 minutes to achieve temperature equilibration and establish blank rate if any. Add 0.1 ml of appropriately diluted enzyme and record increase in A_{460} for 4 - 5 minutes. Calculate ΔA_{460} from the initial linear portion of the curve.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{460}}{\text{min}}}{\frac{11.3 \times \text{mg enzyme}}{\text{ml reaction mixture}}}$$

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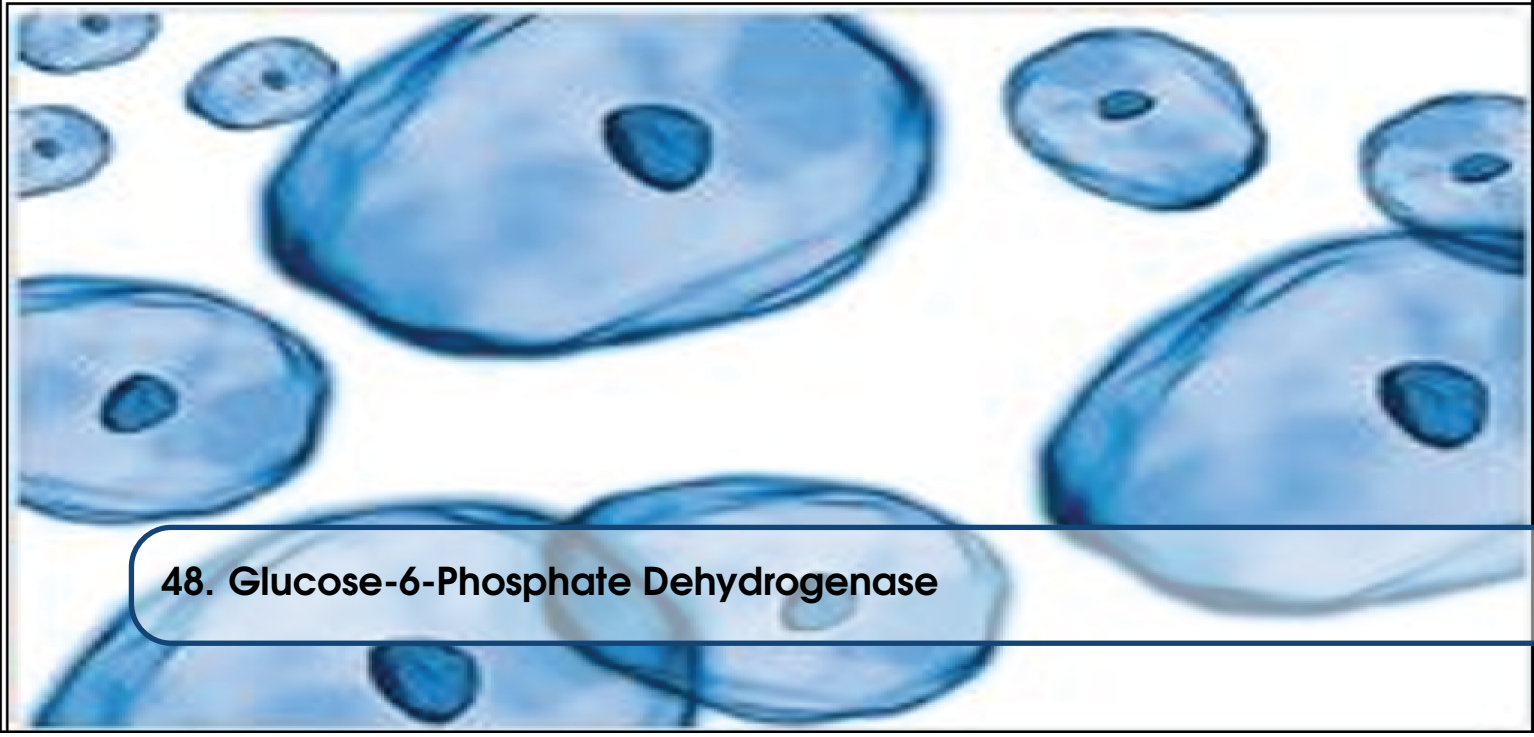

48. Glucose-6-Phosphate Dehydrogenase

D-Glucose-6-Phosphate: NADP$^+$ 1-Oxidoreductase

The G6PD of *L. mesenteroides* has been studied in great detail because of its unique dual-coenzyme specificity. Most dehydrogenases prefer either NAD$^+$ or NADP$^+$, but *L. mesenteroides* G6PD can use either. Glucose-6-phosphate dehydrogenase (G6PD) is a regulatory enzyme catalyzing the first step of the pentose phosphate pathway: oxidation of glucose-6-phosphate using NADP$^+$ and/or NAD$^+$.

$$\text{D-glucose-6-phosphate} + \text{NAD(P)} \rightleftarrows \text{D-glucono } \delta\text{-lactone-6-phosphate} + \text{NAD(P)H}_2 \qquad (48.1)$$

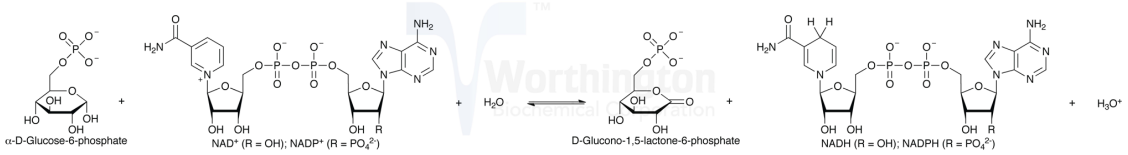

Figure 48.1: Enzymatic Reaction - Glucose-6-Phosphate Dehydrogenase

History

The bacterial genus Leuconostoc first gained interest in the late 1920s and early 1930s as a means of studying lactic fermentation. Pederson determined that glucose fermentation yields equal quantities of lactate, ethanol, and CO_2, which was later confirmed by Friedemann (Pederson 1929, and Friedemann 1939).

In 1951, De Moss *et al.* first isolated glucose-6-phosphate dehydrogenase from *L. mesenteroides*. De Moss *et al.* also demonstrated its dual nucleotide specificity in 1953, and in 1955 showed that fermentation by *L. mesenteroides* proceeds via a mechanism differing from the classical Embden-Meyerhof glycolytic scheme.

In the 1960s, Kemp and Rose demonstrated that the two reduced coenzyme products have different metabolic roles *in vivo*: NADPH is used in biosynthetic reactions and NADH is used in ATP-generating reactions in the production of ethanol and lactate (Kemp and Rose 1964, and White and Levy 1987).

In the late 1980s and into the 1990s, critical amino acid residues were identified, and it was shown that the kinetic mechanisms of NAD- and NADP-linked reactions differ (Levy 1989). In 1991, Lee *et al.* published the complete amino acid sequence of the monomer.

Current research has shown that in the case of G6PD from *L. mesenteroides* the enzyme is active as both a homodimer and monomer, whereas the enzyme from yeast is active only in the native, homodimeric form (Ravera *et al.* 2010). G6PD has also been coupled to other enzymatic reactions, where it functions to regenerate coenzyme (NADH) (Ohno *et al.* 2008). The stability of the enzyme and its resistance to inactivation while bound to substrates also has recently been investigated (Duggleby 2007).

Molecular Characteristics

The *L. mesenteroides* G6PD gene encodes a polypeptide of 485 amino acids. G6PD is unique in that it contains no cysteine residues. The sequences of human and *Escherichia coli*, which use only $NADP^+$ under physiological conditions, have been determined. The sequence of *Z. mobili*, which like *L. mesenteroides* can use either $NADP^+$ or NAD^+, has also been determined. There is approximately 30-36% sequence identity between any two of these species. A conserved sequence begins at Arg175, and contains a lysine residue thought to function in glucose-6-phosphate binding (Lee *et al.* 1991).

Specificity

Either NAD or NADP will serve as coenzyme with the intrinsic reaction velocity of NAD being approximately 1.8 times greater than that of NADP (Olive and Levy 1967). D-glucose-6-phosphate is considered to be the natural substrate, although D-glucose reacts slowly (Metzger *et al.* 1972).

Asp177 and His240 form the catalytic dyad of G6PD. His240 acts as a base to extract the C-1 proton from glucose-6-phosphate; Asp177 stabilizes the resulting positive charge (Cosgrove *et al.* 1998).

Composition

L. mesenteroides G6PD is a dimer of identical subunits with two active sites per dimer putatively located in a pocket between the coenzyme binding site and the large beta+alpha domain in each subunit (Rowland *et al.* 1994). This pocket contains several completely conserved amino acids including an eight residue peptide (Cosgrove *et al.* 1998).

IUB

- 1.1.1.49

CAS

- 9001-40-5

Protein Accession Number

- P11411

CATH: Classification (v. 3.3.0)

Class:

- Alpha Beta and Alpha Beta

Architecture:

- 3-Layer(aba) Sandwich and 2-Layer Sandwich

Topology:

- Rossmann fold and Dihydrodipicolinate Reductase (domain 2)

Molecular Weight

- 108.6 kDa

Optimal pH

- 7.8, Tris · HCl buffer, 0.05 M and NAD as coenzyme

Isoelectric Point

- 4.6 (Olive and Levy 1971)

Extinction Coefficient

- 133,420 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280.5} = 11.5,$ pH 7.2 in 0.1 M Tris · HCl (Olive and Levy 1971)

Active Residue

- Aspartic acid (D177)
- Histidine (H240)

Activators

- Stabilized by BSA
- Modestly stimulated by HCO_3^- ($\leq$ 0.3 M)

Inhibitors

- Acetyl-CoA (Olive and Levy 1975)
- High concentrations of palmitoyl-CoA
- ATP (Olive and Levy 1975)
- CoA (Olive and Levy 1975)
- 1-Fluoro-2,4-dinitrobenzene (irreversible)
- Pyridoxal 5'-phosphate with respect to ZF (competitively) and NAD or NADP (noncompetitive)
- *L. mesenteroides* G6PD, unlike mammalian G6PDs, is unaffected by steroids (Lee *et al*. 1991)

Applications

- Systems where NADPH or NADH is measured (Bhattacharya and Ali 1988)
- Determination of glucose and ATP (when coupled with HK), fructose, glucose-6-phosphate, NAD(P)+, and CK

Assay Information

Method

- This dehydrogenase is rather unique in that it possesses dual coenzyme specificity. When assayed under conditions that are optimal for the particular coenzyme, the ratio of observed catalytic activity is NAD/NADP = 1.8. The reaction velocity is determined by measuring the increase in absorbance at 340 nm resulting from the reduction of NAD or NADP. One unit reduces one micromole of pyridine nucleotide per minute at 30°C and pH 7.8 under the specified conditions.

Reagents

- 0.055 M Tris · HCl buffer pH 7.8 containing 0.0033 M magnesium chloride
- 0.006 M Nicotinamide adenine dinucleotide phosphate, monosodium salt, (NADP), (TPN). Note: NADP may vary in salt form and degree of hydration. Care must be exercised to use an analytical grade and the correct molecular weight. When using NAD, prepare 0.06 M solution.
- 0.1 M Glucose-6-phosphate. As above, an analytical grade and the correct molecular weight must be used.
- 5 mM Glycine buffer, pH 8.0
- 5 mM Glycine buffer, pH 8.0 containing 0.1% bovine serum albumin

Enzyme

- Dissolve lyophilized enzyme or dilute enzyme suspension to 1.0 $\frac{\text{mg}}{\text{ml}}$ in 5 mM glycine buffer, pH 8.0. Care should be taken not to shake the solution as precipitation may result. Dilute further immediately before use in 5 mM glycine buffer, containing 0.1% albumin, to obtain a rate of 0.02 - 0.045 $\frac{\Delta A}{\text{min}}$.

Procedure

Spectrophotometer Settings: Wavelength: 340 nm Temperature: 30°C

Pipette into each cuvette as follows:

- 0.055 M Tris · HCl buffer, pH 7.8 with 0.0033 M $MgCl_2$ - 2.7 ml

- 0.006 M NADP (or 0.06 M NAD) - 0.1 ml
- 0.1 M Glucose-6-phosphate - 0.1 ml

Incubate in spectrophotometer at 30°C for 7 - 8 minutes to achieve temperature equilibration and establish blank rate, if any. Add 0.1 ml diluted enzyme and record increase in $\frac{A_{340}}{min}$ for 4 to 5 minutes. Calculate $\frac{\Delta A_{340}}{min}$ from the initial linear portion of the curve.

Calculation

- $\frac{Units}{mg} = \frac{\frac{\Delta A_{340}}{min}}{\frac{6.22 \text{ x mg enzyme}}{\text{ml reaction mixture}}}$

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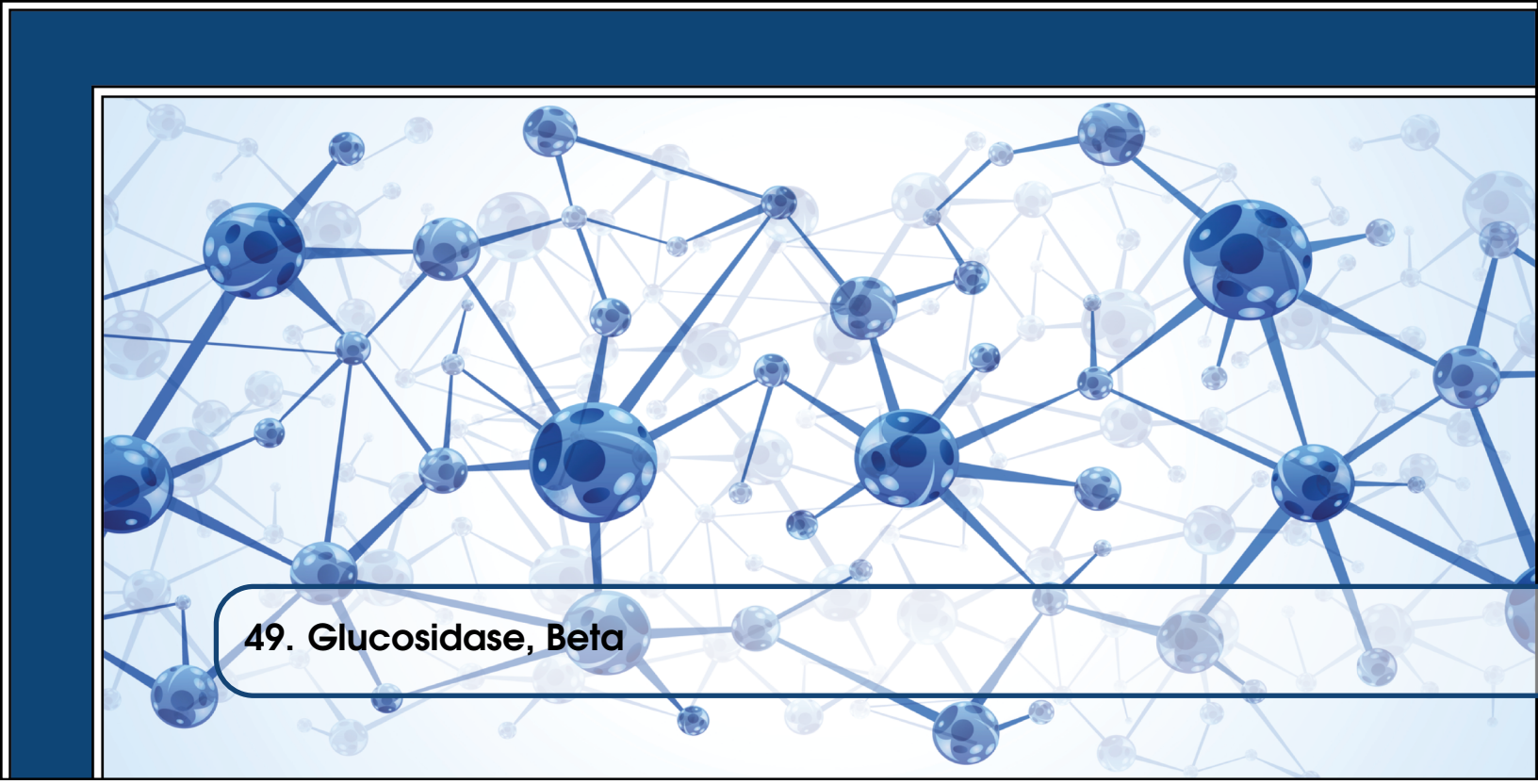

49. Glucosidase, Beta

β-D-Glucoside Glucohydrolase

Emulsin originally was the term applied to the extract of sweet almonds. It remains as the historical prototype of β-glucosidase. Many other sources of the enzyme have been described. Emulsin also shows significant D-galactosidase activity and there is some question as to whether or not it is one enzyme with two activities, because non-parallel response to inhibitors points to two separate enzymes. D-mannosidase has also been reported in almond emulsin (Schwartz *et al*. 1970). The enzyme has been reviewed by Veibel (1950), Larner (1960), and Nisizawa and Hashimoto (1970).

There has been interest in the mammalian form of the enzyme, particularly in relation to Gaucher's disease. Losman (1974) reported that an iso-enzyme of β-glucosidase is lacking in the lymphocytes of homozygotes and a significant decrease is found in heterozygotes. Spleen and liver enzyme activity was lowered in Gaucher's patients when assayed with the synthetic substrate, 4-methylumbelliferal β-D-glycoside. β-Glucosidases catalyze the hydrolysis of glucosides:

$$\beta\text{-D-glucoside} + H_2O \rightarrow \text{D-glucose} + \text{alcohol} \qquad (49.1)$$

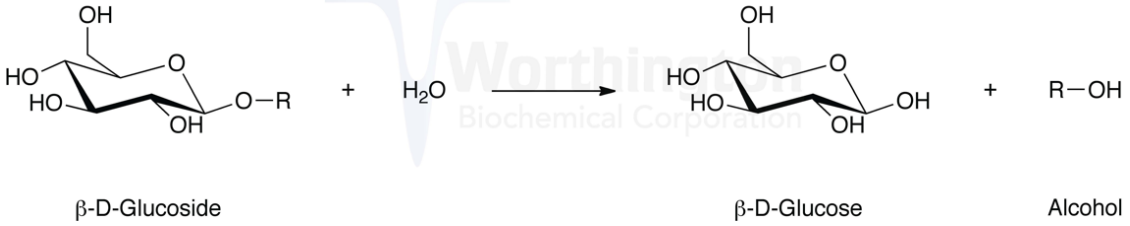

Figure 49.1: Enzymatic Reaction - Glucosidase, Beta

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Almond

Specificity

β-glucosides and β-galactosides are reportedly substrates for the enzyme (Kelemen and Whelan 1966). β-D-fucosides are also attacked (Conchie *et al.* 1967). This is questioned by Schwartz *et al.* (1970) who offer data supporting the existence of separate enzymes.

IUB

- 3.2.1.21

Molecular Weight

- 2 active components, 117,000 and 66,500 (Helferich and Kleinschmidt 1965).

Optimal pH

- Butyryl glycoside in acetate, pH 4.4 (Veible 1950), p-nitrophenyl glycoside 5.2 - 6.0 (Schwartz *et al.* 1970)

Extinction Coefficient

- $E^{1\%}_{280}$ = 17.0 (Plapp and Cole 1966)

Inhibitors

- $HgCl_2$, other heavy metal ions, sulfhydryl-binding compounds and polyols.

Stability/Storage

- The enzyme is stable for 1 - 2 years at 2 - 8°C.

Constants

- K_m (M) Glu-I 0.072 (Schwartz *et al* 1970.)
- Glu-II 0.080 (Schwartz *et al*. 1970)

Assay Information

Method

- Salicin is hydrolyzed by β-glucosidase to yield saligenen and β-D-glucose. The rate of formation of glucose is measured in a hexokinase/glucose-6-phosphate dehydrogenase system. One unit releases one micromole of glucose per minute at 37°C and pH 5.0 under the specified conditions.

Reagents

- 0.1 M Acetate buffer, pH 5.0
- 1% Salicin solution. Prepare by dissolving 1 g salicin in 100 ml 0.1 M acetate buffer, pH 5.0. Incubate at 37°C for 6 - 8 minutes before using.
- Glucose reagent system: 0.1 M Tris · HCl buffer pH 7.6, containing hexokinase (Worthington Code HKQL) $\geq 1.5 \frac{\text{units}}{\text{ml}}$, ATP: 0.77 $\frac{\mu\text{mol}}{\text{ml}}$, NAD: 0.91 $\frac{\mu\text{mol}}{\text{ml}}$, and glucose-6-phosphate dehydrogenase (Worthington Code ZFL) $\geq 1.9 \frac{\text{units}}{\text{ml}}$

Enzyme

- Dissolve at one $\frac{\text{mg}}{\text{ml}}$ in reagent grade water. Immediately prior to use dilute to 0.1 - 0.05 $\frac{\text{mg}}{\text{ml}}$ in reagent grade water.

Procedure

Pipette 1.0 ml of respective enzyme dilutions into a series of numbered test tubes. Include a blank with 1.0 ml reagent grade water. Incubate tubes at 37°C for 6 - 8 minutes to achieve temperature equilibrium. At timed intervals add 4 ml of the salicin solution. Mix well. Incubate each sample exactly 10 minutes then stop reaction at timed intervals by immediately placing each tube in a boiling water bath for at least 5 minutes. Cool in ice bath.
Pipette 3.0 ml Glucose reagent system into cuvettes.
Determine A_{340} of each solution before adding blank or enzyme-salicin reaction mixture. Add 0.1 ml blank and record change in A_{340}. To other cuvettes add 0.1 ml of the heated enzyme-salicin reaction mixture and record change in A_{340}. Record A_{340} until no further change occurs in 3 - 5 minutes. Read final A_{340}.

Calculation

- $\Delta A_{340} = \underset{\text{Final}}{A_{340}} - \underset{\text{Initial}}{A_{340}}$
- $$\frac{\text{Units}}{\text{mg}} = \frac{(\underset{\text{Sample}}{\Delta A_{340}} - \underset{\text{Blank}}{\Delta A_{340}}) \text{ x } 3.1 \text{ x } 5}{6.22 \text{ x } 10 \text{ min x } 0.1 \text{ x mg enzyme in reactions mixture}}$$

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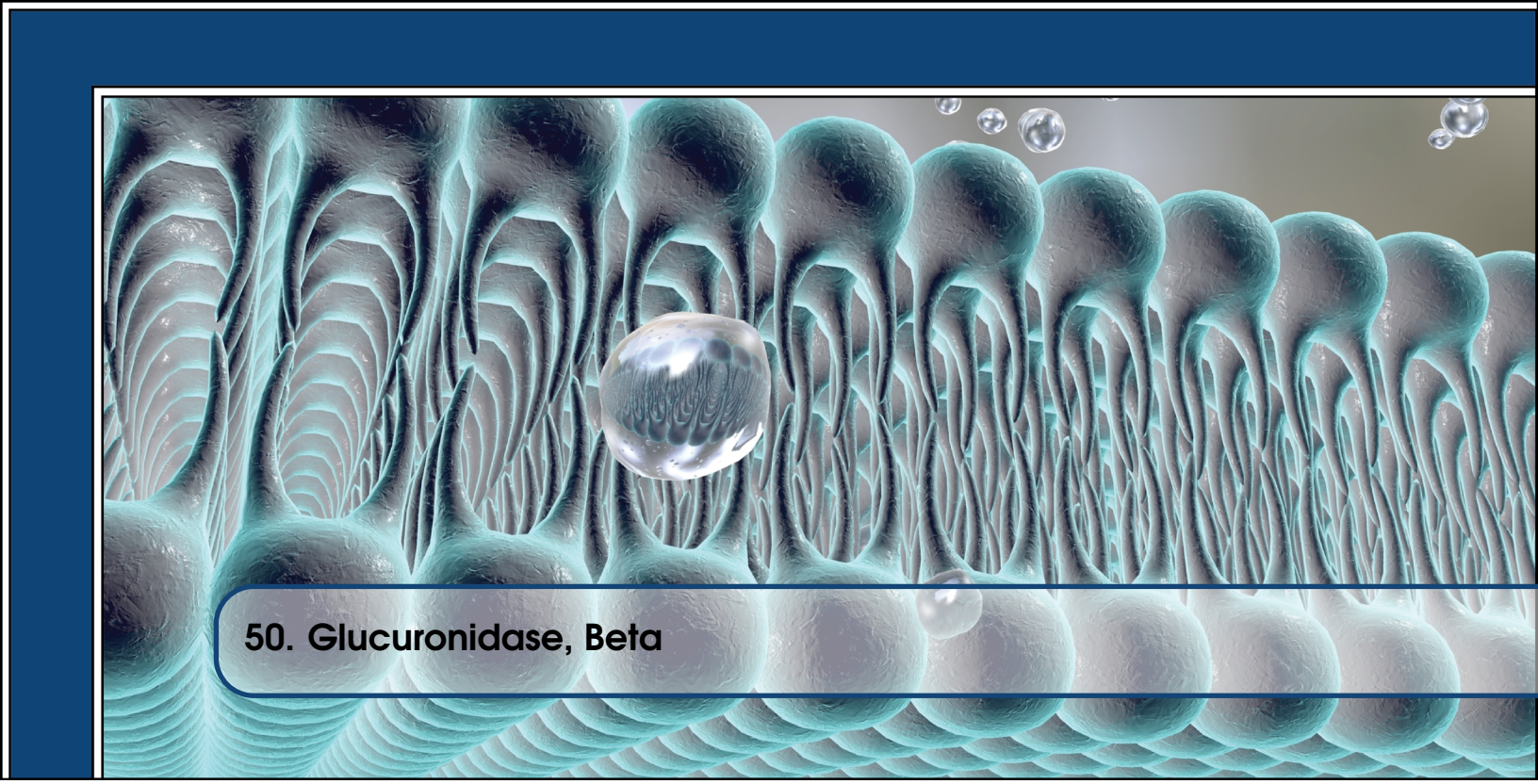

50. Glucuronidase, Beta

β-D-Glucuronoside Glucuronosohydrolase

It is ubiquitious in animal lysozymes.

The enzyme has found wide application in determining urinary steroids. β-Glucuronidase hydrolyzes conjugated glucuronides.

$$\beta\text{-D-glucuronide} + H_2O \rightarrow \text{Alcohol} + \text{D-glucuronic acid} \tag{50.1}$$

Figure 50.1: Enzymatic Reaction - Glucuronidase, Beta

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Bovine Liver

Specificity

The enzyme hydrolyzes a large variety of conjugated glucuronides but not α- or β-glucosides. See report by Tomasic and Keglevic (1973).

Composition

It is a sialic acid containing glycoprotein. The amino acid and carbohydrate content have been reported by Himeno *et al*. (1974). It is probable that the heterogeneity reported by Plapp and Cole (1967) is due to differing carbohydrate content. Wang and Touster (1972) report on an active center study using the rat liver enzyme which may be similar to that from beef liver.

IUB

- 3.2.1.31

Molecular Weight

- 290,000 (Himeno *et al*. 1974)

Optimal pH

- 4.4 (Wang and Touster 1972)

Isoelectric Point

- 5.1 (Himeno *et al*. 1974)

Inhibitors

- It is strongly, reversibly inhibited by various organic peroxides (Christner *et al*. 1970).

Stability/Storage

- Lyophilized, Worthington glucuronidase is stable 6 - 12 months at 2 - 8°C.

Assay Information

Method

- As described by Fishman et al. (1948) utilizing phenolphthalein glucuronidate as substrate. One unit hydrolyzes one micromole of phenolphthalein glucuronidate per minute at 37°C and pH 4.5 under the specified conditions. One Worthington unit is equivalent to 19,000 Fishman units.

Reagents

- 0.15 M Sodium chloride. Keep on ice.
- 0.1 M Sodium acetate, pH 4.5
- 0.01 M Phenolphthalein glucuronidate, pH 7.0
- 0.2 M Glycine with 0.2 M Sodium chloride, pH 10.4

Enzyme

- Dissolve enzyme at a concentration of 2 $\frac{\text{mg}}{\text{ml}}$ in cold 0.15 M sodium chloride. For assay dilute enzyme to a concentration ranging from 0.25 to 2 $\frac{\text{mg}}{\text{ml}}$.

Procedure

Into a series of numbered tubes pipette as follows: (include one tube as a blank).

- 0.1 M Sodium acetate - 0.8 ml
- 0.01 M Phenolphthalein glucuronidate - 0.1 ml

Incubate in 37°C water bath for five minutes to attain temperature equilibration. At timed intervals add to each tube, 0.1 ml of an enzyme dilution. To the blank, add 0.1 ml water.
Incubate for 30 minutes. Stop the reaction by adding 5.0 ml of 0.2 M glycine, 0.2 M NaCl, pH 10.4. Remove from the water bath, cool to room temperature and read A_{540} vs. the blank.

Calculation

- $\frac{\text{Units}}{\text{g}} = \frac{\text{micromoles phenolphthalein liberated x 1000}}{\text{30 minutes x mg enzyme in reaction mixture}}$

Determine micromole phenolphthalein released from a standard curve.

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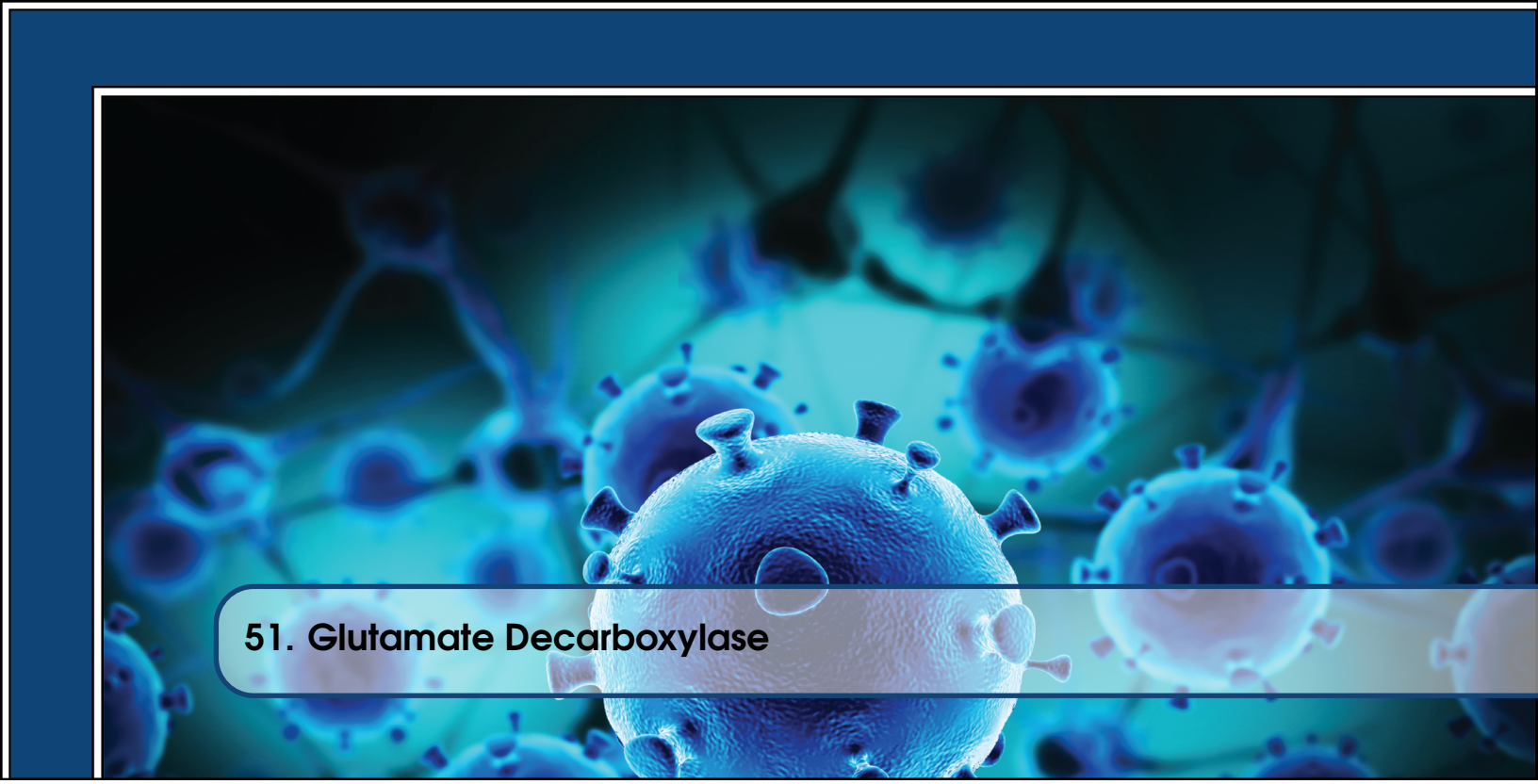

51. Glutamate Decarboxylase

Escherichia coli GAD maintains cellular pH under acidic conditions in the intestines, allowing *Escherichia coli* to survive gastric acidity and volatile fatty acids produced during fermentation in the intestines. As a result, *Escherichia coli* can cause disease even when a small amount of cells are ingested. In humans and higher mammals, the enzyme fulfills important physiological functions in the nervous system by balancing excitation and inhibition of neurons. It also acts as a autoantigen in insulin-dependent diabetes (Dutyshev *et al*. 2005). The following information applies to GAD of *Escherichia coli*. Glutamate decarboxylase (GAD) is a pyridoxal enzyme that catalyzes the removal of the carboxyl group of L-glutamic acid adjacent to the α-amino group to produce γ-aminobutyric acid and carbon dioxide:

$$HOCO\text{-}CH_2CH_2CH(NH_2)CO_2H \rightarrow HOCO\text{-}CH_2CH_2CH_2NH_2 + CO_2 \qquad (51.1)$$

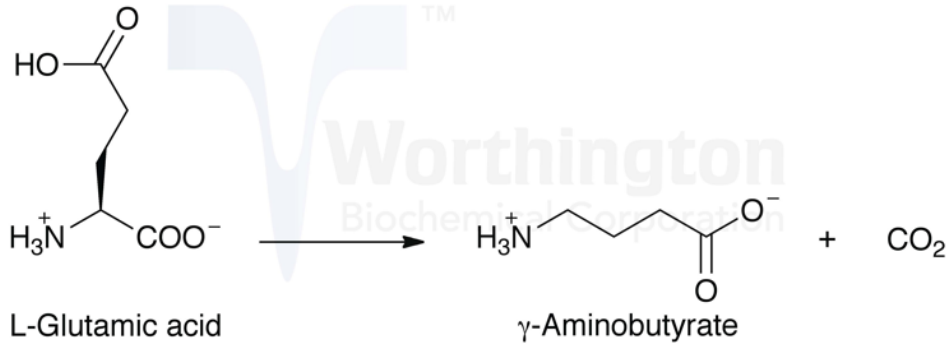

Figure 51.1: Enzymatic Reaction - Glutamate Decarboxylase

History

Investigations in the early 1900s showed the formation of amines from amino acids by mixing cultures of microorganisms, without knowing the processes, organisms, or enzymes involved (Ellinger 1900, Abderhalden *et al.* 1913, and Ackermann 1910, 1911). Later work was done using pure strains of identified organisms from the intestine, and the amino acids formed by each organism were determined (Berthelot and Bertrand 1911, 1912, Sasaki 1914, and Arai 1921).

Owing mainly to the work of Gale's laboratory (Gale 1947) several amino acid decarboxylase preparations were described that were specific for particular amino acids. Gale studied the enzymes involved in production of amino acids by certain bacteria, their properties, and the environmental conditions required for production of these enzymes (Gale 1940). Gale also determined early applications for these enzymes including estimation of particular amino acids (Gale 1954). In 1945, Taylor and Gale made the first attempt to isolate GAD, which was followed by Umbreit and Gunsalus in 1945, and Najjar and Fisher in 1953. In 1960, Shukuya and Schwert described procedures for higher purity (90%) and identified additional properties of the enzyme (Shukuya and Schwert 1960a). Umbreit and Gunsalus were the first to propose the idea that all amino acid decarboxylases are pyridoxal phosphate (PLP) dependent (Umbreit and Gunsalus 1945). After much debate (Mardashev and Semina 1964, and Cozzani 1965), Lawson and Quinn firmly established the enzyme as being PLP dependent in 1967. Conflicting studies on GAD activity and glutamate use by *Escherichia coli* led to the mapping of the GAD gene (gad) and its regulation by both closely and loosely linked genes (Halpern and Umbarger 1961, and Marcus and Halpern 1969).

In the late 1980s, the enzyme was crystallized (Markovic-Housley *et al.* 1987, and Sukhareva *et al.* 1989). In the 1990s, its amino acid sequence and genes were compared to those of other species, and it was determined GADs of various organisms probably share a common origin (Maras *et al.* 1992, and Smith *et al.* 1992). Recent research has investigated the regulation of GADa and GADb expression during acid stress (Castanié-Cornet *et al.* 2010) and the residues important for pH-dependent activity of the enzyme (Pennacchietti *et al.* 2009).

Molecular Characteristics

Two virtually identical genes have been found (gadA and gadB), which encode the two isozymes (GADa and GADb) (Smith *et al*. 1992, De Biase *et al*. 1996, and Shul'ga *et al*. 1999). The protein products differ in only five amino-acid residues, and their functional properties are identical (McCormick and Tunnicliff 2001). A short length of bacterial, cat, and drosophila GAD (a 55 residue segment around the lysine responsible for covalent binding to the coenzyme) has been shown to have significant similarity (25% identity), suggesting the enzymes arose from a common origin (Maras *et al*. 1992). GAD from *Streptococcus pneumoniae* has been found to exhibit 28% homology with GAD65 from human brain (García and López 1995).

Specificity

Glutamate decarboxylase is unusually specific, showing significant activity only on L-glutamic acid and α-methyl glutamic acid (Fonda 1972a). The same report shows the following compounds to be neither substrates nor inhibitors: D-glutamate, D- and L-aspartate, α-amino adipic and α-aminopimelic acids. Pure L-glutamine is not a substrate. The enzyme of *Escherichia coli* shows different physical and catalytic properties from the mammalian enzyme, but has shown some similarities in inhibition studies (Youngs and Tunnicliff 1991). Inhibition studies have suggested cysteinyl residues play a role in glutamate binding to the enzyme (McCormick and Tunnicliff 2001).

Composition

GAD of *Escherichia coli* exists as a hexamer of approximately 50 kDa identical subunits, each containing one molecule of pyridoxal phosphate (PLP) (To 1971).

Escherichia coli has four inducible acid resistance systems to survive extreme acidity. GAD is part of *Escherichia coli*'s second acid resistance system (AR2). This system requires extracellular glutamate and consists of the two isoforms (GADa and GADb) and a putative glutamate:g-aminobutyric acid (GABA) antiporter called GADc (Malashkevich *et al*. 1998, De Biase *et al*. 1999, Richard and Foster 2003, and Richard and Foster 2004). It is believed that this system protects the cell from acid stress by consuming extracellular protons during each decarboxylation reaction to maintain the internal pH of the cell (Castanié-Cornet *et al*. 1999). Richard and Foster suggested that it may be more important that the system reverses the change in transmembrane potential (Richard and Foster 2004).

IUB

- 4.1.1.15

CAS

- 9024-58-2

Protein Accession Number

- P69908

Molecular Weight

- 316 kDa (Dutyshev *et al.* 2005)

Optimal pH

- 3.8 (Shukuya and Schwert 1960b; Strausbauch and Fischer 1970a)

Isoelectric Point

- 5.22

Extinction Coefficient

- 505,620 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 17.0 (Strausbauch and Fischer 1970a)

Activators

- Pyridoxal phosphate is a necessary but firmly bound coenzyme. Because of the reversible configurational change at about pH 5.5 (O'Leary and Brummund 1974), chloride ions may be stimulatory to activity and acetate ions may inhibit. The crystalline enzyme has a maximum specific activity of 100-120 units/mg (pH 4.5, 38°C).

Inhibitors

- L-isoglutamic acid, and aliphatic dicarboxylic acids, especially glutaric, pimelic and adipic acids (Fonda 1972a)
- α-(fluoromethyl)glutamic acid (Kuo and Rando 1981)
- Some sulfhydryl-group reagents: Mercuric chloride (most effective), pCMB, and DTNB (McCormick and Tunnicliff 2001)

Applications

- Detection of *Escherichia coli* in food and water samples (Grant *et al.* 2001)
- Determination of glutamate and aspartic acid (Gale 1947)

Assay Information

Method

- The reaction velocity is measured by a conventional Warburg manometric technique where glutamate is converted to aminobutyrate. One unit releases one micromole of CO_2 per minute at 37°C and pH 5.0 under the specified conditions.

Reagents

- 0.5 M Sodium acetate buffer, pH 5.0
- 0.05 M L-glutamate in 0.5 M sodium acetate, pH 5.0
- 3.0 M Sodium chloride

Enzyme

- Immediately prior to use, dilute the enzyme with reagent grade water to a concentration of 0.5 - 3.0 $\frac{\text{units}}{\text{ml}}$.

Procedure

Into the main well of the Warburg flask pipette the following:

- 0.5 M sodium acetate, pH 5.0 - 1.0 ml
- 3.0 M sodium chloride - 0.1 ml
- Reagent grade water - 1.3 ml
- Enzyme dilution - 0.1 ml

Pipette 0.5 ml of 0.05 M L-glutamate into side arm. Include one flask with no enzyme as a blank and one flask with 3.0 ml water as a thermal barometer. Assemble flasks and incubate for 10 - 15 minutes to achieve temperature equilibration. Tip in substrate and measure CO_2 evolution at 2 - 3 minute intervals for 30 minutes.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\text{microliters } CO_2 \text{ liberated}}{\text{min}}}{22.4 \text{ x mg enzyme in reaction mixture}}$

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52. Glyceraldehyde-3-Phosphate Dehydrogenase

D-Glyceraldehyde-3-Phosphate: NAD^+ Oxidoreductase (Phophorylating)

GAPD is a key enzyme in intermediary metabolism catalyzing the oxidation and subsequent phosphorylation of substrate aldehydes to acyl phosphate. Buehner *et al*. (1973) have indicated its structural similarity to LDH, MDH and ADH. It occurs in high concentrations in mammalian, fish, and fowl muscle, yeast and bacteria. The human enzyme has been reported on by Watson *et al*. (1972) and Girotti (1976); that of pig muscle by Harris and Perham (1965) and Batke *et al*. (1974); lobster GAPD the amino acid sequence of which is reported to be like that of rabbit, by Davidson *et al*. (1967). Yeast GAPD amino acid-sequence was determined by Jones and Harris (1972). A report on rabbit muscle GAPD purification is that by Hill *et al*. (1975b). Nagradova and Grozdova (1974) report on the close structural similarity of the enzymes from mammals. The hybridization of GAPD subunits of different origin forms mixed tetramers, even between such diverse sources as rabbit muscle and Ascaris (Kochman *et al*. 1974), and rabbit muscle and yeast (Spotorno and Hollaway 1970; Osborne and Hollaway 1974 and 1975). See also Markovich and Krapivinskaya (1975). Suzuki and Harris (1975) report on hybridization of GAPD subunits from rabbit, pig, lobster, yeast and *Escherichia coli* and indicate that individual subunits retain their activity even within tetramers formed with subunits from other species.

The enzyme is used for the determination of D-Gly-3-P, NAD^+, Pi, ATP, glycerate-3-P and glycerate-1,3,diP. It is also used for the determination of phosphoglycerate kinase activity.

See Hill *et al*. (1975a), Fife and Szabo (1973), and Francis *et al*. (1973). Glyceraldehyde-3-phosphate dehydrogenase (GAPD) catalyzes the following reaction:

$$\text{glyceraldehyde-3-phosphate} + NAD^+ + \text{Pi} \rightleftarrows \text{1,3-diphosphoglycerate} + NADH + H^+ \qquad (52.1)$$

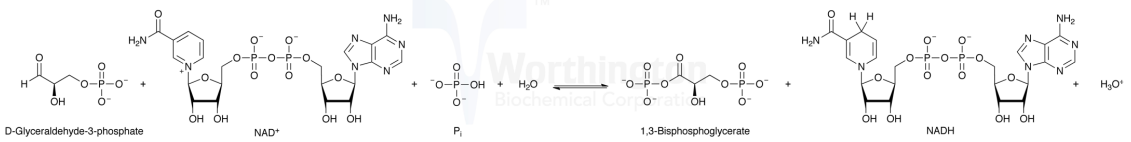

Figure 52.1: Enzymatic Reaction - Glyceraldehyde-3-Phosphate Dehydrogenase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Rabbit Muscle

Specificity

The normal dehydrogenase reaction involves NAD as coenzyme, phosphate and D-glyceraldehyde-3-phosphate as substrates. Arsenate is often used to replace phosphate in assays. D-glyceraldehyde is very slowly oxidized.

Composition

The enzyme consists of four identical polypeptide chains (MW 36,000) each accommodating one molecule of NAD^+ as coenzyme (Reisler *et al.* 1975). See also Price and Radda (1974). The monomers are joined noncovalently. Hoagland and Teller (1969) indicate that as a dimer GAPD exhibits esterase activity and that the dimer and tetramer may be in equilibrium. See also Constantinides and Deal (1970).

Allison *et al.* (1973) report on a sulfenic acid form of GAPD that functions as an acetyl phosphatase. The active site involves cysteine 149 which, in the presence of NAD^+ and the substrate aldehyde, forms a thiol ester intermediate (Bode *et al.* 1975). See also Reisler *et al.* (1975). Bloch *et al.* (1971) indicate that GAPD prepared according to Ferdinand (1964) binds ADP-ribose rather than NAD^+. See also Hill *et al.* (1975). Other active site reports include Dwek *et al.* (1975), Boers and Verhoeven (1973), Buehner *et al.* (1973) and Levitzki (1973) on the similarity of binding sites to LDH, MDH, and ADH.

IUB

- 1.2.1.12

Molecular Weight

- 144,000 (See Bode *et al.* 1975)

Isoelectric Point

- pH 6.55 (Cori *et al.* 1948). See also Velick and Furfine (1963)

Extinction Coefficient

- $E^{1\%}_{278}$ = 10.2 (Murdock and Koeppe 1964)

Activators

- EDTA, cysteine, and Cleland's reagent have activating or protective effects. NAD binding is considered to have allosteric effects (Kirschner *et al.* 1966), to cause conformational changes (Havsteen 1965; Listowsky *et al.* 1965; Jaenicke and Gratzer 1969), to reverse inhibition by multivalent anions (Fenselau 1970), and to decrease susceptibility to proteolysis (Fenselau 1970).

Inhibitors

- Reagents reacting with or oxidizing the catalytic site sulfhydryl group inhibit the enzyme. Heavy metal ions, pCMB, iodoacetate, o-iodosobenzoate, and tetrathionate are among known inhibitors. ATP, cyclic AMP, and other adenine-containing compounds have been indicated as resulting in reversible dissociation, competitive inhibition, and increased susceptibility to proteolysis (Stancel and Deal 1968, 1969; Constantinides and Deal 1969; Yang and Deal 1969; Fife and Szabo 1973; Oguchi *et al.* 1973; Hixson and Hixson 1975; Smith *et al.* 1975).

Stability/Storage

- Crystalline GAPD is stable for one year when refrigerated as a suspension in 2.9 M ammonium sulfate solution.

Assay Information

Method

- The initial reaction velocity is measured as an increase in absorption at 340 nm resulting from the reduction of NAD. The assay system is a modification of the procedures described by Krebs (1955) and Velick (1955). One unit causes an initial rate of reduction of one micromole of NAD per minute at 25°C and pH 8.5 under the specified conditions.

Reagents

- 0.015 M Sodium pyrophosphate buffer, pH 8.5 containing 0.03 M sodium arsenate
- 7.5 mM NAD. Note: NAD may vary in salt form and degree of hydration. Care should be exercised to use an analytical grade and the correct molecular weight.
- 0.015 M DL-glyceraldehyde-3-phosphate (7.5 mM D-glyceraldehyde-3-phosphate). See note below.
- 0.1 M Dithiothreitol (DTT)

Enzyme

- Immediately prior to use, dilute in pyrophosphate/arsenate buffer to a concentration of 10 - 30 $\frac{\mu g}{ml}$.

Procedure

Spectrophotometer Settings: Wavelength: 340 nm Temperature: 25°C

Pipette into each cuvette as follows:

- Pyrophosphate/arsenate buffer - 2.6 ml
- 7.5 mM NAD - 0.1 ml
- 0.1 M DTT - 0.1 ml
- Enzyme - 0.1 ml

Incubate in spectrophotometer at 25°C for 3 - 5 minutes to achieve temperature equilibrium and establish blank rate, if any. At zero time, add 0.1 ml of 0.015 M DL-glyceraldehyde-3-phosphate and record A_{340} for 3 - 5 minutes. Determine $\frac{\Delta A_{340}}{\text{min}}$ from the initial linear portion of the curve.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{340}}{\text{min}}}{6.22 \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$$

Notes

- Note: D-glyceraldehyde-3-phosphate can be prepared from its DL-glyceraldehyde-3-phosphate diethylacetal derivatives as follows:
- Suspend 1.5 grams of Dowex-50 hydrogen form resin in 6 ml of reagent grade water in a test tube.
- Add 100 mg of DL-glyceraldehyde-3-phosphate diethylacetal, barium salt, and mix thoroughly.
- Place the test tube in a boiling water bath for 3 - 5 minutes with occasional mixing. Cool quickly in an ice bath.
- Centrifuge and decant the supernatant which contains the free acid form of DL-glyceraldehyde-3-phosphate.
- Wash the resin several times with 2 ml aliquots of water to complete the extraction. Combine the supernatants and determine the D-glyceraldehyde-3-phosphate concentration. If the hydrolysis and extraction are complete the pooled supernatants should contain approximately 100 micromoles of the D-isomer.
- The concentration of the D-isomer can be measured by the above assay utilizing an excess of enzyme.

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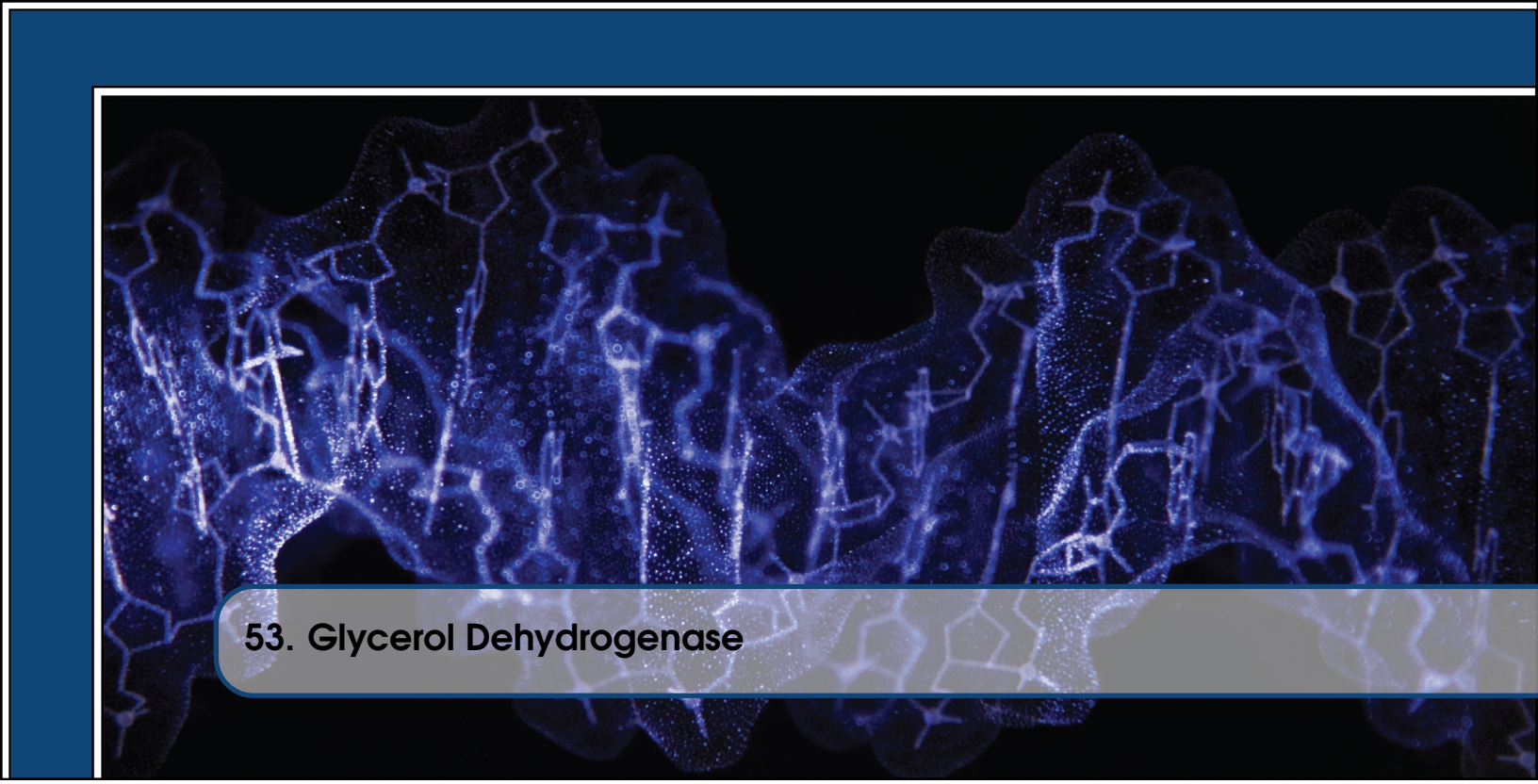

53. Glycerol Dehydrogenase

Glycerol: NAD^+ 2-Oxidoreductase

The primary interest in this enzyme is for the determination of glycerol in biological fluids: reduction of NAD is measured by change in absorbance at 340 nm. Glycerol dehydrogenase catalyzes the following reaction:

$$\text{glycerol} + \text{NAD} \rightleftarrows \text{dihydroxyacetone} + \text{NADH} \tag{53.1}$$

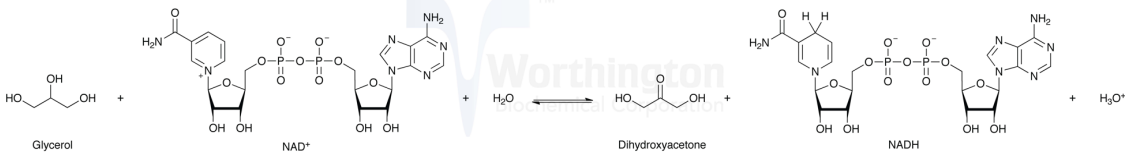

Figure 53.1: Enzymatic Reaction - Glycerol Dehydrogenase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Enterobacter aerogenes*

Specificity

The greatest oxidizing activity is on glycerol and 1,2-propandiol. A table of substrate specificity is found in Lin and Magasanik (1960).

IUB

- 1.1.1.6

Optimal pH

- 9.0 (Lin and Magasanik 1960). At pH 11, the enzyme is nearly inactive.

Inhibitors

- The enzyme is inhibited by zinc, (Lin *et al*. 1960); Li^+ and Na^+ and also by high ionic strength solutions (Strickland and Miller 1968).

Stability/Storage

- The Worthington preparation is stable 6 - 12 months at 2 - 8°C. Concentrated solutions (10 $\frac{mg}{ml}$) are also relatively stable; on dilution to 1 $\frac{mg}{ml}$ the activity rapidly disappears.

Activity

- The K_m for glycerol was found by Lin and Magasanik (1960) to be 0.017 M and 0.0056 M in the presence of 0.0033 M and 0.033 M ammonium chloride repectively. Vmax is increased by NH_4^+ also. They report that K^+ and Rb^+ activate the enzyme.

Assay Information

Method

- The activity is measured using the procedure of Lin and Magasanik (1960) by determining the increase in absorbance at 340 nm resulting from the reduction of NAD. One unit reduces one micromole of NAD per minute at 25°C and pH 10.0 under the specified conditions.

Reagents

- 1.0 M Glycerol
- 0.125 M Potassium carbonate
- 0.125 M Sodium bicarbonate
- 0.125 M Carbonate/bicarbonate buffer, pH 10.0. Prepare by mixing potassium carbonate and sodium bicarbonate to reach pH 10.0.
- 1.0 M Ammonium sulfate
- 0.05 M Potassium phosphate, pH 7.6, containing 0.1 M ammonium sulfate and 0.1 mM manganese chloride
- 0.1 M NAD. Note: NAD may vary in salt form and degree of hydration. Care should be exercised to use an analytical grade and the correct molecular weight.

Enzyme

- Dissolve enzyme at a concentration of one $\frac{mg}{ml}$ in 0.05 M potassium phosphate buffer solution. Immediately prior to use dilute further in this buffer to obtain a rate of 0.02 - 0.05 $\frac{\Delta A}{min}$.

Procedure

Spectrophotometer Settings: Wavelength: 340 nm Temperature: 25°C

Pipette into each cuvette as follows:

- 1.0 M Ammonium sulfate - 0.1 ml
- 0.1 M NAD - 0.1 ml
- 0.125 M Carbonate/bicarbonate buffer, pH 10.0 - 2.4 ml
- 1.0 M Glycerol - 0.3 ml

Incubate in the spectrophotometer for 4 - 5 minutes to achieve temperature equilibration and establish blank rate, if any. At zero time, add 0.1 ml of appropriately diluted enzyme and mix thoroughly. Record increase in A_{340} for 3 - 5 minutes. Determine $\frac{\Delta A_{340}}{min}$ from initial linear portion of the curve.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{340}}{\text{min}}}{6.22 \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$$

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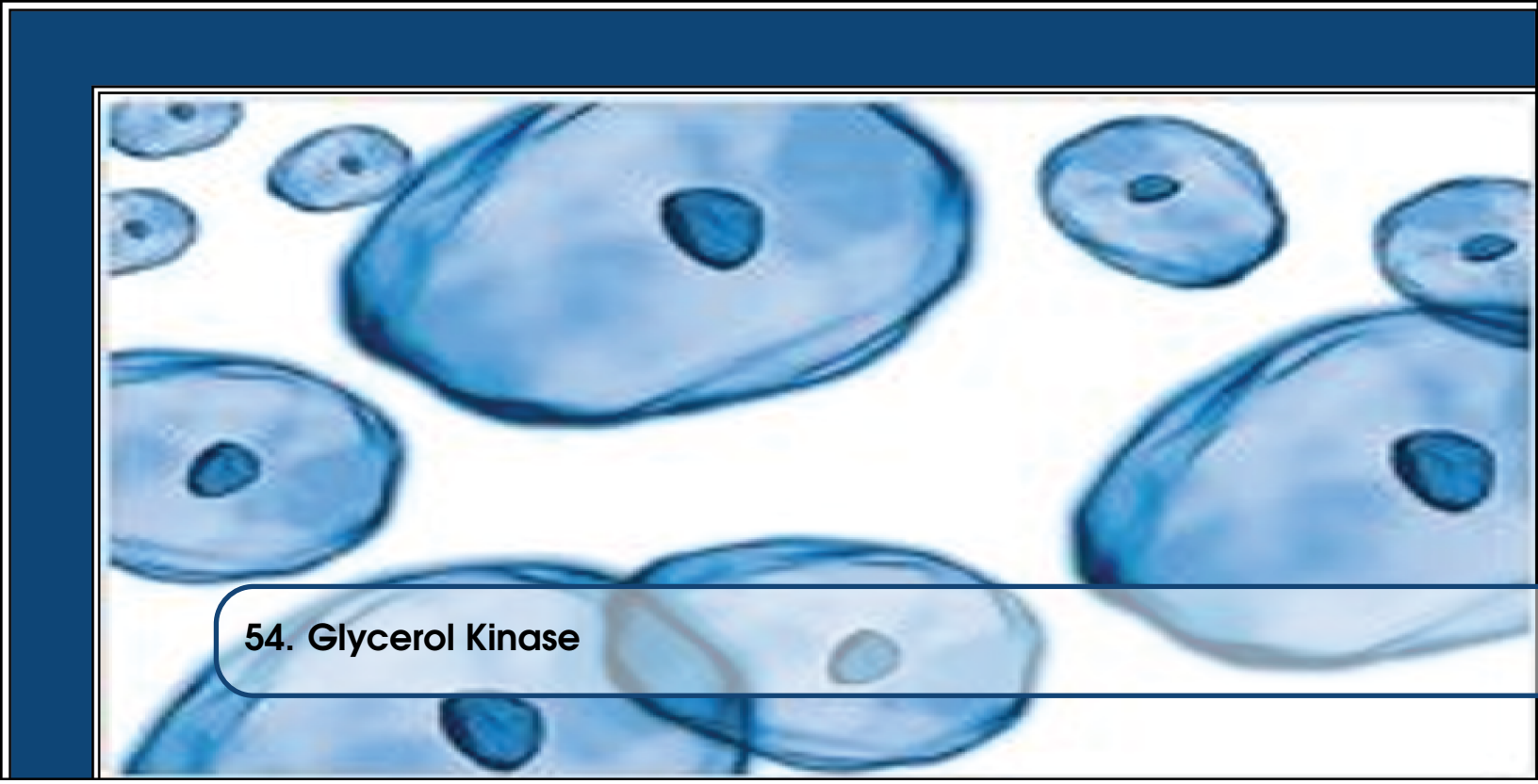

54. Glycerol Kinase

ATP: Glycerol 3-Phosphotransferase

Glycerol kinase has been reviewed by Thorner and Paulus (1973a). The activity is found widely in nature. In microorganisms GK makes possible the utilization of glycerol as a carbon source. According to Kida *et al.* (1973), in mammals the enzyme represents a juncture of sugar and fat metabolism; however, the mammalian enzymes are quite different from the microbial. Yeast GK from Candida mycorderma has been reported on by and Cleland (1974) and Eisenthal *et al.* (1974).

The enzyme is important to the clinical chemist in the determination of glycerol. Pinter *et al.* (1967) also indicate that GK is useful in the assay of glyceraldehyde and dihydroxyacetone following their quantitative reduction to glycerol with sodium borohydride. Mallon and Dalton (1971) report on an automated glycerol assay. Glycerol kinase (GK) catalyzes the following reaction:

$$\text{Glycerol} + \text{ATP} \xrightarrow{Mg^{2+}} \text{L-glycero 1,3-phosphate} + \text{ADP} \qquad (54.1)$$

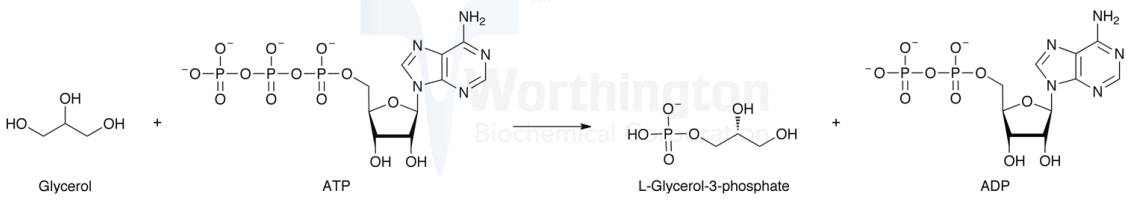

Figure 54.1: Enzymatic Reaction - Glycerol Kinase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Escherichia coli*

Specificity

Of all nucleotides tested, only ATP can act as phosphate donor. Dihydroxyacetone and glyceraldehyde are phosphorylated by a highly purified preparation but at greatly reduced rates (See Thorner and Paulus review, 1973).

Composition

Thorner and Paulus (1973b) indicate the enzyme to be composed of four identical subunits. They also report the purification of GK from a mutant strain with double the specific activity but of identical size and subunit components. (See also Berman-Kurtz *et al.* 1971). The amino acid composition has been reported (Thorner and Paulus 1971).

IUB

- 2.7.1.30

Molecular Weight

- 217,000 (Thorner and Paulus 1971)

Optimal pH

- 9.0 - 9.8. Variations in the assay procedure may shift the optimum pH somewhat. (Hayashi and Lin 1967)

Stability/Storage

- GK is protected by the presence of EDTA and mercaptoethanol and especially by the presence of glycerol. Maximum stability is at pH 7.0; it is relatively poor below pH 6.0 and above pH 7.6. The enzyme is insoluble in pure water and in buffers of low ionic-strength. Excellent stability has been reported for the crystalline enzyme in half-saturated ammonium sulfate at 0°C.

Activity

- GK phosphorylates glycerol exlusively to L-α-glycerophosphate. The reaction is essentially irreversible and Mg^{2+} is required. Although manganous ions may be substituted the activity is reduced to about one third. (Hayashi and Lin 1967). The catalytic rate is slowed by fructose biphosphate (Zwaig and Lin 1966). Thorner and Paulus (1973) report on the allosteric properties of the enzyme.

Assay Information

Method

- The reaction velocity is measured in a coupled system with pyruvate kinase and lactate dehydrogenase. One unit results in the oxidation of one micromole of NADH per minute at 25°C and pH 8.9 under the specified conditions.
- Glycerol + ATP $\overset{GK}{\rightleftarrows}$ α-Glycerophosphate + ADP
- ADP + PEP $\overset{PK}{\rightleftarrows}$ ATP + Pyruvate
- Pyruvate + NADH + H^+ $\overset{LDH}{\rightleftarrows}$ Lactate + NAD^+

Reagents

- 0.4 M Glycine, pH 8.9 containing 45 mM potassium carbonate
- 0.1 M Triethanolamine·HCl buffer, pH 7.4
- 0.1 M Glycerol

Reagent solution - the required amount of solution should be prepared containing:

- ATP - 8.5 mM
- NADH - 1.22 mM
- PEP - 2.0 mM
- Lactate Dehydrogenase (Worthington code: LADCL) - 15.3 $\frac{\text{units}}{\text{ml}}$
- Pyruvate kinase (Worthington code: PKL) - 7.0 $\frac{\text{units}}{\text{ml}}$
- $MgSO_4 \cdot 7H_2O$ - 28.0 mM
- Reduced Glutathione - 26.0 mM
- pH adjusted to - 7.4

Enzyme

- Dissolve at a concentration of one $\frac{\text{mg}}{\text{ml}}$ in 0.1 M TEA buffer, pH 7.4. Immediately prior to use, dilute further in TEA buffer to obtain a rate of 0.02 - 0.04 $\frac{\Delta A}{\text{min}}$.

Procedure

Spectrophotometer Settings: Wavelength: 340 nm Temperature: 25°C

Pipette into cuvettes as follows:

- Carbonate-glycine buffer - 2.1 ml
- Reagent solution - 0.7 ml
- 0.1 M Glycerol - 0.1 ml

Incubate in spectrophotometer for 3 - 4 minutes to achieve temperature equilibration and establish blank rate, if any.

Add 0.1 ml of appropriately diluted enzyme and record ΔA_{340} for 6 - 8 minutes. Determine $\frac{\Delta A_{340}}{\text{min}}$ from the linear portion of the curve. A short lag period may be observed.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{340}}{\text{min}}}{6.22 \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$

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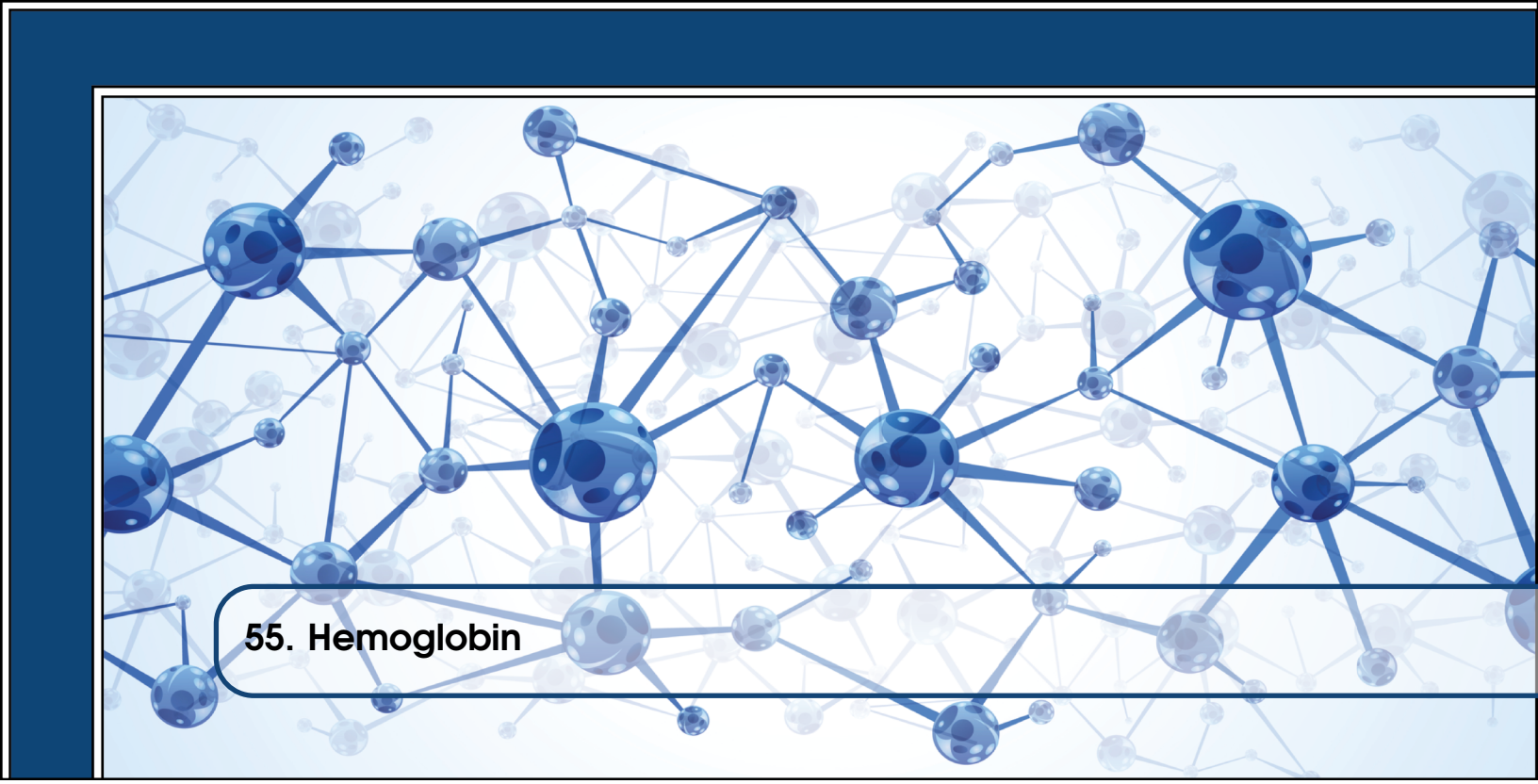

55. Hemoglobin

Bovine erythrocyte hemoglobin is used as a substrate for pepsin and other proteases.

Hemoglobin is composed of one globin plus four hemes; heme consists of protoporphyrin IX and ferrous iron. The iron content of human hemoglobin is 0.338% (w/w) which gives a minimal molecular weight of 16,520; however, ultracentrifugal and osmotic pressure measurements indicate an actual molecular weight four times the minimum molecular weight (Henry 1968).

Pauling (1948) has shown that the chemical characteristics of hemoglobin result from the electrical and magnetic interaction of the iron and the porphyrin ring which prevents the ferrous iron from forming further bonds with most substances other than O_2 and CO, which form covalent bonds with the ferrous ion. Determination of the hemoglobin content of whole blood has been the most common quantitative clinical analysis for at least a half century.

CAS

- 9008-02-0

Stability/Storage

- Stable for years if kept dry.

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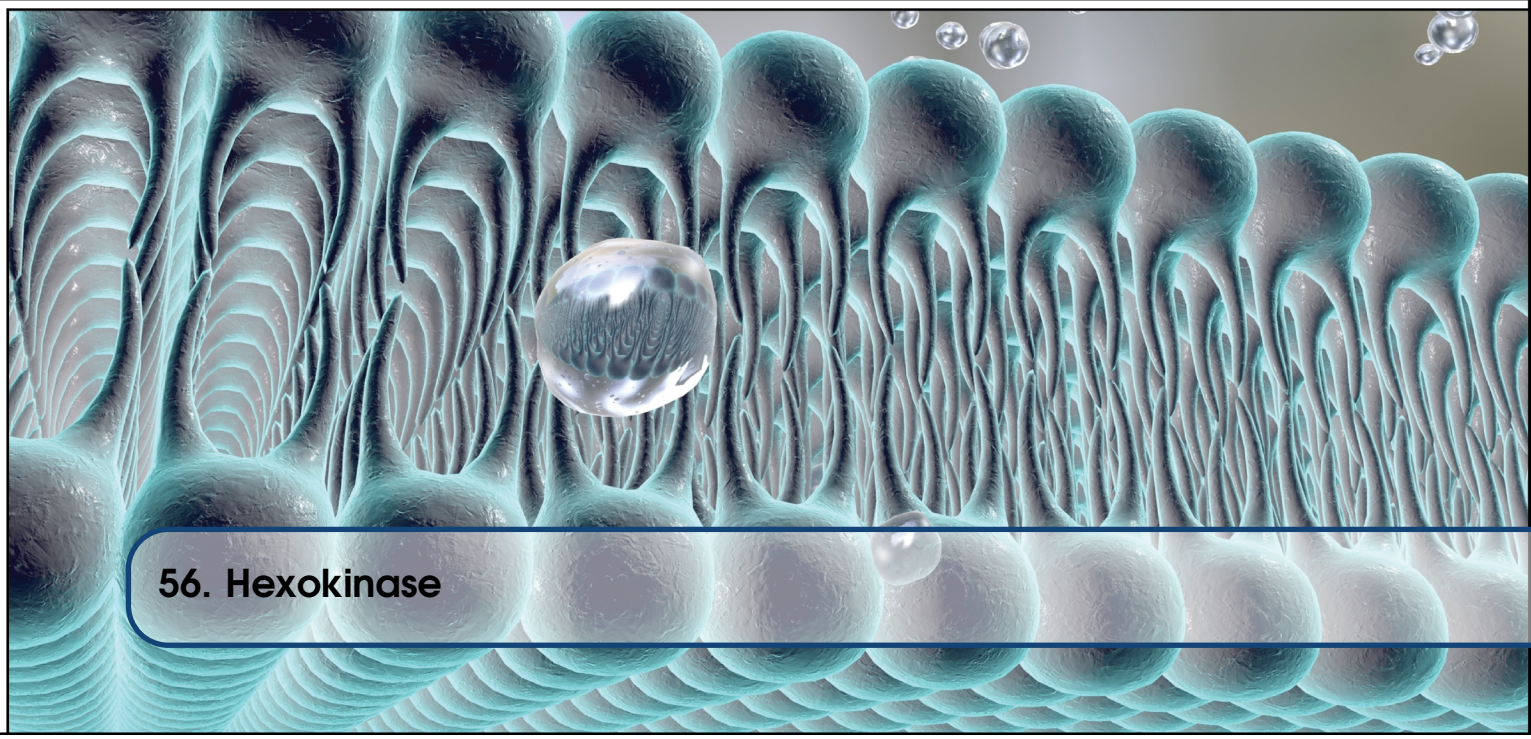

56. Hexokinase

Hexokinases have been isolated from the yeast cell in two distinct forms, designated P-I and P-II (Schulze *et al*. 1969). These are separate, noninterconvertible isozymes (Womack *et al*. 1973).

Hexokinase is used to determine glucose, fructose, mannose and ATP. Hexokinase catalyzes the reaction:

$$\text{D-hexose} + \text{ATP} \rightleftarrows \text{D-hexose 6-phosphate} + \text{ADP} \tag{56.1}$$

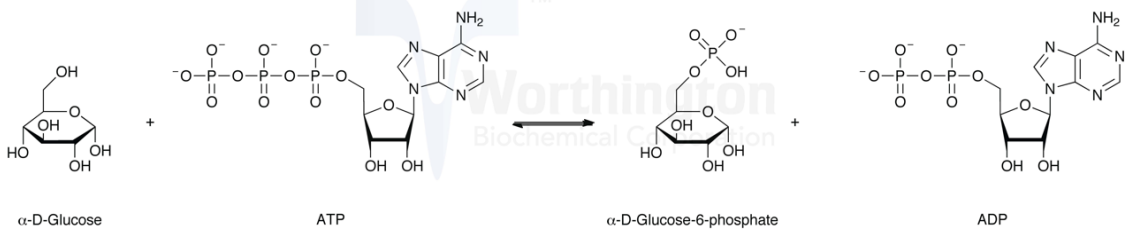

Figure 56.1: Enzymatic Reaction - Hexokinase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Yeast

Specificity

The enzyme phosphorylates D-fructose, 5-keto-D-fructose (Avigrad *et al.* 1968), D-glucose, 2-deoxy-D-glucose, D-mannose and D-glucosamine. ATP and ITP have been demonstrated to transphosphorylate in the yeast hexokinase reaction (Martinez 1961). The activity of P-I with fructose is 2.6 times that with glucose, whereas with P-II, a fructose:glucose ratio of 1:3 is obtained (Lazarus *et al.* 1966). The substrate specificities of yeast hexokinase have been extensively studied by Bessell *et al.* (1972).

Composition

Both P-I and P-II contain the same amino terminus, valine, and the same carboxy terminus, alanine. Amino acid composition has been reported by Schmidt *et al.* (1973b).

IUB

- 2.7.1.1

CAS

- 9001-51-8

Molecular Weight

- The native forms have molecular weights of about 100,000 (Schulze *et al.* 1969) and consist of polypeptide chains of molecular weights slightly higher than 50,000 (Schmidt *et al.* 1973).

Optimal pH

- 7.5 - 9.0 (Sols *et al.* 1958)

Isoelectric Point

- P-I, 5.25 and P-II, 4.93 (Schmidt *et al.* 1973)

Extinction Coefficient

- $E^{1\%}_{280}$ = 8.85 for P-I and $E^{1\%}_{280}$ = 9.47 for P-II (Schmidt *et al.* 1973)

Activators

- Hexokinase requires magnesium ions for its catalytic activity. It is activated by catecholamines and related compounds (Harrison *et al.* 1972). Calcium ions do not affect the enzymatic activity.

Inhibitors

- The enzyme is inhibited by compounds which react with SH groups. It is also inhibited by sorbose-1-phosphate, polyphosphates, 6-deoxy-6-fluoroglucose, 2-C-hydroxy-methylglucose, xylose and lyxose (Sols *et al.* 1958 and McDonald 1955).

Stability/Storage

- Both the lyophilized preparation and the crystalline suspension are stable for 6-12 months at 2 - 8°C.

Assay Information

Method

- The assay is based upon the reduction of NAD+ through a coupled reaction with glucose-6-phosphate dehydrogenase and is determined spectrophotometrically by measuring the increase in absorbance at 340 nm.
- D-glucose + ATP $\overset{HK}{\rightarrow}$ Glucose-6-P + ADP
- Glucose-6-P + NAD $\overset{ZF}{\rightarrow}$ Gluconate-6-P + NADH + H^+
- One unit of activity reduces one micromole of NAD+ per minute at 30°C and pH 8.0 under the specified conditions.

Reagents

- 0.05 M Tris · HCl buffer, pH 8.0 with 13.3 mM $MgCl_2$
- 0.67 M Glucose in above Tris·$MgCl_2$ buffer
- 16.5 mM Adenosine 5'Triphosphate in above Tris·$MgCl_2$ buffer
- 6.8 mM NAD in above Tris·$MgCl_2$ buffer
- Note: NAD may vary in salt form and degree of hydration. Care should be exercised to use an analytical grade and the correct molecular weight.
- *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (Worthington Code: ZF or ZFL). Dissolve at a concentration of 300 $\frac{IU}{ml}$ in above Tris·$MgCl_2$ buffer. Store at 0 - 4°C during use.

Enzyme

- Dissolve in Tris·$MgCl_2$ buffer, pH 8.0 to obtain a rate of 0.02 - 0.04 $\frac{\Delta A}{min}$.

Procedure

Spectrophotometer Settings: Wavelength: 340 nm Temperature: 30°C

Pipette into each cuvette as follows:

- Tris-$MgCl_2$ buffer - 2.28 ml
- 0.67 M Glucose - 0.50 ml
- 16.5 mM ATP - 0.10 ml
- 6.8 mM NAD - 0.10 ml
- G-6-PDH - 0.01 ml

Incubate in the spectrophotometer at 30°C for 6 - 8 minutes to achieve temperature equilibration and establish blank rate, if any. At zero time, add 0.1 ml of diluted hexokinase solution and mix thoroughly. Record increase in A_{340} for 3-4 minutes. Determine $\frac{\Delta A}{min}$ from initial linear portion of curve.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{340}}{\text{min}}}{6.22 \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$$

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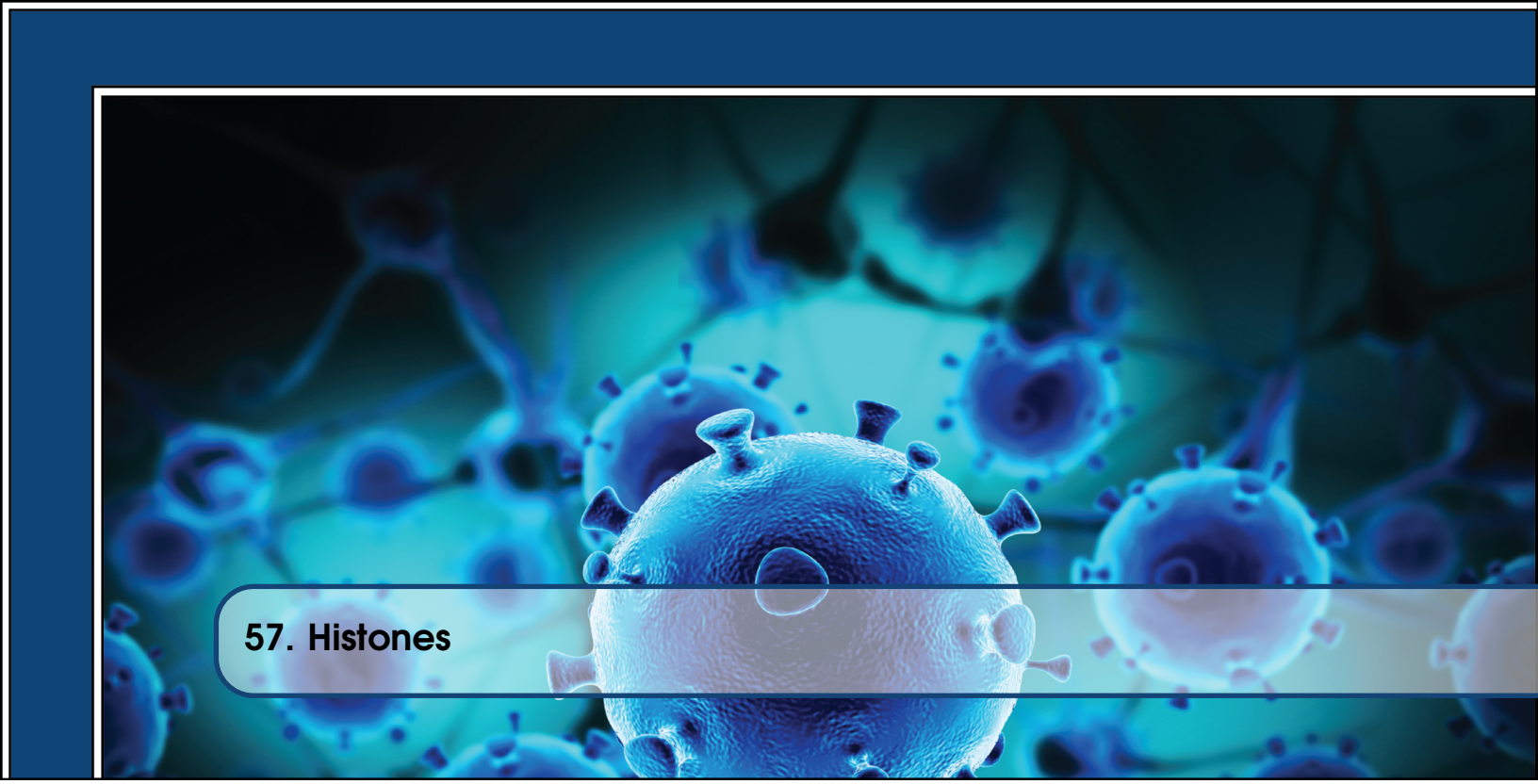

57. Histones

The histones are a group of water and dilute acid soluble basic proteins found associated with DNA in chromosomes. They are characterized by relatively high levels of lysine and arginine. Although histones are classified into a limited number of types of fractions (see below) with each particular fraction having a fundamentally distinct amino acid composition and sequence, numerous subfractions are observed due to the acetylation, methylation, and phosphorylation of various amino acid residues. Microheterogeneity or alteration of structure is dynamic such that the histones of a single cell type are found to vary during development. They are believed to play a role in gene activity and cellular metabolism. See "The Structure and Function of Chromatin," Ciba Foundation Symposium 28, American Elsevier, N.Y. (1975).

Although the classic nomenclature for histones is that of Johns and Butler (1962), the nomenclature of Bradbury (1975) was submitted to the IUPAC.

Characteristics of Histones from Calf Thymus (Elgin and Weintraub 1975):

Class	Bradbury	Johns	Molecular Weight
Lysine Rich	H1	f1	about 21,500
Slightly Lysine Rich	H2a	f2a2	14,004
Slightly Lysine Rich	H2b	f2b	13,774
Arginine Rich	H3	f3	15,324
Arginine Rich	H4	f2a1	11,282

With the exception of H1, the primary structures of the calf thymus histones have been determined.

Comparisons with the structures for histones from other sources indicate that the histones rank among the most highly conserved (low mutation rate) proteins in nature. Naturally occurring histones are often found to be partially acetylated, methylated, or phosphorylated. These modifications may contribute to the electrophoretic microheterogeneity of the histone fractions. The nucleoprotein complex of histone and deoxyribonucleic acid is referred to as nucleohistone or deoxyribonucleoprotein. It is important as a source of its two components as well as an entity in itself for physical studies. Intracellularly, these complexes may be important factors in chromosomal structure and gene transcription. Kornberg (1974) has proposed a model for chromatin in which 200 DNA base pairs are coiled on the outside of a histone unit composed of $(H3)_2(H4)_2$ tetramer and two each of H2a and H2b. Bradbury (1976) suggests that H1 may be involved in generating higher order chromatin structures and that the initiation of mitosis may in part be mediated by H1 phosphorylation. Worthington histones are water soluble at pH 7.0 and are characterized by gel electrophoresis and solubility.

CAS

- 37244-51-2

Stability/Storage

- Stable for years at 2 - 8°C. when stored dry.

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58. Hyaluronic Acid

The hyaluronic acids (HA) are a class of macromolecular proteoglycans characterized by a highly polymerized chain of glucuronic acid and N-acetylglucosamine units bonded to protein. They exist in nature as a hydrated gel, usually closely associated with other tissue components such as chrondroitin sulfate. (Arkins and Sheehan 1972 and Bettelheim and Philpott 1959).

Occurring in intercellular ground substance of connecting tissue (Blumberg *et al.* 1955), they have an important role in controlling tissue permeation, bacterial invasiveness and macromolecular transport between cells (Laurent *et al.* 1975). HA is also one of the proteoglycans found in cartilage (Hardingham and Muir 1973). It has been indicated that hyaluronic acid is important in controlling tissue hydration (Block and Bettelheim 1970). In synovial fluid and heart valve its purpose would appear to be primarily that of a lubricant (Meyer *et al.* 1969). HA is present in the vitreous humor of the eye and fluids of the inner ear. Its ability to act as a mechanoelectric transducer makes possible intriguing speculation in regard to its function (Barrett 1975).

The ratio of glucuronic acid to glucosamine varies with the source as does the protein or peptide residue remaining after exhaustive protease digestion (Meyer *et al.* 1969; Silpananta *et al.* 1968; Taussig 1965; Marcante 1965; Pigman 1961; and Preston *et al.* 1965).

Characteristics of Hyaluronic Acid from Bovine Vitreous Humor:

The molecular weight of HA preparations varies with purification procedures, i.e., the extent of degradation as well as the source. According to Bettelheim (1958) when the protein is completely removed a molecular weight on the order of 70,000 is obtainable as compared with 2-4 million in a highly polymerized preparation (Laurent and Gergely 1955). It has been reported (Laurent *et al.* 1960) that bovine vitreous humor hyaluronic acid has a lower molecular weight than most other sources

and that from human umbilical cord has the largest molecular weight (Jensen and Carlsen 1954). Pigman *et al.* (1961) report that HA is degraded in the presence of oxygen and reducing agents and by merthiolate, often used in its preparation. Ascorbic acid likewise has this effect (Swann 1967). The carbohydrate polymer is negatively charged. When it is mixed with a cationic protein such as albumin at low pH, a precipitate is formed. If the glycosidic bonds have been split, i.e., the chain depolymerized, this precipitation no longer occurs. This phenomenon is the basis for the turbidimetric assay of hyaluronidase.

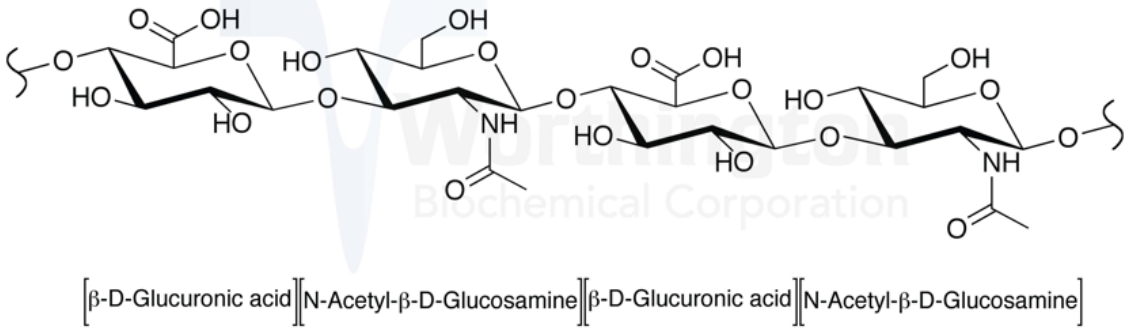

Figure 58.1: Enzymatic Reaction - Hyaluronic Acid

CAS

- 9004-61-9

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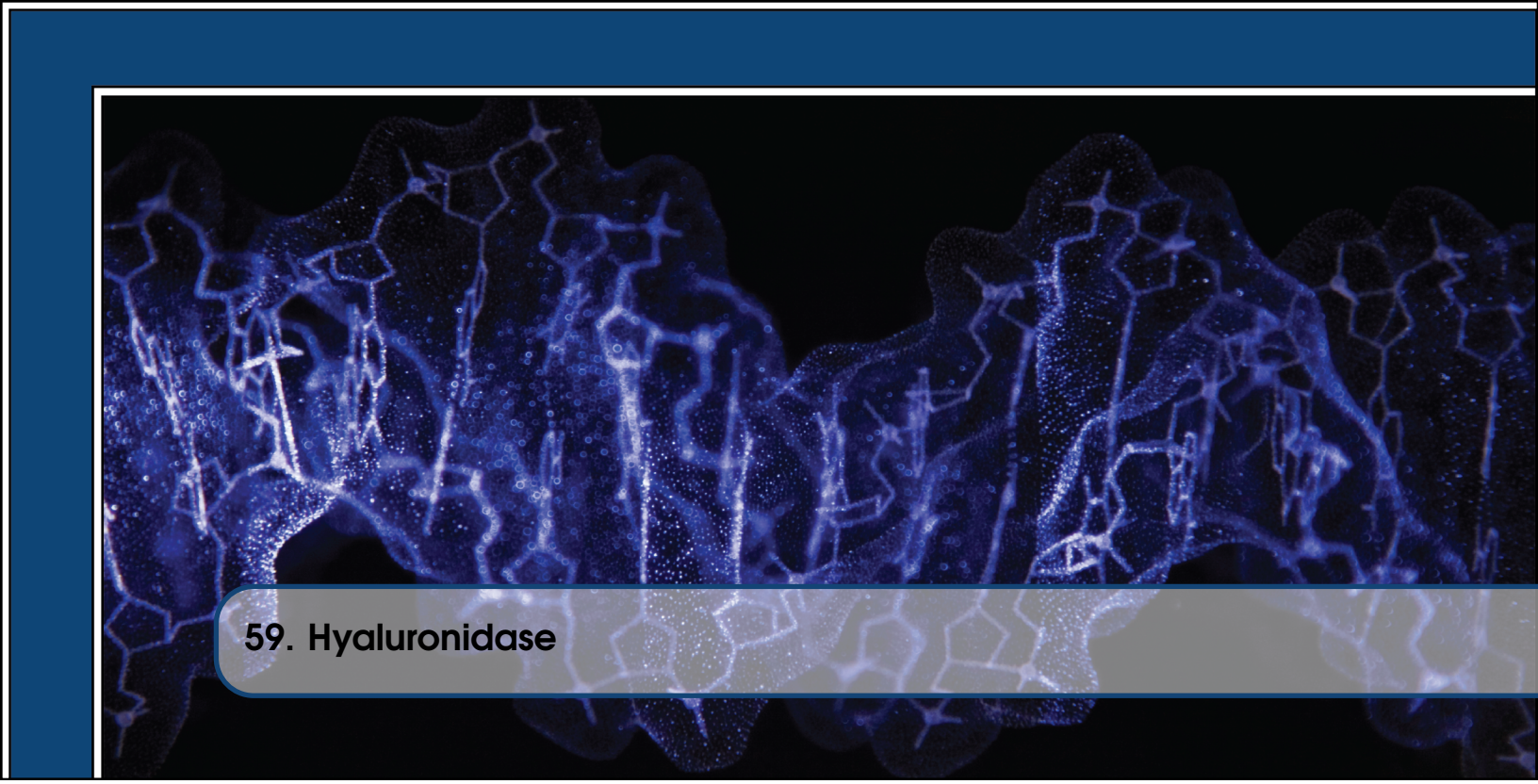

59. Hyaluronidase

Hyaluronate 4-Glycanohydrolase

Hyaluronidase catalyzes the random hydrolysis of 1,4-linkages between 2-acetamido-2-deoxy-b-D-glucose and D-glucose residues in hyaluronate.

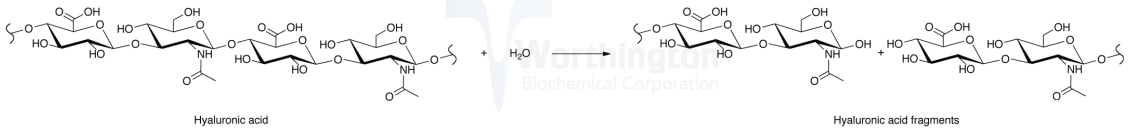

Figure 59.1: Enzymatic Reaction - Hyaluronidase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Bovine Testes

Specificity

Testicular hyaluronidase hydrolyzes the endo-N-acetylhexosaminic bonds of hyaluronic acid and chondroitin sulfuric acids A and C (but not B), primarily to tetrasaccharide residues (Ludowieg *et al.* 1961). Monosaccharides are not liberated (Rappaport *et al.* 1951).

Composition

The enzyme is a glycoprotein containing 5% mannose and 2.17% glucosamine. The amino acid composition has been determined (Borders and Raftery 1968).

IUB

- 3.2.1.35

CAS

- 37326-33-3

Molecular Weight

- Borders and Raftery (1968) reported a molecular weight of 61,000. Khorlin *et al.* (1973) report four subunits of 14,000 each and a total molecular weight of 55,000.

Optimal pH

- 4.5 - 6 (DeSalequi *et al.* 1967)

Extinction Coefficient

- $E^{1\%}_{280} = \approx 8$

Inhibitors

- Fe^{2+} and Fe^{3+} are inhibitory as are Mn^{2+} and Cu^{2+} (Warren *et al.* 1962)

Stability/Storage

- Worthington hyaluronidase is stable for 1 - 2 years and the purified preparation for 6 - 12 months when stored at 2 - 8°C.

Stabilizers

- Yang and Srivastav (1975) report that sodium chloride acts as a stabilizer.

Assay Information

Method

- Based on that of Tolksdorf et al. (1949) and Kass and Seastone (1944). Hyaluronic acid is measured by its ability to form turbidity with an acid albumin solution. Turbidity is a function of hyaluronic acid concentration and can hence be related to enzyme activity. One unit is based on the change in absorbency (turbidity) at 540nm of an internal standard assayed concurrently with each lot. Internal standard replaces USP/NF reference no longer available

Reagents

- 0.1 M Sodium phosphate buffer, pH 5.3 with 0.15 M sodium chloride (HSE buffer)
- 0.5 M Sodium acetate buffer, pH 4.2
- Albumin reagent: Prepare by dissolving 2.5 grams of bovine serum albumin, Fraction V in 250 ml of 0.5 M sodium acetate buffer, pH 4.2. Adjust pH to 3.0 with 2 N HCl and heat at 93°C for 30 minutes. Cool and adjust final volume to 1000 ml with 0.5 ml sodium acetate buffer, pH 4.2.
- Standard: Prepare stock solutions of 1.0 and 0.5 $\frac{mg}{ml}$.
- Hyaluronic acid (HA): Dissolve 10 mg Worthington hyaluronic acid (Code: VHHA) in 25 ml 0.1 M sodium phosphate buffer: pH 5.3 with 0.15 M sodium chloride. Note: This solution can be prepared by allowing VHHA to dissolve overnight. Heating in a boiling water bath for 10 - 15 minutes is the preferred method if the material is not immediately soluble.

Standard Curve

Tube # 1	ml HA	mg HA	ml HSE buffer
1	0.00	0.00	1.00
2	0.10	0.04	0.90
3	0.20	0.08	0.80
4	0.25	0.10	0.75
5	0.30	0.12	0.70
6	0.40	0.16	0.60
7	0.50	0.20	0.50
8	0.60	0.24	0.40
9	0.70	0.28	0.30
10	0.80	0.32	0.20

Place all tubes in a boiling water bath for 5 minutes. Cool to room temperature. Add 9.0 ml of albumin reagent and allow to stand for 10 minutes. Read absorbance at 540 nm. Plot absorbance at 540 nm versus mg HA to form standard curve. Hyaluronic acid should be soluble under the defined conditions and should produce a standard curve with a slope of 1.5 or greater.

Enzyme

- Prepare stock solution of enzyme at one $\frac{mg}{ml}$ in 0.1 M sodium phosphate buffer pH 5.3 with 0.15 M sodium chloride. Immediately prior to use dilute further in the same buffer. For crude grade material concentrations of 0.01 - 0.05 $\frac{mg}{ml}$ are recommended. For purified grade concentration of 0.001 - 0.01 $\frac{mg}{ml}$ are recommended.

Procedure

Pipette 0.5 ml of a 0.4 $\frac{mg}{ml}$ hyaluronic acid solution into a series of test tubes. Incubate at 37°C for 4 - 5 minutes to achieve temperature equilibrium. Incubate one blank tube with one ml of 0.1 M sodium phosphate buffer, pH 5.3 with 0.15 M sodium chloride. At timed intervals add 0.5 ml of appropriately diluted enzyme or standard to respective tubes. Incubate each tube exactly 10 minutes and cool in

an ice bath to room temperature. Add 9.0 ml of albumin reagent to each tube and incubate at room temperature for 10 minutes. Read A_{540} of each tube versus the blank.

Calculation

- Determine the amount of hyaluronic acid remaining after digestion from the standard curve.
- Calculate the amount of hyaluronic acid digested as follows:
- mg HA digested = 0.2 mg - mg HA remaining
- Calculate turbidity reducing $\frac{\text{units}}{\text{mg}}$ of enzyme or standard as follows:
- $\frac{\text{TRU}}{\text{mg}} = \frac{\text{mg HA digested x 3.0}}{\text{mg enzyme in reaction}}$
- Calculate $\frac{\text{units}}{\text{mg}}$ enzyme as follows:
- $\frac{\frac{\text{USP/NF units}}{\text{mg}} \text{ of standard}}{\frac{\text{TRU}}{\text{mg}} \text{ of standard}} = \frac{\frac{\text{USP/NF units}}{\text{mg}} \text{ of sample}}{\frac{\text{TRU}}{\text{mg}} \text{ of sample}}$

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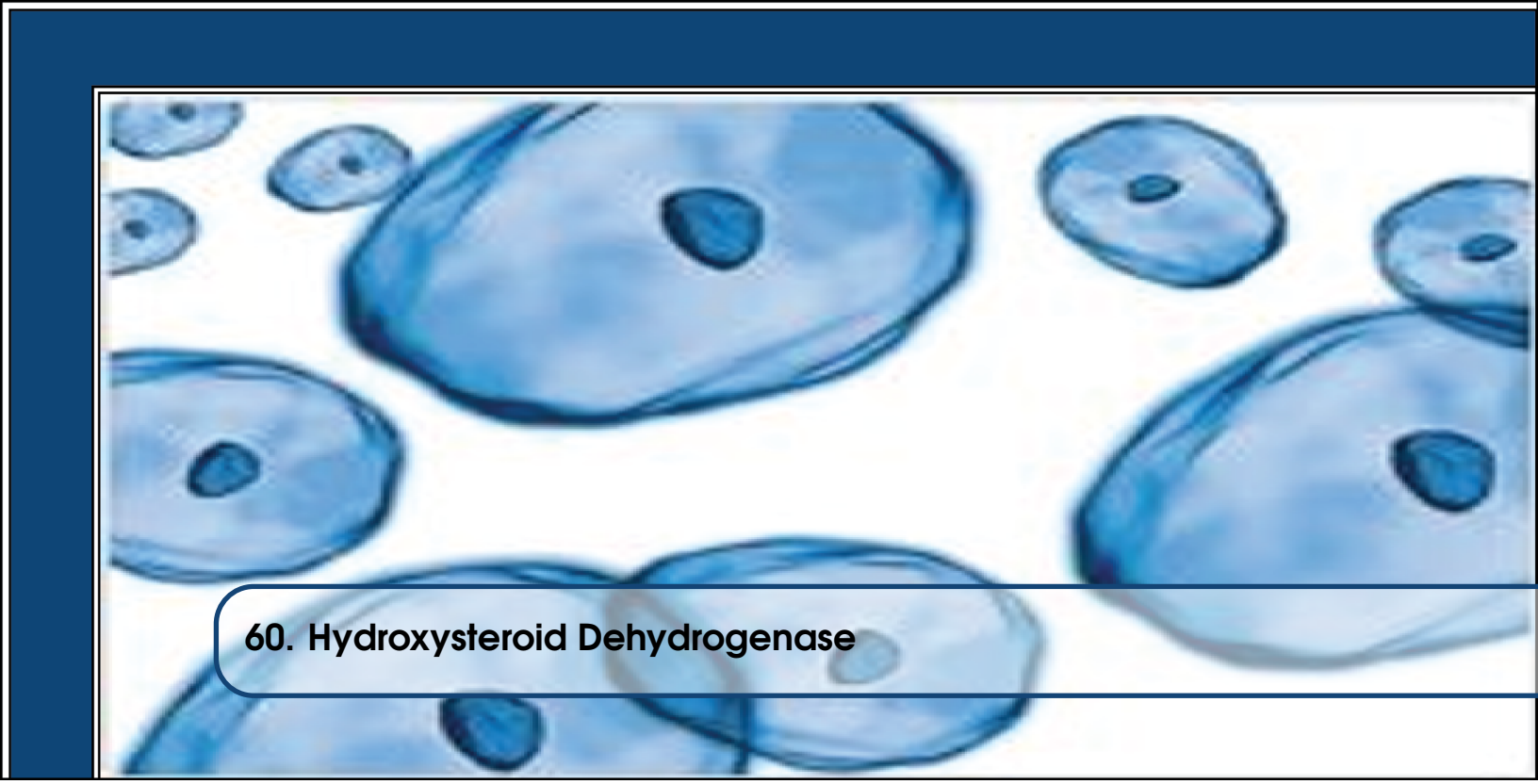

60. Hydroxysteroid Dehydrogenase

3-α-Hydroxysteroid: NAD(P^+) Oxidoreductase

3 (or 17) β-Hydroxysteroid: NAD(P^+) Oxidoreductase

The enzymes have useful application in the assay of steroids (Hurlock and Talalay 1958; Stempfel and Sidbury 1964; Moore 1972). They have been used for the determination of bile acids in blood (Iwata and Yamasaki 1964; Palmer 1969; Leslie 1969; Murphy *et al.* 1970; Schwarz *et al.* 1974).

It should be noted that Worthington supplies two preparations: one from the regular Pseudomonas testosteroni (ATCC 11966) culture which produces both the α and β enzymes, and a second from a mutant strain which produces almost only the α enzyme. By using both, the β-hydroxysteroid can be determined by difference.

Teller and Bongiavanni (1963) reported only slight activity of the mutant strain on epiandrosterone indicating but a trace of the β enzyme. Roe and Kaplan (1969) have investigated the specificities of the mutant and wild type strains, and this reference should be consulted for specific details. Hydroxysteroid dehydrogenases catalyze the interconversion of hydroxyl and carboxyl groups of steroids. Those from Pseudomonas testosteroni are of two types:

1. 3α-Hydroxysteroid Dehydrogenase (1.1.1.50), (α enzyme):

2. 3β- and 17β-Hydroxysteroid Dehydrogenase:

$$\begin{aligned}&\text{Androsterone + NAD} \rightleftarrows \text{5-}\alpha\text{-Androsterone-3,17-dione + NADH}\\&\text{Testosterone + NAD} \rightleftarrows \text{4-}\alpha\text{-Androsterone-3,17-dione + NADH}\end{aligned} \qquad (60.1)$$

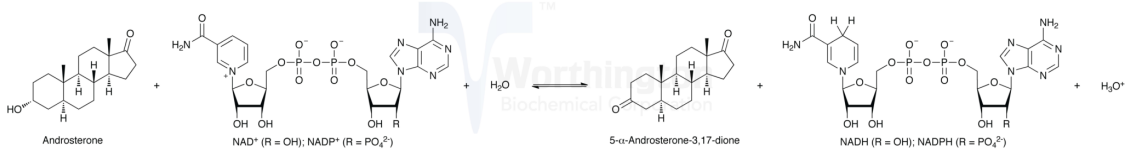

Figure 60.1: Enzymatic Reaction: 3α-Hydroxysteroid Dehydrogenase

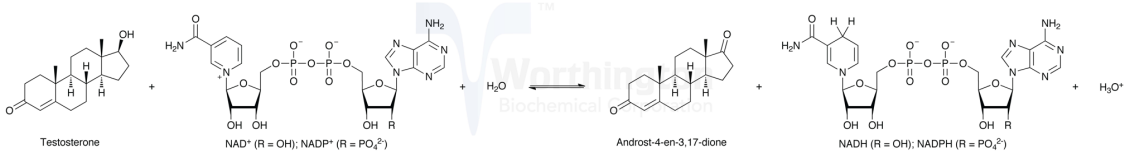

Figure 60.2: 3β- and 17β-Hydroxysteroid Dehydrogenase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Pseudomonas testosteroni*

Specificity

3α-Hydroxysteroid Dehydrogenase: α enzyme oxidizes only 3α-hydroxysteroids of the C19, C21, C24 series (Talalay 1963).

3β- and 17β-Hydroxysteroid Dehydrogenase: Catalyzes the oxidation of 3β-hydroxysteroids of the C19 and C21 series, 17β-hydroxysteroids of the C18, C19, and C21 series, as well as certain 16β-hydroxysteroids (Talalay 1963).

Composition

3α-Hydroxysteroid Dehydrogenase: Amino acid composition has been reported by Squire *et al.* (1964).

3β- and 17β-Hydroxysteroid Dehydrogenase: Amino acid composition has been reported by Squire *et al.* (1964).

IUB

- 1.1.1.50, 1.1.1.51

CAS

- 9028-56-2, 9015-81-0

Molecular Weight

- 3α-Hydroxysteroid Dehydrogenase: 47,000 (Squire *et al*. 1964; Sklhegg 1974)
- 3β- and 17β-Hydroxysteroid Dehydrogenase: 100,000 (Squire *et al*. 1964)

Isoelectric Point

- 3α-Hydroxysteroid Dehydrogenase: pH 6.1 (Squire *et al*. 1964)
- 3β- and 17β-Hydroxysteroid Dehydrogenase: pH 6.5 (Squire *et al*. 1964)

Inhibitors

- 3α-Hydroxysteroid Dehydrogenase: Heavy metals and sulfhydryl-binding reducing agents (Talalay 1963).
- 3β- and 17β-Hydroxysteroid Dehydrogenase: Heavy metals and reducing agents. The oxidation of testosterone is inhibited by 3,17α-estradiol and other 1,3,5-estratriene derivatives (Talalay 1963).

Stability/Storage

- 3α-Hydroxysteroid Dehydrogenase: The Worthington preparation of a enzyme is stable for 6-12 months when stored at -20°C.
- 3β- and 17β-Hydroxysteroid Dehydrogenase: The Worthington preparation of mixed α and β enzyme is stable for 6-12 months when stored at -20°C.

Assay Information

Method

- The assay is that of Marcus and Talalay (1956) in which the reaction velocity is measured as an increase in absorbance at 340 nm resulting from the reduction of NAD. One unit reduces one micromole NAD per min at 25°C and pH 9.0, using androsterone or testosterone as a substrate under the specified conditions.

Reagents

- 0.03 M Tris · HCl buffer, pH 7.2 with 0.001 M EDTA
- 0.166 M Sodium pyrophosphate buffer, pH 9.0
- 0.0043 M NAD in reagent grade water. Note: NAD may vary in salt form and degree of hydration. Care should be exercised to use an analytical grade and the correct molecular weight.
- 0.015% Androsterone. Prepare by dissolving 15 mg androsterone in 100 ml absolute methanol.
- 0.015% Testosterone. Prepare by dissolving 15 mg testosterone in 100 ml absolute methanol.

Enzyme

- Dissolve the purified enzyme at a concentration of 1 $\frac{\text{mg}}{\text{ml}}$ in 0.03 M Tris · HCl pH 7.2 buffer with 0.001 M EDTA. Further dilutions are also made with this buffer.
- Crude enzyme: Extraction of the enzyme from the cells can be accomplished by sonication of a 50 $\frac{\text{mg}}{\text{ml}}$ cell suspension in 0.03 M Tris · HCl buffer, pH 7.2 with 0.001 M EDTA. Clarify by centrifugation and assay the supernatant.

- For assay, dilute the enzyme to obtain a rate of 0.02-0.04 $\frac{\Delta A}{min}$.

Procedure

Spectrophotometer Settings: Wavelength: 340 nm Temperature: 25°C

Pipette into each cuvette as follows:

- 0.166 M Sodium pyrophosphate - 0.6 ml
- 0.0043 M NAD - 0.2 ml
- Reagent grade water - 2.0 ml
- Enzyme - 0.1 ml

Incubate in spectrophotometer for 3-4 minutes to achieve temperature equilibration and establish blank rate, if any. At zero time, add 0.01 ml testosterone solution. Record A_{340} for 3-4 minutes. Calculate ΔA_{340} per minute from the initial linear portion of the curve. Repeat, using androsterone as substrate.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{340}}{\text{min}}}{6.22 \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$$

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61. Lactate Dehydrogenase

L-Lactate: NAD^+ Oxidoreductase

Human LDH has been reported on by Ringoir and Plum (1975), Emes *et al.* (1974), Markel and Janich (1974), McQueen (1974), Burd and Usatequi-Gomez (1973), and McKee *et al.* (1972); that from pig by Hinz and Jaenicke (1975), Bloxham *et al.* (1975), Chen and Engel (1975), Eventoff *et al.* (1974), Jaenicke (1974), Whitaker *et al.* (1974), Holbrook and Ingram (1973), Holbrook and Stinson (1973), and Stinson and Holbrook (1973). Adams *et al.* (1973) and Taylor *et al.* (1973) have reported on dogfish LDH, and Carlotti *et al.* (1974) and Ryan and Vestling (1974) on that of rat liver and hepatomas. Fritz *et al.* (1973) report on different rates of tissue turnover of the rat isozymes. Kabura and Konvich (1972) extracted LDH isozymes from mouse brain. Ehmann and Hultin (1973) studied chicken breast LDH M5. Eby *et al.* (1973) report on frog LDH and Lim *et al.* (1975) on that from salmonid fish. Long and Kaplan (1973) report on horseshoe crab and sea worm LDH. That from potatoes has been studied by Rothe (1974) and Davies and Davies (1972). Brown *et al.* (1975) and Allsopp and Matthews (1975) report on the Actinomyces and Mycoplasma enzymes.

Mammalian lactate dehydrogenase (LDH) exists as five tetrameric isozymes composed of combinations of two different subunits. The isozymes differ in catalytic, physical and immunological properties. Cahn *et al.* (1962) refer to the polypeptide subunits as "H" and "M", which combine to form two pure types of isozymes, H4 and M4, and three hybrids, H3M, H2M2, and HM3. Type H4 is the most negatively charged at pH 7 and in zone electrophoresis appears nearest the anode. Subunit "H" predominates in heart muscle LDH which is geared for aerobic oxidation of pyruvate. The "M" subunit predominates in skeletal muscle and liver and is concerned more with anaerobic metabolism and pyruvate reduction (Fritz 1965).

A sperm isozyme (isozyme x) has been characterized from testes and spermatozoa (Zinkham *et al.*

1964; Stambaugh and Buckley 1967). McKee *et al.* (1972) indicate there to be several. LDH-X differs immunologically and enzymatically from LDH 1-5. (Spielman *et al.* 1973; Goldberg 1972).

LDH isozymes in the developing fetus have been reported on by Werthamer *et al.* (1973) and their variations with age by Gerlach and Fegler (1973). See also Ringoir and Plum (1975), Mitsutaka (1974), O'Carra *et al.* (1974), Glass and Doyle (1972), and Wilkinson and Walter (1972). Silverstein and Boyer (1964) compared kinetics of beef heart and rabbit muscle LDH.

LDH is of interest clinically in that the serum level of certain isozymes reflects pathological condition in particular tissues.

Studies on structure, binding sites and kinetics include the following: Adams *et al.* (1973), Bartholmes *et al.* (1973), Bishop *et al.* (1972), Bloxham *et al.* (1975), Cho and Swaisgood (1974), Dudman and Zerner (1973), Ehmann and Hultin (1973), Hinz and Jaenicke (1975), Holbrook and Ingram (1973), Holbrook and Stinson (1973), Jaenicke (1974), Levetzki (1972), Low *et al.* (1973), Millar (1974), Mitsutaka (1974), Saito (1972), Stinson and Holbrook (1973), Tienhara and Meany (1973). Whitaker *et al.* (1974) report on immobilized LDH. Lactate dehydrogenase (LDH) catalyzes the following reaction:

$$\text{Pyruvate} + \text{NADH} \rightleftarrows \text{Lactate} + \text{NAD} \tag{61.1}$$

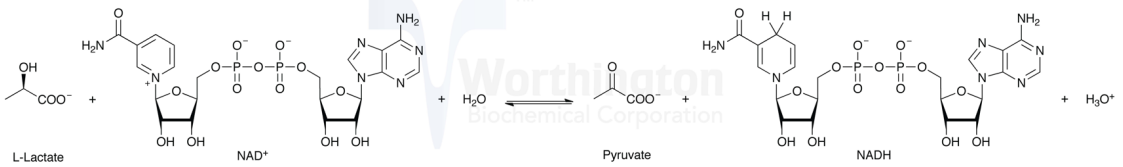

Figure 61.1: Enzymatic Reaction - Lactate Dehydrogenase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Beef Heart and Rabbit Muscle

Specificity

The enzyme is specific for L(+)lactate. Meister (1950) reports it reduces several α-keto and α,β-diketo acids but at about one-tenth the rate of reduction of pyruvate.

Composition

Beef Heart: Vallee and Williams (1975) have reported on its subunit dissociation at low pH. See also Yang and Schwert (1972) and Gold and Segal (1965).

Rabbit Muscle: Lovell and Winzor (1974) report that the tetramer dissociates completely into two dimers (molecular weight 70,000) in acetate-chloride buffer pH 5 (conditions without effect on beef heart LDH). Phosphate and pyridine nucleotides stabilize the quarternary structure of the tetramer. Phosphate has an activation effect. See also Cho and Swainsgood (1973).

IUB

- 1.1.1.27

CAS

- 9001-60-9

Molecular Weight

- Beef Heart: 35,000/subunit (Fosmire and Timasheff 1972)
- Beef Heart: 136,700 ± 2,100/tetramer (Huston *et al.* 1972)
- Rabbit Muscle: 140,000

Extinction Coefficient

- $E_{280}^{1\%} = 14.9$

Activators

- A number of organic compounds which stabilize the enzyme, such as dimethyl sulfoxide, ethanol, and methanol, are reported by George *et al.* (1969). Diethylstilbestrol and several of its derivatives also stabilize the enzyme (Cohen *et al.* 1969).

Inhibitors

- LDH is quite stable. It is inactivated by iodide. Inhibition by p-mercuribenzoate is slow. See Schwert and Winer (1963); also Anderson *et al.* (1974) and Bloxham *et al.* (1975).

Activity

- Beef Heart: See Borgmann *et al.* (1974)
- Rabbit Muscle: Reaction kinetics have been reported by Stambaugh and Post (1966) and Zewe and Fromm (1965).

Assay Information

Method

- The reaction velocity is determined by a decrease in absorbance at 340 nm resulting from the oxidation of NADH. One unit causes the oxidation of one micromole of NADH per minute at 25°C and pH 7.3, under the specified conditions.

Reagents

- 0.2 M Tris · HCl, pH 7.3
- 6.6 mM NADH in above 0.2 M Tris · HCl buffer, pH 7.3
- 30 mM Sodium pyruvate in above 0.2 Tris · HCl buffer, pH 7.3

Enzyme

- Dissolve at 1 $\frac{\text{mg}}{\text{ml}}$ in 0.2 M Tris · HCl buffer. Dilute enzyme prior to use to obtain a rate of 0.02-0.04 $\frac{\Delta A}{\text{min}}$. in Tris buffer and keep cold.
- Determination of Protein Concentration:

Procedure

Spectrophotometer Settings: Wavelength: 340 nm Temperature: 25°C

Pipette into cuvette as follows:

- Tris · HCl, 0.2 M pH 7.3 - 2.8 ml
- 6.6 mM NADH - 0.1 ml
- 30 mM Sodium pyruvate - 0.1 ml

Incubate in the spectrophotometer 4-5 minutes to achieve temperature equilibration and establish a blank rate, if any.

Add 0.1 ml of appropriately diluted enzyme and record $\frac{\Delta A_{340}}{\text{min}}$ from initial linear portion.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{340}}{\text{min}}}{6.22 \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$$

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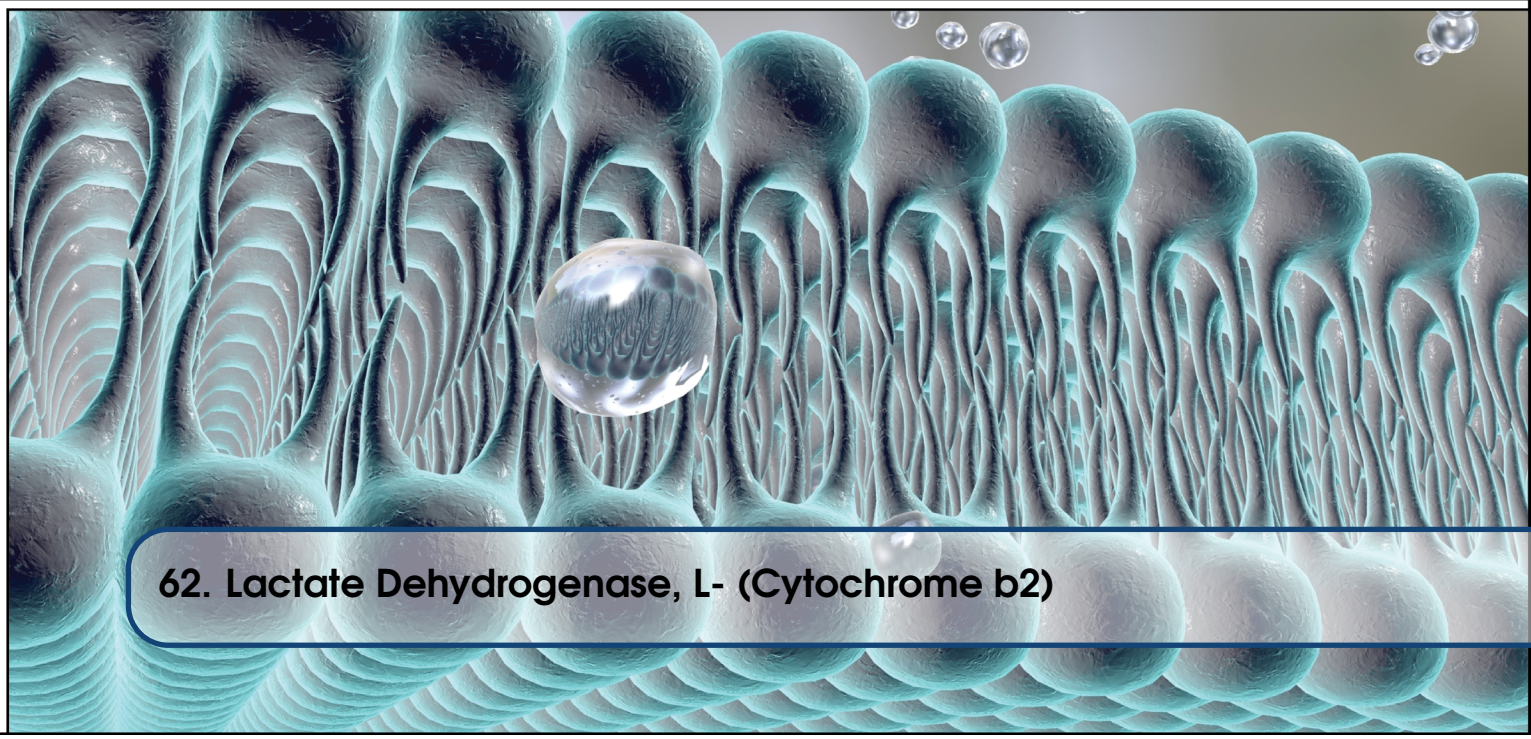

62. Lactate Dehydrogenase, L- (Cytochrome b2)

L-Lactate: Ferricytochrome-C Oxidoreductase

Other known oxidants which can be used *in vitro* are ferricyanide, phenozine methosulfate, certain redox dyes and quinone. A review has been written by Nygaard (1963). Preparation and properties have been summarized by Symons and Burgoyne (1966). An important use of this enzyme is in the specific, enzymatic determination of L-lactate. Schon (1965) describes a method utilizing Fe^{3+} phenanthroline complex. He indicates it to be 3 X more sensitive than that using NAD-dependent LDH from muscle. Another method is given below. L-lactic acid dehydrogenase from baker's yeast (cytochrome b2), in nature, catalyzes the following reaction:

$$\text{L-(+)lactate} + 2\ \text{ferricytochrome c} \rightarrow \text{pyruvate} + 2\ \text{ferrocytochrome c} \qquad (62.1)$$

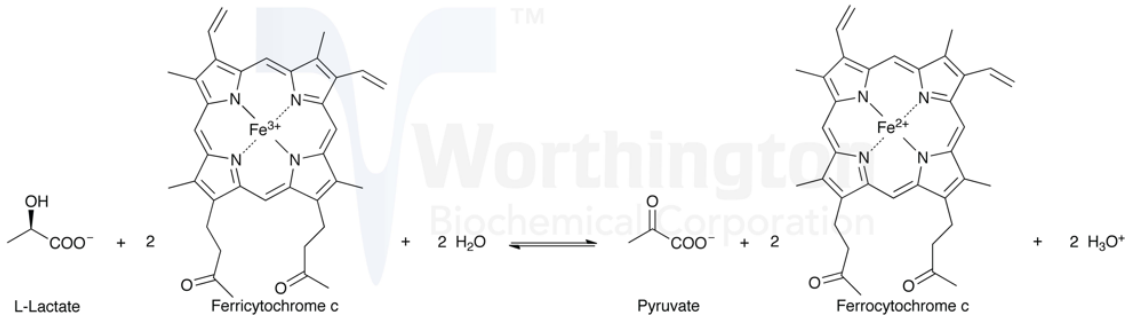

Figure 62.1: Enzymatic Reaction: - Lactate Dehydrogenase, L- (Cytochrome b2)

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Baker's Yeast

Specificity

The enzyme acts upon L-(+)-lactate, but not the D-isomer, and α-hydroxybutyrate. Electrons are transferred to ferricytochrome c, ferricyanide, methylene blue, 2,6-dichlorophenol indophenol and 1,2-naphthoquine-4-sulfate. Kinetic studies have been reported by Iwatsubo and Capeillere (1967).

Composition

Jacq and Lederer (1972 and 1974) and Guiard *et al*. (1973) indicate that the intact enzyme is tetrameric. Each of the identical subunits consists of a single polypeptide chain with one protoheme IX and one flavin mononucleotide as prosthetic groups. The latter have been reported on by Risler and Groudinsky (1973). Guiard *et al*. (1974) have determined the amino acid sequence in the heme binding region. The enzyme prepared by the crystallization method of Appleby and Morton (1959), without a protease inhibitor, shows a single cleavage of each of the four polypeptide chains; thus each monomer consists of an α-chain of molecular weight 36,000 and a β-chain, molecular weight 21,000 (Jacq and Lederer 1974).

IUB

- 1.1.2.3

Molecular Weight

- 228,000 (Jacq and Lederer 1974) (See also Guiard and Lederer 1975)

Optimal pH

- For cytochrome C: 5.5-9.0; for lactate and ferricyanide: 7.0-8.5

Extinction Coefficient

- $E^{1\%}_{424}$ = 31.2 (Pajot and Groudinsky 1970)

Inhibitors

- Heavy metals and oxygen (Armstrong *et al*. 1963) as well as glycerate, oxalate, malate, phenylpyruvate and fatty acids (Nygaard 1963)

Stability/Storage

- The 0.65 saturated ammonium sulfate suspension is stable for at least six months at 2 - 8°C

Stabilizers

- EDTA prevents metal inhibition

Constants

- K_m=1.6 mM for lactate with ferricyanide

Assay Information

Method

- Essentially that described by Appleby and Morton (1959). The rate of reduction of potassium ferricyanide is determined spectrophotometrically at 420 nm. The molar absorbancy of potassium ferricyanide is 1.04 x 10^3 at this wavelength whereas the reduced ferricyanide has negligible absorption. One unit reduces one micromole of ferricyanide per minute at 25°C and pH 8.4 under specified conditions.

Reagents

- 0.067 M Sodium phosphate, pH 7.4 with 0.001 M EDTA
- 0.01 M EDTA
- 0.0083 M Potassium ferricyanide. Caution, read product label for handling instructions.
- 0.1 M Sodium pyrophosphate, pH 8.4
- 0.5 M DL Sodium lactate, pH 7.0

Enzyme

- Immediately prior to assay, dilute enzyme in 0.067 M phosphate buffer, pH 7.4 with 0.001 M EDTA to obtain a rate of 0.02-0.04 $\frac{\Delta A}{min}$.

Procedure

Spectrophotometer Settings: Wavelength: 420 nm Temperature: 25°C

Pipette into cuvettes:

- 0.1 M sodium pyrophosphate - 2.0 ml
- 0.5 M DL sodium lactate - 0.5 ml
- 0.01 M EDTA - 0.3 ml
- Potassium ferricyanide - 0.1 ml

Incubate in spectrophotometer for 3-4 mintutes at 25°C to reach temperature equilibrium and establish blank rate if any. Add 0.1 ml of diluted enzyme and record A_{420} for 4-5 minutes. Calculate $\frac{\Delta A_{420}}{min}$ from initial linear portion of curve.

Calculation

- $\frac{\text{Units}}{\text{ml}} = \frac{\frac{\Delta A_{420}}{min} \text{ x } 1000 \text{ x dilution x reaction volume}}{1040 \text{ x sample volume}}$

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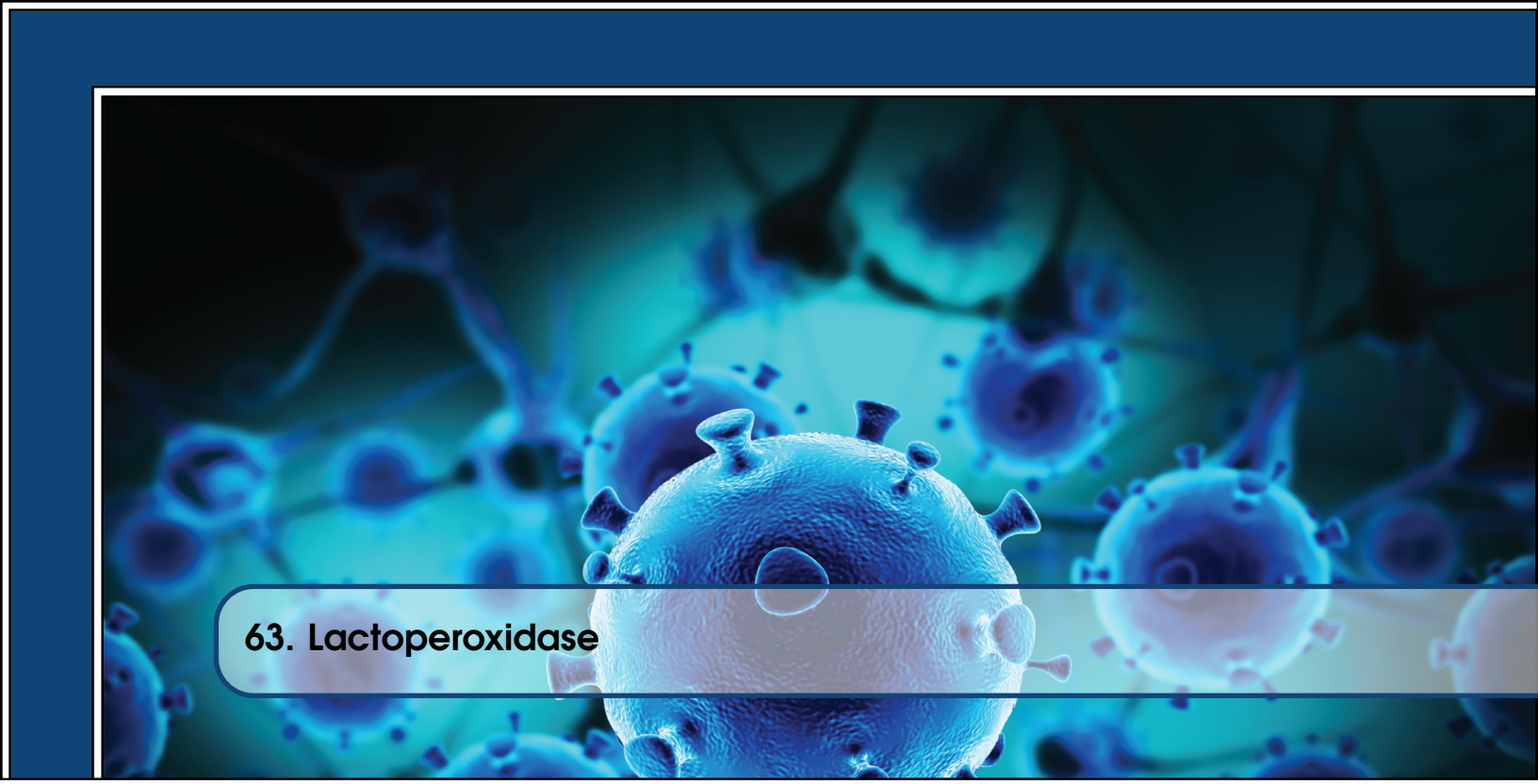

63. Lactoperoxidase

Lodid: Hydrogen Peroxide Oxidoreductase

The bovine milk enzyme is identical to that formed in bovine lacrimal and salivary glands (Morrison *et al.* 1965; and Morrison and Allen, 1963). It is possible that LPO may be important in controlling bacterial flora (Bjorck *et al.* 1970; Gothefors and Marklund, 1975; and Morrison and Allen, 1966). LPO is useful for labelling proteins with radioiodine (Gow and Wardlaw 1975; Holohan *et al.* 1973; Morrison and Bayse 1973; Bayse *et al.* 1972; Frantz and Turkington 1972; and Marchalonis 1969). For membrane studies the large LPO molecule limits labelling to the exposed surface. Poduslo and Braun (1975) report on the topography of membrane proteins of the myelin sheath. Shin and Carraway (1974) and Phillips and Morrison (1971) report on erythrocyte and Nachman *et al.* (1973) on platelet membranes. Haustein (1975) studied the cell-surface protein of lymphoma cells and Huber *et al.* (1975) mitochondrial membranes. Chloroplast membranes were reported on by Arntzen *et al.* (1974). Jone and Hager (1976), and Hogg (1974) compare membrane protein of normal and transformed cell. Immobilized LPO has also proven of interest (Johnson *et al.* 1975; Karonen *et al.* 1975; and David 1972).

$$2I^- + H_2O_2 + 2H^+ \rightarrow I_2 + 2H_2O \qquad (63.1)$$

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Bovine Milk

Composition

LPO is a glycoprotein with a single hemin prosthetic group per molecule (Hultquist and Morrison, 1963). It may consist of two isozymes (Rombauts *et al.* 1967; Morrison *et al.* 1957; and Polis and Schmukler, 1953). See also Morrison and Hultquist (1963).

IUB

- 1.11.1.7

CAS

- 9003-99-0

Molecular Weight

- 77,500 (Rombauts *et al.* 1967)

Extinction Coefficient

- $E^{1\%}_{412} = 13.9$

Inhibitors

- LPO is inhibited by hydrazines (Allison *et al.* 1973). Dolman *et al.* (1968) report on the kinetics of cyanide inhibition.

Activity

- LPO like other peroxidases, in the presence of H_2O_2 catalyzes the oxidation of many phenols and aromatic amines (pyrogallol, ascorbate, guiacol, etc.). See Morrison (1970) page 657 for a comparison of specific activity of a variety of peroxidases, also Maguire and Dunford (1971). Morrison and Bayse (1973) indicate that the iodide reacts directly with the heme group, the complex then iodinates the substrate on adding H_2O_2. Bayse *et al.* (1972) and Morrison and Bayse (1970) report on iodination kinetics. Chung and Wood (1970) report on the oxidation of thiocyanate and Maguire and Dunford (1972) on that of iodide.

Purity

- $\frac{A_{412}}{A_{280}}$ = 0.93-0.96 Allen and Morrison 1963)

Assay Information

Method

- The assay procedure is a slight modification of that described by Morrison (1970). The reaction velocity is determined by measuring the increase in A_{350} resulting from the production of triiodide. One unit results in the formation of one micromole of triiodide per minute at 25°C and pH 7.0 under the specified conditions.

Reagents

- 0.033 M Sodium phosphate buffer pH 7.0
- 0.005 M Potassium iodide in phosphate buffer
- 0.090 M Hydrogen peroxide. Prepare by diluting 0.1 ml hydrogen peroxide (Merck Superoxol or equivalent) to 10 ml with reagent grade water.

Enzyme

- Dissolve at one $\frac{\text{mg}}{\text{ml}}$ in phosphate buffer. Immediately prior to use, dilute further in phosphate buffer to obtain a rate of 0.02-0.04 $\frac{\Delta A}{\text{min}}$.

Procedure

Spectrophotometer Settings: Wavelength: 350 nm Temperature: 25°C

Prepare reaction mixture by diluting 0.15 ml of 0.090 M hydrogen peroxide to 30 ml with 0.005 M potassium iodide. Store no longer than 30 minutes at room temperature. Pipette 3.0 ml reaction mixture into cuvette and incubate at 25°C for 3-4 minutes to achieve temperature equilibrium and establish a blank rate, if any. Add 0.01 ml diluted enzyme and record increase in A_{350} for 3-4 minutes. Calculate $\frac{\Delta A_{350}}{\text{min}}$ from initial linear portion of curve. Reaction remains linear for no longer than 1-2 minutes.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{350}}{\text{min}}}{26 \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$$

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64. Leucine Aminopeptidase

Leucine aminopeptidase (LAP) is an exopeptidase that catalyzes the hydrolysis of amino acid residues from the amino terminus of polypeptide chains. LAPs are widely distributed, ubiquitous in nature, and are of critical biological importance because of their role in protein degradation (Burley *et al.* 1990).

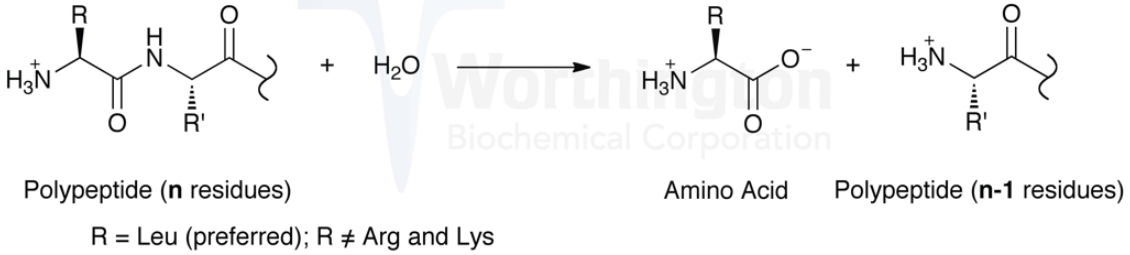

Figure 64.1: Enzymatic Reaction - Leucine Aminopeptidase

History

In 1929 it was reported that an enzyme found in the mucosa of pig intestine cleaved leucylglycine approximately 20 times faster than it cleaved glycylglycine. This enzyme was named dipeptidase II (Linderstrm-Lang 1929). It was later referred to as aminoleucylpeptidase (Holter 1979, and Str*ä*ter and Lipscomb 2004).

In 1936, the enzyme was partially purified and called leucylpeptidase (Johnson *et al.* 1936). Johnson *et*

al. also demonstrated that LAP is activated by magnesium and manganese.

Through the 1950s and 1960s, studies on swine kidney LAP determined chemical and physical properties of the enzyme and the zinc-metalloenzyme nature of LAP (Spackman *et al*. 1955, Smith and Spackman 1955, and Himmelhoch 1969).

In 1937, Sumner and Dounce first reported on the crystallization of catalase from beef liver. Along with the needles of catalase there were nearly always colorless crystals that took the shape of footballs, which lead to this protein being called football protein (FTBL protein). In unpublished work Dounce separated it from catalase. It was not until the late 1980s, fifty years after its initial discovery, that the FTBL protein was finally identified as beef liver leucine aminopeptidase (Dounce and Allen 1988).

In 1990, leucine amino peptidase became the first di-zinc enzyme for which a crystal structure at atomic resolution was determined (Burley *et al*. 1990).

Recent work with LAP has included determining the crystal structure of LAPs from bacterial sources (Jia *et al*. 2010, and Kale *et al*. 2010). Sus scrofa LAP has also been used as a model system for the development of molecular chaperones (Laslo *et al*. 2009). In addition, elevated serum levels of LAP have been found in obstructive jaundice, liver cirrhosis, liver carcinoma, and later stages of pregnancy.

Molecular Characteristics

The primary structure of porcine leucine aminopeptidase has not been determined. However, the sequence from bovine eye lens was determined by Cuypers *et al*. in 1982. Bovine kidney LAP cDNA studies have indicated that a 26 amino acid extension at the amino terminus is present, and not found in the mature, purified lens LAP (Wallner *et al*. 1993).

Specificity

The enzyme liberates amino acids from the N-terminal end of a number of proteins and polypeptides, reacting most rapidly with leucine residues (Spackman *et al*. 1955, and Smith and Spackman 1955). Many aliphatic amides are also hydrolyzed, as are thioesters (Metrione 1972).

Composition

Cattle eye lens LAP is a homohexamer. The monomer has a mixed alpha + beta structure. The interior of the hexamer contains six active sites that line a disk shaped cavity. Accessibility to this cavity is provided by solvent channels. This feature explains why the enzyme does not cleave longer peptides or proteins (Kim *et al*. 1974). Each active site contains two zinc ions, with one water ligand bridging the two ions (Burley *et al*. 1991, and Sträter and Lipscomb 1995).

IUB

- 3.4.11.1

CAS

- 9001-61-0

Protein Accession Number

- P28839 (Sus scrofa sequence fragment)
- P00727 (Bos taurus full sequence)

Molecular Weight

- 326 kDa (Kretschmer *et al*. 1965)

Optimal pH

- 9.0-9.5 (Spackman *et al*. 1955, and Smith and Spackman 1955)

Isoelectric Point

- 6.07 (Theoretical, Bos taurus)

Extinction Coefficient

- 347,280 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$ (Theoretical, Bos taurus)
- $E^{1\%}_{280}$ = 10.28 (Theoretical, Bos taurus)

Activators

- Mg^{2+} or Mn^{2+} (3-4 mM) is essential for activity (Bryce and Rabin 1964b)

Inhibitors

- Cd^{2+}, Cu^{2+}, Hg^{2+}, and Pb^{2+}
- EDTA
- Alcohols
- p-Chloromercuribenzoate
- Bestatin (Burley *et al*. 1991)
- Orthophenanthroline (Himmelhoch 1969)
- Bipyridyl (Himmelhoch 1969)
- Cupferron (Himmelhoch 1969)
- Sodium diethyldithiocarbamide (Himmelhoch 1969)
- Sodium sulfide and sodium cyanide (Himmelhoch 1969)

Applications

- Sequence analysis
- Serum control in protein studies

- Determination of L-peptides and amino acid amides containing N-terminal leucine or proline
- Cleavage of deferriform of albomycins
- Resolution of gamma-methyl and gamma-fluoroglutamic acids (White and White 1997)

Assay Information

Method

- Based on that of Mitz and Schlueter (1958), the hydrolysis of the peptide bond of leucinamide is measured spectrophotometrically at 238 nm. One unit of enzyme activity is equal to one micromole of L-Leucinamide hydrolyzed per minute at 25°C and pH 8.5.

Reagents

- 0.025 M Manganese chloride
- 0.125 M Magnesium chloride
- 0.5 M Tris · HCl buffer, pH 8.5
- 0.0625 M L-Leucine, pH 8.5. Note: The extinction coefficient of L-leucinamide should be checked upon each use by the technique outlined in the procedure.

Enzyme

- Prior to assay, the enzyme must be activated for two hours at 37°C in the following:
- 0.025 M Manganese Chloride - 0.1 ml
- 0.5 M Tris · HCl buffer - 0.1 ml
- Enzyme - The volume of the sample activated may vary, depending upon the material to be assayed; however, no more than 0.4 mg of the purified product should be added to the activation mixture.
- Reagent grade water - q.s. to 2.3 ml

Procedure

Determination of extinction coefficient:

Prepare cuvettes as follows:

Description	Control	Test
0.0625 M L-leucine	2.0 ml	—
0.125 M L-leucinamide	—	1.0 ml
0.5 M Tris · HCl pH 8.0	0.1 ml	0.1 ml
0.125 M Magnesium chloride	0.1	0.1 ml
Reagent grade water	0.2 ml	1.2 ml

Using the control as an absorbance blank, determine A_{238} for test cuvette.

$$E_m = \frac{A_{238}(\text{test})}{0.05\ \text{M (Final concentration of L-Leucinamide)}}$$

Determination of reaction rates:

Incubate test cuvettes in the spectrophotometer at 25°C for 4-5 minutes to achieve temperature equilibration and establish a blank rate, if any. Add 0.1 ml of enzyme activation mixture and record decrease

in A_{238} from the linear portion of the curve. Some lag may occur during the initial period and should not be used in the calculation.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{238}}{\text{min}} \text{ x } 1000}{a_m \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$$

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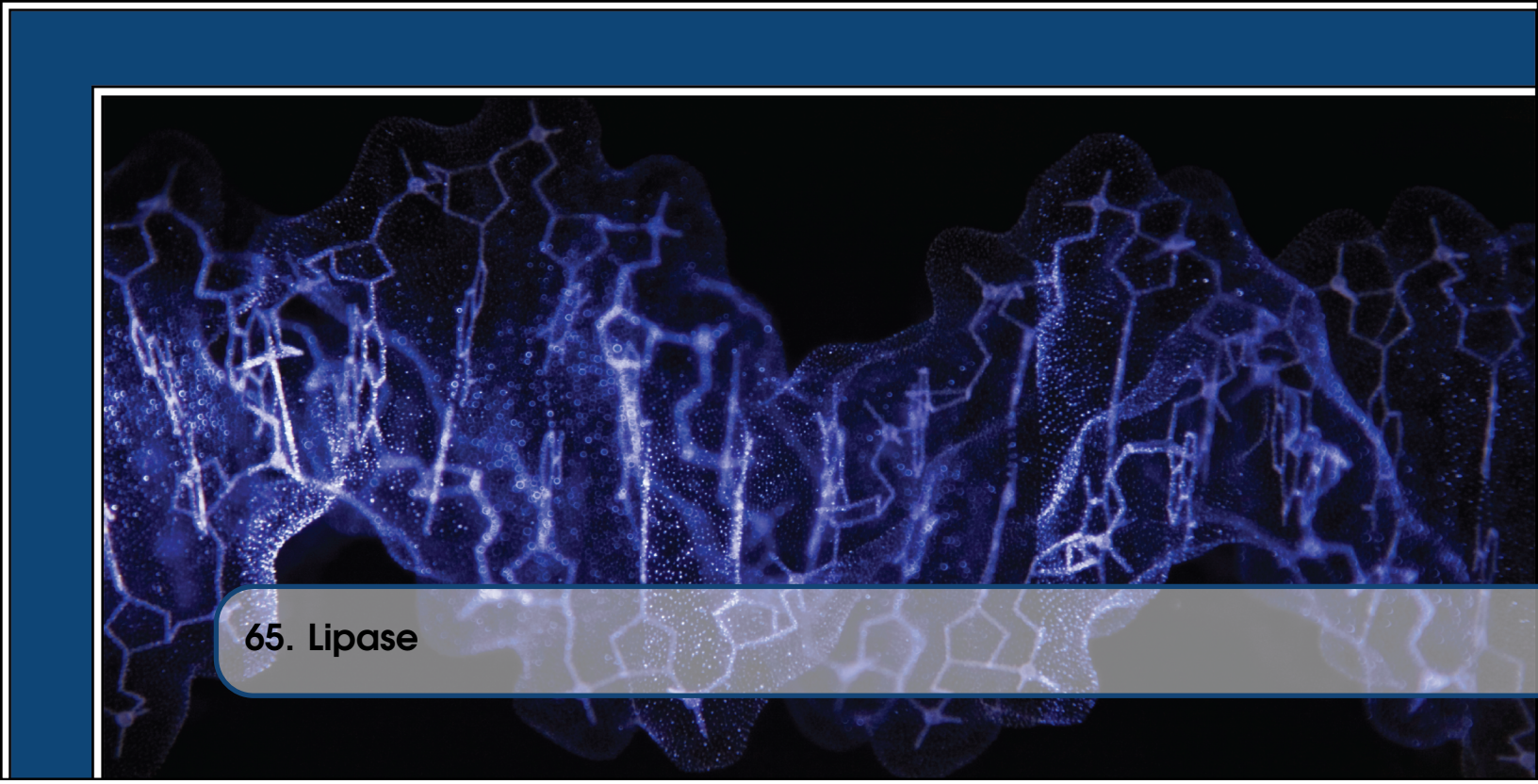

65. Lipase

Triacylglycerol acylhydrolase

Pancreatic lipase (PL),one of the exocrine enzymes of pancreatic juice, catalyzes the hydrolysis of emulsified esters of glycerol and long chain fatty acids. The substrate is not a single molecule but a nonaqueous phase of aggregated lipid (Brockerhoff and Jensen 1974). The operative substrate characteristic is aggregates of ester molecules, micelles or monomolecular film, interfacing an aqueous medium. Enzyme activity is directly related to the concentration of substrate molecules on the interface (Esposito *et al* 1973; Lagocki *et al* 1973. PL attacks the primary ester groups most readily. Monoglycerides are poor substrates (it is the 2-monoglycerides that are absorbed through the intestinal wall and reformed into lymph chlyomicrons). Pancreatic lipases have been thoughly reviewed by Brockerhoff and Jensen (1974), and Desnuell (1972). Liberman and Ollis (1975) have reported on lipase immobilized on stainless steel and polyacrylamide beads. Using a fluidized bed recycle reactor it is indicated that enzyme-substrate affinity is not altered.

Characteristics of Lipase from Porcine Pancreas:

Two lipases are present. Lipase A is more acidic than Lipase B; otherwise, the two isoenzymes are nearly the same (Verger *et al* 1969). Normally, a cofactor is bound to the enzymes (Maylie *et al* 1971). Two co-lipases were purified by Erlanson *et al* (1973). They were quite similar polypeptide chains with a molecular weight of 11,000. See also Borgstrom *et al* (1974). Borstrom and Earlanson (1973) indicated that co-lipase might be classified as a co-enzyme for lipase in that they interact in a stoichiometrical relationship.

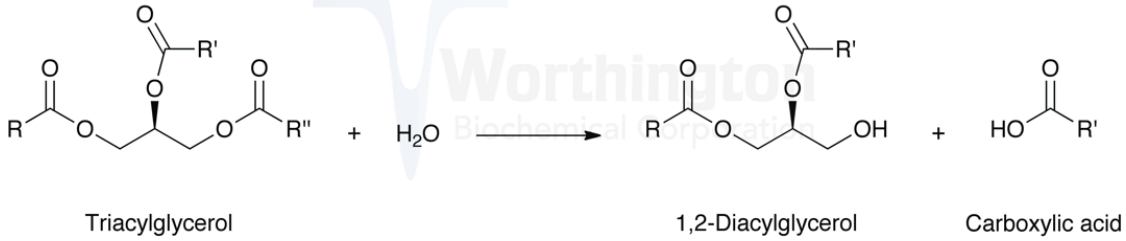

Figure 65.1: Enzymatic Reaction - Lipase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Porcine Pancreas

Specificity

PL has a broad spectrum of side chain specificity (Lagocki *et al* 1973). See also Savary (1972) and Brockerhoff (1969a)

Composition

The amino acid composition, which is almost identical except for isoleucine, is shown in Brockerhoff and Jensen (1974)-(Table IV-3, pg 43). Both contain a carbohydrate moiety (Garner and Smith 1972). Histidine is involved in the active site (Semeriva *et al* 1971). See Hultin (1992). Modification of the free carboxyl group by amide formation inactivates the enzyme (Semeriva *et al* 1972). According to Desnuelle (1972) the carboxyl in lipase stabilizes the active enzyme, i.e., the enzyme conformation resulting from adsorption at a hydrophobic interface. Although PL contains two disulfide groups, they are not involved in enzymatic activity (Verger *et al* 1971). Diisopropylphosphofluoridate (DFP) binds to a tyrosine residue but it is not inhibitory (Maylie *et al* 1969). See also Rovery *et al*. (1973)

IUB

- 3.1.1.3

Molecular Weight

- 45,000-50,000 (Verger *et al* 1969)

Isoelectric Point

- Lipase A= 4.9 (Brockerhoff and Jensen 1974) and Lipase B=5.0

Extinction Coefficient

- $E^{1\%}_{280}$ = 13.3 (Desnuelle 1972)

Activators

- Ca^{2+} is required for activity [Sr^{2+} and Mg^{2+} are less effective activators (Sarda *et al* 1957)]

Inhibitors

- Versene, Zn^{2+}, Cu^{2+}, Hg^{2+}, iodine, PCMB (Willis 1960). DFP does not inhibit.

Stability/Storage

- Highly purified, homogenous preparations of hog pancreas lipase are extremely labile.

Activity

- See Desnuelle (1972) on "Catalytic Properties" (page 586). Momsen and Brockman (1976a and b) report the effects of taurodeoxycholate and co-lipase. At low concentrations, up to 0.3mM, the bile salt increases the stability of the lipase to 5 fold. At higher levels (0.3-0.8mM), but below the critical micelle concentration, it interferes with enzyme adsorption on the substrate interface, thus inhibiting lipolysis. Co-lipase counters this inhibitory effect by providing high affinity binding sites at the surface of the lipase-bile salt complex. See also Borgstrom and Elanson (1973), Borgstrom *et al* (1974), and Kaimal and Saroja (1989). Co-lipase without bile-salts only mildly stimulates activity. Brockman *et al* (1973) report on PL activity toward soluble triglycerides such as tripropionin. It is stimulated in the presence of hydrophobic surfaces. Santhanam and Wagle (1971) indicate that protein kinase, Mg^{2+} , ATP and cAMP stimulate PL activity.

Stabilizers

- DFP may be used to stabilize impure preparations containing proteinases in solutions.

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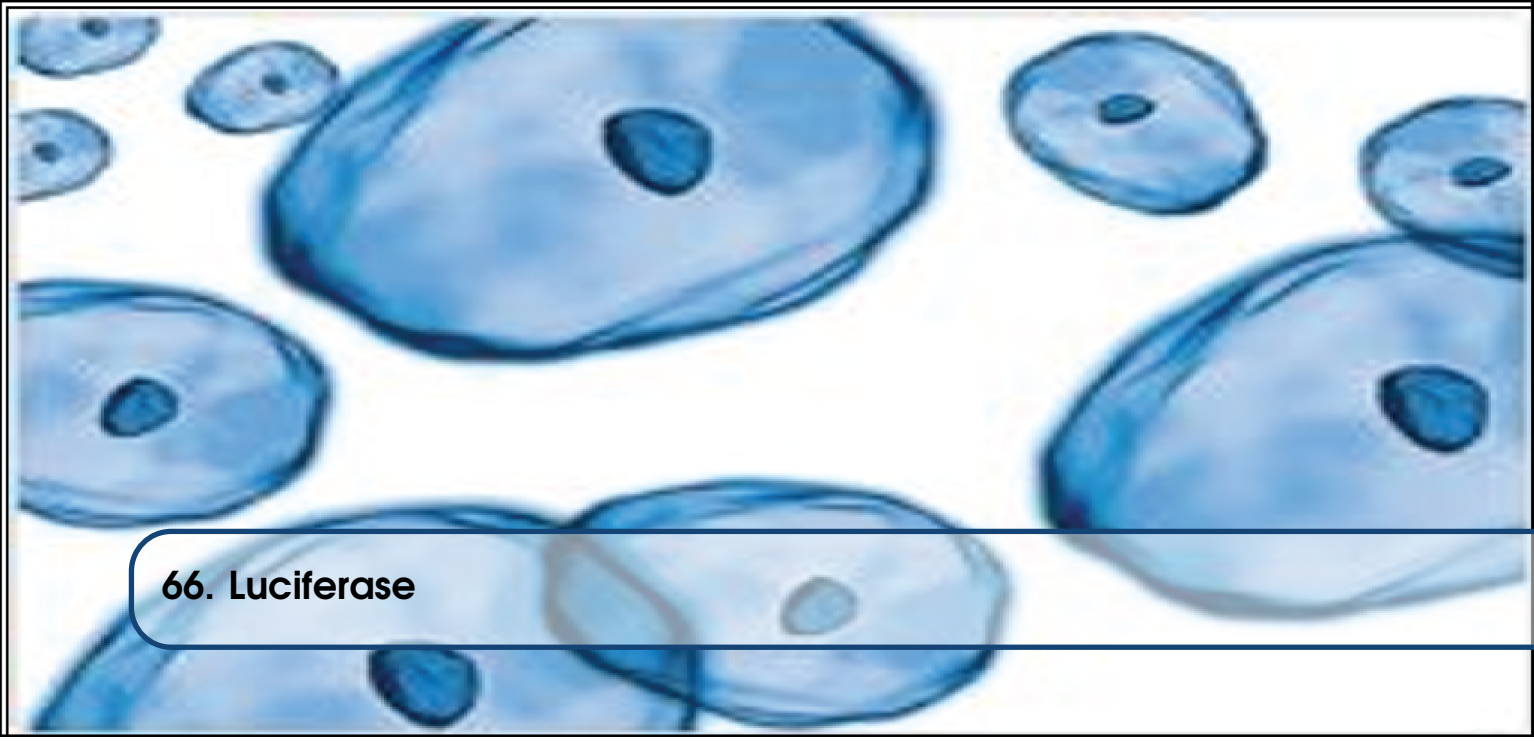

66. Luciferase

See also Hastings *et al.* (1966); the aldehyde is not essential to the reaction but has a striking effect upon the luminescence. The FMN can be reduced by molecular hydrogen with Pt or Pd catalyst, or by treatment with dithionite, but because $FMNH_2$ is so rapidly oxidized by air, the use of NADH is a more workable laboratory method and more useful in analytical applications. The mechanism by which bacterial luciferase functions to produce light was studied by Dr. J. Woodland Hastings' group. In an article describing partial and rapid purification of LU using affinity chromatography, Waters *et al.* (1974) describe the specific activity of highly purified luciferase as being 1.4×10^{14} quanta per second per milligram. Brolin *et al.* (1971) developed a very sensitive assay for trace metabolic intermediates which can be involved in pyridine nucleotide dependent dehydrogenase reactions using bacterial luciferase. See also Chappelle and Picciolo (1971) for its use in assaying FMN and FAD. See Aflalo and DeLuca (1987) for its use monitoring ATP. A review of the use of firefly luciferase as a tool in molecular and cell biology is offered by Gould and Subramani (1988). A review of clinical applications for the enzyme is given by Kricka (1988).

Characteristics of Luciferase from *Photobacterium fischerii*:

Note: In Hastings' laboratory a strain of bacteria of uncertain origin, but with a different luciferase, was isolated and labelled MAV. A number of papers refer to the MAV enzyme. Hastings *et al.* (1969) have compared the two luciferases. The MAV strain has recently been identified as bacterium Beneckea harveyi (Tu *et al.* 1975). Bacterial luciferase (LU) catalyzes the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long chain aldehyde by molecular oxygen to yield FMN, the corresponding acid, H_2O and light. (Baldwin *et al.* 1975; Balny and Hastings 1975; Becvar and Hastings 1975).

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + RCO_2H + H_2O + \approx 0.2h\nu\ 495nm \qquad (66.1)$$

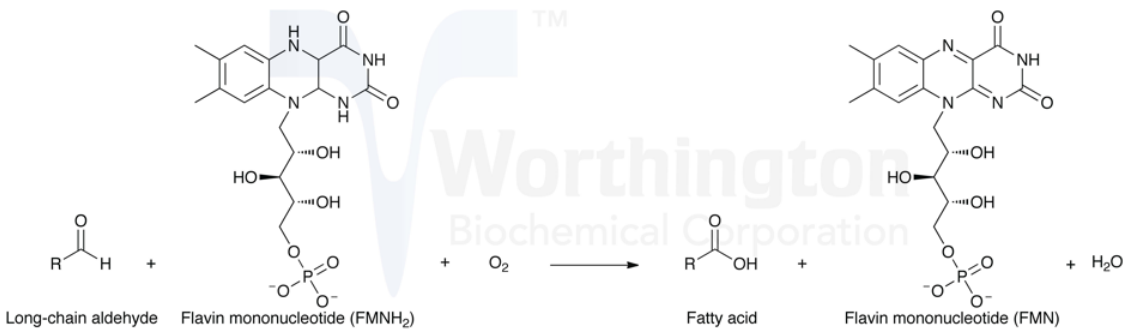

Figure 66.1: Enzymatic Reaction - Luciferase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Photobacterium fischerii*

Specificity

The enzyme is specific for $FMNH_2$. The aldehyde is not essential for the reaction (Hastings *et al.* 1966).

Composition

The enzyme is composed of two distinct subunits, a and b, each of approximately 40,000 molecular weight. Their amino acid content has been reported (Hastings *et al.* 1969). See also Meighen *et al.* (1970, 1971).

The luciferase α-β dimer has but one reduced flavin binding site (Becvar and Hastings 1975). Nicoli *et al.* (1974) and Nicoli and Hastings (1974) report a sulfhydryl group near the active center of MAV luciferase. See also Cline and Hastings (1972) and Gunsalus-Miguel *et al.* (1972).

Molecular Weight

- Approximately 80,000 (Hastings *et al.* 1969)

Optimal pH

- 6.8 (Hastings *et al.* 1969)

Inhibitors

- Oxidase inhibitors such as SKF (2-diethyl-aminoethyl-2,2-diphenyl valerate) are effective at concentrations as low as $10^{-5} - 10^{-4}$ M. (Nealson and Hastings 1972). p-Chloromercuribenzoic acid and riboflavin are both inhibitors.

Assay Information

Method

- Assay of luciferase activity is based upon the measurement of light produced. Although units of activity have been proposed for the purified enzyme (see discussion) currently, no units have been assigned to the Worthington preparations.

Reagents

- 0.01% Mercaptoethanol
- 0.1 M Sodium phosphate buffer, pH 6.8
- 13 mM Nicotinamide adenine dinucleotide, reduced form (NADH)
- 0.42 mM Flavin mononucleotide (FMN)
- 0.1% Decaldehyde in methanol

Enzyme

- Dissolve at 10 $\frac{\text{mg}}{\text{ml}}$ in reagent grade water.

Procedure

A spectrophotometer capable of a percent transmission readout is used for the determination. With the light source off and the UV detector in position, adjust the readout to 0% transmission.

Add to each cuvette:

- 0.01% Mercaptoethanol - 0.1 ml
- 13 mM NADH - 0.2 ml
- 0.42 mM FMN - 0.2 ml
- Enzyme solution - 0.2 ml

Mix contents well and at zero time add 0.05 ml of 0.1% decaldehyde solution. Read the maximum transmission as a measure of the light produced. Addition of excess enzyme will enable the light produced to be visualized by the dark adapted eye.

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67. Lysozyme

Lysozyme is an antimicrobial enzyme that is found in a wide variety of organisms including birds, mammals, plants, insects, and bacteria (Newman *et al.* 1974). The lysozyme of chicken egg white has been most extensively studied.

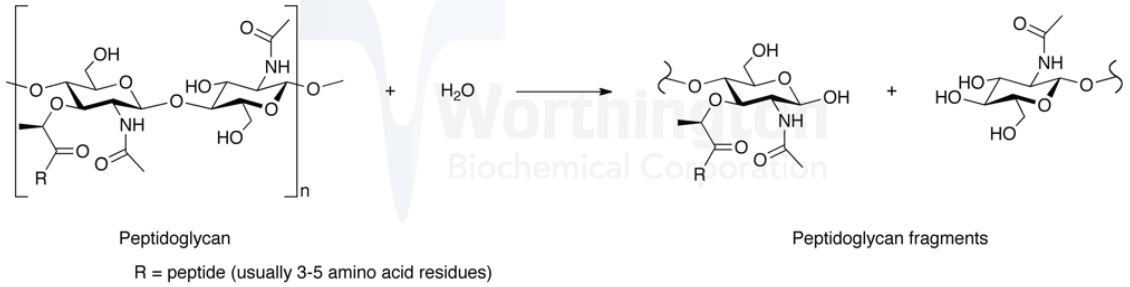

Figure 67.1: Enzymatic Reaction - Lysozyme

History

Lysozyme from chicken egg was first described by Laschtschenko in 1909 (Laschtschenko 1909). It was also reported in saliva by Bloomfield in 1919 (Imoto *et al.* 1972). Lysozyme was not officially named and understood to be present in many biological tissues and secretions until 1922 (Fleming 1922). During these experiments Alexander Fleming discovered *Micrococcus lysodeikticus*, a bacteria especially susceptible to lysozyme, which is still used today for lysozyme activity assays (Imoto *et al.* 1972).

In 1965, Blake *et al.* solved the structure of lysozyme, making it the second protein and first enzyme structure to be solved by X-ray diffraction methods (Blake *et al.* 1965). A year later, the mechanism was explained (Blake *et al.* 1966). Throughout the 1960s and into the 1970s, interest in the enzyme increased as a "natural" antibiotic and aid in the diagnosis of disease (Glynn 1968, Pruzanski and Saito 1969). Elevated lysozyme levels were found to be present in the urine and serum of leukemia patients (Osserman and Lawlor 1966 and Brierre *et al.* 1974), and in the cerebrospinal fluid of patients with a central nervous system tumor (Newman *et al.* 1974).

Lysozyme research in the 1980s included investigating enzyme intermediates (Acharya 1982, Desmadril and Yon 1984, and Ikegudri *et al.* 1986), analyzing the protein structure (Delepierre 1982), and performing binding studies (Nutta *et al.* 1988, Perraudin and Preels 1982, and Smitth-Gill *et al.* 1984). In the 1990s, transcription control, silencers, and additional binding sites were investigated (Bonifer *et al.* 1997, Baniahmad *et al.* 1991, and Madhusudan and Vijayan 1992).

Recent research has focused on obtaining more information about gene regulation of lysozyme both in the hen and other animals (Shimizu *et al.* 2005), gaining a better understanding of the secondary structure (Schwint*é et al.* 2002) and refining its use in biochemical applications (Reischl 2004 and Zhu 2006).

Molecular Characteristics

The mature lysozyme of chicken is composed of 128 amino acids. Amino acid sequences of other avian lysozymes are homologous in sequence and differ in only 4 to 20 amino acids (Arnheim *et al.* 1973). The lysozyme gene in chickens is expressed tissue specifically in the oviduct and in macrophages. Despite there only being one copy of the lysozyme gene, it is regulated differently in the oviduct and macrophages. Regulation of lysozyme in the oviduct utilizes steroid hormones, while a combination of cis-regulatory elements are used during differentiation in macrophages (Shimizu *et al.* 2005). Transcription is controlled by three enhancers, a complex promoter, and a negative regulatory element (Bonifer *et al.* 1997, and Lefevre *et al.* 2008).

Specificity

Lysozyme hydrolyzes the beta-glycosidic linkage between N-acetylmuramic acid and N-acetyl glucosamine in the peptidoglycan of bacterial cell walls and can also bind polymers of N-acetyl glucosamine (Arnheim *et al.* 1972).

Composition

The structure of lysozyme is consistent under a variety of conditions, making it ideal for crystallography studies. The active site of lysozyme consists of a deep crevice, which divides the protein into two domains linked by an alpha helix. One domain (residues 40 to 85) consists almost entirely of beta-sheet structure, whle the second domain (residues 89-99) is more helical (Strynadka and James 1991).

IUB

- 3.2.1.17

CAS

- 9001-63-2

Protein Accession Number

- P00698

CATH: Classification (v. 3.3.0)

Class:

- Mainly Alpha

Architecture:

- Orthogonal Bundle

Topology:

- Lysozyme

Molecular Weight

- 14.3 kDa

Optimal pH

- 6.0-9.0 (Davis *et al*. 1969)

Isoelectric Point

- 9.32 (Theoretical)

Extinction Coefficient

- 38,940 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 27.21

Active Residue

- Glutamic acid (E53)
- Aspartic acid (D70)

Activators

- EDTA (White and White 1997)

Inhibitors

- SDS
- Alcohols
- N-acetyle-D-glucosamine
- Oxidizing agents (White and White 1997)

Applications

- Nucleic acid preparation (Taylor and Utter 1974)
- Protein purification from inclusion bodies (Reischl 2004)
- Plasmid preparation (to break down membranes and cell wall) (Zhu 2006)
- Hydrolysis of chitin (Hayashi *et al*. 1969)
- Hydrolysis of bacterial cell walls (Shockman *et al*. 1996)

Assay Information

Method

- The rate of lysis of *Micrococcus lysodeikticus* is determined as suggested by Shugar (1952). One unit is equal to a decrease in turbidity of 0.001 per minute at 450 nm at pH 7.0 and 25°C under the specified conditions. A wide range of activities are reported for pure lysozyme preparations under these conditions. The Worthington specific activity of 8,000 $\frac{\text{units}}{\text{mg}}$ dw is equivalent to 50,000 $\frac{\text{units}}{\text{mg}}$ dw claimed by other suppliers.

Reagents

- 0.1 M Potassium phosphate, pH 7.0
- *Micrococcus lysodeikticus* cells: Prepare by suspending 9 mg of dried *Micrococcus lysodeikticus* (Worthington code: ML) cells in 25 ml of 0.1 M potassium phosphate buffer, pH 7.0. Dilute to a final volume of 30 ml with the same buffer.

Enzyme

- Dissolve the enzyme at a concentration of one $\frac{\text{mg}}{\text{ml}}$ in cold reagent grade water. Keep cool until assay. Immediately prior to assay, dilute to a concentration of 150-500 $\frac{\text{units}}{\text{ml}}$ with reagent grade water. (The rate should fall between 0.015-0.040 $\frac{\Delta A_{450}}{\text{min}}$).

Procedure

Spectrophotometer Settings: Wavelength: 450 nm Temperature: 25°C

Pipette 2.9 ml of *Micrococcus lysodeikticus* cell suspension into a cuvette and incubate for 4-5 minutes in order to achieve temperature equilibration and to establish blank rate, if any. Add 0.1 ml of appropriately diluted enzyme to cuvette and record the change in A_{450} per minute from the initial linear portion of the curve.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{450}}{\text{min}} \text{ x } 1000}{\text{mg enzyme in reaction mixture}}$

- $\frac{\text{MgP}}{\text{ml}} = A_{280} \times 0.39$

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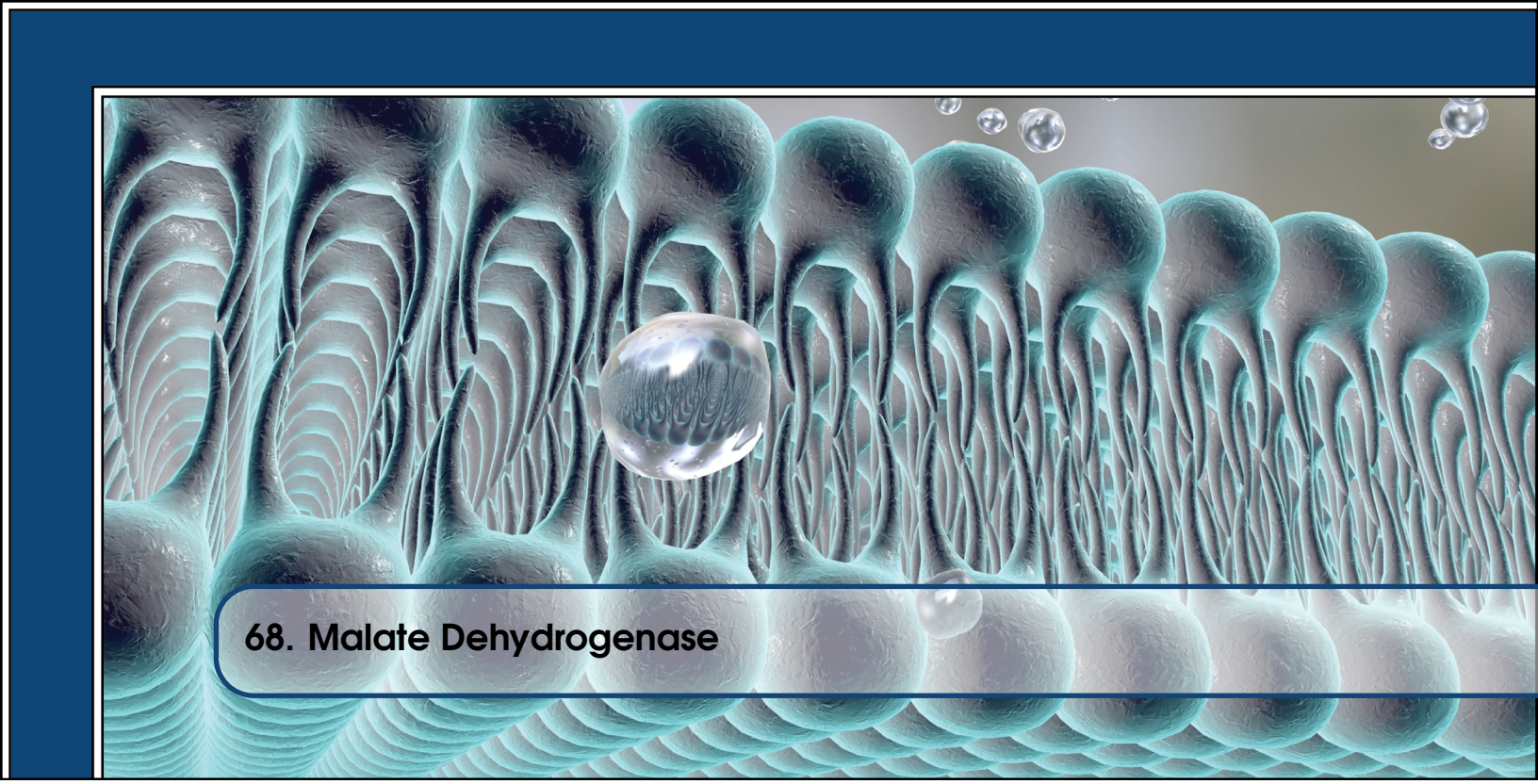

68. Malate Dehydrogenase

L-Malate: NAD^+ Oxidoreductase

MDH is found in all eukaryotic cells as two isozymes: mitochondrial (m-MDH) and cytoplasmic (soluble, s-MDH). Prokaryotes contain only a single form. Pig heart MDH has been extensively studied. The two isozymes, both consisting of two very similar subunits of about 35,000 daltons and having similar enzymatic activity appear as different proteins (Bleile *et al.* 1975). Noyes *et al.* (1974) report on the structural similarity of mitochondrial MDH to L-3-hydroxyacyl CoA dehydrogenase (the cytoplasmic MDH being somewhat similar to lactate dehydrogenase e). Schindler (1975) indicates differences in binding sites. Banaszak and Bradshaw (1975) have reviewed the enzymes and have compared the amino acid compositions of the two isozymes from pig heart with those of other species. They suggest that the subforms of the isozymes that have been reported may be preparative artifacts. A number of non-mammalian malate dehydrogenases have been included in the references.

MDH is of interest to the clinician in that its activity in serum and cerebral/spinal fluid has been shown to be of diagnostic significance (Sharpe *et al.* 1973). See also Schwartz (1973). Malate dehydrogenase (MDH) catalyzes the interconversion of L-malate and oxaloacetate using nicotinamide adenine dinucleotide (NAD) as a coenzyme.

$$\text{L-malate} + NAD^+ \rightleftarrows \text{oxaloacetate} + NADH + H^+ \qquad (68.1)$$

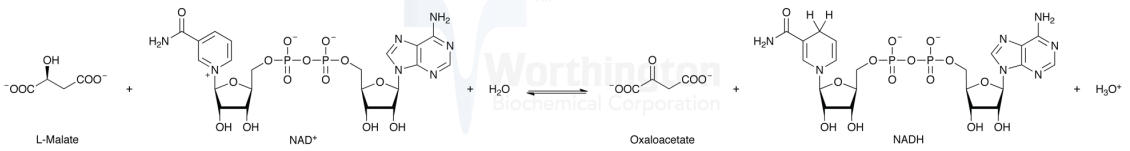

Figure 68.1: Enzymatic Reaction - Malate Dehydrogenase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Pig Heart (mitochondrial)

Specificity

The heart enzyme is specific for L-malic acid, but Hayashi *et al.* (1966) report a D-malate dehydrogenase in Serratia marcescens. Thorne (1962) reports that m-MDH from ox heart, rat liver, rabbit kidney, and those from acetone powders of horse, pig and pigeon heart are similar in specificity and Michaelis constants.

Composition

The molecule consists of two polypeptide chains. There are two coenzyme binding sites per 70,000 daltons (Eberhardt and Wolfe 1975). Active center studies include those of Chen and Engel (1975), Foster and Harrison (1974 and 1975), *et al.* (1975), Wimmer *et al.* (1975), Holbrook and Wolfe (1972). See also Codd (1972) who reports a possible binding site for FMN.

IUB

- 1.1.1.37

Molecular Weight

- 70,000 (Thorne and Kaplan 1963). According to Devenyi *et al.* (1966) the molecule is composed of similar subunits of molecular weight 35,000

Optimal pH

- 7.4 (NADH $\rightarrow$ NAD)

Extinction Coefficient

- $E^{1\%}_{280}$ = 2.8 (Thorne 1962)

Activators

- Phosphate, arsenate, and zinc ions are stimulatory (Blonde *et al.* 1967). Mercuribenzoate has also been shown to be an activator at low concentrations (Silverstein and Sulebele 1970; Kuramitsu 1968).

Inhibitors

- Several iodinated agents, thyroxine, iodine, cyanide, and molecular iodine also inactivate the enzyme by oxidizing the -SH groups (Varrone 1970). 2-Thenoyl-trifluoroacetone (TTFA) (Gutman and Hartstein 1974) and chlorothricin (Schindler 1975) also inhibit MDH. See also Kun *et al.* (1967) and Wedding *et al.* (1967).

Stability/Storage

- Stable for one year when stored at 2 - 8°C as a suspension in ammonium sulfate.

Stabilizers

- Various organic compounds have been shown to have a significant stabilizing effect (George *et al.* 1969).

Assay Information

Method

- The reaction velocity is determined by measuring the decrease in absorbance at 340 nm resulting from the oxidation of NADH. One unit oxidizes one micromole of NADH per minute at 25°C and pH 7.4 under the specified conditions.

Reagents

- 0.1 M Potassium phosphate buffer, pH 7.4
- 0.006 M Oxaloacetic acid, freshly prepared in 0.1 M phosphate buffer pH 7.4. This reagent is unstable and should be stored in an ice bath during use.
- 0.00375 M NADH, freshly prepared in 0.1 M potassium phosphate buffer, pH 7.4
- Note: NADH may vary in salt form and degree of hydration. Care should be exercised to use an analytical grade and to use the correct molecular weight.

Enzyme

- Immediately before use, dilute in 0.1 M phosphate buffer, pH 7.4 to obtain a rate of 0.02-0.04 $\frac{\Delta A}{min}$.

Procedure

Spectrophotometer Settings: Wavelength: 340 nm Temperature: 25°C

Pipette into each cuvette as follows:

- 0.1 M Phosphate buffer - 2.6 ml
- NADH - 0.2 ml

- Oxaloacetate - 0.1 ml

Incubate cuvettes in spectrophotometer for 3-4 minutes to achieve temperature equilibration and establish blank rate, if any. Add 0.1 ml diluted enzyme to cuvette and record decrease in ΔA_{340} for 3-5 minutes. Calculate ΔA_{340} per minute from the initial linear portion of the curve.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{340}}{\text{min}}}{6.22 \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$$

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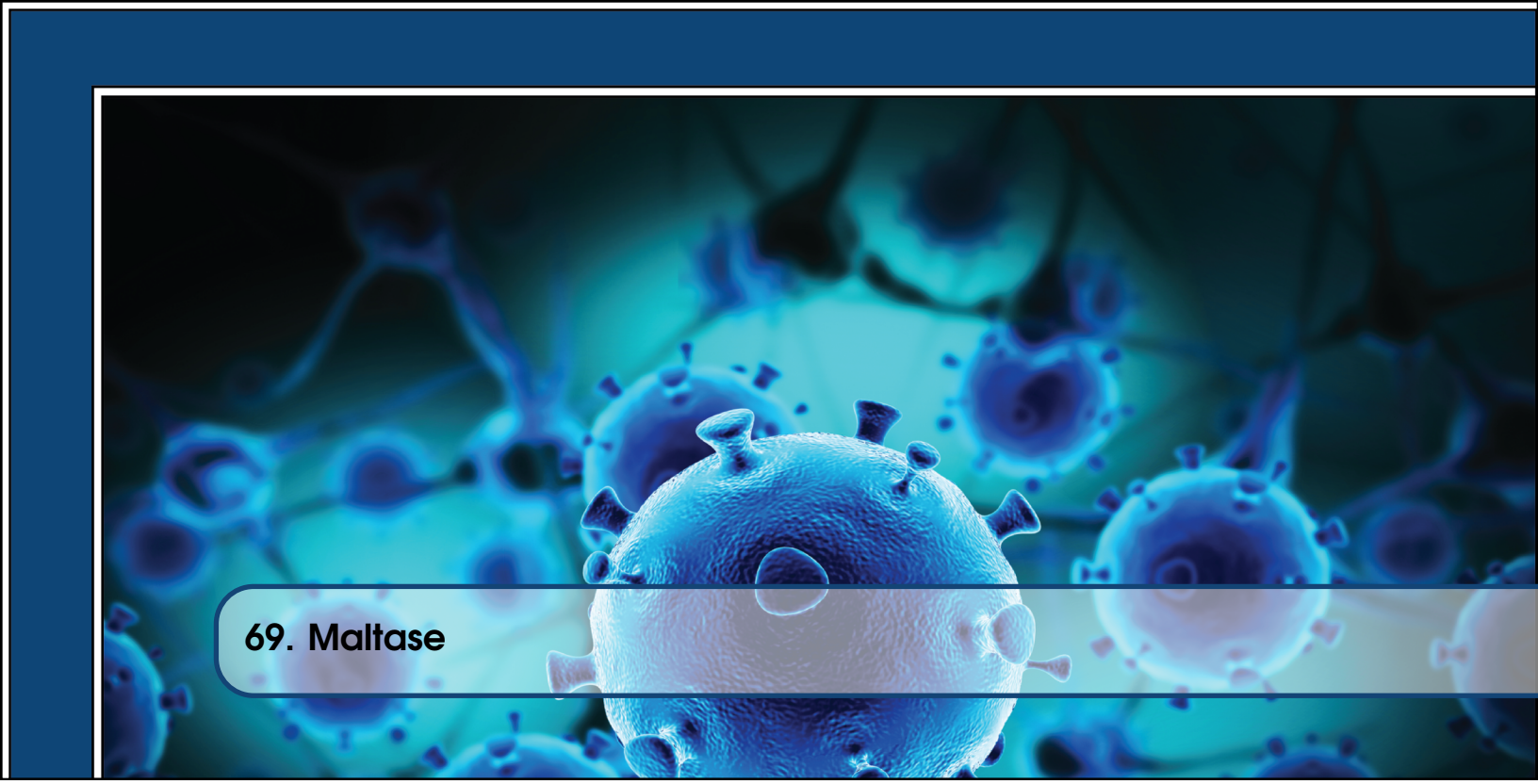

69. Maltase

Glucosidase, Alpha

α-Glucosidase

α-Glucosidase hydrolyzes the terminal, non-reducing 1,4-linked α-D-glucose residues with release of α-D-glucose. α 1,2 and α 1,3 bonds are cleaved at a much slower rate. The rate of hydrolysis decreases substantially with increasing substrate size. α-glucosidase will cleave sucrose but will not hydrolyze α-D-glucosides such as cellobiose.

Maltase, located in the brush border membrane of the enterocytes, was first separated in homogenates of pig intestinal mucosa and later in human preparations and exhibits quite complicated cross-specificity for different substrates. (Dahlqvist 1984).

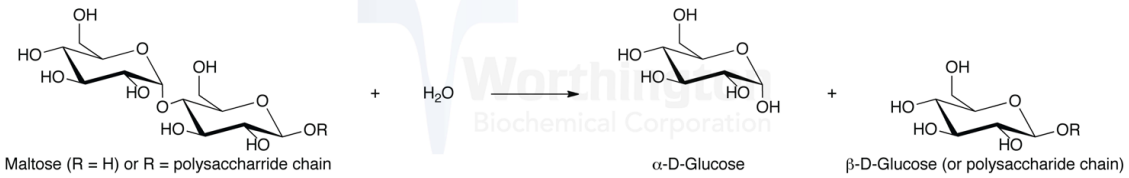

Figure 69.1: Enzymatic Reaction - Maltase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Baker's Yeast

IUB

- 3.2.1.20

Molecular Weight

- 68,500 daltons

Optimal pH

- 7.0 - 7.5 using maltose as the substrate

Inhibitors

- Thiol blocking compounds, heavy metal ions, histidine, and certain amines. Tris should not be used as a buffer due to its inhibitory effect.

Assay Information

Method

- The enzymatic activity is determined by measuring the increase in absorbance at 400 nm caused by the hydrolysis of p-nitrophenyl-α-D-glucopyranoside. One unit hydrolyzes one μmole of p-nitrophenyl-D-glucopyranoside (PNPG) at 37°C, pH 6.8, under the specified conditions.

Reagents

- 0.067 M Potassium phosphate, pH 6.8 with 0.001 M dithio-threitol (DTT).
- 0.01 M p-nitrophenyl-α-D-glucopyranoside (PNPG). Substrate may be aliquoted into vials and frozen for future use. Once thawed, the substrate may not be re-frozen.

Enzyme

- Dilute immediately before use in 0.067 M Potassium phosphate to obtain a rate of 0.02-0.04 $\frac{\Delta A}{min}$. The protein may be determined as follows:

Procedure

Spectrophotometer Settings: Wavelength: 400 nm Temperature: 37°C

Pipette into cuvettes as follows:

Description	Test	Blank
Phosphate buffer	2.8 ml	2.9 ml
PNPG substrate	0.1 ml	0.1 ml

Incubate in spectrophotometer for 5-7 minutes to achieve temperature equilibrium and establish blank rate, if any. Add 0.1 ml diluted enzyme to test cuvette and mix. Record increase in A_{400} for 5-6 minutes. Calculate $\frac{\Delta A_{400}}{min}$ from the initial linear portion of the curve.

Calculation

- $\frac{units}{ml} = \frac{\frac{\Delta A}{min} \text{ x dilution x reaction volume}}{8.7 \text{ x sample volume}}$

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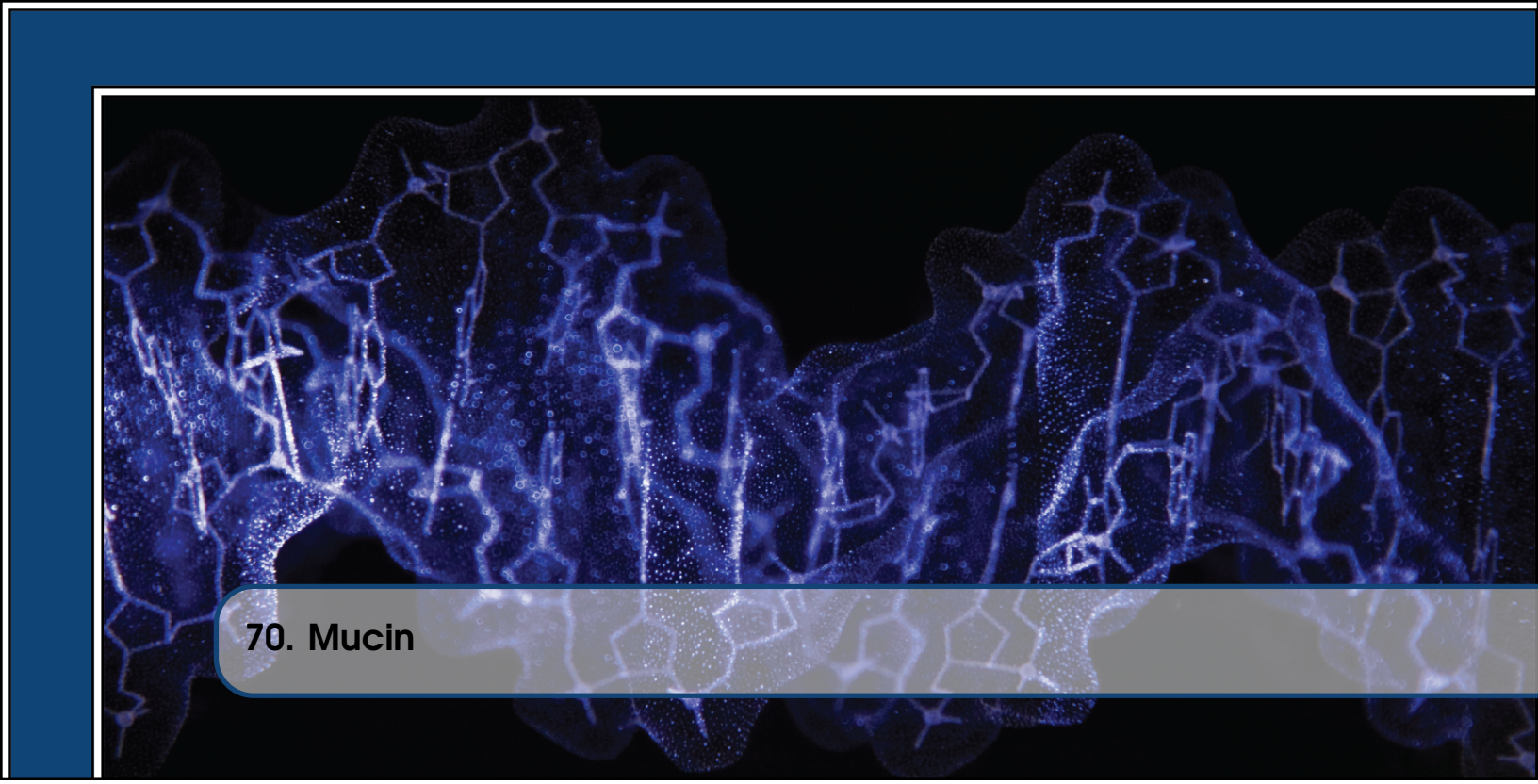

70. Mucin

Mucins are the glycoproteins in epithelial mucous secretions; their protective function is due to their high viscosity. Svennerholm (1963) describes the preparation of sialic acids from mucin.

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Bovine Submaxillary Gland

Composition

Tettamanti and Pigman (1968) separated BSM into major and minor components and determined the amino acids of the protein moiety (36.6% of the molecule). Besides sialic acid and N-acetyl-galactosamine they reported small amounts of fructose and galactose. Bettelheim *et al.* (1972) report the carbohydrate content to be 56.7% and the molecular shape to be that of a stiff rod. Downs and Pigman (1969) and Pigman *et al.* (1973) suggest the possibility of a repeating glycopeptide structure.

CAS

- 84195-52-8

Molecular Weight

- 400,000 (Downs and Pigman 1969)
- 4,000,000 by light-scattering (Bettelheim *et al.* 1962)

Stability/Storage

- Stable indefinately at 2 - 8°C. Protect from moisture.

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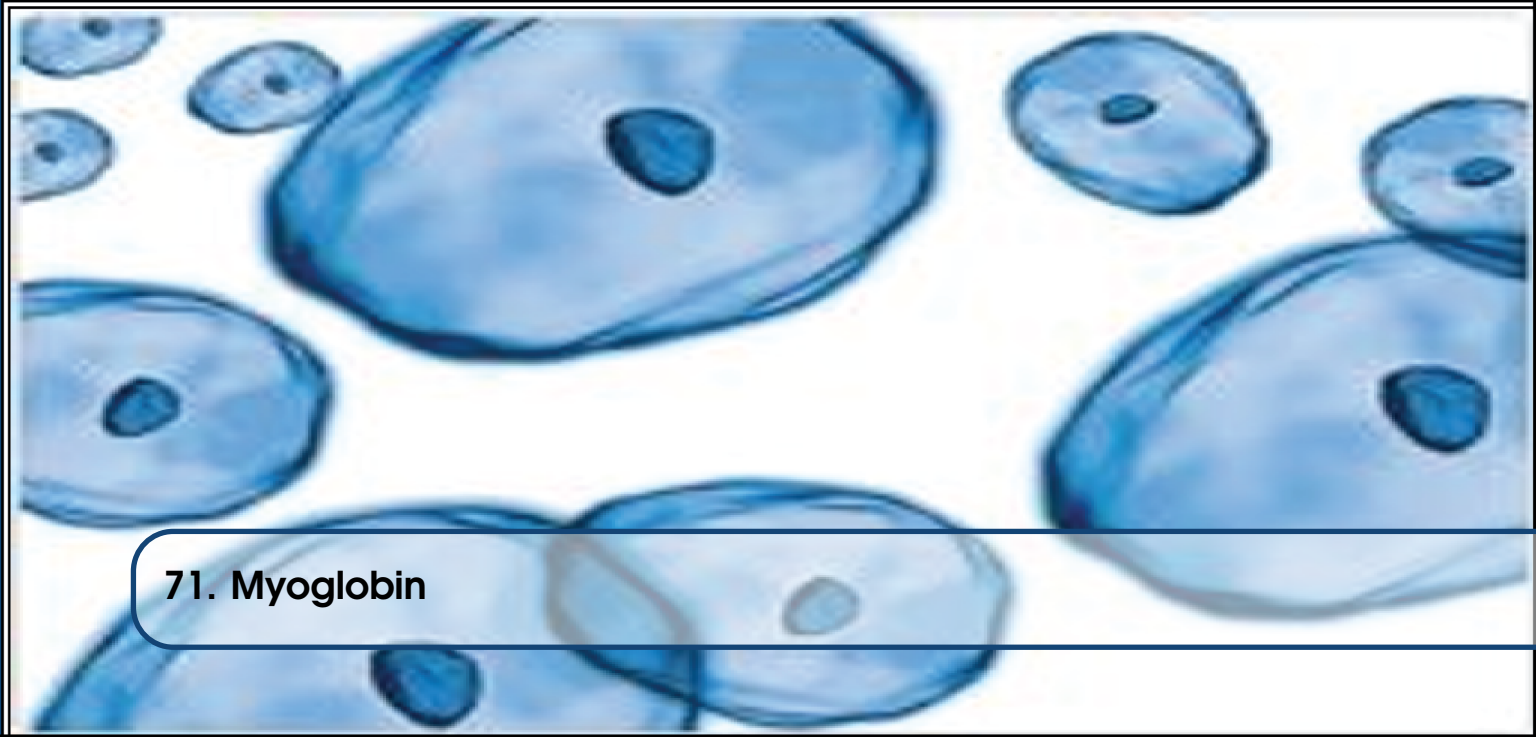

71. Myoglobin

Myoglobin (Mb) is a small, globular protein primarily responsible for oxygen storage in cardiac and skeletal muscle. Mb is involved in the regulation of cellular oxygen tension in respiring tissues, and regulating the bioavailability of the signaling molecule, nitrous oxide (NO) (Wittenberg and Wittenberg 2003). It contains a single heme molecule and has a molecular weight of approximately 17 kDa.

History

In 1897 Mörner described a red protein in muscle, which he called "myochrome".

Early studies of myoglobin are considered to be where the science of protein structure began. John Kendrew and his coworkers determined the atomic structure of sperm whale myoglobin, making it the first protein to have its three dimensional structure revealed by X-ray crystallography. This feat earned Kendrew the Nobel Prize in 1962, shared with Max Perutz who revealed the structure of hemoglobin (Kendrew *et al*. 1958, Perutz *et al*. 1960, and Watson and Kendrew 1969).

Recent research involving myoglobin has included the development of assays for serum myoglobin detection (Osman *et al*. 2013, and Padmanaban *et al*. 2014), as well as the development of additives to bind myoglobin to preserve red coloring of fresh meat (Miura *et al*. 2014, and Suman *et al*. 2014).

Molecular Characteristics

The bovine mb gene is located on chromosome 5. It is conserved in human, chimpanzee, Rhesus monkey, dog, mouse, rat, chicken, and zebrafish. It appears to be deleted in amphibians and a few species of Notothenioid icefish of the Antarctic Ocean (Fuchs *et al*. 2006, and Helbo *et al*. 2013). In addition to heart and skeletal muscle, Mb has been identified in vascular smooth muscle cells, where

it may contribute to vasodilation under hypoxic conditions by converting nitrite to NO (Totzeck *et al*. 2012). Mb has also been identified in non-muscle tissues of fish species and mammalian cancer cells (Fraser *et al*. 2006, Roesner *et al*. 2008, Cossins *et al*. 2009, Flonta *et al*. 2009, and Gorr *et al*. 2011). Mb transcription upregulation is a complex process involving hypoxia in conjunction with intracellular calcium release, which activates calcineurin signaling pathways, transcription factors, and transcriptional co-activators (Kanatous *et al*. 2009, Kanatous and Mammen 2010, and Helbo *et al*. 2013). In skeletal muscle, expression increases with exposure to hypoxic stress together with muscle contraction (Kanatous *et al*. 2009).

Composition

Myoglobin contains one protein chain, a heme group with water bound to the iron, and a sulfate ion. The protein chain consists of 8 spring-shaped alpha helices, linked together by short loops and designated as A-H. The heme prosthetic group is sandwiched between helices E and F. This single heme group is noncovalently bound within a deep cleft of the protein, and is composed of a porphyrin ring and a bound iron. Myoglobin can bind a single oxygen molecule between the distal histidine and the heme iron atom. Hydrogen bonding of the distal histidine and the oxygen crowds the area enough to cause the oxygen to bind at an angle with respect to the plane of the heme group (Pratt and Cornely 2004).

CAS

- 11080-17-4

Protein Accession Number

- P02192

CATH: Classification (v. 3.3.0)

Class:
- Mainly Alpha

Architecture:
- Orthogonal Bundle

Topology:
- Globin-Like

Molecular Weight

- 16.9 kDa

Isoelectric Point

- 6.97

Extinction Coefficient

- 13,940 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280} = 8.23$

Applications

- Protein folding studies
- Molecular weight marker
- Standard for mass spectroscopy
- ELISA

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72. NADase (DPNase)

NAD^+ Glycohydrolase

The enzyme is found primarily in animal tissue. That from pig brain has been purified by Swislocki and Kaplan (1967). See also Kaplan (1955). Bernheimer *et al.* (1957) and Carlson *et al.* (1957) reported on NADase from hemolytic streptococci which Fehrenbach (1971 and 1972) has identified with streptolysin-O. The NADase form *Neurospora crassa* differs from that of the animal tissue enzyme in that it does not form analogs (Kaplan 1955). NADase catalyzes the following reaction:

$$\text{Nicotinamide adenine dinucleotide} + H_2O \rightarrow \text{Nicotinamide} + \text{adenine diphosphoribose} + H^+ \quad (72.1)$$

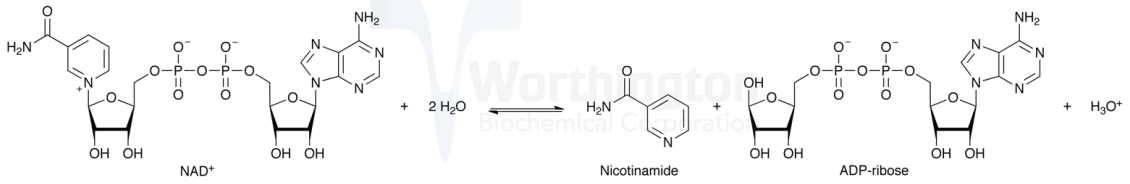

Figure 72.1: Enzymatic Reaction - NADase (DPNase)

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Neurospora crassa*

Specificity

Attacks both NADP and NAD

IUB

- 3.2.2.5

Optimal pH

- 3.0-9.0

Inhibitors

- The *Neurospora crassa* NADase in contrast to the hog brain enzyme is relatively insensitive to nicotinamide. A concentration of 0.1 M nicotinamide is required to bring about inhibition.

Stability/Storage

- Stable when kept frozen, but a slight loss in activity may be noted at 2 - 8°C

Assay Information

Method

- Cyanide reacts with the quaternary nitrogen form of NAD to form an addition product with an absorbance maximum at 340 nm (Colowick et al. 1951). No such reaction occurs with nicotinamide or adenosine-diphosphate-ribose. Therefore, the reaction between cyanide and NAD before and after incubation with NADase will indicate the activity of the enzyme (Kaplan 1955; Nason et al. 1951). One unit will cleave one micromole of NAD per minute at 37°C and pH 7.5. Note: The original method used a unit which was equivalent to the splitting of 0.01 micromole of NAD in 0.5 ml in 7.5 minutes.

Reagents

- 0.1 M Potassium phosphate buffer, pH 7.5
- 1.0 M Potassium cyanide. Caution: Poison, handle with care! Read product label for proper handling instructions.
- 5.4 mM NAD

Enzyme

- Reconstitute vial contents with one milliliter reagent grade water. Immediately prior to use dilute further to a concentration of 0.10-0.50 $\frac{\text{mg}}{\text{ml}}$.

Procedure

Pipette into test tubes as follows:

Description	Test	Blank
0.1 M Potassium phosphate buffer pH 7.5	0.3 ml	0.3 ml
5.4 mM NAD	0.1 ml	0.1 ml
1.0 M KCN	3.0 ml	—
Reagent grade water	0.1 ml	—

Incubate at 37°C for 3-5 minutes to achieve temperature equilibration. At zero time add 0.1 ml appropriately diluted enzyme to assay and blank tube. Incubate for exactly 7.5 minutes at 37°C and add 3.0 ml of 1.0 M KCN to assay tube. Cool to room temperature and read A_{340} of blank and assay tubes.

Calculation

- $$\frac{\text{Units}}{\text{ml}} = \frac{(A_{340\ \text{Blank}} - A_{340\ \text{Test}}) \times 3.5}{5.15 \times 7.5\ \text{min} \times 0.1}$$

Note: If $(A_{340\ \text{Blank}} - A_{340\ \text{Test}})$ approaches 0.7, a further dilution of the enzyme is required

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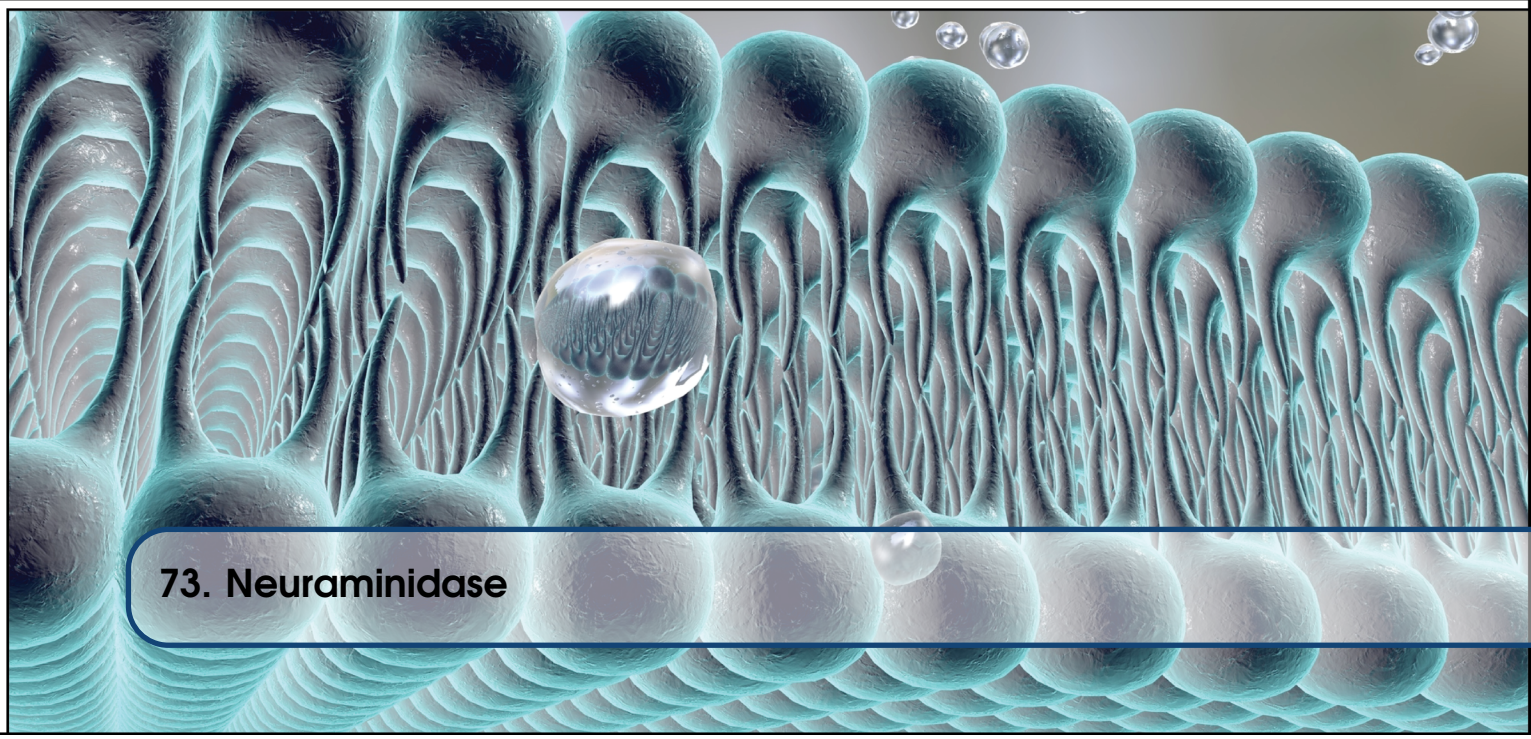

73. Neuraminidase

Acylneuraminyl Hydrolase

Neuraminidases are important tools for the study of glycoproteins in protein chemistry and cell biology (Hatton *et al.* 1973).

Worthington neuraminidase is derived from *Clostridium perfringens*. Chromatographic purification has been developed in this laboratory based on work of Cassidy *et al.* (1965). Wooley and Gommi (1966) describe the use of this neuraminidase in a method for measuring serotonin receptors. It is also employed to determine bound N-acetylneuraminic acid in tissues. Neuraminidase (sialidase) splits off N-acetyl neuraminic acid (sialic acid) from a variety of glycoproteins.

$$\text{Sialyl glycoside} \rightarrow \text{Sialic acid} + \text{aglycon} \qquad (73.1)$$

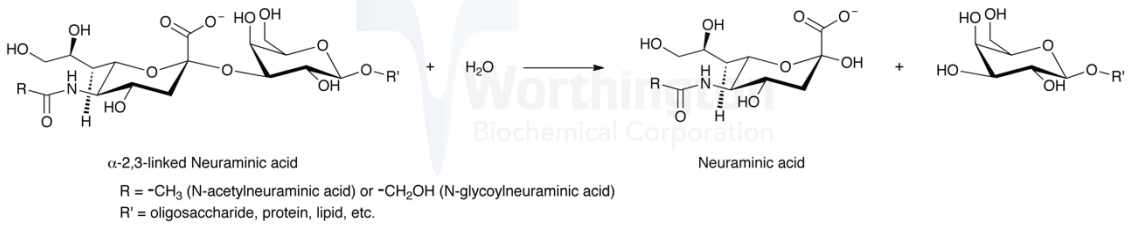

Figure 73.1: Enzymatic Reaction: 2,3-linked Neuraminic acid

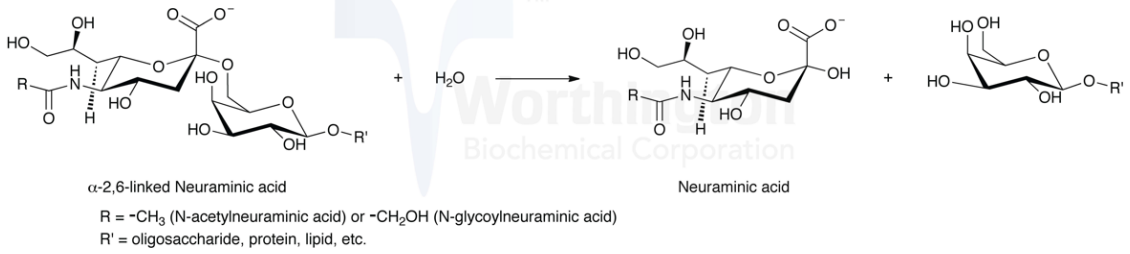

Figure 73.2: Enzymatic Reaction: 2,6-linked Neuraminic acid

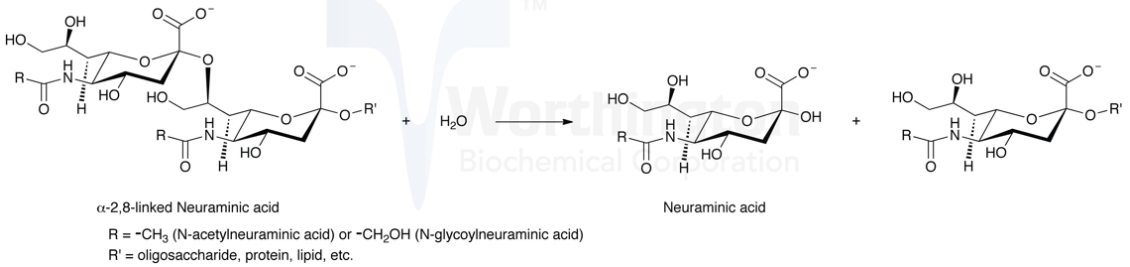

Figure 73.3: Enzymatic Reaction: 2,8-linked Neuraminic acid

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Clostridium perfringens*

IUB

- 3.2.1.18

CAS

- 9001-67-6

Optimal pH

- 5.0-5.1 (Burton 1963)
- Little or no activity at pH 4.0 or above pH 8.0

Isoelectric Point

- 5.1 (Groome and Belyaven 1958)

Activators

- None, in contrast to the neuraminidase from Vibrio which has a divalent metal requirement.

Inhibitors

- Considerable interest has been shown in using neuraminidase inhibitors as possible anti-viral and anti-bacterial agents. (Khorlin *et al.* 1970; Haskell *et al.* 1970; and Tute 1970).

Stability/Storage

- The purified preparation is stable for at least two years when stored at 4°C (Cassidy *et al.* 1965).

Stabilizers

- Serum albumin, 0.3 $\frac{mg}{ml}$ (Cassidy *et al.* 1965).

Assay Information

Method

- The assay is based on the measurement of sialic acid (NANA) released from bovine submaxillary mucin (Worthington Code: MU). One unit causes the release of one micromole of sialic acid per minute at 37°C and pH 5.0, from bovine submaxillary mucin under the specified conditions. A coupled enzyme assay for determining neuraminidase is described by Ziegler and Hutchinson (1972).

Reagents

- 0.2 M Sodium metaperiodate in 9 M phosphoric acid
- 0.5 M Sodium sulfate
- 0.755 M Sodium arsenite in 0.5 M sodium sulfate with 0.1 N H_2SO_4. Prepare by dissolving 25 g sodium arsenite (molecular weight 129.91) in 250 ml of 0.5 M sodium sulfate and add 5 ml 5 N H_2SO_4. Slight heating may be necessary in order to effect solution.
- 2.5 N HCl
- 5% Phosphotungstic acid in 2.5 N HCl
- 0.6% Twice crystallized thiobarbituric acid in 0.5 M Sodium sulfate. Prepare by dissoving 4.5 grams 2x crystallized thiobarbituric acid in 750 ml 0.5 M sodium sulfate. Heat to effect solution and allow to cool. A crystalline precipitate will form. Use only the supernatant for the assay.
- 0.1 M Acetic acid, pH 5.0
- 1% Mucin, pH 5.0. Prepare by dissolving 100 mgs Worthington Submaxillary Mucin (Code: MU) in 9 ml reagent grade water, adjust pH to 5.0 with acetic acid and dilute to a final volume of 10 ml. The solution will appear turbid at pH 5.0.
- Cyclohexanone. Caution: read product label for handling instructions.

Enzyme

- Dissolve enzyme at one $\frac{mg}{ml}$ in reagent grade water. Immediately prior to use prepare four dilutions ranging from 0.1 $\frac{mg}{ml}$ to 0.005 $\frac{mg}{ml}$.

Procedure

Spectrophotometer Settings: Wavelength: 549 nm

Prepare a 37°C water bath.

Into numbered tubes pipette as follows:

- Mucin - 0.4 ml
- 0.1 M acetic acid - 0.5 ml

At timed intervals, add 0.1 ml of the respective enzyme dilution. Include a blank of 0.1 ml of water in place of the enzyme. Incubate for 30 minutes in the 37°C water bath. Stop the reaction at timed intervals by adding 1 ml of 5% phosphotungstic acid to each tube. Centrifuge for 10 minutes. Remove a 0.5 ml aliquot of each supernatant to dry clean test tubes. To each, add 0.1 ml of 0.2 M sodium metaperiodate. Allow to stand for 20 minutes at room temperature, add 1.0 ml of 0.755 M sodium arsenite. Shake the tubes until the brown color disappears, then add 3.0 ml of the 0.6% thiobarbituric acid. Heat for 15 minutes in a boiling water bath, cool in an ice bath for 5 minutes and add 4.6 ml of cyclohexanone to each tube. Extract the color in cyclohexanone layer by vortexing. Centrifuge at high speed for 15 minutes. Remove colored cyclohexanone layer and read A_{549} of the colored cyclohexanone layer versus a reagent grade water blank.

Note: The final A_{549} of sample should not exceed 0.5 absorption unit.

Calculation

- Micromoles NANA released = $(A_{549}{}_{\text{Sample}} - A_{549}{}_{\text{Blank}}) \times 0.075$
- $\frac{\text{Units}}{\text{mg}} = \frac{\text{Micromoles NANA liberated x 4}}{\text{30 x mg enzyme in initial reaction mixture}}$

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74. Neutral Protease (Dispase)

Neutral protease is an extremely stable Zn-metalloendopeptidase that is produced by *Paenibacillus polymyxa*. It is involved in the generation of beta- and alpha-amylases from the large amylase precursor. Although related to trypsin, neutral protease is significantly less harmful to cells and can help prevent unwanted cell clumping without cell membrane damage after one hour incubations (Alvarez *et al.* 2006).

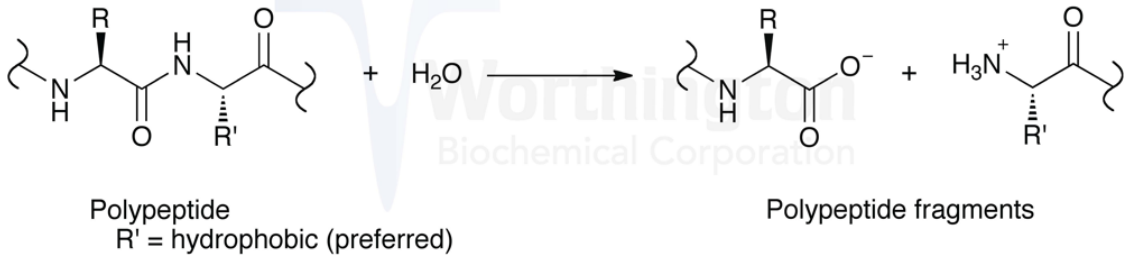

Figure 74.1: Enzymatic Reaction - Neutral Protease (Dispase)

History

In the 1950s neutral protease was first crystallized and characterized from *Bacillus subtilis* and was thought to be an extracellular product (Fukumoto and Negoro 1951, Guntelburg 1954, Ottesen and Spector 1960).

Into the 1970s the proteases of *Bacillus thermoproteolyticus* (Keay *et al*. 1970), *Bacillus megaterium* (Millet *et al*. 1969), *Bacillus cereus* (Feder *et al*. 1971), *Streptomyces griseus* (Nomoto *et al*. 1959), *Aspergillus oryzae* (Misaki *et al*. 1970), and Serratia (Miyata 1971) were preliminarily studied. Subsequently, Griffin and Fogarty investigated the proteolytic and starch degrading enzymes of *Bacillus polymyxa* (Griffin and Fogarty 1971 and 1973), and reported on the effects of different factors influencing the proteolytic activities.

Work done in the early 1980s highlighted the use of neutral protease as a means of separating epidermal sheets (Kitano and Okada 1983).

In 1993 Ash *et al*. (1991 and 1993) compared the 16S rRNA gene sequences of different Bacillus species and defined a new genus named *Paenibacillus*, to which the former *Bacillus polymyxa* now belongs to as *Paenibacillus polymyxa*.

Molecular Characteristics

Neutral protease is produced by the extracellular neutral protease gene, npr (GenBank accession number BAA00734), which shares 62% identity with that of *Bacillus subtilis* (Koide *et al*. 1986). A single 978 bp open reading frame is present and the presence of a pro sequence is common to all secreted proteases of Bacilli. (Takekawa *et al*. 1991). The Npr sequence from Asn-287 to Gly-590 shows considerable homology with sequences of neutral proteases such as thermolysin (55% identical) from *Bacillus thermoproteolyticus*.

Specificity

Neutral protease is a non-specific metalloprotease. It cleaves fibronectin, collagen IV, and to a lesser extent collagen I, but it does not cleave collagen V or laminin. It hydrolyzes N-terminal peptide bonds of non-polar amino acid residues and may preferentially attack denatured and intercellular proteins with exposed hydrophobic amino acid residues. It is believed to bind one zinc ion and four calcium ions per subunit. Unlike other Bacillus species that produce neutral, alkaline, or a mixture of both proteases, *Paenibacillus polymyxa* is one of three species that produces only a neutral protease (Fogarty and Griffin 1973).

Composition

The enzyme is known to contain 1g-atom of zinc per g-mol of purified enzyme. If this zinc component is removed by chelating agents such as EDTA or EGTA, an inactive apoenzyme is obtained. Calcium has been detected in the purified protein and is believed to play a role in maintaining the structure and configuration, and preventing autolysis (Griffin and Fogarty 1973 and Alvarez 2006).

IUB

- 3.4.24.28

CAS

- 9001-92-7

Protein Accession Number

- P29148

CATH: Classification (v. 3.3.0)

Class:

- The protein consists of two distinct domains: the C-terminal is mainly alpha, while the N-terminal is mainly beta.

Architecture:

- The distinct architectures of the C-terminal and N-terminal are orthogonal bundle and roll, respectively.

Topology:

- The C-terminal domain is elastase-like and the N-terminal domain is neutral protease-like.

Molecular Weight

- 32.5 kDa

Optimal pH

- 5.9-7.0 (Fogarty and Griffin 1973)

Isoelectric Point

- 5.14

Extinction Coefficient

- 45,350 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 13.96

Active Residue

- Glutamic Acid (E424)
- Histidine (H507)

Activators

- Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, and Al^{3+}
- Manganese has a greater activating effect in the case of *Paenibacillus polymyxa* than other Bacilli neutral proteases (Griffin and Fogarty 1973).

Inhibitors

- EDTA, EGTA, Hg^{2+}
- Other heavy metals (Griffin and Fogarty 1971)
- Not serum

Applications

- Tissue disaggregation and subcultivation
- Prevention of unwanted cell clumping
- Preparation of cells for culture
- Separation of intact epidermis from dermis and intact epithelial sheet in culture from the substratum (Kurt *et al*. 1989)
- Harvest and transfer of normal, diploid cells and cell lines (Matsumura et. al 1975)
- Gentle and intact detachment of epidermal cells (Kitano and Okada 1983)

Assay Information

Method

- The reaction velocity is determined in a peroxidase coupled system by measuring the increase in A_{436} resulting from the oxidation of D-alanine. One unit oxidizes one micromole of D-alanine per min. at 37°C and pH 8.3 under the specified conditions.

Reagents

- The nature of this enzyme requires the substrate to be prepared accurately. Take care to follow the instructions below.
- 1 M Tris
- Buffer-Substrate (2% casein in 0.05M Tris buffer): Dissolve 1.0 g casein (USB grade or equivalent) in 40.0 ml reagent grade water by stirring for approximately twenty minutes. Add 1.0 ml of 1N NaOH and 2.5 ml of 1.0M Tris. Allow substrate to stir until casein has gone into solution. pH to 7.8 with dilute phosphoric acid and continue stirring for 5 minutes. Bring to a final volume of 50.0 ml with reagent grade water. Prior to pipetting substrate make sure substrate is homogeneous throughout and casein has not fallen out of solution. If casein appears to have fallen out of solution, do not use substrate for assay.
- Enzyme activator - 0.01 M $CaCl_2$: Dissolve 147 mg $CaCl_2$ in 100 ml reagent grade water. When not used keep in refrigerator.
- 5% TCA: Dissolve 5 g TCA in 100 ml reagent grade water. Keep at room temperature.
- Tyrosine standard: Prepare 10 ml at a concentration of 10 $\frac{\mu\text{moles}}{\text{ml}}$ in reagent grade water. (181μg tyrosine = 1μmole). Weigh 18.1 mg tyrosine and dissolve in 10 ml reagent grade water by stirring to initial boil. Remove from heat and cool to approximately 40°C for assay.
- 0.5 M NaOH: Dissolve 1 g in 50 ml reagent grade water, or make a dilution from 1 M or 10 M NaOH.
- Folin & Ciocalteus Phenol Reagent, use neat

Enzyme

- Dissolve in deionized water, at a concentration of 5 $\frac{\text{mg}}{\text{ml}}$, and prepare 1:50 dilution. Use 25 μl and 50 μl in duplicate.

Procedure

To clear glass tubes add 1.0 ml buffer-substrate, 20 μl of 0.01M $CaCl_2$, using a Pasteur pipette, and two levels of the enzyme dilution: 25 μl and 50 μl, each level in duplicate. Also include two tubes for

blank, with no enzyme, and four tubes for tyrosine standard with 1ml of buffer substrate, and 25 μl, 50 μl, 75 μl, and 100 μl of tyrosine standard solution. Incubate for 15 minutes at 37°C in a water bath. Stop reaction by adding 2 ml 5% TCA. Mix each tube, then centrifuge at 2000 rpm for 10 minutes using a bench top Sorvall, or equivalent. From each tube withdraw (quantitatively) 1ml supernatant and add to marked clean glass tubes. Then add 2.0 ml 0.5M NaOH to each tube. Mix, then add 0.1ml Folin-Ciacalteu reagent and read at A_{550} after 10 minutes. Plot A_{550} vs. μmoles tyrosine. Draw best fit line and select a sample reading that falls within the linear portion of the line and calculate as listed below.

Calculation

- $\frac{\text{Activity}}{\text{ml}} = \frac{0.75 \text{ x } 50}{0.05 \text{ x } 15} = \frac{0.75 \text{ x dilution}}{\text{sample volume x time incubated}} = 50 \frac{\text{units}}{\text{ml}}$
- $\frac{\text{Units}}{\text{mg}} = \frac{\mu\text{moles dispase x dilution}}{\text{sample volume x 15 min x 5 } \frac{\text{mg}}{\text{ml}}}$

Draw a standard curve for tyrosine standards. Establish equivalence between A_{550} and μmoles of tyrosine. For example, using 50μl enzyme, diluted 50 times, reading at A_{550} is 0.26. From the tyrosine curve A_{550} of 0.26 is equivalent to 0.75 μmoles tyrosine.

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75. Nitrate Reductase

Ferrocytochrome: Nitrate Oxidoreductase

(Cytochrome)

It is a membrane-bound enzyme closely associated with formate dehydrogenase which, along with NR contains a functional cytochrome b. An electron transport scheme from formate to nitrate and including possible quinone participation has been reported by Enoch and Lester (1974). Despite disruption of the system and removal of cofactors, purified NR remains functional in the formation of nitrite when a suitable reducing agent is supplied. In such cases reduced methyl viologen and benzyl viologen have been found most useful.

The use of viologen dyes which may be reduced chemically serves both in the assay of the enzyme and in the determination of low concentrations of nitrate. Lowe and Gillespie (1975) describe a very sensitive colorimetric nitrate determination. (The nitrite formed reacts with a chromogen reagent to yield a red color.) The test is adaptable for ground and stream water pollution studies. See also McNamara *et al*. (1971). This respiratory or "anaerobic" nitrate reductase (NR) (Cytochrome) from *Escherichia coli* catalyzes the following reaction:

$$\text{Ferrocytochrome + nitrate} \rightarrow \text{Ferricytochrome + nitrite} \qquad (75.1)$$

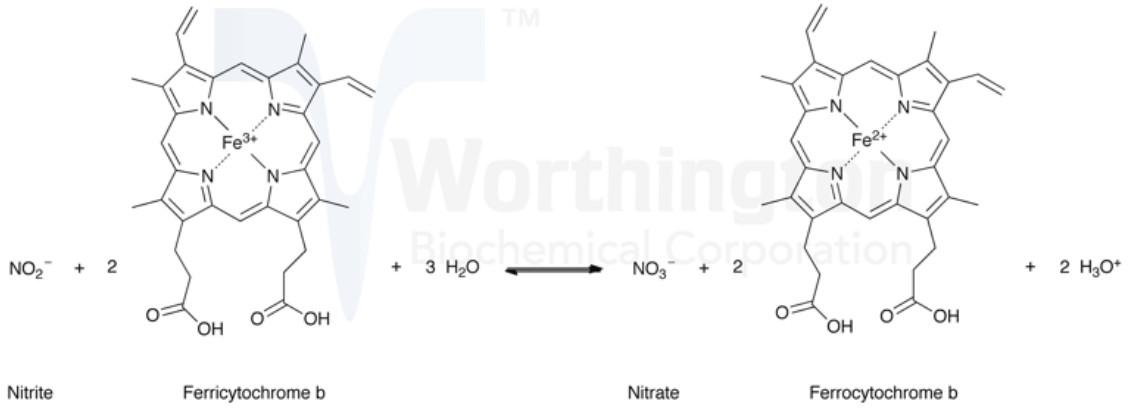

Figure 75.1: Enzymatic Reaction - Nitrate Reductase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Escherichia coli*

Specificity

Both nitrate and chlorate are substrates. The maximum specific activity reported was 0.27 $\frac{\text{mM}}{\text{min}}$ by Der Vartanian and Forget (1975). Reduced benzyl and methyl viologen, $FMNH_2$ and FADH2 may be electron donors but NADH and NADPH are not suitable. (Forget 1974).

Composition

NR contains both iron and molybdenum. MacGregor *et al.* (1974) indicate there to be four atoms of Mo and four active sites per enzyme molecule. Although earlier investigators have claimed that the enzyme contains neither flavin nor heme, Enoch and Lester (1974) have shown heme to be a part of the molecule. A point generally agreed on is that the iron is closely associated with protein sulfur at nearly a 1:1 ratio.

IUB

- 1.9.6.1

CAS

- 9029-42-9

Molecular Weight

- Originally determined by Taniguchi and Itagaki (1960) as 1,000,000, the molecular weight was most recently determined by Enoch and Lester (1975) to be 498,000 in a Triton X100 solution. The latter authors describe the structure as a complex of three polypeptides of molecular weights

155,000, 63,000, and 19,000 (237,000 total, assuming one each) which suggests that they occur together in a dimer to make up most of the 498,000, and perhaps a tetramer to give the one million weight originally described.

- Comparison with the results of Van't Riet and Planta (1975) who studied the nitrate reductase of Klebsiella aerogenes (a bacterium very similar to *Escherichia coli*) may be helpful. They found the Klebsiella aerogenes enzyme to be a one million dalton tetramer of which each monomer was composed of four units: 117,000, 57,000, and two 52,000 molecular weight parts. MacGregor *et al*. (1974) also examined the *Escherichia coli* enzyme and concluded that the molecule is composed of four 142,000 molecular weight polypeptides plus four of 58,000 molecular weight totaling 800,000 daltons.
- In an attempt to clarify the confusion surrounding the molecular weight of *Escherichia coli* nitrate reductase, Clegg (1975) has reexamined the enzyme and concluded that several different forms exist with differing subunit compositions. The suggestion by MacGregor *et al*. (1974) of a protease assisting the release of the enzyme from the membrane may account for some of the disagreement concerning size and make-up.

Optimal pH

- 7.1

Isoelectric Point

- 4.2 (Forget 1974)

Inhibitors

- Cyanide and azide are strong inhibitors. Forget (1974) indicates the cyanide inhibition is competitive, but that the affinity of cyanide to the enzyme is about 1,000 times that of nitrate. Chloromercuribenzoate and iodoacetate are not inhibitory at about 1 mM concentrations (Forget 1974). Chelating agents, o-phenanthroline, 8-hydroxyquinoline, etc., have mild inhibitor effect; EDTA has none. (Taniguchi and Itagaki 1960).

Stability/Storage

- In purified form, the stability is moderate. Forget (1974) recommends storage at 0 - 1°C as a precipitate in 60% saturated ammonium sulfate.

Assay Information

Method

- The method employed is that of Lowe and Evans (1964) wherein the reaction velocity is determined by measuring the production of nitrite in a dithionite/methyl viologen system. One unit produces one micromole of nitrite per minute at 30°C and pH 7.0 under the specified conditions.
- Note: Enzyme and test are destroyed by oxygen. Extreme care must be taken when mixing and adding reagents. The test should be dark blue to show that it is in a reduced conditon until it is stopped by shaking.

- Prepare the following:
- Ice bath (used to slow oxygen gain)
- 30°C dry bath with thermometer
- 12 x 75 test tubes
- Serological pipets, 1 and 2 ml
- Vacuum with 4 ports
- 4 side arm vacuum flasks with stoppers

Reagents

- Reagent grade water: Place 150 ml water in 250 ml side arm and stopper. FLASK #1.
- 0.10 M Sodium nitrate: Dissolve 850 mg of sodium nitrate (MW 84.99) in 100 ml reagent grade water. Put in side arm flask and stopper. FLASK #2.
- 0.15 M Potassium phosphate, pH 7.0: Dissolve 2.04 g potassium phosphate, monobasic (MW 136.1) in 80 ml reagent grade water. pH to 7.0 with NaOH, q.s. to 100 ml. Put 80 ml in side arm flask and stopper. FLASK #3. Save remaining 20 ml.
- 20 ml Phosphate buffer/40 ml water: Place 40 ml reagent grade water and the remaining 20 ml of 0.15 M potassium phosphate, pH 7.0 in side arm flask and stopper. FLASK #4.
- 58 mM Sulfanilamide in 3N HCl: Dissolve 199.8 mg of sulfanilamide (MW 172.21) in 20 ml of 3N HCl.
- 0.10 M Sodium nitrate
- 23 mM Sodium dithionite (MW 174.11) prepared in 48 mM sodium bicarbonate. Prepare fresh and avoid aeration.
- 0.39 mM N-(1-napthyl) ethylenediamine hydrochloride (NED): Dissolve 10.1 mg of NED (MW 259.12) in 100 ml reagent grade water.
- 1000 $\frac{\mu\text{mole}}{\text{L}}$ Sodium nitrite stock solution: Dissolve 69 mg of sodium nitrate (MW 69.0) in 1 L of reagent grade water.
- Dry reagents: Weigh 80 mg Sodium dithionate (MW 174.11), 80 mg sodium bicarbonate (MW 84.01), and 5 mg methyl viologen into a small test tube.

Enzyme

- Prepare enzyme at 10 $\frac{\text{mg}}{\text{ml}}$ in degassed water just prior to use. Use serological pipets to gently add water, cover, and mix gently. Further dilutions are made in degassed KPO_4 buffer. Enzyme is not stable. Run 2x and 4x dilutions.

Procedure

Connect all four filled vacuum flasks to vacuum, place in ice bath and de-gas for 30 minutes. Check 30°C bath. Number tubes and set up in ice bath. Have one tube with 0.5 ml H_2O and thermometer as a temperature check. After 30 minutes under vacuum, release vacuum. Add weighed dry reagents (dithionite, bicarbonate, methyl viologen) to degassed flask containing H_2O/PO_4^{-3}. (FLASK #4) Replace stopper. Swirl gently to dissolve. Should be deep blue and stay that way. Do not put back under vacuum once dry reagents have been added. If color fades discard. Prepare enzyme and then continue procedure.

Set up 9 tubes, labelled as listed in next column. Pipet water, nitrate, blue reagent, nitrite (std) into respective tubes using serological pipettes.

Tube	Degassed Water [ml]	Degassed Nitrate [ml]	Degassed Blue Reagent [ml]	Nitrate [μmoles]
1 - blank	0.01	—	0.30	—
2 - std (0.02)	0.08	—	0.30	0.02
3 - std (0.05)	0.05	—	0.30	0.05
4 - std (0.10)	—	—	0.30	0.10
5 - substrate blk	—	0.10	0.30	—
6 - test 1 2x	—	0.10	0.30	—
7 - test 1 4x	—	0.10	0.30	—
8 - enz blk 2x	0.10	—	0.30	—
9 - enz blk 4x	0.10	—	0.30	—

Transfer to 30°C bath and watch temperature. As soon as temperature reaches 30°C, at timed intervals add 0.1 ml diluted enzyme to tubes 6-9. Incubate for 10 minutes. Reaction mixture should still be deep blue.

After 10 minutes, stop reaction by vigorous mixing until blue color is completely removed. Quickly add 0.5 ml of sulfanilamide solution and 0.5 ml of NED solution. Add 1.5 ml reagent grade water and incubate at room temperature for 10 minutes. Read A_{540}.

Calculation

- $\frac{\text{Units}}{\text{g}} = \frac{(\Delta A_{540} - \Delta A_{540}\ \text{Blank}) \times \text{Dilution} \times 1000}{0.1\ \text{ml} \times 10\ \text{min} \times 10\ \frac{\text{mg}}{\text{ml}} \times \frac{\Delta A_{540}}{\mu\text{mole}}}$

From the standards calculate $\frac{\Delta A_{540}}{\mu\text{mole}}$. (i.e. 0.02 μmole = 540 nm of 0.306 = 0.306/.02 = 15.3). Take average of three points.

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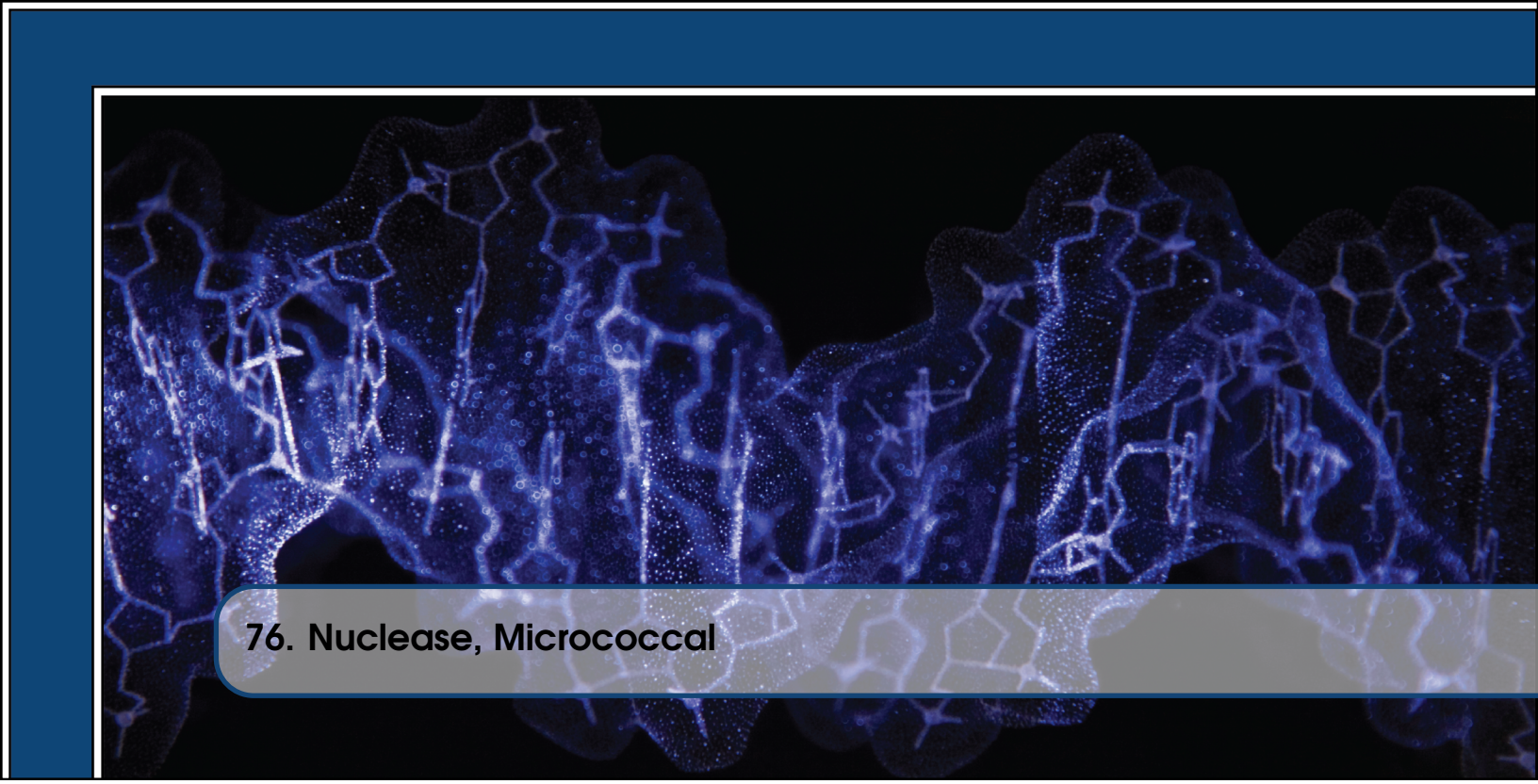

76. Nuclease, Micrococcal

Nucleate 3'-Oligonucleotidohydrolase

Micrococcal nuclease (MN) catalyzes cleavage of both DNA and RNA to yield 3'-nucleotides (Alexander *et al.* 1961).

MN is the extracellular nuclease of *Staphylococcus aureus*. Strains V8 and Foggi yield almost identical enzymes (Cusumano *et al.* 1968). A surface-bound nuclease has been purified by Okabayashi and Mizumo (1974). MN was first reported by Cunningham *et al.* (1956) and crystallized by Cotton *et al.* (1966) and Sulkowski and Laskowski, Sr. (1966). It has been extensively studied by Anfinsen's group.

Of particular interest is the limited tryptic digestion of MN to yield three peptide fragments two of which can reassociate to yield an active complex, nuclease-T (Taniuchi, Anfinsen and Sodja 1967; Taniuchi and Anfinsen 1968). Taniuchi *et al.* (1972) reported the x-ray diffraction pattern of nuclease-T crystals. See also: Sanchez *et al.* (1973), Chaiken (1972), Chaiken and Anfinsen (1971), Parikh *et al.* (1971), Taniuchi and Anfinsen (1971, 1968).

The specificity of hydrolysis attributed to micrococcal nuclease makes it a useful tool in the elucidation of nucleic acid and oligonucleotide sequence studies.

Axel (1975) and Sollner-Webb and Felsenfeld (1975) have reported on MN cleavage of nuclei and chromatin DNA and Bode and Gillin (1971) on its effect on tailless phage heads.

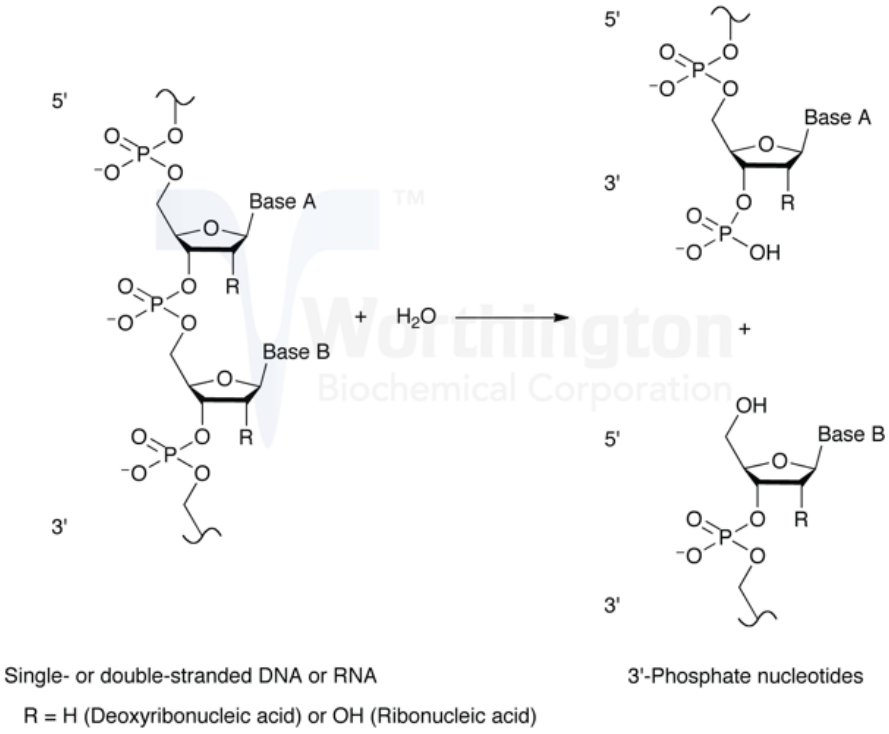

Figure 76.1: Enzymatic Reaction - Nuclease, Micrococcal

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Staphylococcus aureus*

Specificity

Exhibits both exo- and endo-5'-phosphodiesterase activities against both DNA and RNA (Alexander *et al.* 1961). The enzyme catalyzes preferential endohydrolysis of the RNA and DNA at sites rich in adenylate or uridylate and deoxyadenylate or thymidylate (Roberts *et al.* 1962; Rushisky *et al.* 1962).

Composition

The amino acid sequence has been reported by Cone *et al.* (1971). The active site has been reported on by Chaiken and Anfinsen (1971), Cuatrecasas (1970), and Cuatrecasas *et al.* (1968). See also: Furie *et al.* 1973), Bonhert and Taniuchi (1972), Sacks *et al.* (1972), Arnone *et al.* (1971), Epstein *et al.* (1971), Chaiken and Anfinsen (1970), Cohen *et al.* (1970), Markley *et al.* (1970), Putter *et al.* (1970), and Schechter *et al.* (1970).

IUB

- 3.1.31.1

CAS

- 9013-53-0

Molecular Weight

- 16,807 (Taniuchi *et al.* 1967b)

Optimal pH

- The pH optimum is reported as 9.2. However, the enzymic activity has an absolute requirement for Ca^{2+} and the pH optimum varies according to Ca^{2+} concentration (Heins *et al.* 1967).

Extinction Coefficient

- $E^{1\%}_{280}$ = 9.2 (Fuchs *et al.* 1967)

Inhibitors

- 5'-dioxynucleotides and 5'-ribonucleotides are inhibitory with the former demonstrating greatest inhibition.

Stability/Storage

- The Worthington preparation is stable for at least 6-12 months at 2 - 8°C. Concentrations of 4 micrograms per ml of the purified enzyme made in 0.1% albumin are stable for at least 2 days at 2 - 8°C.

Activity

- Ca^{2+} is essential for activity. (See, however, Frank *et al.* 1975). Both RNase and DNase activities are competitively inhibited by deoxythymidine 3',5' diphosphate (Cuatrecasas *et al.* 1967). Sulkowski and Laskowski, Sr. (1970) indicate that the kinetics of degradation of RNA and DNA differ. They found no activation with Sr^{2+}. See also: Dunn *et al.* (1973), Rammler *et al.* (1972), Chaiken and Anfinsen (1971), Markley and Jardetzy (1970) and Mikulski *et al.* (1969).

Assay Information

Method

- Essentially that described by Heins et al. (1966) based upon the release of acid soluble oligonucleotides following nuclease digestion of DNA. One unit corresponds to a change in optical density of 1.0 at 260 nm at 37°C and pH 8.0 under the specified conditions.

Reagents

- 0.1 M Sodium borate, pH 8.8
- 0.01 M Calcium chloride

- DNA, 2.5 $\frac{mg}{ml}$: prepare by dissolving 25 mg Worthington Calf Thymus DNA in 10 ml 0.01 M NaCl. Allow to stand overnight at room temperature and then stir slowly to effect solution.
- 7% Perchloric acid
- 0.1% Bovine serum albumin

Enzyme

- Dissolve at one $\frac{mg}{ml}$ in reagent grade water. Dilute for assay to 0.001-0.002 $\frac{mg}{ml}$ in 0.1% albumin.

Procedure

x5 Numbered Test Tubes

- 0.1 M Sodium borate, pH 8.8 - 0.1 ml
- 0.01 M $CaCl_2$ - 0.05 ml
- 0.25% DNA - 0.1 ml

Incubate in 37°C water bath for 6-7 minutes to achieve temperature equilibration. Prepare a minimum of four different dilutions of enzyme in the range of 0.001-0.0002 $\frac{mg}{ml}$. At timed intervals pipette 0.1 ml of appropriate dilution into respective tubes and replace in the water bath. To the fifth, which will serve as a blank, add 0.1 ml of 0.1% albumin. Incubate for 30 minutes. Stop the reaction at timed intervals by adding 0.5 ml 7% perchloric acid. Place the tubes in an ice bath for 10 minutes, then add 2.7 ml reagent grade water. Centrifuge for 15 minutes at highest speed on a clinical centrifuge. Withdraw clear supernatant and read A_{260} versus the blank.

Note: For best results, sample should be diluted so that the final A_{260} falls between 0.2-0.9.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{(A_{260\ \text{Sample}} - A_{260\ \text{Blank}})}{\text{mg enzyme in reaction mixture}}$$

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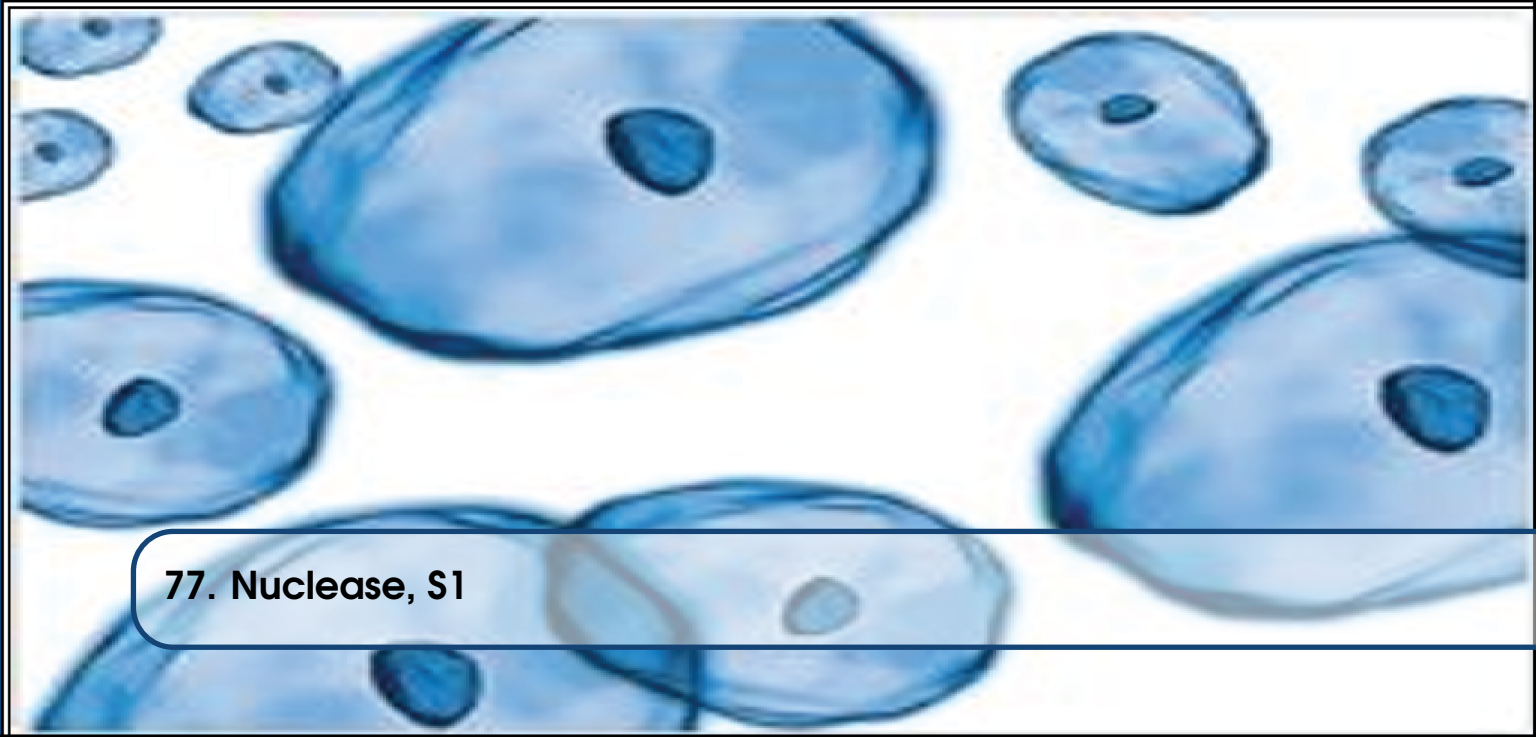

77. Nuclease, S1

Nuclease S1, isolated from certain Neurospora and Aspergillus species, specifically hydrolyzes both terminal and internal phosphodiester bonds of single-stranded DNA and RNA. It is used to eliminate non-annealed polynucleotide tails and hair-pin loops in DNA-RNA or DNA-DNA duplexes in hybridization studies and in genetic recombination experiments.

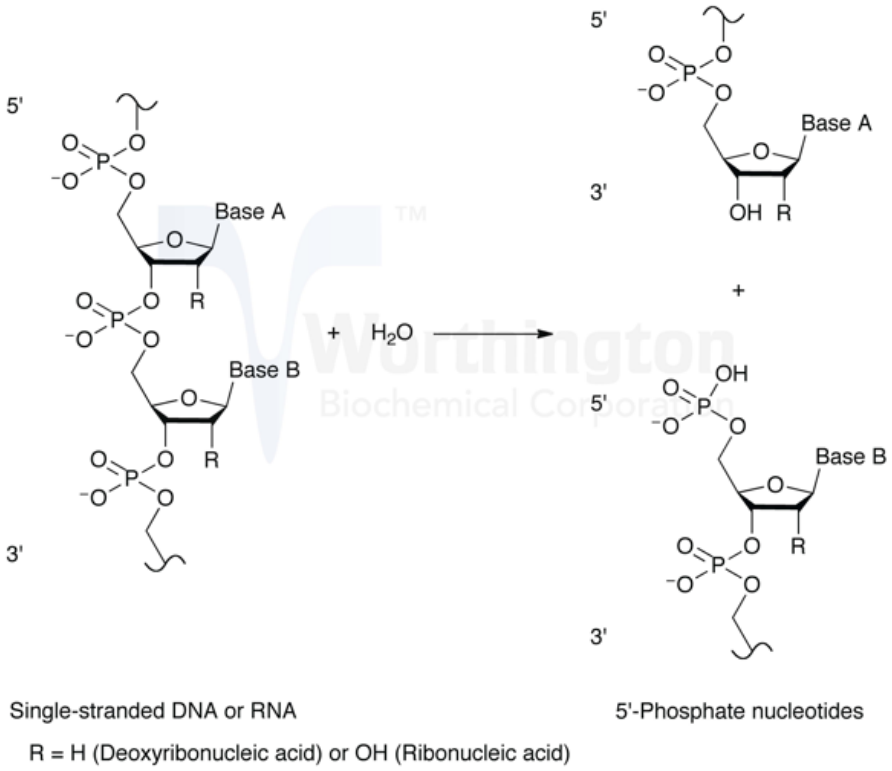

Figure 77.1: Enzymatic Reaction - Nuclease, S1

IUB

- 3.1.30.1

CAS

- 37288-25-8

Molecular Weight

- Approximately 32,000 - 36,000 daltons, exists as a monomer (Vogt, V. 1973)

Optimal pH

- 4.0 - 4.6. (Vogt 1973 and Ando 1966)

Activators

- Zn^{++} and/or Ca^{++} (Vogt 1973 and Ando 1966)

Inhibitors

- EDTA, citrate (Vogt 1973 and Ando 1966) and a high concentration of SDS

Assay Information

One unit is the amount of enzyme liberating 1 μg (0.033 A_{260}) of acid-soluble nucleotides from heat-denatured DNA per minute at 37°C and at pH 4.6

Reagents

- Buffer: 0.2M NaCl, 0.002M $ZnCl_2$, 0.06M $ZnCl_2$, pH 4.6: dissolve 5.844 g NaCl (MW 58.44), 136mg $ZnCl_2$ (MW 136.29) and 1.85ml concentrated glacial acetic acid in 450 ml reagent grade water. Adjust pH to 4.6 with 10M NaOH. Bring to a final volume of 500 ml with reagent grade water.
- Enzyme Diluent: Dissolve 40 mg BSA in 200 ml Buffer
- Substrate: Shred into small fibers, 60 mg calf thymus DNA and dissolve in 50 ml reagent grade water by standing at room temperature for at least 18 hours. Additional stirring may be necessary to effect soluiton. Remove 10ml DNA solution to 10 ml of buffer. This is native calf thymus DNA solution (Substrate B)
- Heat the remaining DNA solution in a large Pyrex test tube with a stir bar in boiling water on a heater/stirrer while stirring for 20 minutes. Immediately pour into PRE-FROZEN 1 liter beaker on ice. Mix equal volumes of the DNA solution and cold buffer. This is heat-denatured calf thymus DNA solution (Substrate A). Use as soon as possible to prevent the blank from elevating.
- 15% Perchloric Acid: Add 21.5 ml concentrated perchloric acid (70%) to 78.5 ml deionized water.

Procedure

To clean glass tubes (two for each point) add 2 ml Substrate B for tests. Include 2 tubes with 2 ml substrate B for blanks (no enzyme added) and 2 tubes with 2 ml Substrate A (native DNA test).
Incubate for 5 minutes before adding the enzyme.
Add 0.1 ml enzyme dilution
Incubate at 37°C for 10 minutes.
Stop reaction by adding 2 ml 15% perchloric acid.
Leave on ice for 10 minutes.
Centrifuge on a bench-top centrifuge for 15 minutes at 2000 rpm.
Withdraw 3 ml supernatant and read A_{260}.

Calculation

- $$\frac{\text{units}}{\text{ml}} = \frac{A_{260\ \text{Sample}} - A_{260\ \text{Blank}} \times \text{dilution} \times 1242}{10}$$

1242 is derived by dividing the reaction volume (4.1 ml) by the A_{260} of 1 μg (0.033) and dividing by the enzyme sample volume used (0.1 ml)

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78. Nucleohistone

The histones are a group of water and dilute acid soluble basic proteins found associated with DNA in chromosomes. They are characterized by relatively high levels of lysine and arginine. Although histones are classified into a limited number of types of fractions (see below) with each particular fraction having a fundamentally distinct amino acid composition and sequence, numerous subfractions are observed due to the acetylation, methylation, and phosphorylation of various amino acid residues. Microheterogeneity or alteration of structure is dynamic such that the histones of a single cell type are found to vary during development. They are believed to play a role in gene activity and cellular metabolism. See "The Structure and Function of Chromatin," Ciba Foundation Symposium 28, American Elsevier, N.Y. (1975).

Although the classic nomenclature for histones is that of Johns and Butler (1962), the nomenclature of Bradbury (1975) was submitted to the IUPAC.

Characteristics of Histones from Calf Thymus Elgin and Weintraub(1975):

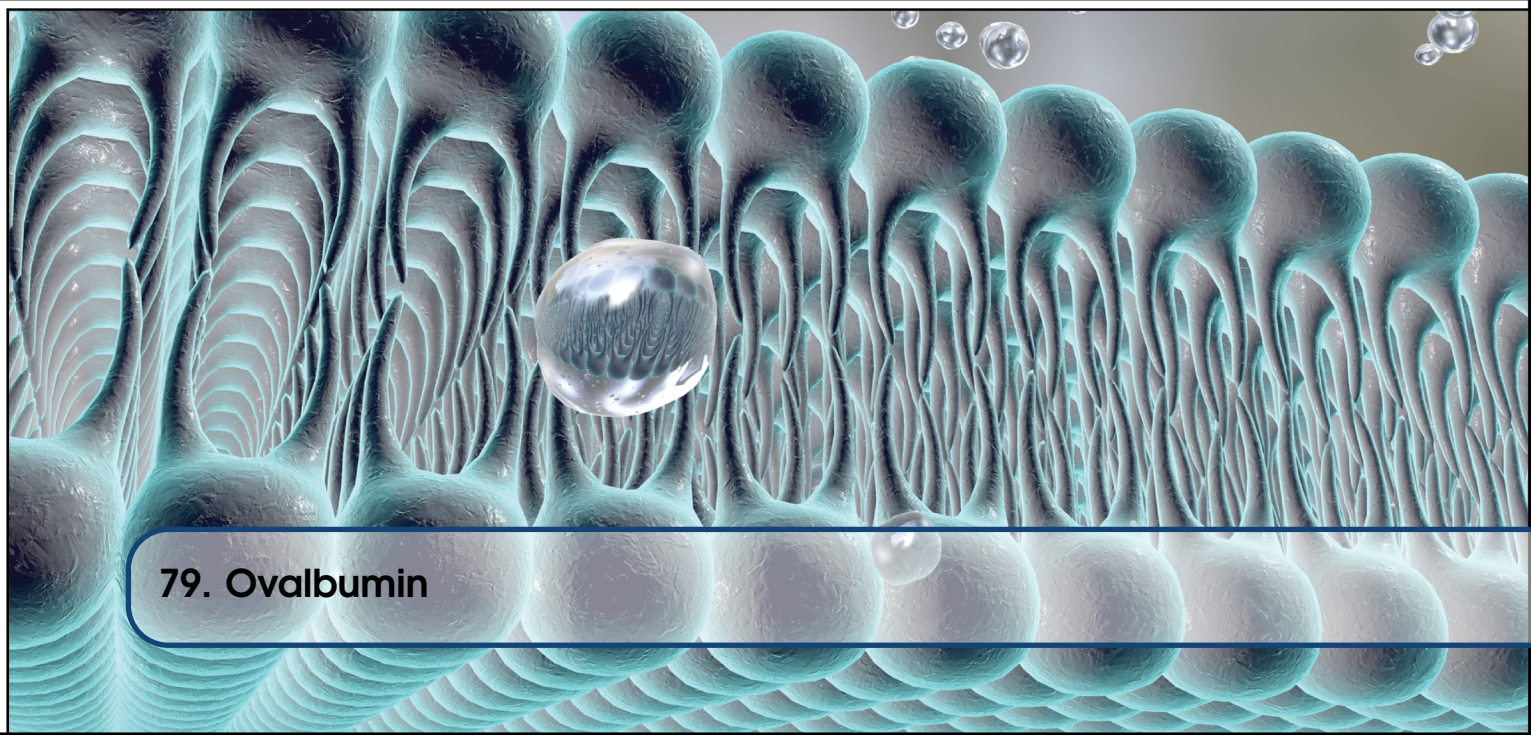
Class	Bradbury	Johns	Molecular Weight
Lysine Rich	H1	f1	about 21,500
Slightly Lysine Rich	H2a	f2a2	14,004
Slightly Lysine Rich	H2b	f2b	13,774
Arginine Rich	H3	f3	15,324
Arginine Rich	H4	f2a1	11,282

With the exception of H1, the primary structures of the calf thymus histones have been determined.

Comparisons with the structures for histones from other sources indicate that the histones rank among the most highly conserved (low mutation rate) proteins in nature. Naturally occurring histones are often found to be partially acetylated, methylated, or phosphorylated. These modifications may contribute to the electrophoretic microheterogeneity of the histone fractions. The nucleoprotein complex of histone and deoxyribonucleic acid is referred to as nucleohistone or deoxyribonucleoprotein. It is important as a source of its two components as well as an entity in itself for physical studies. Intracellularly, these complexes may be important factors in chromosomal structure and gene transcription. Kornberg (1974) has proposed a model for chromatin in which 200 DNA base pairs are coiled on the outside of a histone unit composed of $(H3)_2(H4)_2$ tetramer and two each of H2a and H2b. Bradbury (1976) suggests that H1 may be involved in generating higher order chromatin structures and that the initiation of mitosis may in part be mediated by H1 phosphorylation. Worthington histones are water soluble at pH 7.0 and are characterized by gel electrophoresis and solubility. Stable for years at 2 - 8°C. when stored dry.

CAS

- 37244-51-2



79. Ovalbumin

Ovalbumin is a glycoprotein that comprises 54% of the total proteins of egg white.

History

Ovalbumin and albumin were some of the very first proteins to be studied. Ovalbumin was first crystallized in 1890 by Hofmeister. In 1938, Neuberger reported that the carbohydrate moiety contained two moles of hexosamine, four moles of mannose, and some unidentified nitrogeneous material.

In the 1930s to 1940s, the electrophoretic differences of the three components were discovered (Young 1939, Longsworth 1940, and Linderstrm-Lang and Otteseen 1947). In the 1950s, Perlmann determined that the electrophoretic separation was due to differences in ovalbumin's phosphorous content. The first component having two atoms of phosphorous per mole protein, the second, one atom, and the third minor fraction no phosphorous (Perlmann 1952). The C-terminal amino acid residue was determined in 1955 by Niu and Fraenkel-Contrat, and Narita and Ishii determined portions of the N-terminal sequence in 1962.

In the 1960s, Nuenke and Cunningham believed that the carbohydrate is linked to the protein through an aspartic acid carboxyl group, and they were later able to purify the glycopeptides (Nuenke and Cunningham 1961). It was later determined that an asparagine residue was the site of glycosylation. Because ovalbumin was found to be unstable in aqueous solutions, especially at room temperature, physical studies were difficult. To better study the physical properties and electrophoretic behavior, Winzor and Creeth modified the cysteines residues by reacting the thiol groups with iodine (Creeth and Winzor 1962, and Winzor and Creeth 1962).

In the 1970s, the ovalbumin gene was the first split gene to be discovered (Breathnach *et al*. 1977).

Preliminary X-ray diffraction studies on ovalbumin crystals were performed in the 1980s (Miller *et al*. 1982).

Recent work has used ovalbumin in the development of a microbicide to prevent the sexual transmission of HIV and other sexually transmissible viruses (Li *et al*. 2011). Researchers also continue to investigate the mechanisms for regulation of the ovalbumin gene (Dougherty *et al*. 2009).

Molecular Characteristics

Ovalbumin consists of 385 amino acid residues (Nisbet *et al*. 1981). It is unique in that its signal sequence is in the middle of the polypeptide chain (residues 234-252) (Lingappa *et al*. 1979). Interestingly, ovalbumin has been found to have sequence homology with a group of proteinase inhibitors called serpins (30% homology with the archetype member of the family, alpha1-antitrypsin) (Hunt and Dayhoff 1980). However, it differs from this group of inhibitors in that it does not undergo a conformational change upon proteolytic cleavage. Upon proteolytic cleavage, serpins are converted from the S (stressed) to R (relaxed) conformation, and each conformation exhibits different heat stabilities. Ovalbumin does not exhibit these structural changes or differences in heat stability (Stein *et al*. 1989).

The synthesis of ovalbumin is hormonally induced in the oviduct by the hormone oestrogens (O'Malley *et al*. 1979). The ovalbumin gene (ov) comprises eight exons and seven introns (McReynolds *et al*. 1978). Two genes under steroid hormone control have been found within a 46 kb region that also includes the ovalbumin gene. These genes, gene X and Y of unknown function, also have seven introns but are transcribed at a much lower level than ovalbumin mRNA (Royal *et al*. 1979, and LeMeur *et al*. 1981). The ovalbumin gene has been a model to study tissue-specific, steroid hormone-induced gene expression for decades; however, the regulation mechanisms of this gene are yet to be determined (Dougherty *et al*. 2009).

Composition

Ovalbumin has four cysteine residues and a single cystine disulfide bridge (Stevens 1991). It is a monomeric protein; however, at high concentrations dimeric and, to a lesser extent, trimeric forms are observed. These three bands correspond to the dephosphorylated, monophosphorylated, and diphosphorylated forms. Electrophoretic separation shows three ovalbumin bands (Lush 1961). The phosphorylation sites are Ser68 and Ser344. A carbohydrate moiety is linked through Asn292. The N-terminus is acetylated. Two polymorphic forms of ovalbumin are known (ovalbumin A and ovalbumin B). Ovalbumin A has an asparagine at position 311, while ovalbumin B has an aspartic acid (Stevens 1991).

CAS

- 9006-59-1

Protein Accession Number

- P01012

Molecular Weight

- 42.7 kDa

Isoelectric Point

- 4.5 (Stevens 1991)

Extinction Coefficient

- 30,590 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280} = 7.16$

Applications

- Conjugo-immuno determinations
- Drug and pharmaceutical processing

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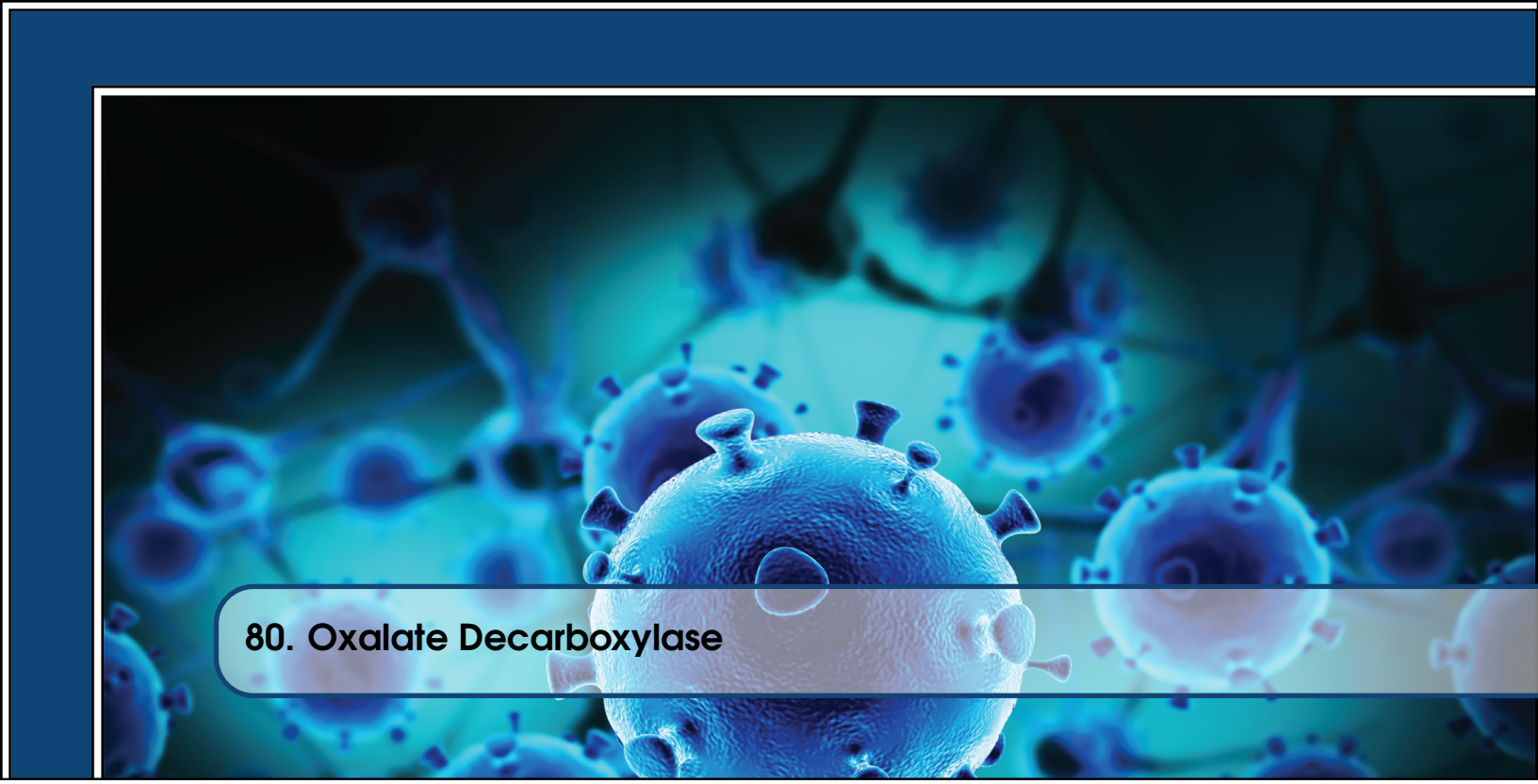

80. Oxalate Decarboxylase

Oxalate Carboxy-lyase

Oxalate decarboxylase has been isolated from *Aspergillus niger* (Emiliani and Bekes 1964) and *Myrothecium verrucaria* (Lillehoj and Smith 1965). That purified by Shimazono and Hayaishi (1957) from the mycelum of *Collybia velutipes*, was purified about 400-fold and is reported most useful in the specific determination of oxalic acid. Hallson and Rose (1974) describe a simple, specific assay for urinary oxalate wherein no preliminary treatment of the urine is required. A change in pH is measured. Mayer *et al*. (1963) have shown the enzyme suitable for urinary oxalate determination. Crawhill and Watts (1961) used it for plasma oxalate content and Haas and Fleischman (1961) for oxalate in beer. Bengtsson (1967) has reported on the enzymatic determination of oxalic acid in plant tissue.

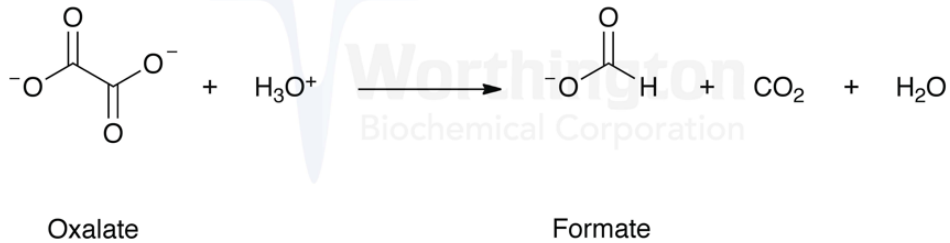

Figure 80.1: Enzymatic Reaction - Oxalate Decarboxylase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Collybia velutipes*

Specificity

It acts specifically on oxalic acid and produces stoichiometric quantities of CO_2 and formic acid. Pyruvate malonate, succinate, glutarate, malate, citrate, oxaloacetate, α-ketomalonate, acetate and formate are not acted upon. (Jakoby 1962).

IUB

- 4.1.1.2

Optimal pH

- 3

Assay Information

Method

- The reaction velocity is measured by a conventional Warburg manometric technique. One unit of activity releases one micromole of CO_2 per minute at 37°C and pH 3.0 under the specified conditions.

Reagents

- 1.0 M Potassium citrate, pH 3.0
- 0.1 M Potassium oxalate, pH 3.0

Enzyme

- Dissolve enzyme at a concentration of 3-5 $\frac{\text{units}}{\text{ml}}$ in reagent grade water.

Procedure

Into the bottom well of the Warburg flasks pipette as follows:

- 1.0 M Potassium citrate, pH 3.0 - 0.4 ml
- Reagent grade water - 2.4 ml
- Diluted enzyme - 0.1 ml

Pipette 0.1 ml of 0.1 M potassium oxalate, pH 3.0 into the side arm. Include one flask containing no enzyme as a blank and a flask containing 3.0 ml of water to serve as a thermal barometer. After ten minutes of equilibration, close the manometers, tip in and mix the substrate and replace the flasks in the bath. Read all the flasks every five minutes for 30 minutes.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\text{microliters } CO_2 \text{ released}}{\text{min}}}{22.4 \text{ x mg enzyme in reaction vessel}}$

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81. Papain

Papain is a cysteine hydrolase that is stable and active under a wide range of conditions. It is very stable even at elevated temperatures (Cohen *et al.* 1986). The latex of Carica papaya is a rich source of four cysteine endopeptidases including papain, chymopapain, glycyl endopeptidase, and caricain. The proteins are synthesized as inactive precursors that become active within two minutes of the plant being wounded and the latex expelled. Papain is a minor constituent, but has been more widely studied because it is more easily purified (Azarkan 2003).

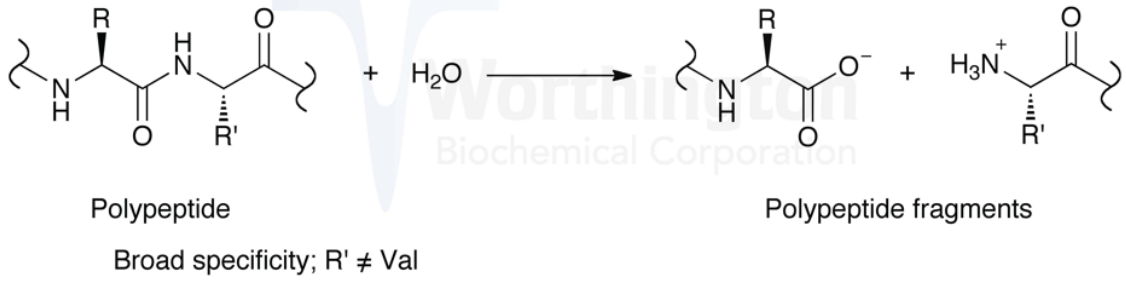

Figure 81.1: Enzymatic Reaction - Papain

History

In 1873 G.C. Roy first investigated the action of papain in an article published in the Calcutta Medical Journal entitled "The Solvent Action of Papaya Juice on Nitrogenous Articles of Food". Papain was first named in the late nineteenth century by Wurtz and Bouchut who partially purified the product from the

sap of papaya (Menard and Storer 1998). When named, it was simply recognized as a proteolytically active constituent in the latex of tropical papaya fruit (Wurtz and Bouchut 1879).

Throughout the mid-1950s and 1960s, purification and separation techniques improved greatly and pure papain was isolated. The study of papain allowed for great advances in understanding enzymes as proteins. In 1968, papain was the second enzyme to be crystallized and its structure determined by x-ray methods. Papain was the first cysteine protease to have its structure identified (Drenth *et al.* 1968).

In the 1980s, the geometry of the active site was reviewed and the three-dimensional structure was determined to a 1.65 Angstrom resolution (Kamphuis *et al.* 1984). The precursors and inhibitors of papain were extensively studied into the 1990s (Vernet *et al.* 1991).

Today's research aims to further understand the specificity (Portaro *et al.* 2000) and the structural perturbations brought about by inhibitors, low pH, metal ions, and fluorinated alcohols (Alphey and Hunter 2006, Huet *et al.* 2006, Kaul *et al.* 2002, Naeem *et al.* 2004).

Molecular Characteristics

The mature forms of all papaya proteinases are between 212 and 218 amino acids, and exhibit a strong degree of homology (Azarkan 2003). X-ray structure analysis has shown that they adopt identical three-dimensional folds (Pickersgill *et al.* 1991, O'Hara *et al.* 1995, and Maes *et al.*1996).

Papain is synthesized as a zymogen with a 133 amino acid N-terminal region that is not part of the active enzyme (Cohen *et al.* 1986). The papain precursor gene, prepropapain, has been cloned and expressed either in parts or as a whole (Cohen *et al.* 1986 and Choudhury *et al.* 2009).

Specificity

Papain has fairly broad specificity; it has endopeptidase, amidase, and esterase activities. The active site consists of seven subsites (S1-S4 and S1'-S3') that can each accommodate one amino acid residue of a substrate (P1-P4 and P1'-P3') (Schechter and Berger 1967). Specificity is controlled by the S2 subsite, a hydrophobic pocket that accommodates the P2 side chain of the substrate. Papain exhibits specific substrate preferences primarily for bulky hydrophobic or aromatic residues at this subsite (Kimmel and Smith 1954). Outside of the S2 subsite preferences, there is a lack of clearly defined residue selectivity within the active site.

Composition

Papain is a single-chained polypeptide with three disulfide bridges and a sulfhydryl group necessary for the activity of the enzyme. Papain is expressed as an inactive precursor, prepropapain. The formation of active papain requires several cleavage steps including an initial cleavage of the 18 amino acid preregion (the signal sequence), followed by further cleavage of the glycosylated 114 amino acid proregion (Vernet *et al.* 1995). This proregion serves as an intrinsic inhibitor and folding template. For further detail see Revell *et al.* 1993, Taylor *et al.* 1992, and Cohen *et al.* 1990.

IUB

- 3.4.22.2

CAS

- 9001-73-4

Protein Accession Number

- P00784

CATH: Classification (v. 3.3.0)

Class:

- Alpha Beta

Architecture:

- Alpha-Beta Complex

Topology:

- Cathepsin B; Chain A

Molecular Weight

- 23.4 kDa

Optimal pH

- 6.0-7.0

Isoelectric Point

- 8.88

Extinction Coefficient

- 53,610 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280} = 22.88$

Active Residue

- Cysteine (C158)
- Histidine (H292)
- Asparagine (N308)

Activators

- Cysteine
- Sulfide and sulfite
- Heavy metal chelating agents like EDTA
- N-bromosuccinimide (White and White 1997)

Inhibitors

- PMSF
- TLCK, TPCK
- alph2-macroglobulin
- Hg^{2+} and other heavy metals
- AEBSF
- Antipain
- cystatin
- E-64
- Leupeptin
- Sulfhydryl binding agents
- Carbonyl reagents
- Alkylating agents (White and White 1997)

Applications

- Cell isolation where it is more gentle than other proteases (i.e.: cortical neurons, retina, and smooth muscle)
- Protein structural studies, peptide mapping
- Red cell surface modification for antibody screening or identification
- Fab preparation from IgG and IgM antibodies
- Solubilization of integral membrane proteins
- Production of glycopeptides from purified proteoglycans
- Enzymatic wound debridement

Assay Information

Method

- A titrimetric determinatin of the acid produced during the hydrolysis of benzoyl-L-arginine ethyl ester (BAEE). One unit will hydrolyze one micromole of benzoyl-L-arginine ethyl ester per minute at 25°C and pH 6.2 under the specified conditions.

Reagents

- Enzyme diluent (Activation buffer): Prepare fresh daily by mixing the following:
- 0.01 M EDTA - 10 ml
- 0.06 M Mercaptoethanol - 0.1 ml
- 0.05 M Cysteine·HCl - 10 ml
- Reagent grade water - 70 ml

- Substrate solution: Prepare fresh daily by mixing the following:
- 0.058 M BAEE - 15.0 ml
- 0.01 M EDTA - 0.8 ml
- 0.05 M Cysteine·HCl - 0.8 ml
- Adjust pH to 6.2 and dilute to a final volume of 21 ml with reagent grade water.
- Titrant: 0.01-0.02 N NaOH, standardized

Enzyme

- Activate enzyme by dissolving in enzyme diluent to a concentration of 0.05-0.1 $\frac{\text{mg}}{\text{ml}}$. Under these conditions activation is complete within 30 minutes.
- Determination of protein concentration

Procedure

The reaction can be measured with either an automatic titrator or a laboratory pH meter. The titration vessel should be maintained at 25°C.
Pipette the following into the titration vessel at 25°C:

- Substrate solution - 5.0 ml
- 3.0 M NaCl - 5.0 ml
- Reagent grade water - 5.0 ml

At zero time add 0.1 ml of appropriately diluted enzyme and adjust the pH to 6.2. Record the amount of standardized NaOH added per minute to maintain the pH at 6.2 after a constant rate is achieved.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\text{ml base added}}{\text{min}} \text{ x normality x } 1000}{\text{mg enzyme in reaction mixture}}$

Notes

- Mercuripapain must be activated before use. Mercury is removed from the enzyme in activation buffer. After 30 minutes in this solution, the enzyme is completely activated and the mercury has been chelated. The mercuripapain suspension contains no free mercury. The product has been extensively dialyzed prior to packaging.

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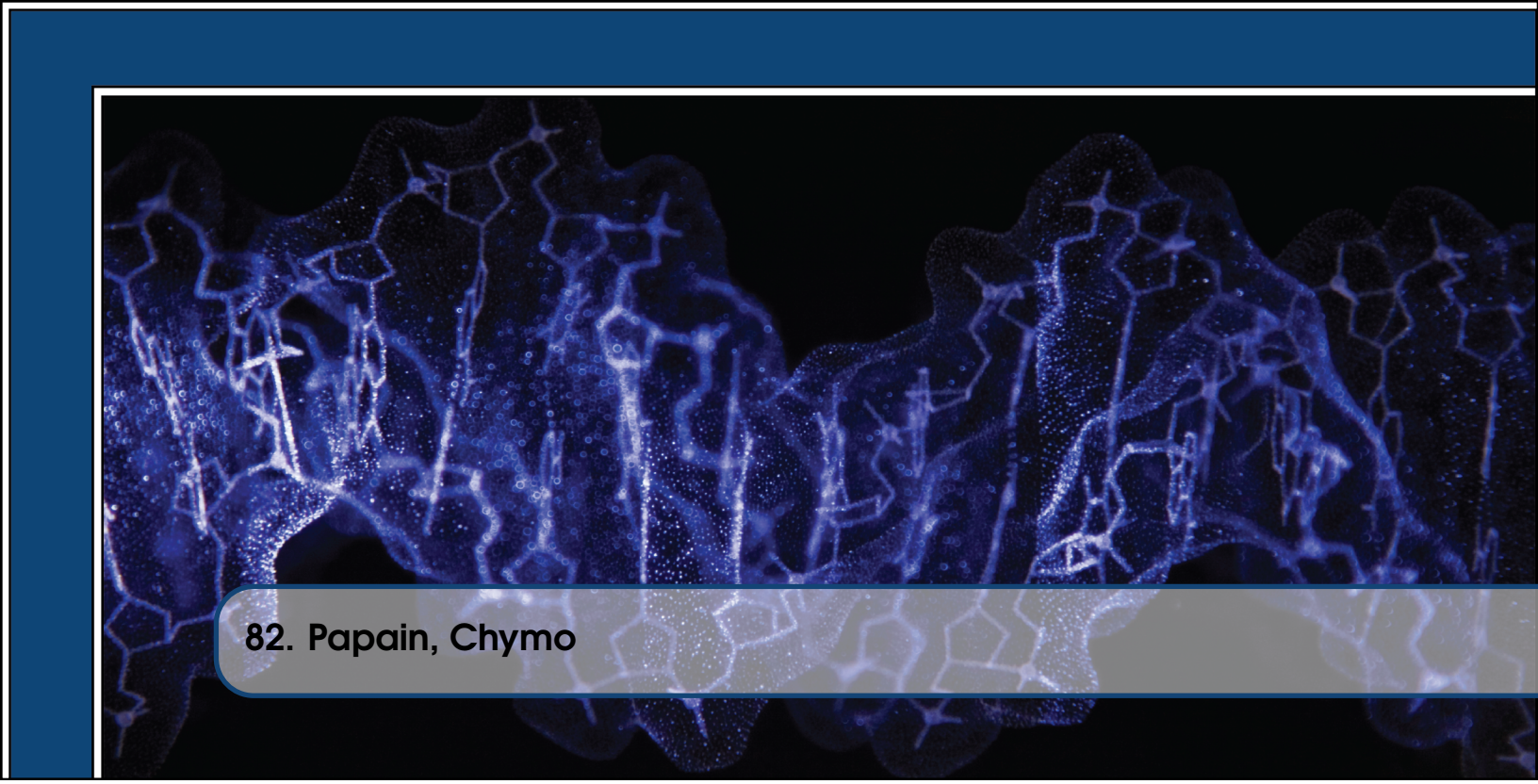


82. Papain, Chymo

Chymopapain is an extracellular cysteine proteinase secreted in the latex of the Carica papaya tree. Chymopapain was thought to exist as two forms, A and B (Maes *et al.* 1996); however, those terminologies were replaced through the results of molecular cloning experiments, which revealed a multiplicity of enzymes differing in only one or two amino acid residues (Taylor *et al.* 1999).

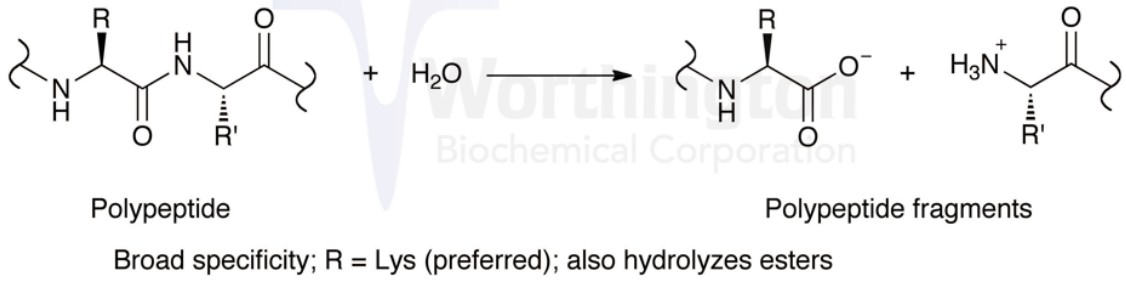


Figure 82.1: Enzymatic Reaction - Papain, Chymo

History

In 1941, Jansen and Balls first described chymopapain, after obtaining it in crystalline form from papaya latex (Guha *et al.* 2006). It was termed chymopapain to distinguish it as the proteolytic activity remaining after the removal of papain (Taylor *et al.* 1999).

In 1967, Kunimitsu and Yasunobo first demonstrated chymopapain's charge heterogeneity through cation-exchange chromatography, creating the notion that two forms of the enzyme existed: chymopapain A and chymopapain B. It has since been determined the separation observed during cation-exchange chromatography was instead due to the charge heterogeneity of chymopapain, and contamination with glycyl endopeptidase (Buttle 2004).

The amino acid sequences for chymopapain were published by Jacquet *et al.* in 1989 (Jacquet *et al.* 1989b) and Watson *et al.* in 1990. In 1996, Maes *et al.* elucidated the structure of chymopapain to a 1.7 resolution, and in 1999 Taylor *et al.*'s molecular cloning studies revealed five isoforms of the enzyme, putting an end to the chymopapain A and chymopapain B terminologies.

Recent research continues to investigate the wound-induced expression of chymopapain, as well as other papaya endopeptidases (Azarkan 2006). Chymopapain is also being used as a control to study human invertebral disc degeneration, as it mimics the natural process of degradation (Chen *et al.* 2009). Chymopapain has also found use in research in rheumatoid arthritis as it mimics the increased activity of proteases found in the joints of patients with rheumatoid arthritis (Sabaratnam *et al.* 2007).

Molecular Characteristics

The enzyme is a polypeptide chain of 218 amino acids. Chymopapain shows 58% sequence identity with papain (Sumner *et al.* 1993, and Buttle 2004). Among the papaya proteinases, V133 and V157 are highly conserved. Chymopapain is unique in that V133 and V157 are replaced by leucine in all five isoforms (Taylor *et al.* 1999, and Buttle 2004).

Specificity

Chymopapain shows a broad substrate specificity (Jacquet *et al.* 1989a). Chymopapain hydrolyzes a wide variety of substrates, similar to papain, but at slower rates. The substrate specificity is primarily controlled by the S2 subsite (Drenth *et al.* 1976).

Composition

Chymopapain is synthesized with N-terminal signal and propeptide regions, which are required for proper folding (McKee *et al.* 1986, Revell *et al.* 1993, Karrer *et al.* 1993, Taylor *et al.* 1995, and Vernet *et al.* 1995). These propeptides have been found to be selective high-affinity inhibitors of the mature enzyme (Taylor *et al.* 1995). Unlike other proteinases that have one free cysteine in the active site, chymopapain has a second non-essential free cysteine residue (C117) (Sumner *et al.* 1993).

IUB

- 3.4.22.6

CAS

- 9001-09-6

Protein Accession Number

- P14080

CATH: Classification (v. 3.3.0)

Class:

- Alpha Beta

Architecture:

- Alpha-Beta Complex

Topology:

- Cathepsin B; Chain A

Molecular Weight

- 27 kDa (Ebata 1962)

Optimal pH

- Broad, centered around 7 (Buttle 2004)

Isoelectric Point

- 10.2-10.6 (Baines and Brocklehurst 1982)

Extinction Coefficient

- 42,600 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 18.14 (Theoretical)

Active Residue

- Cysteine (C159)
- Histidine (H293)
- Asparagine (N313)

Activators

- 2,3-Dimercaptopropanol (Ebata and Yasunobu 1962)
- Cysteine (Ebata and Yasunobu 1962)
- Glutathione (Ebata and Yasunobu 1962)
- NaN3 (Kunimitsu 1970)

Inhibitors

- Ag^{+}, Cu^{2+}, Hg^{2+}, and Zn^{2+} (Kunimitsu 1970)
- Cystatin (Björk and Ylinenjörvi 1990)
- Iodoacetate
- PCMB
- Albumin and succinyl-albumin (Zucker *et al.* 1985)

Applications

- Rheumatoid arthritis research
- Control in disc degeneration studies
- Prior to being discontinued in the United States in 2003, chymopapain was injected directly into herniated disks to dissolve part of the disc and relieve pain

Assay Information

Method

- A titrimetric determinatin of the acid produced during the hydrolysis of benzoyl-L-arginine ethyl ester (BAEE). One unit will hydrolyze one micromole of benzoyl-L-arginine ethyl ester per minute at 25°C and pH 6.2 under the specified conditions.

Reagents

- Enzyme diluent (Activation buffer): Prepare fresh daily by mixing the following:
- 0.01 M EDTA - 10 ml
- 0.06 M Mercaptoethanol - 0.1 ml
- 0.05 M Cysteine·HCl - 10 ml
- Reagent grade water - 70 ml
- Substrate solution: Prepare fresh daily by mixing the following:
- 0.058 M BAEE - 15.0 ml
- 0.01 M EDTA - 0.8 ml
- 0.05 M Cysteine·HCl - 0.8 ml
- Adjust pH to 6.2 and dilute to a final volume of 21 ml with reagent grade water.
- Titrant: 0.01-0.02 N NaOH, standardized

Enzyme

- Activate enzyme by dissolving in enzyme diluent to a concentration of 0.05-0.1 $\frac{\text{mg}}{\text{ml}}$. Under these conditions activation is complete within 30 minutes.
- Determination of protein concentration

Procedure

The reaction can be measured with either an automatic titrator or a laboratory pH meter. The titration vessel should be maintained at 25°C.

Pipette the following into the titration vessel at 25°C:

- Substrate solution - 5.0 ml

- 3.0 M NaCl - 5.0 ml
- Reagent grade water - 5.0 ml

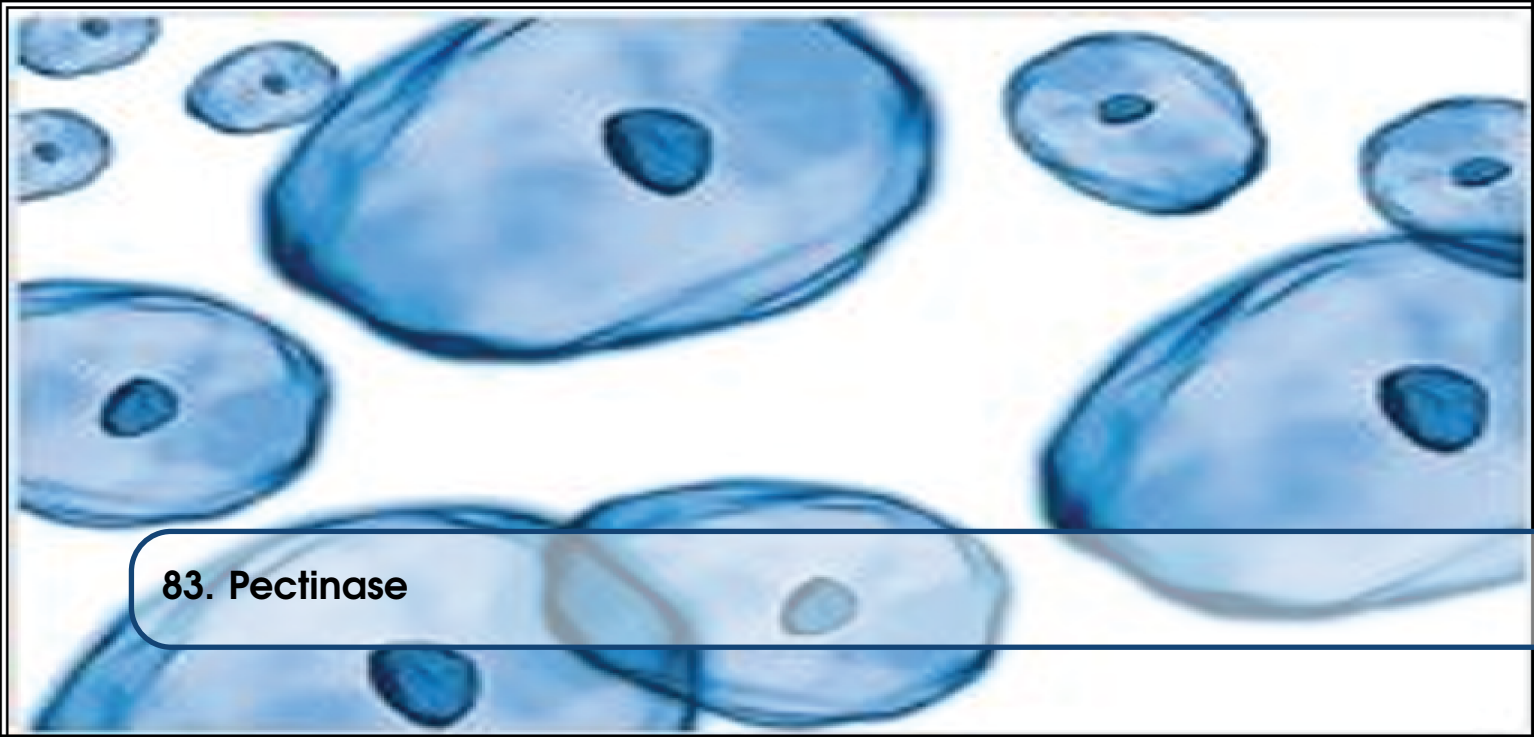
At zero time add 0.1 ml of appropriately diluted enzyme and adjust the pH to 6.2. Record the amount of standardized NaOH added per minute to maintain the pH at 6.2 after a constant rate is achieved.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\text{ml base added}}{\text{min}} \text{ x normality x } 1000}{\text{mg enzyme in reaction mixture}}$

Notes

- Mercuripapain must be activated before use. Mercury is removed from the enzyme in activation buffer. After 30 minutes in this solution, the enzyme is completely activated and the mercury has been chelated. The mercuripapain suspension contains no free mercury. The product has been extensively dialyzed prior to packaging.



83. Pectinase

Purified pectinase is a multi-component preparation highly effective in depolymerizing plant pectins with varying degrees of esterification. Important enzymatic activities include pectin lyase (EC 4.2.2.10) and activities on polygalacturonic acid (pectin) and nonmethylated polygalacturonic acid (pectate). The product contains substantial hemicellulase, cellulase, pectinesterase and xylanase activities which together with pectin lyase and polygalacturonase work synergistically to digest plant cell wall tissues.

Characteristics of Pectinase from *Aspergillus niger*:

In addition to its high enzymatic activites, purified pectinase has been developed specifically for use in plant protoplast culture studies. The enzyme is assayed using a method which quantitatively measures the liberation of D-galacturonic acid from polygalacturonic acid. This method is based upon determination of reducing sugars using a neocuprine hydrochloride color reagent assay. When used with Worthington purified cellulase, purified pectinase has been found to be useful for generating good yields of viable protoplast in several plant systems, e.g., corn, soybean, red beet, sunflower, tomato and citrus. In general, a concentration range of 0.1% to 0.5% pectinase (with accompanying 0.5% to 1.5% cellulase) used at 24°C to 37°C for periods of 1 to 16 hours will yield good results.

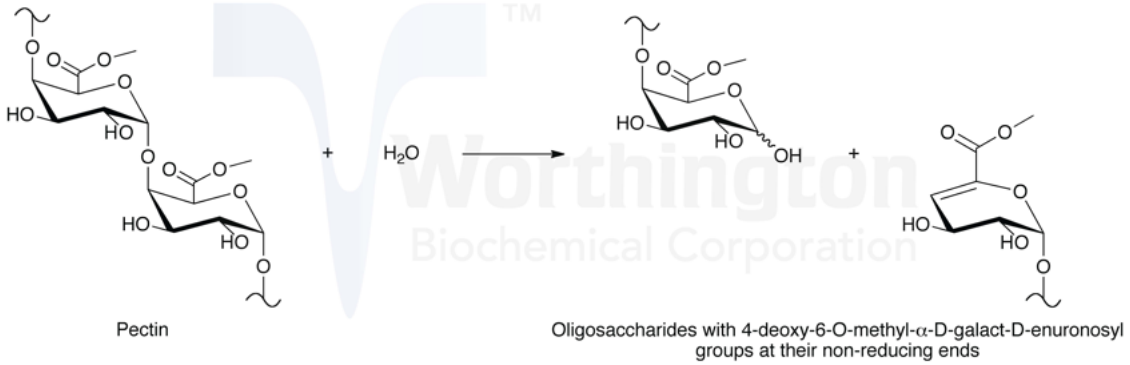


Figure 83.1: Major Enzymatic Reaction: Pectin Lyase

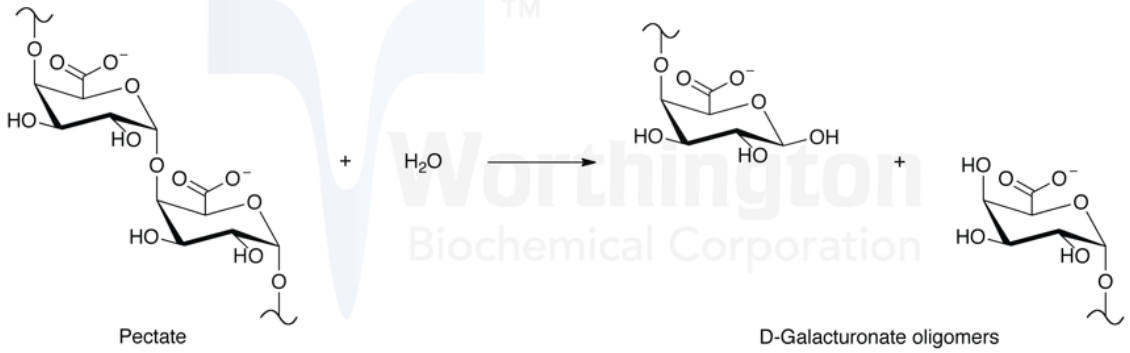


Figure 83.2: Major Enzymatic Reaction: Polygalacturonidase

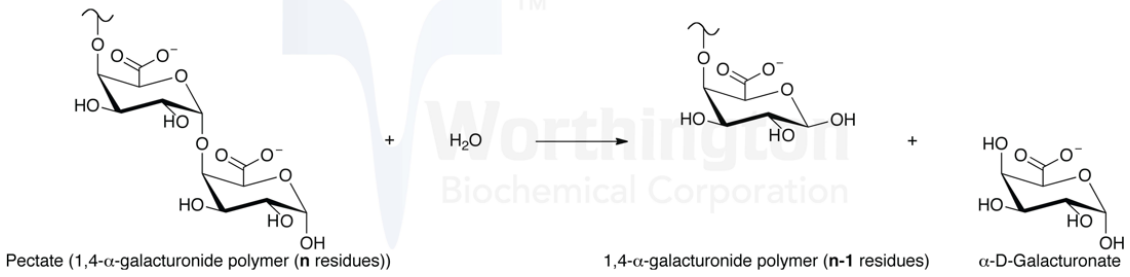


Figure 83.3: Major Enzymatic Reaction: Galacturan 1,4-α-galacturonidase

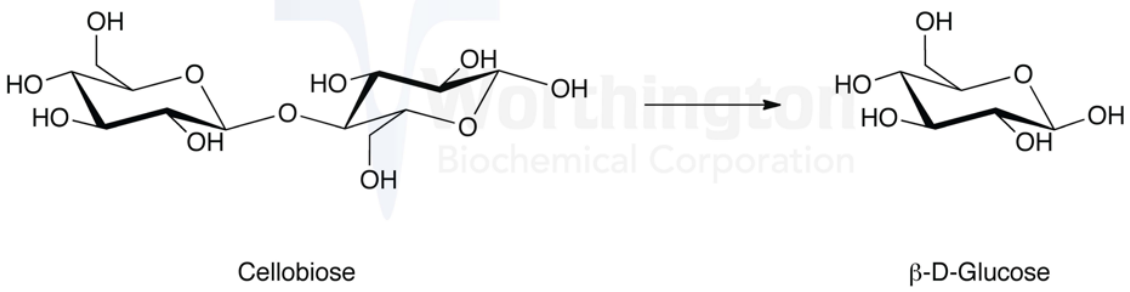


Figure 83.4: Minor Enzymatic Reaction: Cellulase (Cellobiase)

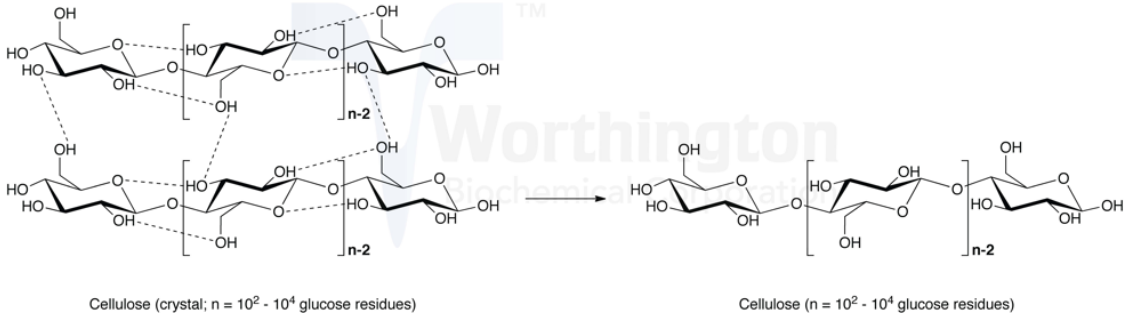


Figure 83.5: Minor Enzymatic Reaction: Cellulase (Endocellulase)

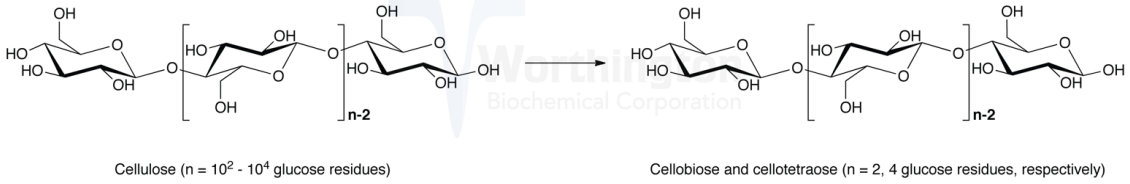


Figure 83.6: Minor Enzymatic Reaction: Cellulase (Exocellulase)

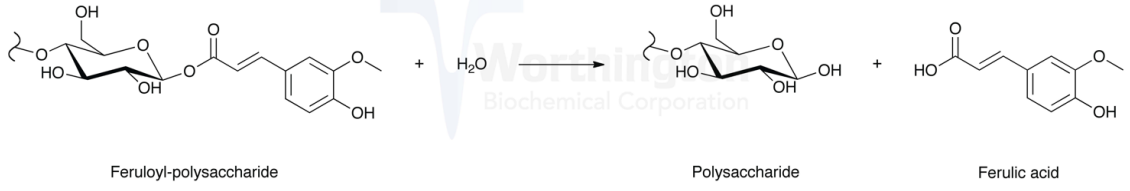


Figure 83.7: Minor Enzymatic Reaction: Hemicellulase

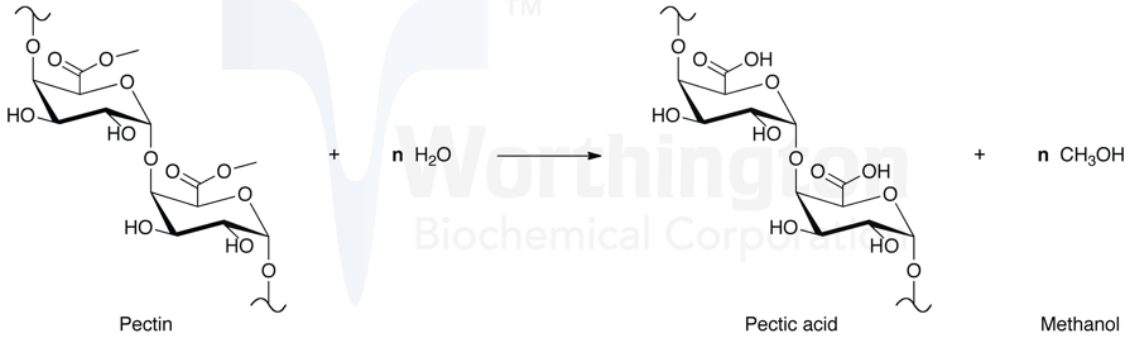


Figure 83.8: Minor Enzymatic Reaction: Pectinesterase

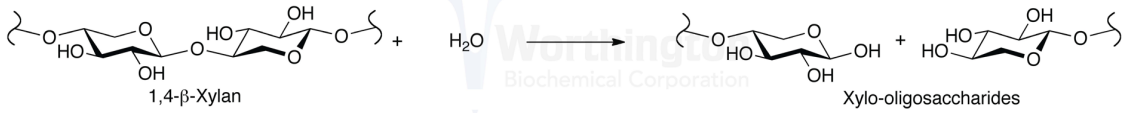


Figure 83.9: Minor Enzymatic Reaction: Xylanase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Aspergillus niger*

IUB

- 4.2.2.10

CAS

- 9033-35-6

Stability/Storage

- Store at 2 - 8°C. Protect from moisture. If not using entire bottle at once, weigh into single-use aliquots on arrival and store tightly covered, dessicated, refrigerated. Material is very hygroscopic and can become tacky and hard-to-weigh if exposed to moisture.

Assay Information

Method

- One unit liberates 1 μmole of D-galacturonic acid from polygalacturonic acid per minute at 37°C, pH 5.0.

Reagents

- 0.1 M Citric Acid/Phosphate Buffer (Assay Buffer), pH 5.0
- Color Reagent A: Dissolve 40g anhydrous Na_2CO_3 in 600 ml reagent grade H_2O. Add 16 g glycine and stir until dissolved. Add 0.450 g $CuSO_4$ $5H_2O$, stir until dissolved. Bring to 1 liter with reagent grade H_2O.
- Color Reagent B: Dissolve 1.2 g neocuprine - HCl in 1 liter reagent grade H_2O. Store at 4°C in a brown bottle.
- D-galacturonic acid standard, 1 $\frac{mg}{ml}$
- 0.5% Polygalacturonic Acid Substrate:
- Heat 500 ml Assay Buffer on a hot plate. While heating and stirring, slowly add 2.5 g polygalacturonic acid. Heat and stir until dissolved. It will be slightly viscous and opaque. Do not allow solution to boil. Cool and bring back up to volume if necessary. Store at 4°C.

Enzyme

- Make up fresh daily. Keep solutions on ice until used. Make serial dilutions to 0.01 $\frac{mg}{ml}$ in assay buffer.

Procedure

Prepare three tubes, each containing 1 ml of buffer and 6 ml of substrate (Reagent Blanks). Prepare three tubes, each containing 6 ml of substrate and 1 ml of enzyme sample at 0.1 $\frac{mg}{ml}$ (Test). Prepare three tubes, each containing 6 ml of buffer and 1 ml of enzyme sample at 0.1 $\frac{mg}{ml}$ (Sample Blanks).

Incubate tubes in 37°C water bath for 60 minutes ± 1 minute. Aliquot the standard curve tubes, in triplicate, using 10 µg to 125 µg of D-galacturonic acid standard.
After the assay tubes have incubated for 60 minutes, place rack immediately into ice water to stop reaction. Aliquot from each tube 100 µl into another set of tubes (also on ice). To each reaction tube and standard tube add 2 ml of Color Reagent A and 2 ml of Color Reagent B. Mix well by inversion. Place rack into boiling water bath for 13 minutes ±1 minute. Cool tubes, then add 2 ml H_2O. Mix well by inversion. Read absorbance at A_{450} against a water blank using disposable cuvettes. Do not use a flowcell; the orange color adheres to glass. Calculate mean absorbances for each set of triplicates.

Calculation

- Plot a standard curve:
- x = µg D-galacturonic acid
- y = A_{450}

Calculate rate of change. The curve should be linear up to 100 µg D-galacturonic acid, if not, repeat curve with fresh reagents.

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84. Pepsin

Pepsin is the principal proteolytic enzyme of vertebrate gastric juice. Its inactive precursor form, pepsinogen, is produced in stomach mucosa. The minor pepsins are designated "B", "C", and "D", while the major component is "A", to which the following data applies.

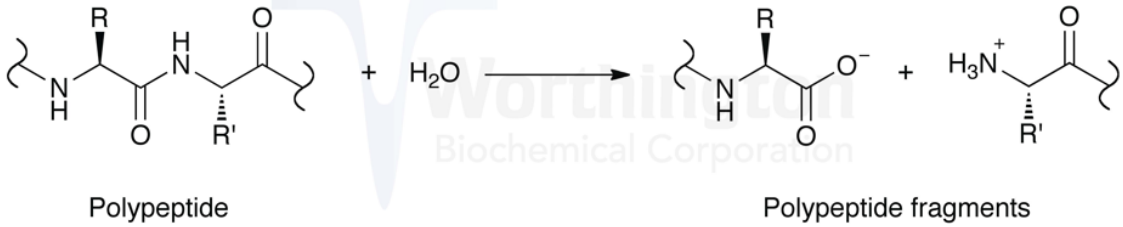

Figure 84.1: Enzymatic Reaction - Pepsin

History

Pepsin is of particular interest as it was the first enzyme to be discovered. The name pepsin was given by Theodor Schwann (1810-1882) in 1836, and came from pepsis, the term for digestion in Hippocratic writings. Into the mid-nineteenth century, scientists showed that pepsin broke down proteins into "peptones" (Fruton 2002).

Pepsin was later found to be an effective treatment for digestive disorders. Through this important application, efforts to produce and purify it greatly increased, and were successful by the end of the nineteenth century (Tang 1998).

At that time, however, the chemical nature and properties of enzymes as proteins were not completely understood. It was not until John H. Northrop crystallized pepsin in 1930, an achievement for which he shared the Nobel Prize in 1946, that the protein nature of enzymes was established (Manchester 2004).

After the Nobel Prize was awarded to Northrop, Sumner, and Stanley in 1946, new separation methods including crystallization and chromatography were further developed. Through these methods, the amino acid sequences of pepsin and pepsinogen were determined (Tang 1973).

Pepsin B and C were first isolated from porcine stomach by Ryle and Porter in 1959.

As X-ray diffraction techniques improved through the mid-1970s, the three-dimensional structure of pepsin was determined, allowing for a better understanding of the catalytic reaction (Fruton 2002).

Recently, interest in pepsin-type enzymes and their inhibitors has been renewed due to the recognition of HIV-protease as a member of this aspartic protease family (Campos 2003).

Molecular Characteristics

The amino acid sequence of porcine pepsin was determined by Tang *et al.* (1973) and Moravek and Kostka (1974), and later confirmed through cDNA analysis by Tsukagoshi *et al.* (1988) and Lin *et al.* (1989).

The pepsinogen A (PGA) gene is divided among nine exons that encompass approximately 9.4 kb of genomic DNA (Sogawa 1983).

There are multiple versions of the PGA genes found in human and chimp populations, but the activities of these various gene products are indistinguishable (Taggart 1985 and Zelle 1988). In contrast, Southern blot analyses of a sampling of pigs suggest that there is only a single PGAgene found in all pigs (Evers 1988).

PGA production is mainly controlled at the transcription level (Sogawa *et al.* 1981 and Ichinose *et al.* 1988). In both humans and pigs, it has been found that the PGA gene is under tissue-specific transcriptional control, with mRNA only detected in gastric fundic mucosa (Ichinose 1991 and Meijerink *et al.* 1993). Transcription of the PGA gene is regulated by transcription-activating proteins acting at 3 major regions in the promoter and initiation regions of the PGA gene (Meijerink *et al.* 1993).

There are four reported pepsin proteins: pepsin A, pepsin B (parapepsin I), pepsin C (gastricsin), and pepsin D (an unphosphorylated version of pepsin A) (Lee and Ryle 1967). Pepsin A is the predominant gastric protease; minor amounts of the other pepsins have been detected. Pepsins B and C share a higher degree of homology with each other. In dog, B and C share 89% identity, A and B share 44% identity, and A and C share 45% identity (calculated based on Thompson *et al.* 1994).

Specificity

Pepsin has broad specificity with a preference for peptides containing linkages with aromatic or carboxylic L-amino acids. It preferentially cleaves C-terminal to Phe and Leu and to a lesser extent Glu linkages. The enzyme does not cleave at Val, Ala, or Gly.

Composition

Pepsin is a monomeric, two domain, mainly beta protein with a high percentage of acidic residues. Porcine pepsin has 4 basic residues, and 42 acidic residues and is O-phosphorylated at S68 (Tang *et al*. 1973). For the protein to be active, one of the two aspartate residues in the catalytic site has to be protonated, and the other deprotonated. This occurs between pH 1 and 5, and above pH 7 pepsin is irreversibly denatured.

IUB

- 3.4.23.1

CAS

- 9001-75-6

Protein Accession Number

- P00791

CATH: Classification (v. 3.3.0)

Class:
- Mainly beta

Architecture:
- Beta Barrel

Topology:
- Cathepsin D, subunit A; domain 1

Molecular Weight

- Pepsin: 34.5 kDa (Theoretical)
- Pepsinogen: 41.4 kDa

Optimal pH

- 1.0-4.0 (At pH 1.5 pepsin exhibits about 90% of maximum activity, and at pH 4.5 about 35% of maximum activity.

Isoelectric Point

- 1.0 (Bovey and Yanari 1960)

Extinction Coefficient

- 49,650 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 14.39

Active Residue

- Aspartic acid (D32 and D215)

Activators

- Pepsinogen

Inhibitors

- Aliphatic alcohols
- Substrate-like epoxides
- Pepstatin A

Applications

- Digestion of antibodies
- Preparation of collagen for cosmeceutical purposes
- Assessment of digestibility of proteins in food chemistry
- Subculture of viable mammary epithelial cells (Riser 1983)

Assay Information

The Worthington assay is based on the stop-point assay of hemoglobin degradation developed by Anson (1938).

Method

- The rate of hydrolysis of denatured hemoglobin is measured. One unit releases 0.001 A_{280} as TCA soluble hydrolysis products per minute at 37°C under the specified conditions.

Reagents

- 1.0 N HCl
- 0.3 N HCl
- 0.01 N HCl
- 2.5% w/v Hemoglobin: Prepare by dissolving 2.5 grams Worthington bovine erythrocyte hemoglobin powder (Code: HB) in 100 ml reagent grade water. Blend in a Waring blender at maximum speed for 3-5 minutes. Filter through gauze. Dilute 80 ml of filtrate with 20 ml of 0.3 N HCl.
- 5% w/v Trichoracetic acid (TCA)

Enzyme

- Pepsin activity: Dissolve pepsin at a concentration of 0.5 $\frac{mg}{ml}$ in 0.01 N HCl. Keep chilled. Immediately prior to assay, dilute further in 0.01 N HCl to 10-20 $\frac{\mu g}{ml}$. Three dilutions are recommended.
- Pepsinogen: Dissolve 25 mg pepsinogen in approximately 40 ml reagent grade water. Adjust the pH to 8.0 with 0.01 N NaOH and allow 10 minutes to inactivate any contaminating pepsin activity. Lower the pH to 2.0 with HCl and dilute to a final volume of 50 ml with reagent grade water. For assay dilute further to 10-20 $\frac{\mu g}{ml}$ with 0.01 N HCl.

Procedure

Into each of six numbered test tubes pipette 2.5 ml hemoglobin substrate. Place in a 37°C water bath to equilibrate. Tubes 1-3 are blanks. Into each, pipette 5 ml of TCA followed by 0.5 ml of respective enzyme dilution. Remove from bath after 5 minutes and filter. Read A_{280} of clear filtrate.
Tubes 4-6 are for test. At timed intervals, add 0.5 ml of respective enzyme dilution to each and incubate at 37°C for exactly 10 minutes, stop the reaction by adding 5 ml of 5% TCA at timed intervals. Remove from bath after 5 minutes and filter. The filtrates should be clear. Record filtrate absorbance at 280 nm and subtract A_{280} of appropriate blank.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{(A_{280\ \text{Filtrate}} - A_{280\ \text{Blank}}) \times 1000}{10\ \text{min} \times \text{mg enzyme in reaction mixture}}$$

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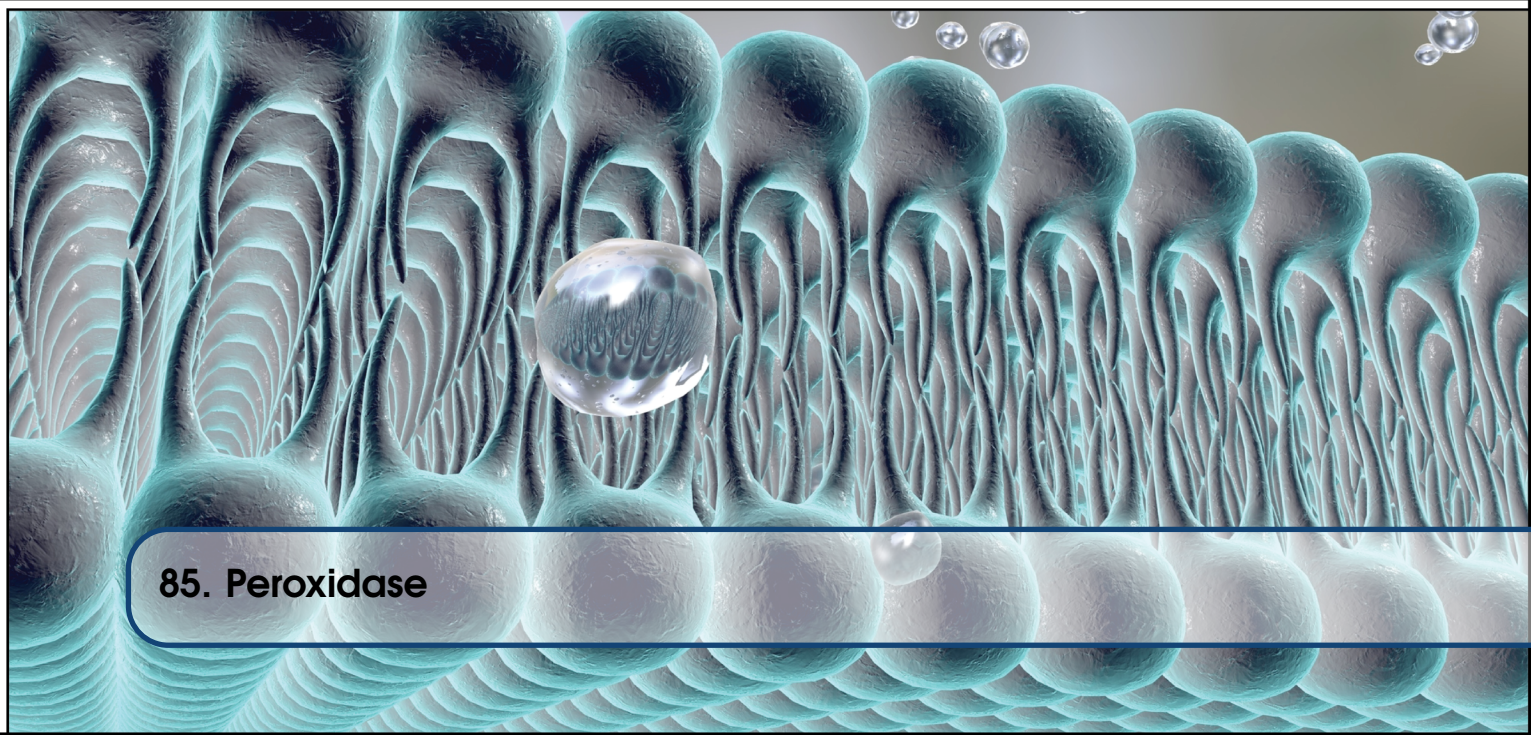

85. Peroxidase

Donor: H_2O_2 Oxidoreductase

Peroxidase has been found well suited for the preparation of enzyme conjugated antibodies, due in part to its ability to yield chromogenic products, and in part to its relatively good stability characteristics. Peroxidase labeled immunoglobulins have been used successfully as immunohistological probes for the demonstration of tissue antigens, and in enzyme amplified immunoassay systems for the quantitative determination of soluble and insoluble antigens (Nakane and Pierce 1967; Avrameas 1969; Kurstak *et al*. 1969; Avrameas and Guilbert 1972; van Weeman and Schuurs 1974; Greenwalt *et al*. 1975). Sternberger *et al*. (1970) and Moriarty *et al*. (1973) have described novel soluble peroxidase-antiperoxidase techniques for immunohistochemistry and immunoassay.

The use of the highly specific, sensitive and very stable horseradish peroxidase with a chromogenic donor has proven very useful for assay systems producing hydrogen peroxide; for example, in the determination of glucose or galactose by their respective oxidases and in the determination of certain L-amino acids in conjunction with L-amino acid oxidase (Malmnstadt and Hadjiioannou 1963). A more generalized application of the principle is as follows:

To a solution of 0.05 M sodium acetate, pH 5.0, add 10 mg o-dianisidine hydrochloride, 18.6 mg EDTA, 5.0 ml of a filtered 0.1% peroxidase solution (equivalent to Worthington Code: HPOD), and 1.0 ml 10% Triton X-100. Dilute to 50 ml with sodium acetate. Protect solution from light. Add 2.0 ml of reagent to 2.0 ml of unknown. After 15 minutes at room temperature, read absorbance at 460 nm. Standard solutions containing from 1 to 3 micrograms of hydrogen peroxide per test are run simultaneously. Alternatively, the extinction coefficient of oxidized o-dianisidine may be used. It has been determined to be 1.13 x $10^4 \frac{1}{\text{cm}} \frac{1}{\text{M}}$.

A fluorometric method of determining H_2O_2 in concentrations as low as 5 x 10^{-12} $\frac{\text{mole}}{\text{ml}}$ has been described by Perschke and Broda (1961). Peroxidase (HRP) is a hemoprotein catalyzing the oxidation by hydrogen peroxide of a number of substrates such as ascorbate, ferrocyanide, cytochrome C and the leuco form of many dyes.

$$\begin{aligned} &\text{HRP} + H_2O_2 \rightarrow \text{Compound I} \\ &\text{Compound I} + AH_2 \text{ (oxidizable substrate)} \rightarrow \text{Compound II} + AH^0 \\ &\text{Compound II} + AH_2 \rightarrow \text{HRP} + AH \\ &2AH \rightarrow \text{Oxidized product} \end{aligned} \tag{85.1}$$

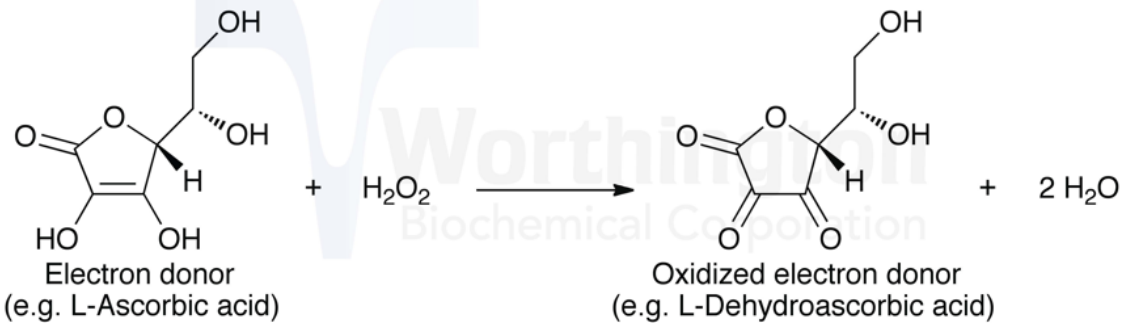

Figure 85.1: Enzymatic Reaction - Peroxidase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Horseradish Roots

Specificity

The enzyme exhibits a high specificity. Activity is observed with H_2O_2, MeOOH, and EtOOH (Maehly and Chance 1954). See also Chmielnicka *et al*. (1971) and Morrison and Bayse (1973).

Composition

Seven isozymes have been described by Shannon *et al*. (1966);Kay *et al*. (1967); and Strickland *et al*. (1968). See also Delincée and Radola (1975) and Shih *et al*. (1971). All contain photohemin IX as prosthetic group. Neutral and amino sugars account for approximately 18% of the enzyme. Weinryb (1966) indicates that the "active site" involves apoprotein as well as the heme group. See also Lanir and Schejter (1975). Dolman *et al*. (1975) have reported on the formation of Compound I. See also Dunford *et al*. (1975), Santimone (1975) and Stillman *et al*. (1975).

IUB

- 1.11.1.7

CAS

- 9003-99-0

Molecular Weight

- 40,000 (Maehly 1955)

Optimal pH

- 7.0 (Maehly 1955)

Isoelectric Point

- 7.2 (Maehly 1955)

Inhibitors

- Horseradish peroxidase is reversibly inhibited by cyanide and sulfide at a concentration of 10^{-5} M (Theorell 1951).

Stability/Storage

- The enzyme is quite stable. As a lyophilized powder, it may be stored several years refrigerated.

Assay Information

Method

- Peroxidase activities have traditionally been expressed in units based upon the rate of oxidation of pyrogallol, a method introduced by Willstalter and Stoll in 1917, and which more recent studies have shown to be somewhat inadequate. (Maehly and Chance 1954.) A wide variety of hydrogen donors have been utilized in peroxidase assay systems including potentially carcinogenic compounds such as o-dianisidine. An improved assay has been adopted using 4-aminoantipyrine as hydrogen donor (Trinder 1966). The reaction rate is determined by measuring an increase in absorbance at 510 nm resulting from the decomposition of hydrogen peroxide. One unit results in the decomposition of one micromole of hydrogen peroxide per minute at 25°C and pH 7.0 under the specified conditions.

Reagents

- 0.2 M Potassium phosphate buffer pH 7.0
- 0.0017 M Hydrogen peroxide. Prepare by diluting 1 ml of 30% hydrogen peroxide (Merck Superoxol or equivalent) to 100 ml with reagent grade water. Further dilute 1 ml of this solution to 50 ml with 0.2 M potassium phosphate buffer pH 7.0. Prepare fresh daily.
- 0.0025 M 4-Aminoantipyrine with 0.17 M Phenol: Prepare by dissolving 810 mg phenol in 40 ml reagent grade water. Add 25 mg 4-aminoantipyrine and dilute to a final volume of 50 ml with reagent grade water.

Enzyme

- Dissolve at one $\frac{mg}{ml}$ in reagent grade water. Immediately prior to use, dilute further to obtain a rate of 0.02-0.04 $\frac{\Delta A}{min}$.

Procedure

Spectrophotometer Settings: Wavelength: 510 nm Temperature: 25°C

Pipette into each cuvette as follows:

- Phenol/aminoantipyrine solution - 1.4 ml
- 0.0017 M Hydrogen peroxide - 1.5 ml

Incubate in spectrophotometer at 25°C for 3-4 minutes to achieve temperature equilibration and establish blank rate, if any. Add 0.1 ml of diluted enzyme and record the increase in A_{510} for 4-5 minutes. Calculate $\frac{\Delta A_{510}}{min}$ from linear portion of the curve.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{510}}{\text{min}}}{6.58 \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$
- $\frac{\text{mgP HPOFF}}{\text{ml}} = A_{275} \text{ x } 1.22$

Notes

- Note: Although the reaction rate obtained with the phenolantipyrine method is 4.5-4.7 times less than previous methods, the peroxidase preparations are the same.
- The RZ (Reinheitzahl) which is the absorbance ratio, $\frac{A_{403}}{A_{275}}$, (RZ) has been used as an indication of purity. However, Shannon *et al*. (1966) report that this ratio for the isozymes varies from 2.50 to 4.19. This, together with the influence exerted by buffer and pH, would seem to render questionable the preciseness of this ratio as a criterion of purity.

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86. Phosphatase, Acid

Orthophosphoric-Monoester Phosphohydrolase (Acid Optimum)

Acid phosphatase is ubiquitous in nature, perhaps one of the most concentrated sources being human prostate gland, a fact exploited by the clinical chemist who measures the serum enzyme level as an index of prostatic cancer. It has also been associated with Gaucher's disease, patients exhibiting unique peaks in their electrophoresed sera (Goldberg *et al.* 1966). Acid phosphatases have been reviewed by Hollander (1971) although the wheat germ enzyme is not included.

Acid phosphatase activity was observed by Teller in 1954 in preparations of a wheat germ lipase described by Singer in 1948. Subsequent work confirmed that the non-specific esterase activity of the wheat germ preparation may be measured both as lipase (tracetin as substrate) and phosphatase.

The enzyme has been purified and its properties described by at least three different laboratories. Joyce and Grisolia (1960) purified it some 3000-fold and measured its activity on a variety of phosphate esters. Brouillard and Ouellet (1965) obtained four separate forms of the enzyme on ion-exchange and gave evidence for the presence of ferric iron in the molecule. Verjee (1969) obtained three active peaks and showed them to have individual properties. Acid phosphatase catalyzes the following reaction at an optimal pH below 7:

$$\text{Orthophosphoric monoester} + H_2O \rightarrow \text{alcohol} + H_3PO_4 \qquad (86.1)$$

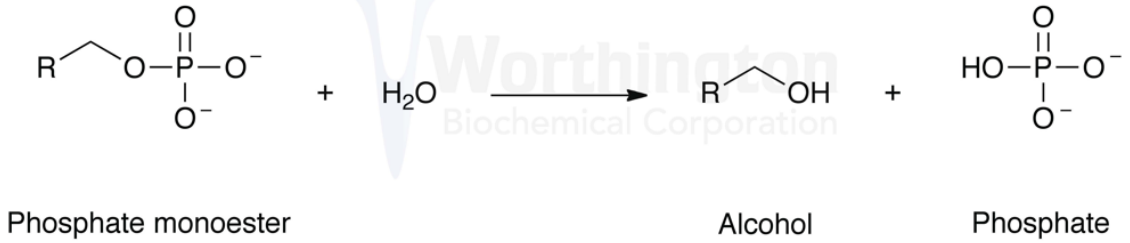

Figure 86.1: Enzymatic Reaction - Phosphatase, Acid

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Wheat Germ

Specificity

The enzyme has a broad esterase activity. See Joyce and Grisolia (1960). It shows highest activity for pyrophosphate.

Composition

Three isozymes of closely similar molecular weights have been reported by Verjee (1969): EI, EII, and EIII. See also Brouillard and Ouellet (1965).

IUB

- 3.1.3.2

CAS

- 9001-77-8

Molecular Weight

- 55,000 ± 5,000 (Verjee 1969)

Optimal pH

- EI - 5.5, EII - 4.5, and EIII - 4.0. (Verjee 1969)

Inhibitors

- Fluoride, molybdate and orthophosphate (Verjee 1969)

Assay Information

Method

- Based on the work of Brandenberger and Hanson (1953) and Hofstee (1954). The initial rate of hydrolysis of o-carboxyphenyl phosphate is determined by following the increase in absorbance at 300 nm resulting from the release of salicylic acid. One unit hydrolyzes one micromole of o-carboxyphenyl phosphate per minute at 25°C, pH 5.0 under the specified conditions. (An equivalent specific activity is obtained under above conditions using p-nitrophenyl-phosphate substrate).

Reagents

- 0.15 M Sodium acetate buffer, pH 5.0
- 3.65 mM o-Carboxyphenyl-phosphate

Enzyme

- Dissolve at a concentration of 1.0 $\frac{\text{mg}}{\text{ml}}$ in reagent grade water.

Procedure

Spectrophotometer Settings: Wavelength: 300 nm Temperature: 25°C

Pipette into each cuvette as follows:

- 0.15 M Acetate buffer, pH 5.0 - 2.0 ml
- 3.65 mM o-Carboxyphenyl-phosphate - 0.5 ml

Incubate in spectrophotometer for 3-4 minutes to reach temperature equilibration and establish blank rate, if any. Add 0.5 ml enzyme and record increase in A_{300} for 4-5 minutes. Calculate $\frac{\Delta A_{300}}{\text{min}}$ from the initial linear portion of the curve.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{300}}{\text{min}} \text{ x } 1000}{3500 \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$ where 3500 is the molar extinction coefficient of salicylic acid at 300 nm

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87. Phosphatase, Alkaline

Orthophosphoric-Monoester Phosphohydrolase (Alkaline Optimum)

Alkaline phosphatase is a broad term associated with non-specific phosphomonesterases with activity optima at alkaline pH.

The mammalian alkaline phosphatases have been reviewed by Fernley (1971).

Escherichia coli alkaline phosphatase is a valuable reagent for removal of terminal monoesterified phosphate from both ribo- and deoxyribo-oligonucleotide. It has been reviewed by Reid and Wilson (1971).

$$\text{Orthophosphoric monoester} + H_2O \rightarrow \text{alcohol} + H_3PO_4 \qquad (87.1)$$

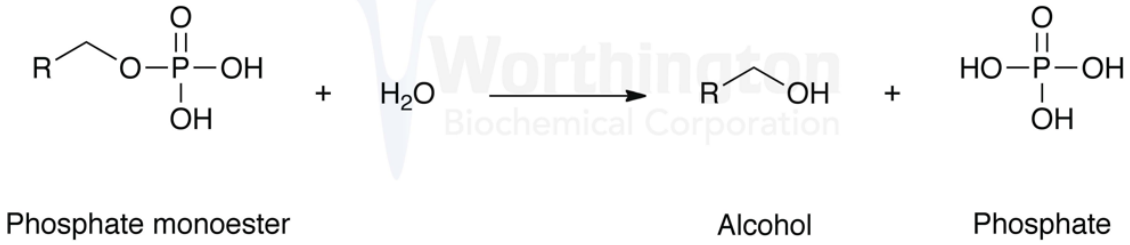

Figure 87.1: Enzymatic Reaction - Phosphatase, Alkaline

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Chicken Intestine, *Escherichia coli*, and Calf Intestine

Specificity

Escherichia coli: See review by Reid and Wilson (1971). Also Mushak and Coleman (1972) and Snyder and Wilson (1972).

Composition

Chicken Intestine: The enzyme is a zinc metallo-enzyme. Schüssler (1968) reports four isozymes. Chang and Moog (1972) found three isozymes in the enzyme from chicken duodenum. (The Worthington preparation is from the whole intestine.)

Escherichia coli: *Escherichia coli* alkaline phosphatase is a dimeric, zinc and magnesium containing protein (Anderson *et al.* 1975; Bosron *et al.* 1975). Though the subunits are believed to be coded by the same gene (Garen and Garen 1963), they develop molecular heterogeneity after translation (Bosron and Vallee 1975; Bridgen and Secher 1973). See also Schlesinger (1974), Halford *et al.* (1972). For amino acid composition see Reid and Wilson (1971) and Kelley *et al.* (1973).

Fernley (1973) and Chappelet-Tordo *et al.* (1974) indicate there to be two active sites, only one is functional at a time. Two Zn^{2+} are needed for activity. A stable apoenzyme can be reactivated with Zn^{2+} , Mn, Co, Ni, Cu, Cd and Hg have been substituted for Zn but only Co restores significant activity (Taylor and Coleman 1972). Magnesium does not activate the apoenzyme but enhances activity of the enzyme containing two gram atoms of zinc (Anderson *et al.* 1975). See also Anderson and Vallee (1975), Lilja *et al.* (1975), Brown *et al.* (1974), Daemen and Riordan (1974), Kaden *et al.* (1972).

IUB

- 3.1.3.1

CAS

- 9001-78-9

Molecular Weight

- *Escherichia coli*: 80,000 Taylor and Coleman (1972). 86,000 Lazdunski and Lazdunski (1969). 89,000 Anderson *et al*. (1975).
- Calf Intestine: 140,000

Optimal pH

- Chicken Intestine: 8 - 9
- *Escherichia coli*: 8.0 (Garen and Levinthal 1960)
- Calf Intestine: 9.8

Isoelectric Point

- *Escherichia coli*: 4.5 (Garen and Levinthal 1960)
- Calf Intestine: 5.7

Extinction Coefficient

- *Escherichia coli*: $E_{278}^{1\%} = 7.2$ (Anderson *et al*. 1975)

Activators

- Chicken Intestine: Schüssler (1968) indicates activation by Mg^{2+}. See Sivanaesan *et al*. (1991).
- Calf Intestine: Zn, Mg^{++}, Ca^{++}

Inhibitors

- Chicken Intestine: Acidification to pH 4.5 reversibly inactivates the enzyme.
- *Escherichia coli*: The enzyme is inhibited by chelating agents and inorganic phosphates.

Stability/Storage

- Chicken Intestine: The lyophilized preparation is stable for 1-2 years at 2 - 8°C.
- *Escherichia coli*: Stable at 2 - 8°C for at least 6 - 12 months.

Activity

- *Escherichia coli*: Bloch and Schlesinger (1974) report on kinetic studies. Bock and Sheard (1975) indicate that the *Escherichia coli* enzyme binds phosphate tightly over a wide range of pH forming complexes that may be intermediate in the hydrolytic action. See also Bosron and Vallee (1975), Zukin and Hollis (1975), and Chlebowski and Coleman (1974).

Assay Information

Chicken Intestine Enzyme

Method

- The measurement of phosphatase activity is based upon the work of Brandenberger and Hanson (1953) and Hofstee (1954). The catalytic effect of the enzyme on the initial rate of hydrolysis of o-carboxyphenyl phosphate is determined. Salicylic acid, unlike the phosphate ester, absorbs light maximally at about 300 nm; hence the rate of hydrolysis can be determined by the increase in absorbance. One unit hydrolyzes one micromole of o-carboxyphenyl phosphate per minute at 25°C and pH 8.8 under the specified conditions.

87.0.1 Reagents

- 0.1 M Tris · HCl, pH 8.5
- 0.2 M Glycine, pH 8.8
- 0.05 M Magnesium chloride
- 3.65 mM o-Carboxyphenyl phosphate (OCPP)

87.0.2 Enzyme

- Dissolve at one $\frac{\text{mg}}{\text{ml}}$ in 0.1 M Tris · HCl, pH 8.5 (Stock solution). A further dilution may be required following activation.

Procedure

Spectrophotometer Settings: Wavelength: 300 nm Temperature: 25°C

Activate the enzyme by incubating the $\frac{\text{mg}}{\text{ml}}$ solution for 20-30 minutes in a 25°C water bath.

Pipette into each cuvette as follows:

- 0.2 M Glycine, pH 8.8 - 2.0 ml
- 3.65 mM OCPP - 1.0 ml
- 0.05 M $MgCl_2$ - 0.5 ml

Incubate in spectrophotometer at 25°C for 3-4 minutes to reach temperature equilibration and establish blank rate, if any. Add 0.1 ml of stock solution and record increase $\frac{\Delta A_{300}}{\text{min}}$ from initial linear portion of the curve.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{300}}{\text{min}} \text{ x } 1000}{3500 \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$ where 3500 is the molar extinction coefficient of salicylic acid at 300 nm

Assay Information

Escherichia coli Enzyme

Method

- The assay is that of Garen and Levinthal (1960) in which the reaction velocity is determined by measuring an increase in absorbance at 410 nm resulting from the hydrolysis of p-nitrophenylphosphate to p-nitrophenol. One unit releases one micromole of p-nitrophenol per minute at 25°C, pH 8, under the specified conditions.

Reagents

- 1.5 M Tris · HCl buffer, pH 8.0
- 0.003 M p-nitrophenylphosphate (PNP). Care must be exercised to use an analytical grade and the correct molecular weight.

Enzyme

- Dilute in reagent grade water to obtain a rate of 0.02 - 0.04 $\frac{\Delta A}{min}$.
- $\frac{mg}{ml} = A_{278}$ x 1.4

Procedure

Spectrophotometer Settings: Wavelength: 410 nm Temperature: 25°C Cuvettes

- 0.003 M PNP - 1.0 ml
- 1.5 M Tris · HCl, pH 8.0 - 2.0 ml

Mix well and incubate in the spectrophotometer for 4-5 minutes to achieve temperature equilibration and to establish blank rate, if any. Add 0.1 ml of diluted enzyme and record. Determine A_{410} for 3-5 minutes from linear portion of the curve.

Calculation

- $\frac{Units}{mg} = \frac{\frac{\Delta A_{410}}{min} \text{ x } 1000}{1.62 \text{ x } 10^4 \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$

Assay Information

Calf Intestine Enzyme

Method

- One unit hydrolyzes 1 μmole of p-nitrophenol phosphate per minute at 37°C, pH 9.8.

Reagents

- 1.0 M Diethanolamine with 0.05 mM $MgCl_2$ buffer, pH 9.8: Dilute 12.4 g diethanolamine (85%) wtih reagent grade water. Add 0.05 ml $MgCl_2$ solution (see below) and adjust the pH to 9.8 (at 37°C) with HCl. Adjust to 100 ml with reagent grade water.
- $MgCl_2$ solution: Dissolve 20.3 g $MgCl_2$°6 H_2O in 100 ml reagent grade water.
- 0.67 M p-Nitrophenyl phosphate solution: Dissolve 250 mg p-nitrophenyl phosphate, Na salt in 1.0 ml reagent grade water.

- Diluent: 0.1 M TEA·HCl. Dissolve 1.86 g TEA·HCl in reagent grade water, add 0.1 ml $MgCl_2$ solution and 0.1 ml 0.1 M $ZnCl_2$, adjust the pH to 7.6 with NaOH and adjust to 100 ml with reagent grade water.

Enzyme

- Use diluent to obtain approximately 0.05 - 0.06 $\frac{\text{units}}{\text{ml}}$. Let stand 15-20 minutes at room temperature.

Procedure

Spectrophotometer Settings: Wavelength: 405 nm Temperature: 37°C Pipette into each cuvette as follows:

Mix and measure the change in absorbance. Calculate $\frac{\Delta A}{\text{min}}$ based on the linear range of the curve.

Description	Test	Blank
Buffer	3.00 ml	3.00 ml
4-nitrophenyl phosphate	0.050 ml	0.050 ml
Mix and incubate to achieve temperature equilibration.		
Diluent	—	0.050 ml
Sample	0.050 ml	—

Calculation

- $$\frac{\text{units}}{\text{ml}} = \frac{\frac{\Delta A}{\text{min}}_{(\text{Sample})} - \frac{\Delta A}{\text{min}}_{(\text{Blank})} \times 3.10}{18.2 \times 0.05}$$

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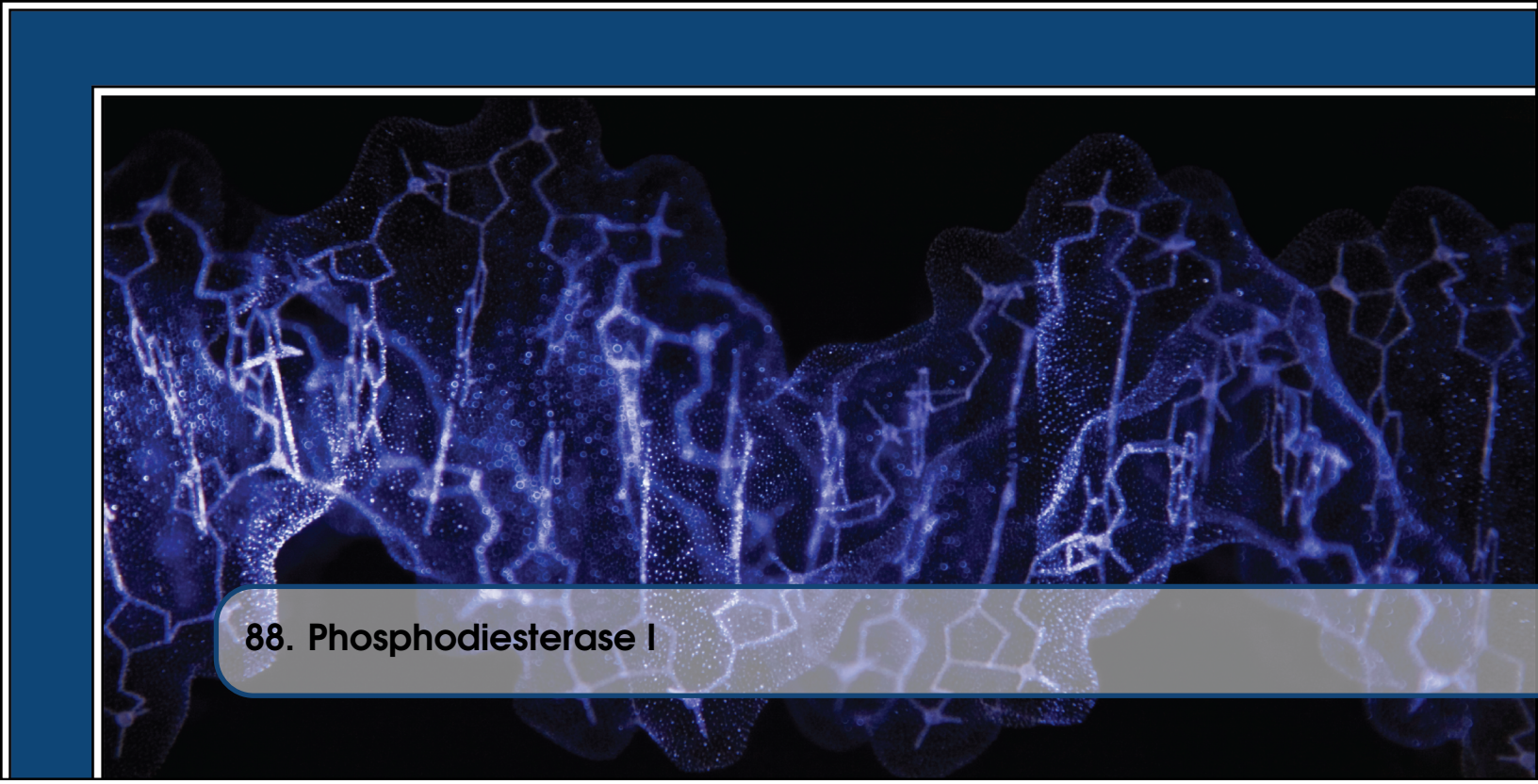
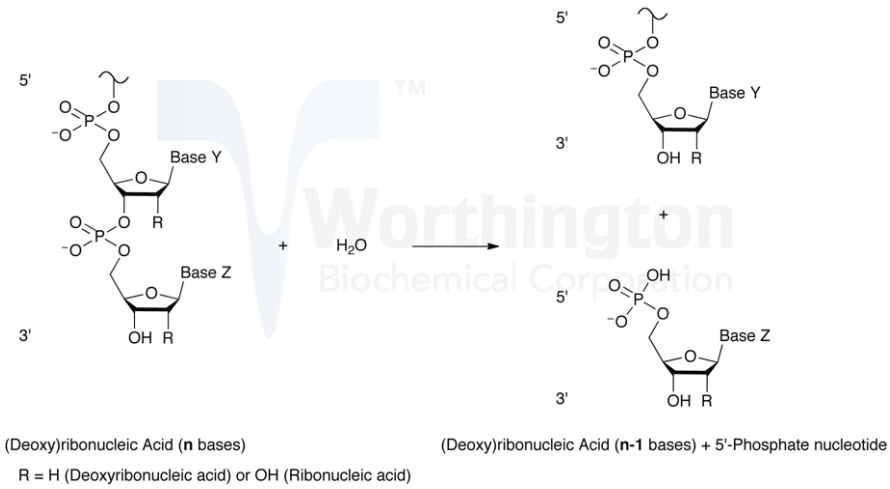


88. Phosphodiesterase I

Oligonucleate 5'-Nucleotidohydrolase

Venom exonuclease (Phosphodiesterase I) successively hydrolyzes 5'-mononucleotides from 3'-hydroxy-terminated ribo- and deoxyribo-oligonucleotides. It has been reviewed by Laskowski, Sr. (1971).

The enzyme has been widely utilized as a tool for structural and sequence studies of nucleic acids (Ho and Gilham 1973).



(Deoxy)ribonucleic Acid (**n** bases)

(Deoxy)ribonucleic Acid (**n-1** bases) + 5'-Phosphate nucleotide

R = H (Deoxyribonucleic acid) or OH (Ribonucleic acid)

Figure 88.1: Enzymatic Reaction - Phosphodiesterase I

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Crotalus adamanteus* venom

Specificity

The enzyme has been purified by Dolapchiev *et al*. (1974) and Sulkowski and Laskowski, Sr. (1961) with endonuclease activity being eliminated as well as 5'-nucleotidase and nonspecific monophosphatase.

It is nonspecific with respect to base or sugar moieties of nucleotides. A variety of synthetic substrates are hydrolyzed. See review by Razell (1963). Ogilvie and Hruska (1976) indicate that exonuclease will not recognize nucleoside units in the syn conformation. See also Philipps and Chiemprasert (1975).

ADP-ribosylated proteins are cleaved at the pyrophosphate linkages by venom phosphodiesterase to yield phosphoribosyl-AMP (Hayaishi 1976).

Composition

A glycoprotein which binds concanavalin A. (Sulkowski and Laskowski 1961)

IUB

- 3.1.4.1

CAS

- 9025-82-5

Molecular Weight

- 115,000 (Philipps 1975)

Optimal pH

- 9.8-10.4 (Philipps 1975)

Activators

- The enzyme has an absolute requirement for Mg^{2+}; Philipps (1975) indicates an optimum concentration of 15 mM.

Inhibitors

- Reducing agents such as glutathione, cysteine and ascorbic acid (Razell 1963). It is completely inhibited by 5 mM EDTA while ATP, ADP and AMP are partial inhibitors.

Assay Information

Method

- The assay is essentially that of Razell and Khorana (1959) where the reaction velocity is determined by an increase in absorbance at 400 nm resulting from the hydrolysis of p-nitrophenyl thymidine-5'-phosphate. One unit hydrolyzes one micromole of p-nitrophenyl thymidine-5'-phosphate per minute at pH 8.9 and 25°C under the specified conditions.

Reagents

- 0.11 M Tris · Salts buffer)
- 5 mM p-nitrophenyl thymidine-5'-phosphate. Note: The purity of commercial preparations varies somewhat and should be considered in preparing this reagent.

Enzyme

- Dissolve at one $\frac{\text{mg}}{\text{ml}}$ in Tris · Salts buffer to obtain a rate of 0.02-0.04 $\frac{\Delta A}{\text{min}}$.

Procedure

Spectrophotometer Settings: Wavelength: 400 nm Temperature: 25°C

Pipette into microcuvettes as follows:

- Tris-Salts buffer - 0.9 ml
- 5 mM p-nitrophenyl thymidine-5'-phosphate - 0.1 ml

Incubate cuvettes in spectrophotometer for 3-5 minutes to reach temperature equilibrium and establish blank rate, if any. Add 10 microliters of diluted enzyme and record increase in A_{400} for 3-5 minutes. The reaction remains linear until A_{400} reaches about 1.2. Calculate $\frac{\Delta A_{400}}{\text{min}}$ from initial linear portion of absorbance curve.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{400}}{\text{min}}}{16 \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$ where 16 is the extinction coefficient of p-nitrophenol under these conditions

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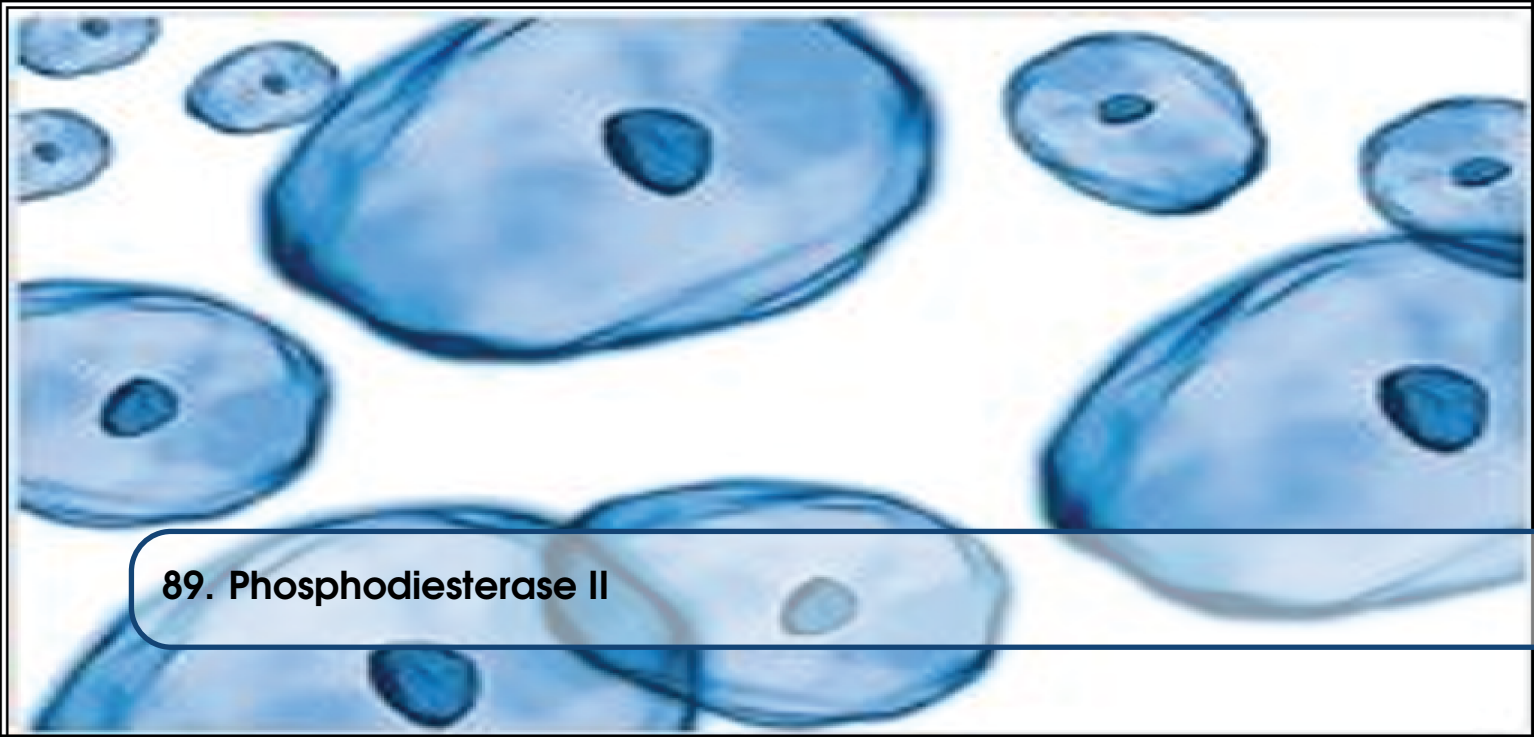

89. Phosphodiesterase II

Oligonucleate 3'-Nucleotidohydrolase

Spleen exonuclease (Phosphodiesterase II) hydrolyses 3'-phosphomononucleotides from oligonucleotides containing a 5'-hydroxyl terminus. See review by Bernardi and Bernardi (1971).

The enzyme's specificity for the 5'-phosphate end of a polynucleotide chain after conversion to 5'-OH by use of *Escherichia coli* alkaline phosphatase makes spleen exonuclease very important in the characterization of polynucleotide chain length, base composition and identity of terminal nucleotide. See Ho and Hilham (1973), Philippsen and Zachau (1971), Roychoudhury *et al.* (1971), and Sneider (1971).

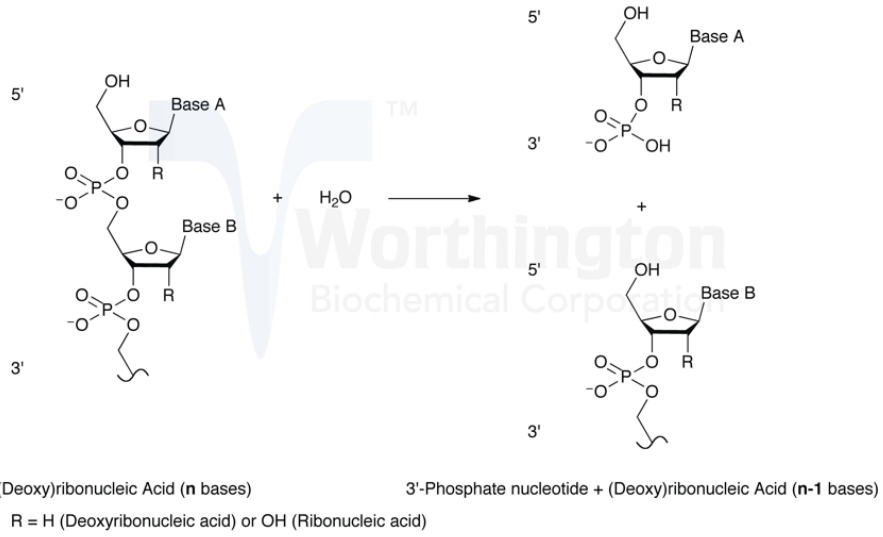

Figure 89.1: Enzymatic Reaction - Phosphodiesterase II

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Bovine Spleen

Specificity

Spleen exonuclease splits off, sequentially, nucleoside 3'-phosphates, starting at the 5'-OH end, from ribo- or deoxyribo-oligonucleotides. RNA "core", alkali-treated DNA, poly(A), poly(U), and poly(I) are acted upon. Prior removal of the 5'-terminal phosphate group is essential.

Native DNA and poly(C) are quite resistant. Bis-(p-nitrophenyl) phosphate is a very poor substrate. (Bernardi and Bernardi 1968).

IUB

- 3.1.16.1

CAS

- 9068-54-6

Optimal pH

- 5.5 using succinate and phosphate buffer and an acid DNase digest; pH 6-7 with 0.1 M acetate buffer. (Bernardi and Bernardi 1971).

Assay Information

Method

- A modification of the assay as described by Hilmoe (1961). One unit is defined as a change in absorbance of 0.2 at 37°C and pH 6.5 under the specified conditions.

Reagents

- 5.9 mM Uranyl acetate in 2.5% perchloric acid. Prepare by dissolving 250 mg uranyl acetate and 3.55 ml 70% perchloric acid in 80 ml reagent grade water. Dilute to a final volume of 100 ml with reagent grade water.
- 0.25 M Sodium succinate·HCl, pH 6.5
- Worthington RNA Core (Code:RNAC). Dissolve at a concentration of 17 $\frac{\text{mg}}{\text{ml}}$ in reagent grade water. Adjust pH to 7.0.

Enzyme

- Dissolve contents of vial with one ml reagent grade water. Prior to use, dilute to 2-8 $\frac{\text{units}}{\text{ml}}$ with reagent grade water.

Procedure

Prepare a 37°C water bath.

Pipette into tubes as follows:

Description	Test	Blank
Sodium succinate buffer	0.4 ml	0.4 ml
RNA core	0.5 ml	0.5 ml
Reagent grade water	0.1 ml	1.1 ml
Incubate tubes in 37°C water bath for 5 minutes to achieve temperature equilibration. At timed intervals add:		
Enzyme dilution	0.1 ml	——

Incubate at 37°C for 30 minutes. Stop the reaction at timed intervals by adding 2.0 ml uranyl acetate/perchloric acid solution. Remove to an ice bath and chill for 10 minutes. Centrifuge at high speed in a clinical centrifuge for 15 minutes. Transfer 0.2 ml aliquot of each supernatant to a clean dry test tube and add 7.8 ml reagent grade water. Mix well and read A_{260} vs water.

Calculation

- $\frac{\text{Units}}{\text{vial}} = \frac{(A_{260\ \text{Test}} - A_{260\ \text{Blank}})}{0.2 \times \text{sample volume}}$

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90. Phosphoenolpyruvate Carboxylase

Orthophosphate: Oxaloacetate Carboxy-lyase (Phosphorylating)

Phosphoenolpyruvate carboxylase (PEPC) catalyzes the fixation of carbon dioxide with phosphoenolpyruvate to produce oxaloacetate and inorganic phosphate.

PEPC is found in most plants and bacteria. It has been reviewed by Utter and Kolenbrander (1972).

$$CH_2 = C(OPO_3H_2)CO_2H + CO_2 + H_2O \rightleftarrows CO_2HCOCH_2CO_2H + Pi \qquad (90.1)$$

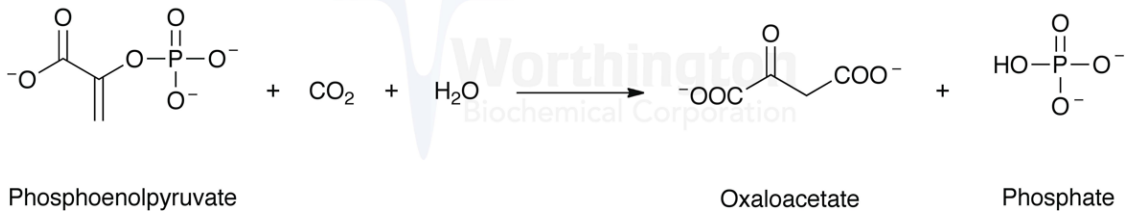

Figure 90.1: Enzymatic Reaction - Phosphoenolpyruvate Carboxylase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Escherichia coli*

Specificity

Apparently only phosphoenolpyruvate and carbon dioxide can participate in the reaction.

Composition

The enzyme is a tetramer composed of identical subunits of about 100,000 molecular weight (Wohl and Markus 1972; Yoshinaga *et al.* 1970). There are thirty-six cysteine residues per mole which exist as free SH groups. See also Izui (1973) for conformation studies and Izui *et al.* (1970) on active site.

IUB

- 4.1.1.31

Molecular Weight

- Approximately 400,000 (Smith 1971)

Optimal pH

- 8-9. pH 8.5 is generally used.

Activators

- Acetyl CoA and fructose-1,6-diphosphate (Utter and Kolenbrander 1972). Acetyl CoA provides maximum activation at about 1 mM concentration, but we were unable to detect significant effects with the fructose diphosphate, guanosine diphosphate, or even the aspartate. Dioxane is very effective, and is used in the assay. However, dioxane generally contains traces of peroxides which must be removed by the dithioerythritol (DTE).
- The enzyme is less active in higher ionic strength solutions, showing about 50% activity in 0.15 M ammonium sulfate or potassium chloride. See also Wohl and Markus (1972).

Inhibitors

- L-aspartate, fumarate and L-malate. (See Izui *et al.* 1970).

Stability/Storage

- Solutions are stable at least six hours in a Mg^{2+} buffer. In dilute solution unless Mg^{2+} or aspartate is present the enzyme dissociates into monomer and dimers and is inactivated Smith and Perry 1973).

Assay Information

Method

- The formation of oxaloacetate is monitored spectrophotometrically in a malate dehydrogenase coupled system. The reaction velocity is measured as a decrease in A_{340} resulting from the

oxidation of NADH. One unit oxidizes one micromole of NADH per minute at 25°C and pH 8.5 under the specified conditions.

Reagents

- 0.11 M Tris sulfate buffer, pH 8.5
- 0.3 M Magnesium sulfate
- 6.0 mM NADH in 0.11 M Tris sulfate buffer, pH 8.5
- Malate dehydrogenase (WBC Code MACDHCL) dissolved to a concentration of 600 $\frac{\text{units}}{\text{ml}}$ in 0.11 M Tris sulfate buffer, pH 8.5
- 0.1 M Sodium bicarbonate in reagent grade water
- 0.03 M Phosphoenolpyruvate (PEP), either potassium or cyclohexylammonium salt in 0.11 M Tris sulfate buffer, pH 8.5
- Dioxane: fresh reagent, low in peroxide content
- 0.3 M Dithioerythritol (DTE) in in 0.11 M Tris sulfate buffer, pH 8.5

Enzyme

- Dissolve at a $\frac{\text{mg}}{\text{ml}}$ in 0.11 M Tris sulfate buffer, pH 8.5 containing 0.005 M magnesium sulfate.

Procedure

Spectrophotometer Settings: Wavelength: 340 nm Temperature: 25°C

Into a cuvette pipette the following:

- 0.11 M Tris sulfate buffer, pH 8.5 - 1.8 ml
- 6.0 mM NADH - 0.1 ml
- 0.3 M Magnesium sulfate - 0.1 ml
- 0.3 M DTE - 0.1 ml
- 0.1 M Sodium bicarbonate - 0.3 ml
- Dioxane - 0.3 ml

Incubate in spectrophotometer for 4-5 minutes to achieve temperature equilibration. Add 0.1 ml of diluted enzyme and 0.01 ml of malate dehydrogenase. Record the decrease in A_{340} to establish blank rate. Add 0.1 ml PEP and record decrease in A_{340} (test rate).

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{(\Delta A_{340\ \text{Test}} - \Delta A_{340\ \text{Blank}})}{6.22 \times \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$$

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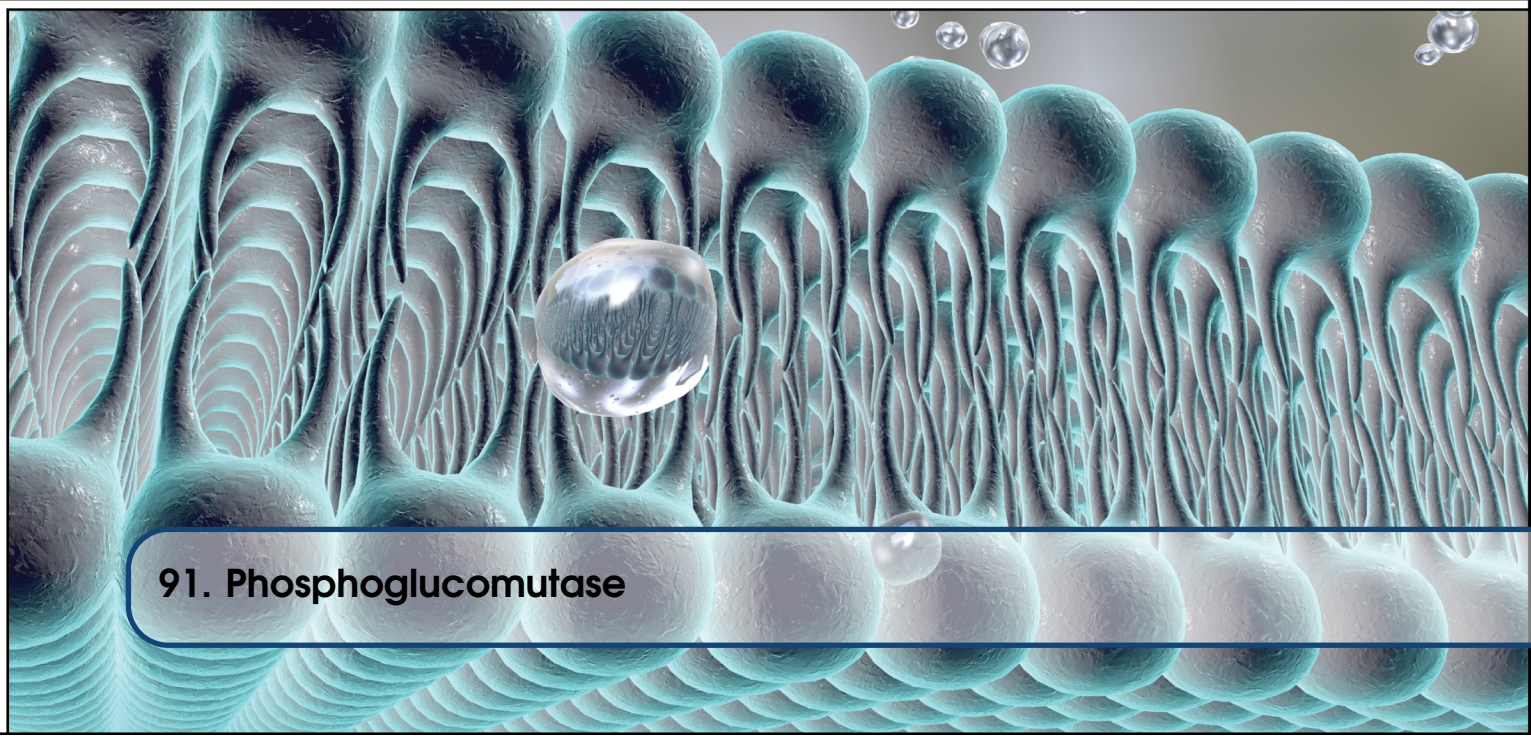

91. Phosphoglucomutase

Phosphoglucomutase (PGM) will interconvert the 1- and 6-phosphate isomers of a-D-glucose. Although it is a reversible reaction, the formation of glucose-6-phosphate is markedly favored. PGM will not convert β-D-glucose-1-phosphate or β-D-glucose-6-phosphate.

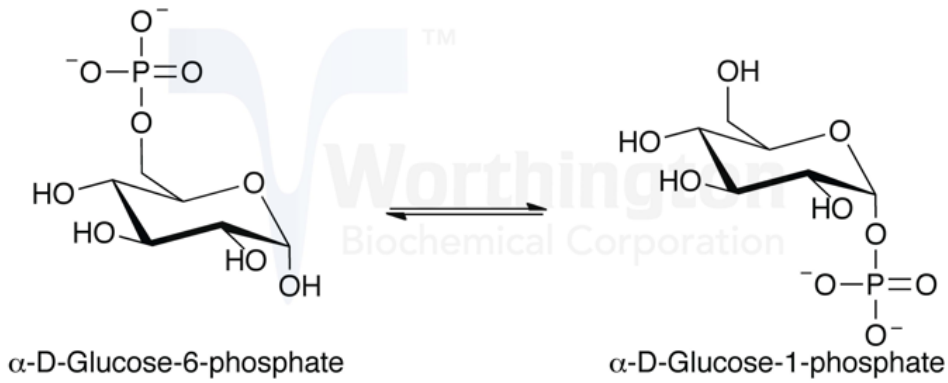

Figure 91.1: Enzymatic Reaction - Phosphoglucomutase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Rabbit Muscle

IUB

- 5.4.2.2

Molecular Weight

- Approximately 62,000 daltons (Bergmeyer 1983).

Optimal pH

- 7-5 - 8.0

Extinction Coefficient

- $E^{1\%}_{278}$ = 7.70 (Najjar 1955)

Activators

- Divalent cations, expecially Mg^{++}, glucose-1,6-bisphosphate and glutathione. Histidine, 8-hydroxyquinoline, and other metal-binding agents also stimulate the enzyme (Sutherland 1949).

Inhibitors

- High concentrations of chelating agents, gluconate-6-phosphate, nucleotides, and acetate.

Stability/Storage

- The enzyme is quite stable when dissolved in acetate buffer at pH 5.0. Its activity can be maintained for about 10 days at 2 - 8°C., then declines slowly over a period of 6 to 8 weeks. Najjar (1955) reports that below pH 4.5 and above pH 8.5 it is readily inactivated. In the crystalline state it is stable for months in 0.60 to 0.65 saturated ammonium sulfate containing 0.05 M acetate buffer, pH 5.0.

Assay Information

Method

- Phosphoglucomutase catalyzes an intra-molecular phosphate transfer between the 1 and 6 positions of glucose and prepares phosphorylated compounds for subsequent energy-producing or biosynthetic reactions. Enzymatic activity is determined by measuring the increase in absorbance at 340 nm caused by the reduction of NAD in the following reactions:
- Glucose-1-phosphate $\underset{glucose-1,6-diphosphate}{\overset{\text{Phosphoglucosmutase}}{\rightarrow}}$ Glucose-6-phosphate
- Glucose-6-phosphate + NAD $\overset{\text{ZF}}{\rightarrow}$ Gluconate-6-phosphate + $NADH^{=}$
- One unit reduces 1 μmole of NAD per minute at 30°C, pH 7.6, using glucose-1-phosphate as the substrate in a glucose-6-phosphate dehydrogenase coupled system.

Reagents

- 6.5 $\frac{\text{mg}}{\text{ml}}$ solution NAD trihydrate (MW 717.5) in reagent grade water. Prepare fresh daily.
- 200 mg Glucose-1-phosphate dipotassium dihydrate (MW 372.4) in 5 ml reagent grade water
- 0.5 $\frac{\text{mg}}{\text{ml}}$ Glucose-1,6-diphosphate, tetracyclohexylammonium salt (MW 808.9)
- Imadazole buffer: Dissolve 3.26 g imidazole (MW 68.08) in 900 ml reagent grade water. Add 0.813 g magnesium chloride and stir to dissolve. Add 0.645 g EDTA disodium dihydrate and stir until completely dissolved. Adjust pH to 7.6 with 5N HCl. Bring to final volume of 1000 ml with reagent grade water.
- 150 $\frac{\text{units}}{\text{ml}}$ Glucose-6-phosphate dehydrogenase (Worthington Code ZF) in imidazole buffer. Prepare fresh daily.

Enzyme

- Dilute in buffer to approximately 0.2 $\frac{\text{units}}{\text{ml}}$, prepared immediately before use.

Procedure

Spectrophotometer Settings: Wavelength: 340 nm Temperature: 30°C

Mix together with stirring, no more than 2 hours before use, the following reaction mixture: 26.0 ml imidazole buffer, 1.0 ml NAD solution, 1.0 ml glucose-1-phosphate solution, 1.0 ml glucose-1,6-diphosphate solution, and 0.1 ml ZF. Place in a 30°C water bath to achieve temperature equilibration. Pipette into cuvettes 2.9 ml reaction mixture. Incubate in spectrophotometer at 30°C for 3-5 minutes to achieve temperature equilibrium and record blank rate, if any. Add 0.1 ml of appropriately diluted enzyme and record increase in absorbance at 340 nm for 5 - 7 minutes. Calculate ΔA_{340} from the initial linear portion of the curve.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{340}}{\text{min}} \text{ x } 3.0 \text{ x dilution}}{6.22 \text{ x } 0.1}$

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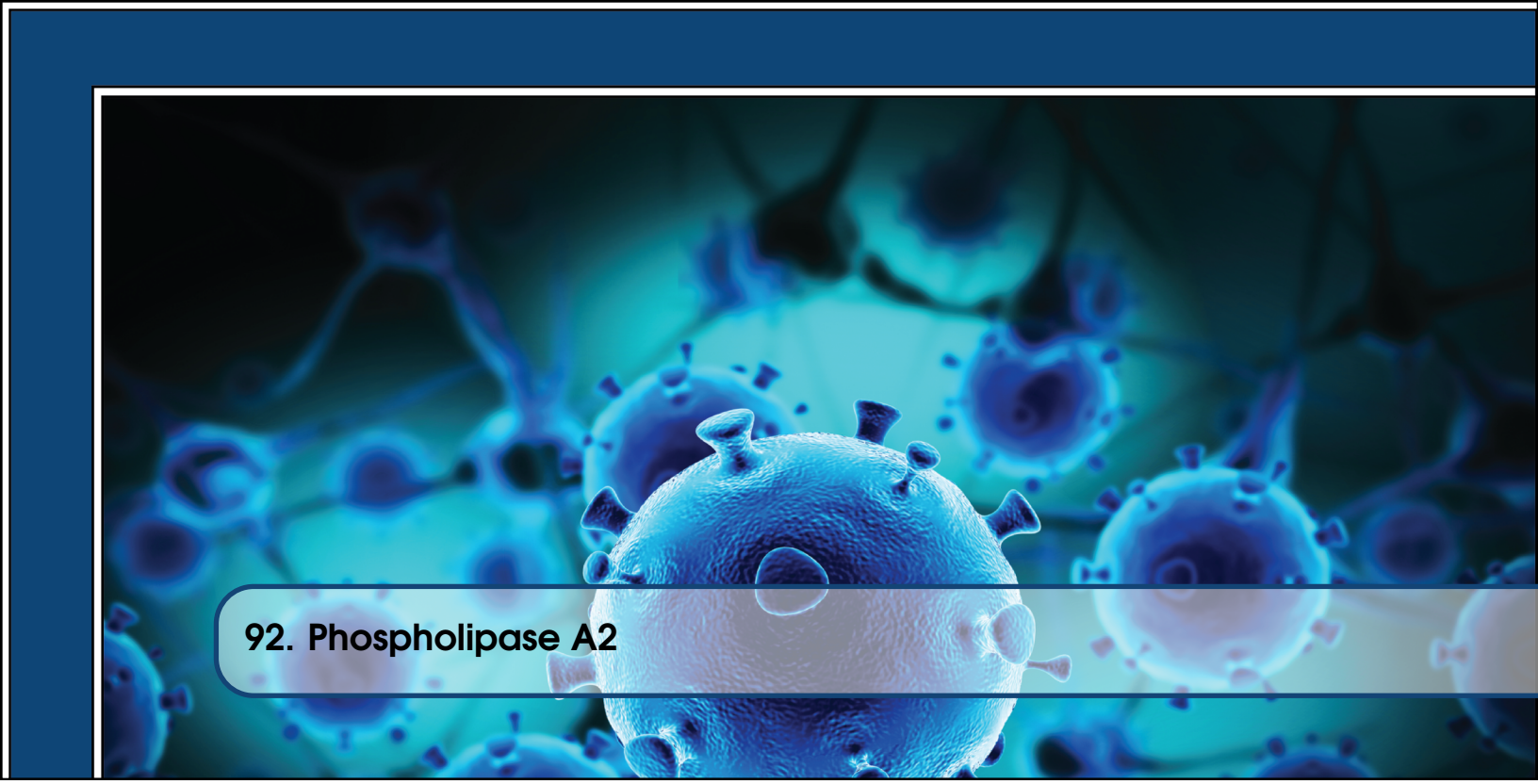

92. Phospholipase A2

Phosphatidylcholine 2-Acetylhydrolase

Phospholipase A2 has been isolated from pancreas, snake and bee venoms. Forst *et al*. (1986), Chan *et al*. (1982), Coulard *et al*. (1987) and Horigome *et al*. (1987) report on platelet phospholipase A2. The primary structure of human pancreatic PLA2 has been reported by Verheij *et al*. (1983).

Phospholipases are involved in lipid metabolism and are important probes of structure-function relationships in biological membranes. Dawson 1973).

Characteristics of Phospholipase A2 from *Crotalus adamanteus* venom:

Two proteins (Aα, Aβ) exhibiting Phospholipase A activity have been isolated from this source. (Saito and Hanahan 1962, Wells and Hanahan 1969). Each protein is composed of dimeric subunits, Aα, Bβ, respectively (Wells 1971). The two proteins have similar activities but are chromatographically and electrophoretically distinct. Heinrikson *et al*. (1977) report on the distinctive structural features of the two enzymes and the complete amino acid sequence of phospholipase A2α. Phospholipase A2 represents a class of heat-stable, calcium-dependent enzymes catalyzing the hydrolysis of the 2-acyl bond of 3-n-phosphoglycerides. [This enzyme is named Phospholipase A2 to denote its 2-acyl specificity (Uthe 1971).]

$$\text{Phosphatidylcholine} + H_2O \rightarrow \text{1-Acyl-glycerophosphocholine} + \text{Carboxylic acid} \qquad (92.1)$$

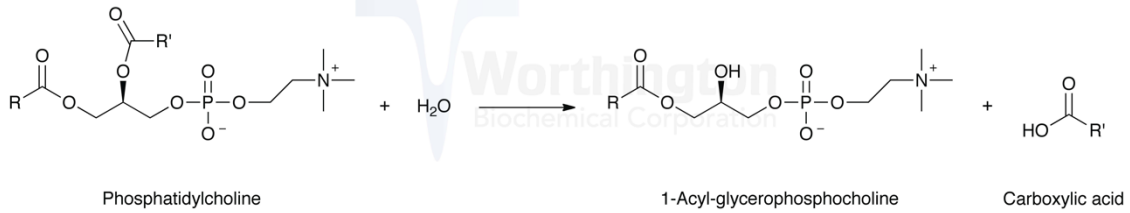

Figure 92.1: Enzymatic Reaction - Phospholipase A2

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Crotalus adamanteus* venom

Specificity

Substrate specificity has been investigated (VanDeenen 1963).

IUB

- 3.1.1.4

CAS

- 9001-84-7

Molecular Weight

- 30,000 (Wells 1969)

Isoelectric Point

- 4.55 and 4.40 for Aα and Aβ respectively (Saito 1962)

Extinction Coefficient

- Phospholipase A2: $E^{1\%}_{280}$ = 22.7 (Wells 1969)

Activators

- Calcium ion (Dennis 1973)

Inhibitors

- Zinc, barium, and manganese ions (Uthe 1971). Also see Golec *et al.* (1992).

Stability/Storage

- The enzyme is stable at 90°C and pH 3.0 for at least five minutes. (Uthe 1971; Saito 1962).

Assay Information

Method

- Activity values for phospholipase A2 preparations which are derived from titrimetric assay procedures can be quite dependent on source and type of lecithin, its preparation as a substrate emulsion, other components of the reaction mixture, and the methodology/instrumentation utilized. Values reported in the literature for highly purified venom phospholipase A2 have ranged from 200 to 3000 $\frac{\text{units}}{\text{mg}}$. Comparison between assay systems is difficult. The following assay has been found to be reproducible in our laboratory. One unit releases one micromole titratable fatty acid per minute from lecithin emulsion at pH 8.9 and 25°C under the specified conditions.

Reagents

- 0.1 M Calcium chloride
- 1.0 M Sodium chloride
- Lecithin emulsion: Weigh out 4.0 grams reagent grade soybean lecithin in a 250 ml beaker and add 30 ml of 1.0 M sodium chloride, 10 ml of 0.1 M calcium chloride and 100 ml of reagent grade water. After stirring for 30 minutes at 4°C, sonicate the mixture for 10 minutes at maximum power on a Branson sonicator or its equivalent. Dilute with reagent grade water to a final volume of 200 ml.
- 0.01-0.02 N NaOH - Standardized

Enzyme

- Dissolve enzyme at a concentration of 1.0 $\frac{\text{mg}}{\text{ml}}$ in reagent grade water. Keep this stock solution on ice while assay is being run. Further dilutions are made in reagent grade water immediately prior to use.

Procedure

The titration can he carried out with an automatic titrator or on a laboratory pH meter. The reaction vessel should be maintained at 25°C.

Blank rate determination: Pipette 15 ml of lecithin emulsion into a reaction vessel at 25°C. Adjust the pH to 8.9 and record the volume of titrant required to maintain the pH at 8.9 for 3-5 minutes after a constant rate is obtained. Determine the blank rate as the volume of titrant added per minute from the final linear portion of the curve.

Sample determination: Add appropriately diluted enzyme to the above emulsion. Record the amount of titrant required to maintain the pH at 8.9 for 4-5 minutes. Determine the sample rate as the volume of titrant added per minute from the linear portion of the curve.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\text{(sample rate - blank rate) x normaility of base x 1000}}{\text{mg enzyme in reaction mixture}}$

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93. Phospholipase C

Phosphatidylcholine Cholinephosphohydrolase

Phospholipase C (PLC) is found in culture media of *Clostridium perfringens*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Ottolenghi 1969). PLC is hemolytic, dermonecrotic and lethal. Because it hydrolyzes lecithin of biological membranes it has been found useful in membrane structure studies (O'Toole 1975; Sabban and Loyter 1974; Rottem *et al*. 1973; Stahl 1973; VanSchijndel *et al*. 1973). A recent, in-depth report by Low and Saltiel (1988) is of interest.

Other reports on the action of PLC in a number of physiological systems are as follows: Effect on red blood cells and ghosts (Ikezawa and Murata 1964; Coleman *et al*. 1970; Berengo and Simpkins 1972; DeBoer and Loyter 1971; Lenard and Singer 1968); Effect on muscle preparations (Finean and Martinosi 1965; Martonosi 1968); Inhibition of viral action (Mitzutani and Mitzutani 1964; Freedman and Pastan 1968); Prevention of cholesterol esterification in human serum (Rowen 1964); Effect on glucose transport in free fat cells (Blecher 1965; Rodbell and Jones 1966); Physicochemical studies on the gelatin of hen's egg yolk; Delipidation of yolk plasma by treatment with phospholipase C and extraction with solvents (Kumar and Mahadevan 1970); Effect on nerve membrane (Simpkens *et al*. 1971); effect on purified myelin (McIlwain and Rapport 1971); Study of bacterial cells (Davie and Brock 1966); Effect on thyroid (Macchia and Pastan 1966); Effect on brain microsomes (Stahl 1973). Phospholipase C (originally called lecithinase and also referred to as α-toxin) catalyzes the hydrolysis of the linkage between glycerol and phosphate in lecithin and other phosphatides.

$$\text{Phosphatide} + H_2O \rightarrow \text{Diglyceride} + \text{Phosphoryl-R(lecithin)} \qquad (93.1)$$

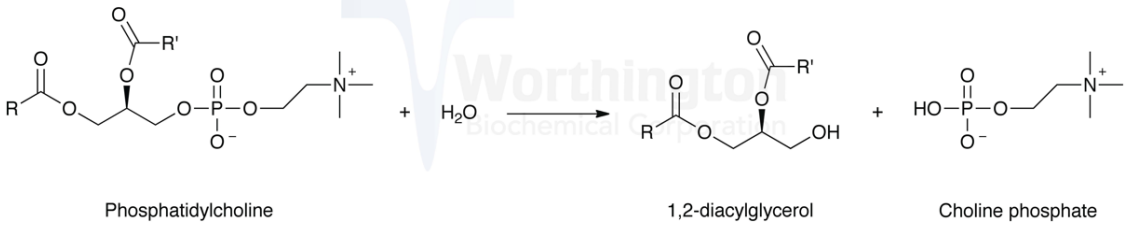

Figure 93.1: Enzymatic Reaction - Phospholipase C

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Clostridium perfringens*

Specificity

Splits lecithin to choline phosphate and diglyceride. It also hydrolyzes sphingomylin, phosphatidylethanolamine and the synthetic water soluble L-α-dicaproyl lecithin. See comparison of possible substrates by Stahl (1973, Table II).

IUB

- 3.1.4.3

Molecular Weight

- 46,000 $\pm$ 500 (Stahl 1973)

Optimal pH

- 7.0 - 7.6

Activators

- Ca^{2+}. Ottolenghi (1967, 1969) reports that the phospholipid substrate must have a net positive charge for enzyme action to occur and that the widely reported Ca^{2+} activation is a reflection of this requirement. See Renard *et al.* (1987).

Inhibitors

- Phosphonate analogs of glycerophosphatides (Rosenthal and Pousada 1968); ferricyanide (Bangham 1962); bleomycin and polymixin B (Saito *et al.* 1972); basic proteins such as lysozyme and cytochrome C (Lenaz *et al.* 1972). Inactivation by EDTA or phenanthroline may be reversed by adding Zn^{2+} but not Ca^{2+} (Möllby and Wadstrom 1973).

Stability/Storage

- Worthington partially purified phospholipase C is stable for at least 5 years when stored at 2 - 8°C. Worthington's chromatographically purifed phospholipase is stable at least 6 months when stored at 2 - 8°C.

Assay Information

Method

- Phospholipase activity values which are derived from titrimetric assay procedures can be quite dependent upon the source and the type of substrate, the preparation of the substrate emulsion, other components of the reaction mixture and the methodology and instrumentation used. Comparative investigation has resulted in the standardized method described below. Values obtained by this procedure have been found to be both reproducible and reliable. The reaction velocity is measured titrimetrically as the rate of hydrolysis of soybean lecithin emulsion. One unit is that amount of enzyme which releases one micromole of acid from soybean lecithin per minute at 25°C and pH 7.4 under the specified conditions.

Reagents

- 6 mM Imidazole · HCl buffer, pH 7.4, containing 2.2 mM calcium chloride
- 0.15 M Sodium chloride
- 0.01 N NaOH, standardized
- 2% Soybean lecithin. Prepare fresh daily by suspending 2.0 grams soybean lecithin in 100 ml of 0.15 M NaCl. Stir at moderate speed for 20 minutes on a magnetic stirrer. Sonicate for 10 minutes at maximum power.
- 0.64 M Calcium chloride

Enzyme

- Dissolve at a concentration of 0.5 $\frac{\text{mg}}{\text{ml}}$ in cold 6 mM imidazole buffer, pH 7.4 with 2.2 mM $CaCl_2$ just before the assay is performed.

Procedure

The titration can be measured with either an automatic titrator or with a laboratory pH meter. The reaction vessel should be maintained at 25°C.

Blank rate determination - Pipette into reaction vessel as follows:

- 2% Soybean lecithin - 7.5 ml
- 0.64 M Calcium chloride - 0.5 ml

Adjust pH to 7.4 and record the volume of the titrant required to maintain the pH at 7.4 for 4-5 minutes after a constant rate is obtained. Determine the blank rate as the volume (ml) of titrant added per minute from the final linear portion of the curve.

Sample determination - Add appropriately diluted enzyme to the above lecithin-$CaCl_2$ mixture. Record the amount of titrant required to maintain the pH at 7.4 for 4-5 minutes. Determine the sample rate as the volume (ml) of titrant added per minute from the initial linear portion of the curve.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\text{(sample rate - blank rate) x normaility of base x 1000}}{\text{mg enzyme in reaction mixture}}$

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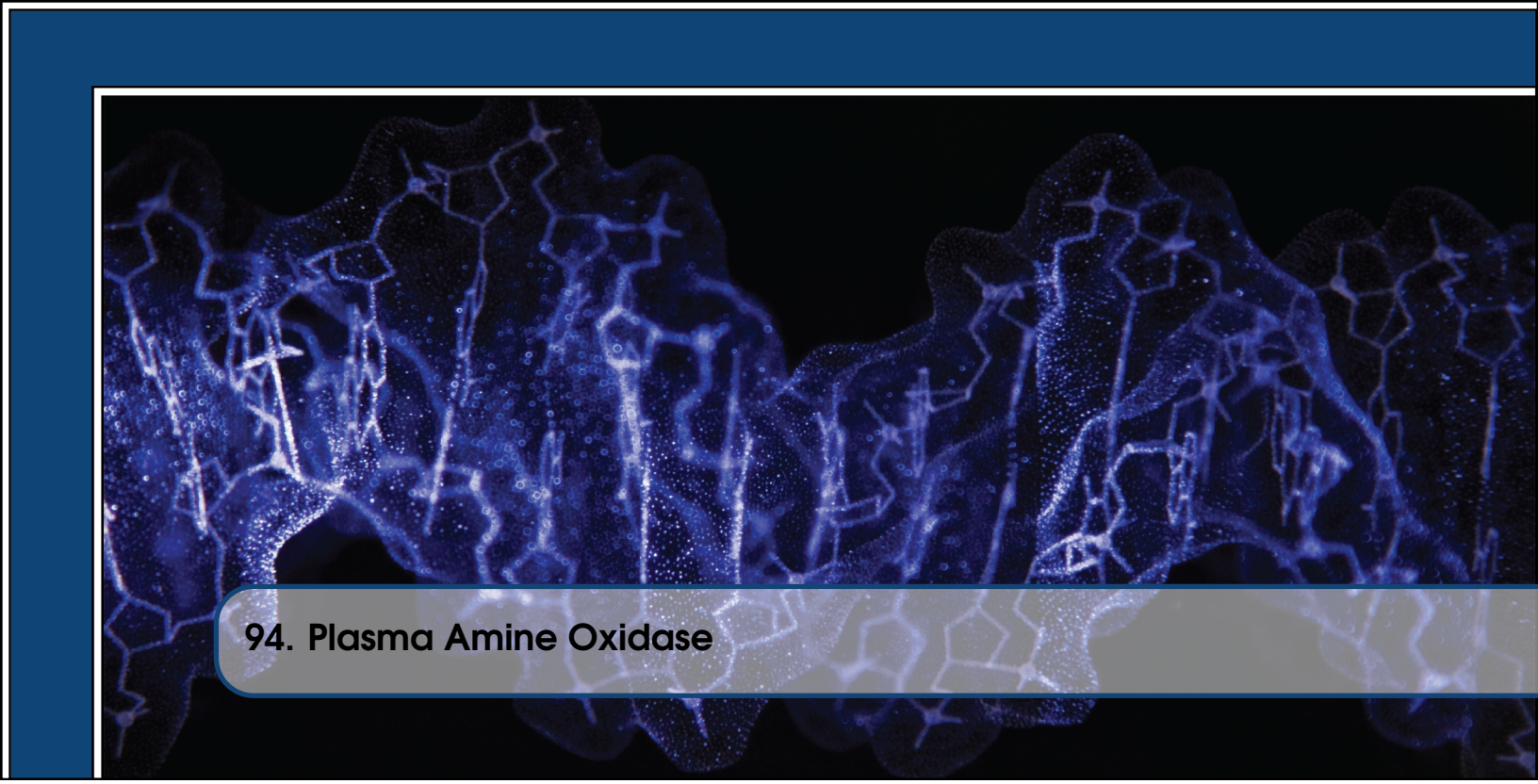

94. Plasma Amine Oxidase

Amine: Oxygenoxidoreductase (Deaminating)

There are two classes of amine oxidases: the pyridoxal and copper-containing enzyme to which plasma amine oxidase belongs and the FAD-containing amino oxidases. They play an important biological role. See review by Blaschko (1963). Natural substrates include catecholamines, tryptamine derivatives and other physiologically active amines. Amine oxidases from different sources have different substrate specificities. They have been reviewed by Malmström *et al.* (1975). Considerable literature exists concerning amine oxidases derived from various organs and animals. The amines spermidine and spermine, most specifically oxidized by bovine plasma oxidase, are significant biochemicals; and have been reviewed by Tabor and Tabor (1972) and by Russell (1973). Russell (1973, 1971; and Russell *et al.* 1971) report an increased urinary excretion of polyamines by cancer patients. This was further expanded on by Lipton *et al.* (1975), and by Russell, Durie and Salmon (1975). Analysis of low concentrations of the polyamines can be done by gas chromatography and amino acid analysis (Russell 1973), and electrophoresis (Lipton *et al.* 1975), but older reports showed utility of plasma amine oxidase for fluorometric (Unemoto *et al.* 1963) and spectrophotometric analysis (Bachrach and Reches 1966). Similar peroxide-detecting systems Guilbault and Kramer 1964; Gochman and Schmitz 1971) could also be adapted. Bachrach (1970, 1973) deals with this subject in detail along with many other aspects of polyamine functions. Plasma amine oxidase (PAO) catalyzes the reaction:

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2 \qquad (94.1)$$

Figure 94.1: Enzymatic Reaction - Plasma Amine Oxidase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Bovine Plasma

Specificity

The major physiological amines oxidized are spermine and spermidine (Yamada and Yasunobu 1962; Yasunobu and Smith 1970; Tabor *et al.* 1954) and some activity is also seen with benzylamine, homosulfanilamide, furfurylamine and simple aliphatic monoamines. (See also Malmström 1975). There is only slight activity with tyramine, and none with tryptamine, epinephrine, serotonin, or agmatine (Yamada and Yasunobu 1962; Tabor *et al.* 1954).

Composition

Achee *et al.* (1968) indicate that the enzyme is composed of two identical polypeptide chains. There are two pyridoxal phosphates and two atoms of Cu^{2+} per molecule (Yamada and Yasunobu 1962 and 1963). The amino acid composition is listed by Malmström *et al.* (1975).

IUB

- 1.4.3.21

CAS

- 9001-53-0

Molecular Weight

- 170,000 (Achee *et al.* 1968)

Optimal pH

- For spermine, 6.2, for spermidine, 7.2. Other amines may have other pH optima (Tabor *et al.* 1954).

Extinction Coefficient

- $E^{1\%}_{280}$ = 9.8 (Yamada and Yasunobu 1962)
- For spectra at various pH values, see Yamada and Yasunobu (1963).

Inhibitors

- Copper chelating agents, many carboxyl reagents such as cuprizone and hydroxylamine; cyanide (Yamada and Yasunobu 1963). Benzoic acid and benzyl alcohol are both non-competitive inhibitors (Ki=30 and 34 mM respecitvely), (Wang *et al*. 1968). Tabor *et al*. (1954) give good summary of inhibitors and the paper by Bardsley *et al*. (1974) dealing with human placental amine oxidases has a large amount of inhibitor data that may be relevant to this bovine plasma amine oxidase also.

Stability/Storage

- Yamada and Yasunobu (1962) report that impure enzyme in 0.03 M phosphate buffer between the pH values of 6.0 to 8.0 is fairly stable when refrigerated, but that highly purified preparations are less stable. Both forms require electrolytes to prevent precipitation of amorphous protein. It was recommended that the enzyme be stored as a suspension in 55% saturated ammonium sulfate in 0.03 M phosphate buffer, pH 7.0, at -5 - 2°C. Lyophilization causes 15-20% loss of activity, but provides a product of adequate stability (Yamada and Yasunobu 1962).

Assay Information

Method

- Determination of amine oxidases has been thoroughly reviewed by Kapeller-Adler (1971). The method employed at Worthington is essentially that of Tabor et al. (1954) with the reaction temperature reduced to 25°C.
- The reaction velocity is determined as an increase in absorbance at 250 nm resulting from the oxidation of benzylamine. One unit results in the oxidation of one micromole of benzylamine per minute at 25°C and pH 7.2 under the specified conditions. One International Unit so defined is equivalent to 4330 Tabor units (Tabor and Tabor 1972).

Reagents

- 67 mM Potassium phosphate buffer pH 7.2
- 1.0% v/v (0.10 M) Benzylamine (if at all colored, re-distill) in 67 mM Potassium phosphate buffer, pH 7.2 (Substrate)

Enzyme

- Dissolve to a concentration of 10 $\frac{\text{mg}}{\text{ml}}$ in 67 mM potassium phosphate buffer, pH 7.2.

Procedure

Spectrophotometer Settings: Wavelength: 250 nm Temperature: 25°C

Into each cuvette pipette 2.8 ml of buffer and 0.1 ml of substrate solution.

Incubate in spectrophotometer for 3-4 minutes to reach temperature equilibration and to establish a blank rate, if any. Add 0.1 ml of enzyme solution and record increase in A_{250} for 6-8 minutes. Calculate $\frac{\Delta A_{250}}{\text{min}}$ from the linear portion of the curve. A 2-3 minute lag may occur after which the reaction should be linear to an A_{250} of 0.75.

Calculation

- $$\frac{\text{Units}}{\text{g}} = \frac{\frac{\Delta A_{250}}{\text{min}} \text{ x } 1000}{13.0 \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$$

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95. Pokeweed Antiviral Toxin

Pokeweed antiviral protein is prepared from the leaves of *Phytolacca americana* It has been shown to have both antiviral (Irvin, 1983) and anticellular (Irvin 1983, Irvin and Aron 1982, and Cawley *et al.* 1979) activity. The site of action for the toxin has been shown in cell-free lysates to be at the ribosomes and specifically the EF-2 mediated translocation step of the elongation cycle during protein synthesis. The protein isolated from leaves exists as two forms, Pokeweed Antiviral Protein (PAP) at a molecular weight of 29,000 and PAP II at a molecular weight of 30,000 (Irvin *et al* 1980)

Classical plant toxins such as ricin or abrin are composed of two functionally active fractions. The A chain contains the toxic activity and the B chain gives the toxin a cell recognition and binding function to facilitate transport across the cell membrane. The A chain is not active until it is internalized by the cell, where it halts protein synthesis. Pokeweed antiviral protein is a hemitoxin which has the A chain activity but lacks a B chain. Without this B chain to mediate cell membrane binding, the toxin cannot enter the cell. By conjugating the protein to a monoclonal antibody or a lectin, the substance can be bound to a cell membrane and internalized where it acts to inhibit protein synthesis.

When used with a cell-free lysate, Worthington Pokeweed Antiviral Toxin can inhibit protein synthesis *in vitro*. When coupled to a monoclonal antibody the toxin can be internalized and be used to inhibit cellular or viral protein synthesis. This data can be further used to illustrate specific steps in the regulation of protein synthesis.

CAS

- 63231-57-2

Stability/Storage

- Should be aliquotted upon receipt and stored frozen at -20°C. Stability at 4°C is approximately two weeks.

Activity

- The activity is defined as that concentration of pokeweed antiviral toxin which will inhibit protein synthesis by 50% in a nuclease treated wheat germ cell-free translation system.

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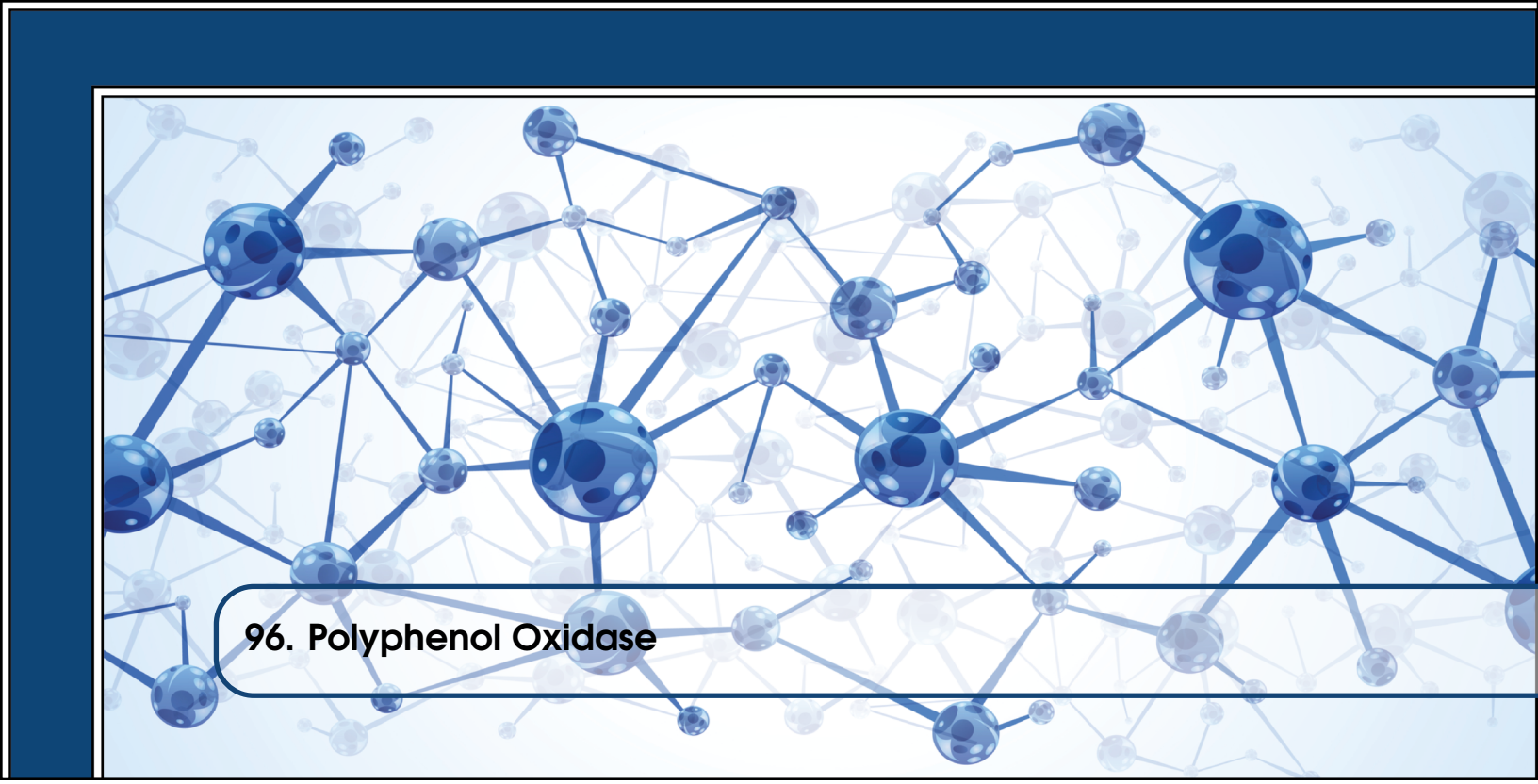

96. Polyphenol Oxidase

Monophenol, Dihydroxyphenylalanine: O_2 Oxidoreductase

Tyrosinase

Polyphenol oxidase (tyrosinase) (TY) is a bifunctional, copper-containing oxidase having both catecholase and cresolase activity (Malström and Rydén 1968) (See Reaction).

Jolley *et al.* (1974) refer to it as an oxygen and 4 electron-transferring phenol oxidase. It is responsible for browning reactions throughout the phylogenetic scale.

Although a tyrosinase from *Neurospora crassa* has been purified (Fling *et al.* 1963), most work has been done with the mushroom enzyme, even though yields and consistency are poor; its multiplicity was shown by Smith and Krueger (1962). Bouchilloux *et al.* (1963) obtained four enzymes. See review by Nelson and Mason (1970).

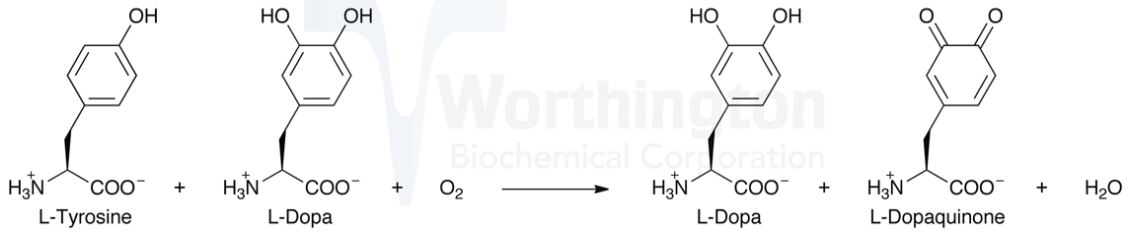

Figure 96.1: Enzymatic Reaction - Polyphenol Oxidase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Mushroom

Specificity

A large number of parasubstituted catechols areoxidized (Duckworth and Coleman 1970).

Composition

The enzyme is a tetramer containing four gram atoms of copper per molecule (Jolley *et al.* 1974), and two binding sites for aromatic compounds including phenolic substrates. There is also a distinctly different binding site for oxygen, the copper site (Duckworth and Coleman 1970). The copper is probably in the cuprous state; inactivation of the enzyme is associated with increase in Cu^{2+}. (Kertész *et al.* 1972). The amino acid composition has been determined. Extensive structural studies have been reported by Jolley *et al.* (1969); and Duckworth and Coleman (1970). See also Jolley *et al.* (1972, 1973, and 1974).

IUB

- 1.14.18.1

CAS

- 9002-10-2

Molecular Weight

- 128,000 (Duckworth and Coleman 1970)

Optimal pH

- 6.0-7.0

Extinction Coefficient

- $E^{1\%}_{280}$ = 24.9 (immediately after purification) (Duckworth and Coleman 1970)

Inhibitors

- Compounds that complex with copper. The enzyme is also inhibited competitively by benzoic acid with respect to catechol and by cyanide with respect to oxygen (Duckworth and Coleman 1970).

Stability/Storage

- The lyophilized preparation is stable for 6-12 months when stored at -20°C.

Activity

- Polyphenol oxidase is an oxygen transferring enzyme. Besides using O_2 to catalyze the dehydrogenation of catechols to orthoquinones and the orthohydroxylation of phenols to catechols, a peroxidase activity has been reported on by Strothkamp and Mason (1974). Kinetic studies have been reported by Kert*ész et al*. (1971). See also the review by Malstr*ö*m and Rydé*n*.

Assay Information

Method

- Polyphenol oxidase oxidizes tyrosine to dihydroxyphenylalanine which in turn is oxidized to o-quinone. The latter is accompanied by an increase in absorbance at 280 nm. The rate of increase is proportional to enzyme concentration and linear during a period of 5-10 minutes after an initial lag. One unit causes a change in absorbance at 280 nm of 0.001 per minute at 25°C, pH 6.5 under the specified conditions.

Reagents

- 0.5 M Potassium phosphate buffer, pH 6.5
- 0.001 M L-Tyrosine

Enzyme

- Dissolve at a concentration of 1 $\frac{\text{mg}}{\text{ml}}$ in reagent grade water. Dilute further in reagent grade water to a concentration of 200-400 $\frac{\text{units}}{\text{ml}}$.

Procedure

Spectrophotometer Settings: Wavelength: 280 nm Temperature: 25°C

Pipette into each cuvette as follows:

- 0.5 M Phosphate buffer, pH 6.5 - 1.0 ml
- 0.001 M L-Tyrosine - 1.0 ml
- Reagent grade water - 0.9 ml

Oxygenate this reaction mixture by bubbling oxygen into cuvettes through a capillary tube for 4-5 minutes. Transfer cuvettes to the spectrophotometer and record A_{280} for 4-5 minutes to achieve temperature equilibration and to establish blank rate, if any. Add 0.1 ml of appropriately diluted enzyme and record A_{280} for 10-12 minutes. Determine ΔA_{280} from the linear portion of the curve. A non-linear lag of 2-3 minutes can be expected.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{280}}{\text{min}} \text{ x } 1000}{\text{mg enzyme in reaction}}$$

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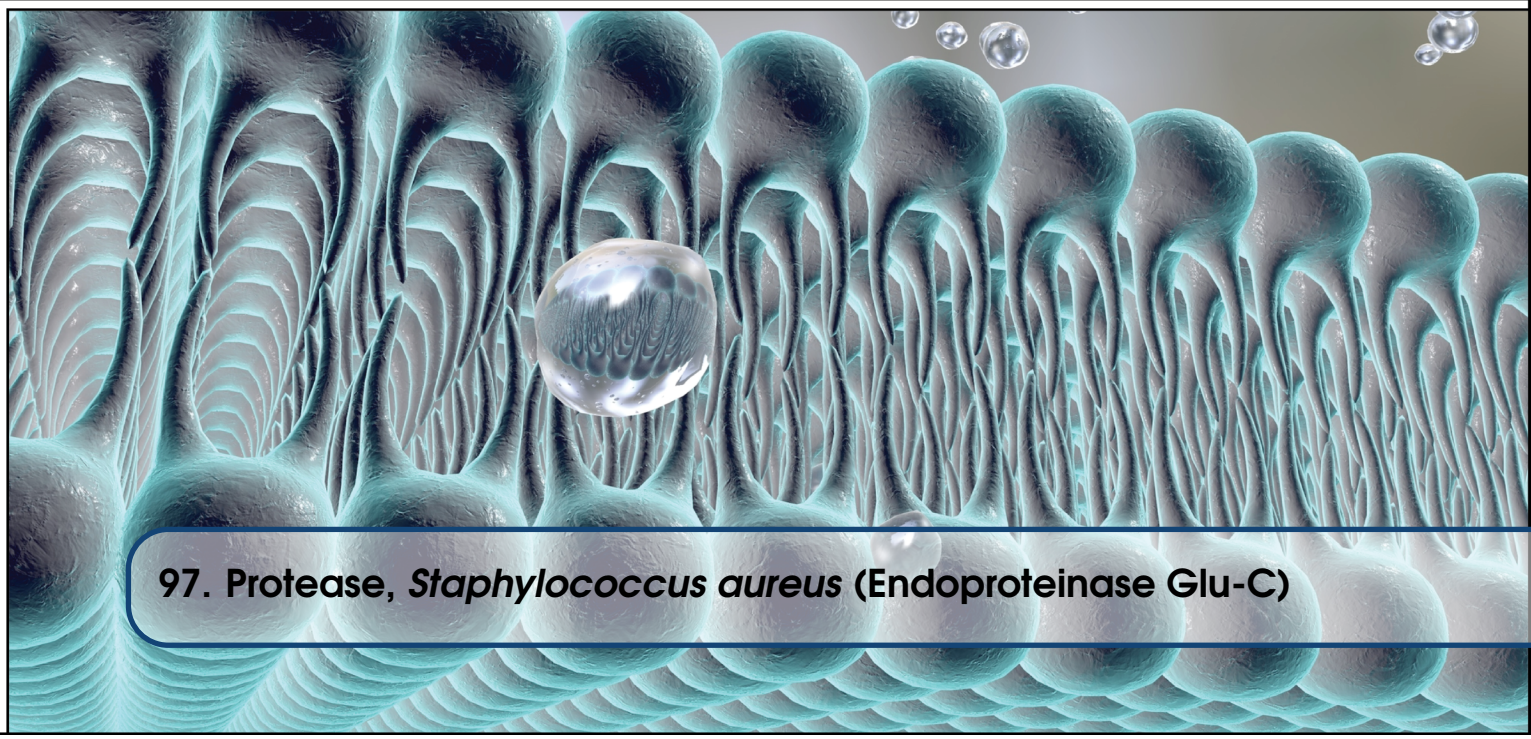

97. Protease, *Staphylococcus aureus* (Endoproteinase Glu-C)

Protease *Staphylococcus aureus*, V8 (endoproteinase-Glu-C) specifically cleaves peptide bonds on the COOH-terminal side of either aspartic or glutamic acids (Drapeau *et al.* 1972). Houmard and Drapeau (1972) report that in the presence of ammonium buffers the enzyme specificity can be limited to glutamoyl bonds. Its rather unique specificity, which can be considered as opposite to that of trypsin, makes it a useful tool for protein chemistry and peptide mapping studies (Cleveland *et al.* 1977) and (Hall *et al.* 1978).

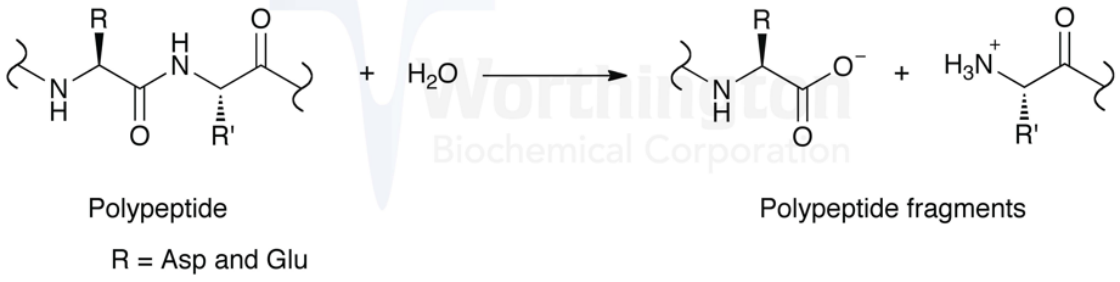

Figure 97.1: Enzymatic Reaction - Protease, *Staphylococcus aureus* (Endoproteinase Glu-C)

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Staphylococcus aureus*, V8

IUB

- 3.4.21.19

CAS

- 66676-43-5

Molecular Weight

- 27,000 (Drapeau 1978)

Optimal pH

- 4.0 and 7.8 with hemoglobin substrate. (Drapeau *et al.* 1972)

Extinction Coefficient

- $E_{280}^{1\%} = 4.26$ (Houmard 1976)

Inhibitors

- Diisopropyl fluorophosphate (DFP) and monovalent anions such as F^-, Cl^-, Br^-, CH_3COO^-, and NO_3^- (Houmard 1976).

Assay Information

Method

- Enzyme activity is determined by the casein digestion assay described by Drapeau (1976). Since substrate grade caseins can vary from lot to lot and according to the manner produced, the standardization of casein digestion assays has been difficult to achieve. The use of a reference enzyme preparation is suggested. One unit is that amount of enzyme which releases acid soluble fragments equivalent to 0.001 A_{280} per minute at 37°C and pH 7.8 under the specified conditions.

Reagents

- 1% Casein in 0.05 M Tris·PO_4 buffer, pH 7.8. (Dissolve 1 gram Hammersten casein in 50 ml 0.01 N NaOH with gentle heating and stirring. Add 40 ml reagent grade water and 5.0 ml 1.0 M Tris. Adjust pH to 7.8 with H_3PO_4 and q.s. to 100 ml.)
- 10% Trichloroacetic acid (TCA)

Enzyme

- Dissolve at 1 $\frac{mg}{ml}$ in reagent grade water.

Procedure

Equilibrate a series of tubes with 5.0 ml of 1% casein at 37°C for 5 minutes. At zero time add 10 μl or 20 μl of enzyme. Mix. Include a reagent blank. Exactly ten minutes after adding sample, stop reaction by adding 5.0 ml TCA. Mix. Allow tubes to stand ten minutes and then filter. Read A_{280} of the filtrate.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{(A_{280\ \text{Test}} - A_{280\ \text{Reagent Blank}})}{10 \text{ min x mg enzyme in reaction}}$$

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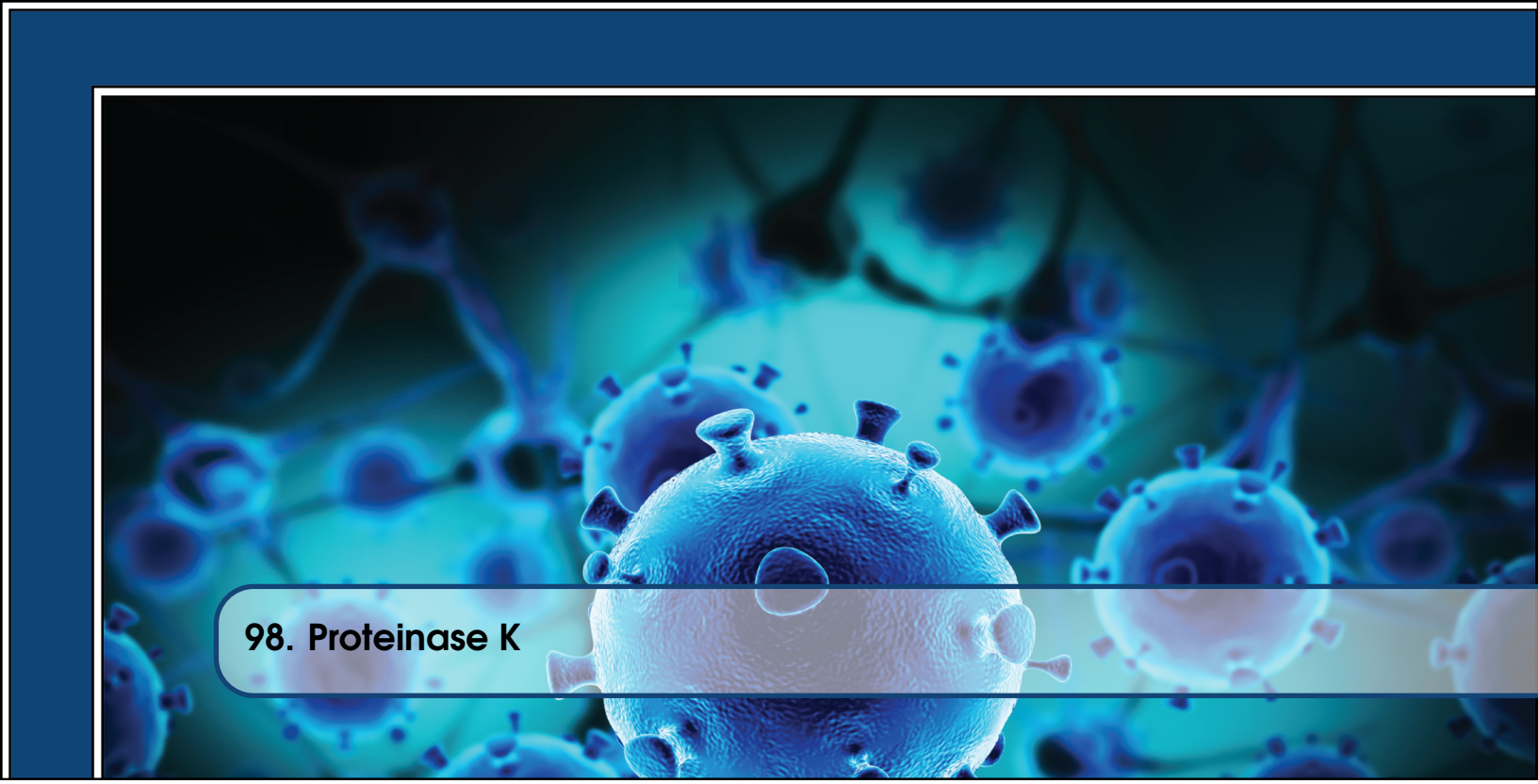

98. Proteinase K

Tritirachium Alkaline Proteinase

Proteinase K (PROK) is a serine endopeptidase with a broad spectrum of action, isolated from the filtrate of the fungus *Tritirachium album limber*.

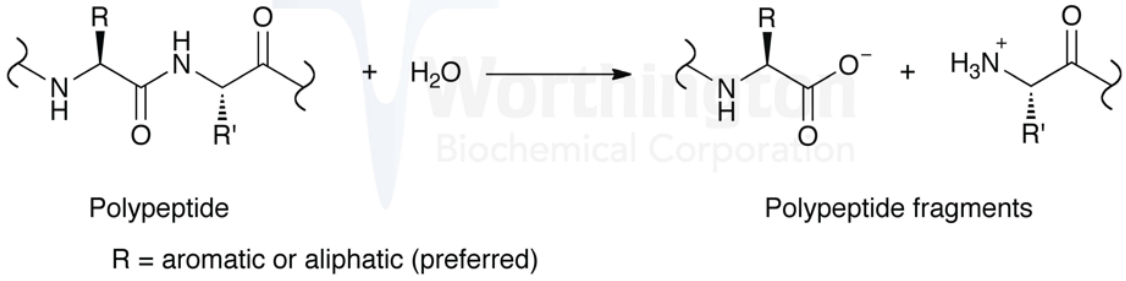

Figure 98.1: Enzymatic Reaction - Proteinase K

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Tritirachium album limber*

Specificity

In addition to cleavage of peptide bonds, it is able to catalyze peptide amide hydrolysis. Proteinase K is inactivated by diisopropyl fluorophosphate (DFP) or phenyl methane sulphonyl fluoride (PMSF). Chelating agents such as citrate and EDTA have no affect on the enzyme activity.

IUB

- 3.4.21.64

CAS

- 39450-01-6

Molecular Weight

- 28,900 daltons

Optimal pH

- 7.5 - 12, using denatured hemoglobin as substrate

Applications

- Proteinase K is very useful in the isolation of highly native, undamaged DNAs or RNAs, since most microbial or mammalian DNases and RNases are rapidly inactivated by the enzyme, particularly in the presence of 0.5 - 1% SDS.

Stability/Storage

- Although calcium ions do not affect the enzyme activity, they do contribute to its stability when present at a concentration of 1 - 5 μmoles. An interesting characteristic of proteinase K is that it retains its activity in the presence of sodium dodecyl sulphate (SDS) or urea. (0.5 - 1% SDS and 1 - 4 M urea). Raising the temperature of the reaction from 37°C to 50 - 60°C can increase the activity several folds. A special feature of proteinase K is its ability to digest native proteins, thereby inactivating enzymes such as DNase and RNase without recourse to a denaturation process.
- PROK: Store at 2 - 8°C; PROKS: Store at -20°C
- Worthington PROK is supplied as a highly purified lyophilized powder. Code PROKS is a 20$\frac{\text{mg}}{\text{ml}}$ solution containing 50% glycerol. Both are tested to be free of DNase and RNase.

Assay Information

Method

- Proteinase K hydrolyzes hemoglobin denatured with urea, and liberates Folin Postive amino acids and peptides, which are determined as tyrosine equivalents. 1 unit releases 1 μmole of Folin positive amino acid at 37°C, pH 7.5, using denatured hemoglobin as substrate.

Reagents

- 0.05 N HCl - Dilute 0.82 ml conc. HCl to 200 ml with reagent grade water.
- 0.5 M NaOH - Dissolve 4.0 g NaOH in 200 ml reagent grade water.
- Buffer-Substrate - Dissolve 2.0 g hemoglobin in 35 ml reagent grade water, add 36.0 g urea and 16 ml 0.5 M NaOH. Stir for 30 - 60 minutes at room temperatue. Add 0.618 g boric acid and stir. Adjust the pH to 7.5 with 5 N HCl and q.s. to 100 ml.
- Tyrosine standard (2.5 nmol/L) - Dissolve 45.3 mg tyrosine in 100 ml of 0.05 N HCl.
- 0.3 M Trichloroacetic acid - Dissolve 9.8 g trichloroacetic acid in 200 ml reagent grade water.
- Folin Reagent - Add 10 ml Folin-Ciocalteus Phenol Reagent to 20 ml reagent grade water.

Enzyme

- Dissolve 10 mg lyophilized material in 1 ml reagent grade water. Prepare a 1:1000 dilution with water immediately before use.

Procedure

Label clear glass test tubes for blank, standard, and test. Add 2.5 ml buffer-substrate and incubate for 5 minutes at 37°C. Start reaction by adding 0.2 ml tyrosine standard to the standard tube, 0.2 ml of sample to the test, and 0.2 ml of 0.05 N HCl to the blank. Incubate for 10 minutes at 37°C. Stop reaction by the addition of 5.0 ml trichloroacetic acid. Mix, then add 0.2 ml of sample to the blank and standard, and add 0.2 ml of 0.05 N HCl to the test. Mix and let stand for 10 minutes at room temperature, filter and pipette into test tubes 1.0 ml of filtrate, 2.0 ml of 0.5 N NaOH, and 0.6 ml of Folin Reagent. Mix well. Let stand for 15 minutes and read A_{578}.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{0.5\ \mu\text{moles tyrosine}}{0.2\text{ ml x }10\text{ min}} \text{ x } \frac{(A_{578}\text{ of sample - }A_{578}\text{ of blank})}{A_{578}\text{ of standard}} \text{ x Dilution}$

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99. Pyruvate Kinase

ATP: Pyruvate O^2-Phosphotransferase

Pyruvate kinase (PK) is a key enzyme in glycogen metabolism. Mammalian PK of different tissues are distinct, their characteristics being related to tissue metabolic requirements (Bigley *et al.* 1974; Boivin and Galand 1974). The enzyme has been well reviewed by Kayne (1973).

The pyruvate kinase most readily obtainable and hence most widely studied, is that from rabbit muscle. It has found extensive use in the quantitative determination of ADP and of enzymes that catalyze the formation of ADP. Pyruvate kinase catalyzes the reaction:

$$\text{Phosphoenolpyruvate} + \text{ADP} \rightleftarrows \text{pyruvate} + \text{ATP} \tag{99.1}$$

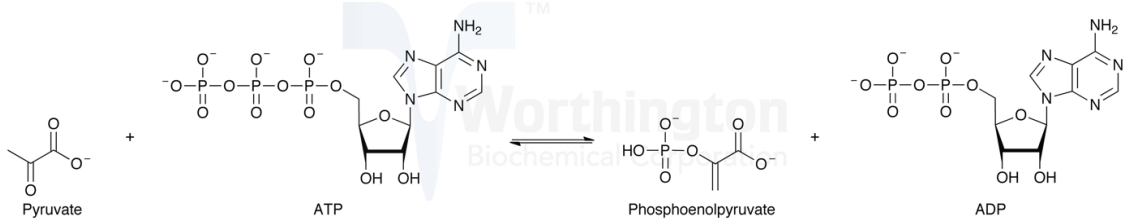

Figure 99.1: Enzymatic Reaction - Pyruvate Kinase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Rabbit Muscle

Specificity

Hohnadel and Cooper (1973) report on specificity with respect to the nucleotide substrate. Phosphate from phosphoenolpyruvate (PEP) is transferred to ADP, GDP, UDP, or CDP with activity decreasing in this order. (See also Kayne 1973)

Composition

The enzyme is tetrameric (subunit M.W.: 57,000) with four metal binding sites (Melamud and Mildvan 1975). On subunit dissociation, a dimeric intermediate is formed (Davies and Kaplan 1972). There is no covalently bound prosthetic group. Cottam *et al.* (1969) determined the constituent amino acids and McPherson and Rich (1972) an x-ray structural analysis. Kwan *et al.* (1975) have reported on the metal-binding sites. PK has an absolute requirement for a divalent metal ion and a monovalent metal ion. Mg^{2+} and K^{+} probably fill these needs *in vivo*; (Kayne and Price 1972). Erhard and Davis (1975) indicate that the same active site catalyzes hydrolysis of phosphoenolpyruvate. Flashner *et al.* (1972 and 1973) indicate that four sulfhydryl groups have a role at the active site. (See also Nowak and Mildvan 1972). Brevet *et al.* (1975) characterize two binding sites, one for the nucleoside substrate (metal dependent) and one for the acceptor.

IUB

- 2.7.1.40

Molecular Weight

- 237,000 (Cottam *et al.* 1969)

Optimal pH

- 7.5 (B*ü*cher and Pfeiderer 1955)

Extinction Coefficient

- $E^{1\%}_{280}$ = 5.4 (Cottam *et al.* 1969)

Inhibitors

- Activity is inhibited by Ca^{2+} (Betts *et al.* 1968; Mildvan and Cohn 1965). Fluorophosphate inhibits the enzyme reaction competitively with respect to PEP (Mildvan and Cohn 1967). ATP inhibits the reaction by removal of Mg^{2+} from the substrate MgADP-. ATP also appears to inhibit the reaction competitively with respect to both ADP and PEP if the Mg^{2+} concentration is higher than that of ATP (Holmsen and Storm 1969). Inactivation is also observed in the presence of pyridoxal 5'-phosphate (Johnson and Deal 1970). Creatine phosphate appears to be

competitive with PEP (Kemp 1973). Bondar and Pon (1969) reported evidence of an endogenous inhibitor removable by chromatography. See also Davidoff and Carr (1973) and Kayne and Price (1972).

Activity

- The kinetics of the reaction have been dealt with by Ainsworth and MacFarlane (1973). PK, in addition to its phosphorylation of ADP by phosphoenolpyruvate, also catalyzes the phosphorylation of fluoride (Tietz and Ochoa 1958), hydroxylamine and glycolate (Kayne 1974) by ATP. Anderson and Randall (1975) indicate that rabbit muscle enzyme is not activated by fructose-1,6-diphosphate as in sturgeon muscle and rabbit liver PK. Giles *et al*. (1975) have reported on the kinetics of the reverse reaction. See also Erhard and Davis (1975).

Assay Information

Method

- The reaction velocity is determined in a lactate dehydrogenase coupled assay system by measuring the decrease in absorbance at 340 nm resulting from the oxidation of NADH.
- NADH + Pyruvate $\rightarrow$ Lactate + NAD
- One unit of activity causes the oxidation of one micromole of NADH per minute at 25°C and pH 7.6 under the specified conditions.

Reagents

- 0.05 M Imidazole · HCl buffer, pH 7.6, containing 0.12 M potassium chloride and 0.062 M magnesium sulfate
- 45 mM Adenosine diphosphate
- 45 mM Phosphoenolpyruvate
- 6.6 mM NADH
- Lactate dehydrogenase (Code: BHLDHC). Dilute lactate dehydrogenase to a concentration of 1300-1400 $\frac{\text{units}}{\text{ml}}$ in above imidazole buffer. Keep cold during use.

Enzyme

- Dilute immediately before use to obtain a rate of 0.02-0.04 $\frac{\Delta A}{\text{min}}$.
- The protein concentration of a solution of the purified enzyme may be determined as follows:

Procedure

Spectrophotometer Settings: Wavelength: 340 nm Temperature: 25°C

Pipette into cuvettes as follows:

- 0.05 M Imidazole-HCl buffer, pH 7.6 - 2.7 ml
- 45 mM Adenosine diphosphate - 0.1 ml
- 6.6 mM NADH - 0.1 ml
- 45 mM Phosphoenolpyruvate - 0.1 ml
- Lactate dehydrogenase - 0.01 ml

Mix well and incubate in spectrophotometer for 4-5 minutes to achieve temperature equilibrium and establish blank rate, if any. Add 0.01 ml of diluted enzyme and record decrease in A_{340} for 4-5 minutes. Calculate $\frac{\Delta A_{340}}{\text{min}}$ from the initial linear portion of the curve.
Note: Initial absorbance at 340 nm should be 1.4 ± 0.1. If not, the NADH may be impure or improperly prepared and should not be used.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{340}}{\text{min}}}{6.22 \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$$

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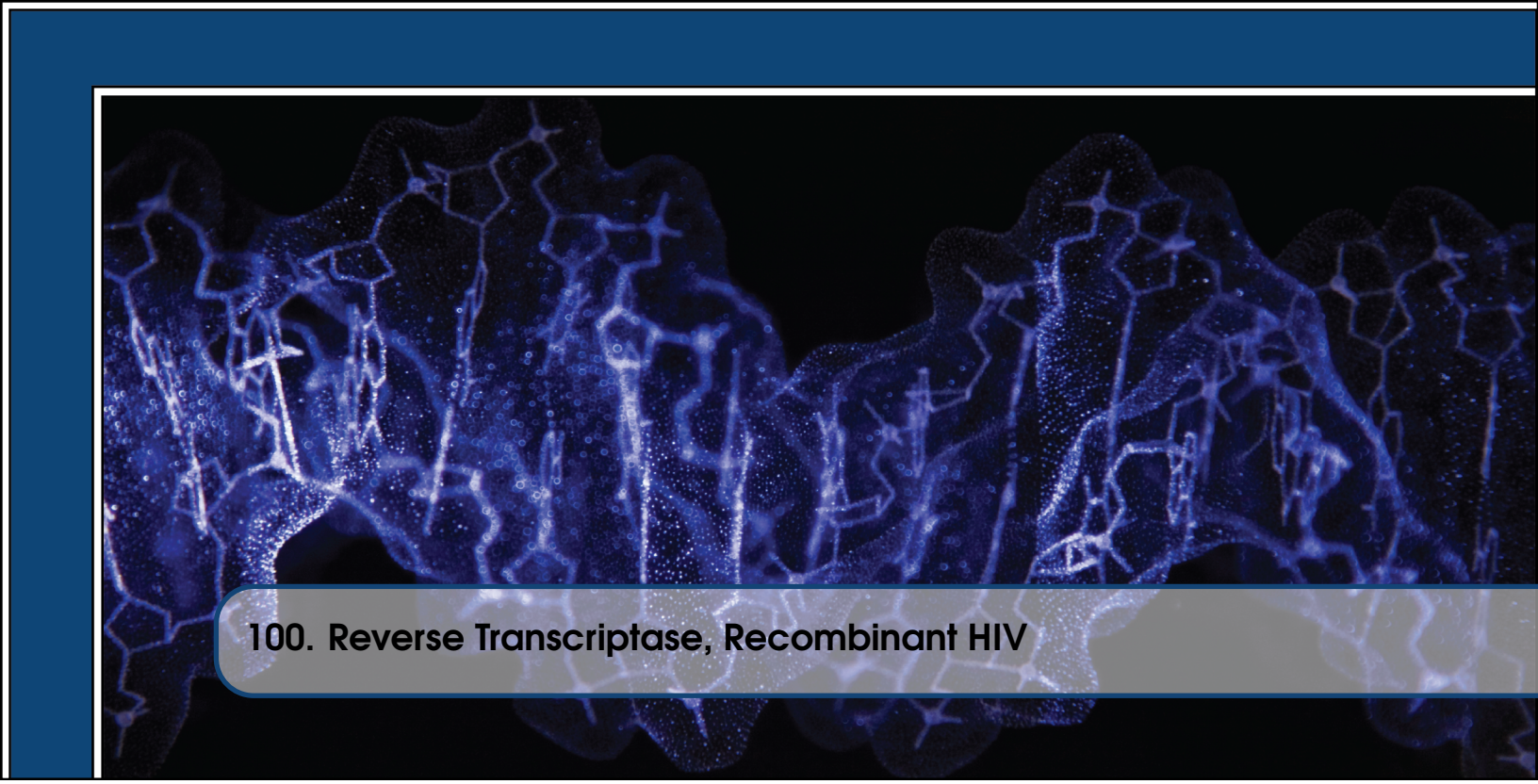

100. Reverse Transcriptase, Recombinant HIV

Deoxynucleoside-Triphosphate: DNA Deoxynucleotidyltransferase (RNA-Directed)

Reverse transcriptases are enzymes encoded in retroviruses viral genome. The enzyme is responsible for transcription of the viral RNA to produce a dsDNA that can be inserted into the host genome.

Reverse transcriptases are multifunctional enzymes. These enzymes exhibit an RNA and DNA directed polymerase activity. In addition reverse transcriptases catalyze the degradation of RNA in an RNA-DNA hybrid. The exonucleolytic activity proceeds in a $5' \rightarrow 3'$ and in a $3' \rightarrow 5'$ direction. The RNA or DNA directed activity requires a template (RNA or DNA) and a primer (See Reaction Images).

Characteristics of Reverse Transcriptase from recombinant *Escherichia coli*

The recombinant HIV reverse transcriptase consists of a heterodimer , with subunits having a MW of 66,000 and 51,000 daltons (p66/p51 heterodimer)

Optimal activity is obtained at pH 8. Mg^{++} ions and sulfhydryl reagents are required. For RNA transcription a RNA template and deoxyoligonucleotides primer are required. The primer may be oligo dT 8-12 or a mixture of DNAs with random or specific sequences. The resulting DNA transcribed is referred to as complementary DNA (cDNA). Reverse transcriptases exhibit a DNA directed DNA polymerase. This activity lacks the $3' \rightarrow 5'$ exonuclease activity usually associated with bacterial DNA dependent DNA polymerase.

A third activity of the HIV reverse transcriptase is RNase H. RNase H degrades RNA in RNA:DNA hybrid. Analysis of the structure-function relqationship between the polymerase and RNase H activities

indicates that the two activities reside in separate segments of the molecule. While the polymerase domain is contained within the NH2-terminal 51KDa, the RNase H activity is located in COOH-terminal 15 KDa of the p66 subunit.

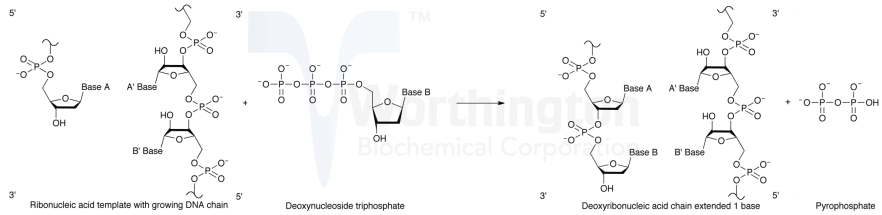

Figure 100.1: Enzymatic Reaction: Reverse Transcription

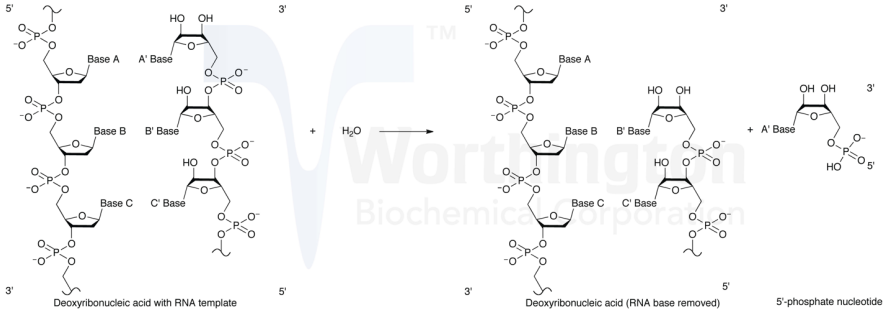

Figure 100.2: Enzymatic Reaction: 3' to 5' Exonuclease Activity

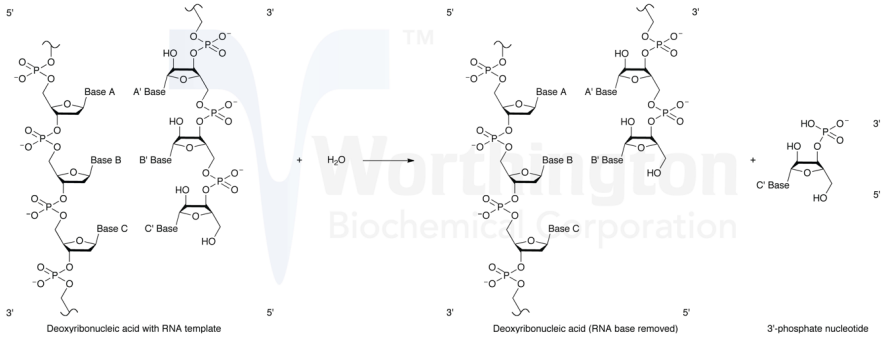

Figure 100.3: Enzymatic Reaction: 5' to 3' Exonuclease Activity

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Escherichia coli*

IUB

- 2.7.7.49

CAS

- 9068-38-6

Applications

- HIV reverse transcriptase is used for research on the AIDS primer. However it can be substituted for AMV reverse transcriptase, which is mainly used to transcribe mRNA into double stranded cDNA, that can be inserted into prokaryotic vectors. The enzyme can also be used with either single stranded DNA or RNA templates to make probes for use in hybridization experiments. It can be used for labeling the termini of DNA fragments with protruding 5' termini. The enzyme can also be used to sequence DNAs by the dideoxy chain termination method of Sanger when the Klenow fragment of *Escherichia coli* DNA polymerase I, or the T7 DNA polymerase yield unsatisfactory results.

Assay Information

Reagents

- 0.05 M Tris, pH 8.3, containing 0.008 M $MgCl_2$
- 1 $\frac{mg}{ml}$ polyadenylic acid in water (poly A)
- DNA primer: Oligo d(T)12-18
- 1 μmole dTTP/mL stock solution
- (methyl-3H)-Thymidine 5'-triphosphate (^{3}H-dTTP)
- dTTP-^{3}H-dTTP working mix: Add 1-2 μl ^{3}H-dTTP per mL of 100 nmol/mL dTTP in order to obtain 1 to 1.5 x 10^5 cpm/mL
- 1% bovine serum albumin
- 10% perchloric acid
- 1% perchloric acid

Buffer substrate reaction mixture. Prepare immediately before use. For each 1mL of reaction mixture required mix the following:

- 0.7 mL Tris/HCl, pH 8.3, 0.008M $MgCl_2$
- 0.3 mL 1 $\frac{mg}{ml}$ poly(A) RNA template
- 0.005 mL 0.02 $\frac{mg}{ml}$ oligo d(T)12-18 DNA primer
- 0.02mL 1% BSA

Enzyme

- dilute as needed wtih 0.05M Tris $\cdot$ HCl, pH 8.3, 0.008M $MgCl_2$ containing 0.1 $\frac{mg}{ml}$ (1%) BSA

Procedure

Pipette into each tube as follows:

- Buffer substrate mix - 0.1 ml

- dTTP-^{3}H-dTTP - 0.1 ml
- Enzyme - 5 to 10 μl

Incubate 20 minutes at 37°C. Stop reaction by adding 1 ml 10% cold perchloric acid. Filter through 0.2 μ manifold filters used with Millipore vacuum manifold. Wash four times using 2 mL 1% cold perchloric acid/wash. Transfer filter to scintillation vials. Add 2 mL Cellosolve® (or 2-methoxyethanol) to dissolve filter. Filters become opaque upon addition of Cellosolve® . Make sure filters are dissolved before proceeding. Add 10 mL scintillation cocktail and count.

Calculation

- $\frac{\text{Units}}{\text{ml}} = \frac{\text{reaction cpm x 10}}{\text{total cpm x reaction volume (ml)}}$
- $\frac{\text{Units}}{\text{mgP}} = \frac{\frac{\text{Units}}{\text{ml}}}{\frac{\text{mgP}}{\text{ml}}\ \text{(Lowry)}}$

Notes

- One unit incorporates 1 nanomole of tritiated dTMP into acid insoluble productsusing poly(A)·oligo(dT) 12-18 as the template-primer in 20 minutes at 37°C.

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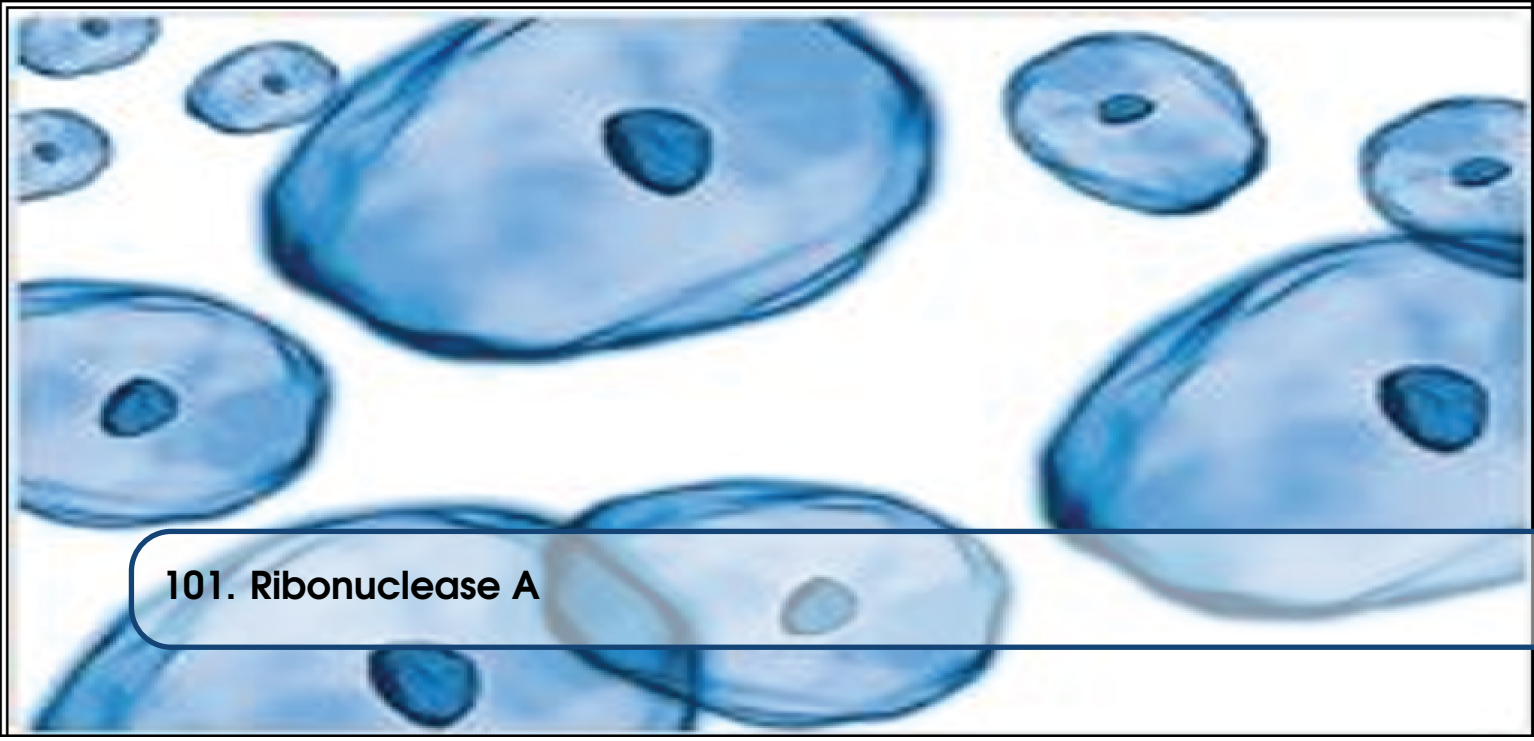

101. Ribonuclease A

Pancreatic ribonuclease (RNase) is an endoribonuclease. It catalyzes the cleavage of the phosphodiester bond between the 5'-ribose of a nucleotide and the phosphate group attached to the 3'-ribose of an adjacent pyrimidine nucleotide. This cleavage forms a 2',3'-cyclic phosphate, which is then hydrolyzed to the corresponding 3'-nucleoside phosphate.

RNase is found in greatest quantity in ruminant pancrease (Barnard 1969). The major component of the crystalline enzyme is RNase A; a minor component is RNase B. RNase B is the glycosylated form of RNase A (Beintema *et al.* 1976).

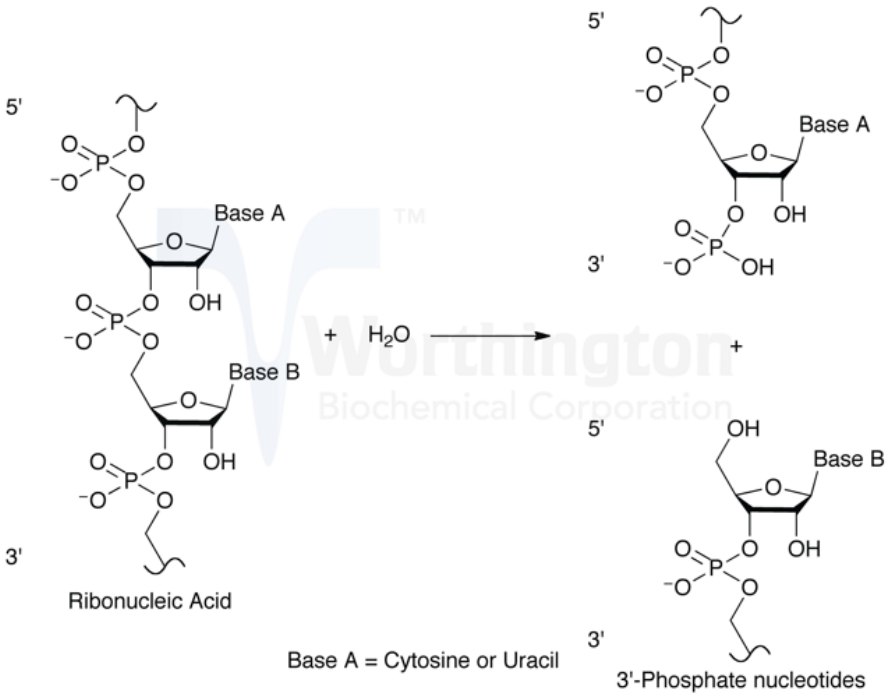

Figure 101.1: Enzymatic Reaction - Ribonuclease A

History

The work of Jones in 1920 is usually cited as the "beginning" of pancreatic ribonuclease (Richards and Wycoff 1971). RNase was isolated by Dubos and Thompson in 1938 and crystallized by Kunitz in 1940.

In 1947 Worthington was the first company to manufacture highly purified crystalline RNase. In the early 1950s, the company Armour prepared crude crystalline enzyme, and offered it at a very affordable price. Through the 1960s and 1970s, RNase A was a favorite to study primarly because it is remarkably thermostable and present at high concentration in an accessible source, bovine pancreas. These studies led to the elucidation of the crystal structure (Anfinsen 1959, Groves 1966, and Scheraga 1967), determination of the amino acid sequence (Smyth *et al.* 1963), identification of the catalytic mechanism (Beers 1960), and clarification of folding pathways (Hantgan *et al.* 1974). RNase A was the first enzyme and third protein for which a correct amino acid sequence was determined (Raines 1998).

Four Nobel prizes have been awarded for work associated with studies of RNase (Anfinsen, Moore, Stein, and Merrifield). The vast literature and numerous studies have made RNase the most extensively studied enzyme of the 20th century (Raines 1998).

Recent work continues to investigate the synthesis and maturation of RNase in the endoplasmic reticulum of live cells (Geiger *et al.* 2010). Much work is also still being dedicated to studying the folding and aggregation of RNase (Benito *et al.* 2008, Iwaoka *et al.* 2008, and Arai *et al.* 2010). The enzyme's role in cancer development and gene regulation is being studied (Shlyakhovenko 2009), and it is being developed into cancer chemotherapeutic agents (Chao *et al.* 2010).

Molecular Characteristics

RNase A is a small protein, the mature enzyme only having 124 amino acid residues, with no carbohydrate attached. RNase A contains 19 of the 20 amino acids, lacking only tryptophan (Nogués *et al.* 1995, and Raines 1998). The three dimensional structure of RNase A is fully encoded by its amino acid sequence (White and Anfinsen 1959, and Raines 1998). All eight human RNase A-like genes are located on chromosome 14. Each encodes a secretory signal sequence and contains an invariant catalytic triad of two histidines and one lysine with a conserved motif (CKXXNTF) (Marshall *et al.* 2008).

The amino acid sequences of many RNase A homologues have been identified, making RNase A a model system for vertebrate molecular evolution (Dyer and Rosenberg 2006). From the sequences and their distribution over a range of species it has been established that RNase A is a modern protein that is evolving rapidly (Doolittle 1992, and Raines 1998).

Specificity

RNase A is specific for pyrimidine nucleoside linkages (Volkin and Cohn 1953). The reaction is believed to take place in two steps. In the first step, the 3',5'-phosphodiester bond is cleaved, while generating a 2',3'-cyclic phosphodiester intermediate. In the second step, the cyclic phosphodiester is hydrolyzed to a 3'-monophosphate group. The first step is nonspecific with respect to the nitrogenous base of the substrate; however, the second step is absolutely specific for pyrimidine nucleotides with terminal 2',3'-cyclic phosphates. RNase B has the same specificity as RNase A toward both cyclic cytidylate and yeast RNA (Plummer and Hirs 1963). RNase A shows a preference for larger substrates (Nogués *et al.* 1995).

The enzyme cleaves at cytidine residues twice as fast as at uridyl residues (Richards and Wyckoff 1971). Thr45 has been found to be most important for mediating the pyrimidine specificity, both by forming hydrogen bonds with pyrimidine bases and sterically excluding purine bases (del Cardayré and Raines 1994). The side chain of Asp83 is important for stabilizing the transition state during the cleavage of uridine-containing substrates; this residue has no effect on the kinetics of cytidine cleavage (del Cardayré and Raines 1995).

Composition

The shape of the protein resembles a kidney, with the active site residues laying in the cleft (Richardson 1981, and Raines 1998). The secondary structure contains long four-stranded anti-parallel beta-sheets and three short alpha-helices (Raines 1998). RNase A contains four disulfide bonds, which are critical to the stability of the native enzyme. Two of these disulfide bonds lie between an alpha-helix and a beta-sheet and contribute more to the thermal stability than do the other two (Klink *et al.* 2000). RNase B is a glycoprotein containing at Asn34 a single oligosaccharide composed of six residues of mannose and two residues of N-acetylglucosamine (Tarentino *et al.* 1970).

IUB

- 3.1.27.5

CAS

- 9001-99-4

Protein Accession Number

- P61823

CATH: Classification (v. 3.3.0)

Class:

- Alpha-Beta

Architecture:

- Roll

Topology:

- P-30 Protein

Molecular Weight

- 13.7 kDa (Hirs *et al.* 1956b)

Optimal pH

- 7.0-7.5 (Brown and Todd 1955)

Isoelectric Point

- 9.3 (Ui 1971)

Extinction Coefficient

- 8,640 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 7.3 (Worthington RNase A)

Active Residue

- Histidine (H12, H119)
- Lysine (K41)

Activators

- Sodium chloride (Weickmann *et al.* 1981)
- Sulfate (Moosavi-Movahedi *et al.* 2006)

Inhibitors

- Heavy metal ions
- Ribonuclease inhibitor (RI), a 50 kDa protein that constitutes $\leq 0.01\%$ of the protein in the cytosol of mammalian cells (Takahashi 1967)
- Uridine-vanadate complexes (Lindquist *et al.* 1973)

Applications

- RNA removal during DNA isolation
- RNA sequence analysis
- RNase protection assays
- RNA quantification or mapping
- Purifying plasmid DNA
- Genomic DNA isolation
- Molecular weight marker

Assay Information

Method

- Standardization of ribonuclease activity has been difficult due to varying rates at which reactions occur as well as to the significant differences in nucleotide patterns in RNA isolated from biological sources. Crook et al. (1960) have published an assay using a synthetic substrate, cytidine 2', 3'-phosphate. Zimmerman and Sandeen (1965) described a sensitive assay using polycytidylic acid.
- The method of Kalnitsky et al. (1959) is used at Worthington. The rate of hydrolysis of yeast RNA at pH 5.0 is determined by measuring the amount of acid soluble oligonucleotide released under defined conditions. One unit causes an increase in absorbance of 1.0 at A_{260} at 37°C and pH 5.0 under the specified conditions.

Reagents

- 0.10 M Sodium acetate buffer, pH 5.0
- 25% Perchloric acid containing 0.75% Uranyl acetate
- 1% Worthington yeast RNA in 0.10 M sodium acetate, pH 5.0. Equilibrate to37°C prior to assay.

Enzyme

- Prepare stock solution at 1 $\frac{\text{mg}}{\text{ml}}$ in reagent grade water. Immediately prior to assay, dilute further to 2, 4, and 6 $\frac{\mu\text{g}}{\text{ml}}$ in 0.10 M sodium acetate, pH 5.0.

Procedure

Pipette one ml of respective enzyme dilution into centrifuge tubes. Include a blank containing one ml of 0.10 M sodium acetate buffer, pH 5.0. Incubate all tubes at 37°C for 5-8 minutes. At timed intervals, add one ml of 1% RNA. Incubate each tube exactly 4 minutes and stop reaction by the addition of one ml of uranyl acetate-perchloric acid solution. Transfer to an ice bath and cool for 5 minutes. Clarify by

centrifugation and dilute 0.1 ml of clear supernatant to 3.0 ml with reagent grade water. Read A_{260} versus blank.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\Delta A_{260} \text{ x } 30}{\text{mg enzyme in reaction mixture}}$

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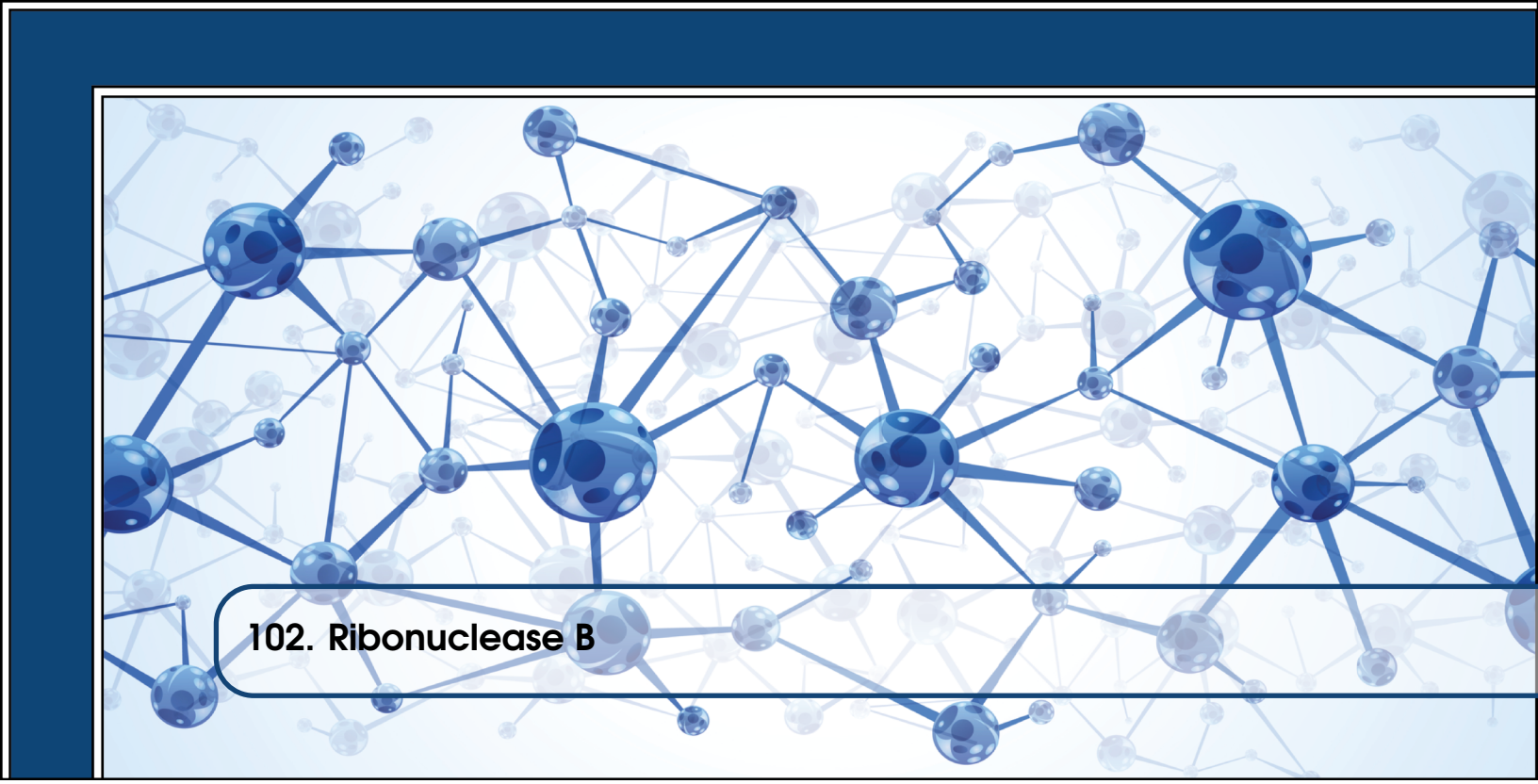

102. Ribonuclease B

Pancreatic ribonuclease (RNase) is an endoribonuclease. It catalyzes the cleavage of the phosphodiester bond between the 5'-ribose of a nucleotide and the phosphate group attached to the 3'-ribose of an adjacent pyrimidine nucleotide. This cleavage forms a 2',3'-cyclic phosphate, which is then hydrolyzed to the corresponding 3'-nucleoside phosphate.

RNase is found in greatest quantity in ruminant pancrease (Barnard 1969). The major component of the crystalline enzyme is RNase A; a minor component is RNase B. RNase B is the glycosylated form of RNase A (Beintema *et al.* 1976).

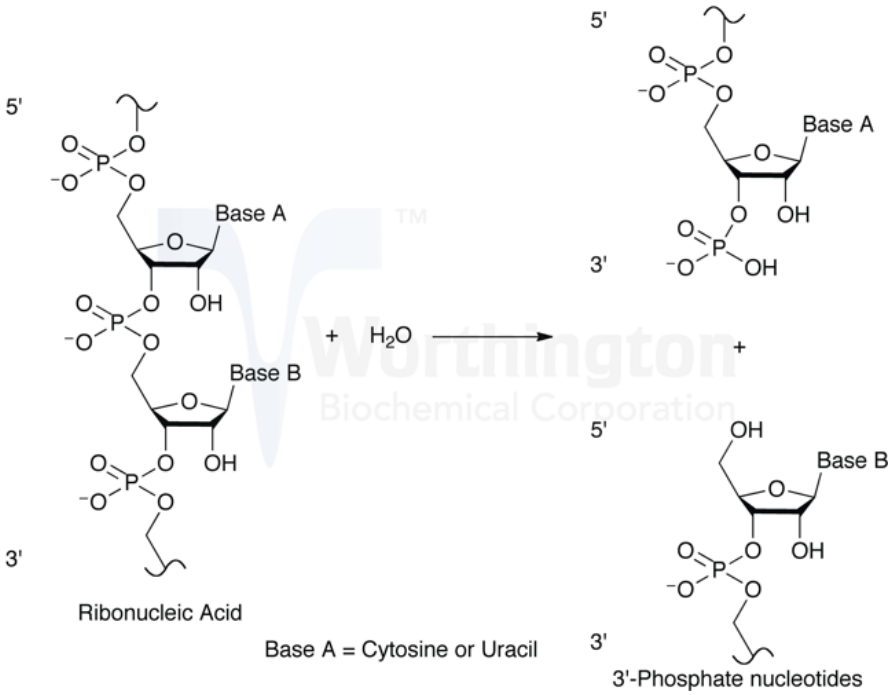

Figure 102.1: Enzymatic Reaction - Ribonuclease B

History

The work of Jones in 1920 is usually cited as the "beginning" of pancreatic ribonuclease (Richards and Wycoff 1971). RNase was isolated by Dubos and Thompson in 1938 and crystallized by Kunitz in 1940.

In 1947 Worthington was the first company to manufacture highly purified crystalline RNase. In the early 1950s, the company Armour prepared crude crystalline enzyme, and offered it at a very affordable price. Through the 1960s and 1970s, RNase A was a favorite to study primarly because it is remarkably thermostable and present at high concentration in an accessible source, bovine pancreas. These studies led to the elucidation of the crystal structure (Anfinsen 1959, Groves 1966, and Scheraga 1967), determination of the amino acid sequence (Smyth *et al.* 1963), identification of the catalytic mechanism (Beers 1960), and clarification of folding pathways (Hantgan *et al.* 1974). RNase A was the first enzyme and third protein for which a correct amino acid sequence was determined (Raines 1998).

Four Nobel prizes have been awarded for work associated with studies of RNase (Anfinsen, Moore, Stein, and Merrifield). The vast literature and numerous studies have made RNase the most extensively studied enzyme of the 20th century (Raines 1998).

Recent work continues to investigate the synthesis and maturation of RNase in the endoplasmic reticulum of live cells (Geiger *et al.* 2010). Much work is also still being dedicated to studying the folding and aggregation of RNase (Benito *et al.* 2008, Iwaoka *et al.* 2008, and Arai *et al.* 2010). The enzyme's role in cancer development and gene regulation is being studied (Shlyakhovenko 2009), and it is being developed into cancer chemotherapeutic agents (Chao *et al.* 2010).

Molecular Characteristics

RNase A is a small protein, the mature enzyme only having 124 amino acid residues, with no carbohydrate attached. RNase A contains 19 of the 20 amino acids, lacking only tryptophan (Nogués *et al.* 1995, and Raines 1998). The three dimensional structure of RNase A is fully encoded by its amino acid sequence (White and Anfinsen 1959, and Raines 1998). All eight human RNase A-like genes are located on chromosome 14. Each encodes a secretory signal sequence and contains an invariant catalytic triad of two histidines and one lysine with a conserved motif (CKXXNTF) (Marshall *et al.* 2008).

The amino acid sequences of many RNase A homologues have been identified, making RNase A a model system for vertebrate molecular evolution (Dyer and Rosenberg 2006). From the sequences and their distribution over a range of species it has been established that RNase A is a modern protein that is evolving rapidly (Doolittle 1992, and Raines 1998).

Specificity

RNase A is specific for pyrimidine nucleoside linkages (Volkin and Cohn 1953). The reaction is believed to take place in two steps. In the first step, the 3',5'-phosphodiester bond is cleaved, while generating a 2',3'-cyclic phosphodiester intermediate. In the second step, the cyclic phosphodiester is hydrolyzed to a 3'-monophosphate group. The first step is nonspecific with respect to the nitrogenous base of the substrate; however, the second step is absolutely specific for pyrimidine nucleotides with terminal 2',3'-cyclic phosphates. RNase B has the same specificity as RNase A toward both cyclic cytidylate and yeast RNA (Plummer and Hirs 1963). RNase A shows a preference for larger substrates (Nogués *et al.* 1995).

The enzyme cleaves at cytidine residues twice as fast as at uridyl residues (Richards and Wyckoff 1971). Thr45 has been found to be most important for mediating the pyrimidine specificity, both by forming hydrogen bonds with pyrimidine bases and sterically excluding purine bases (del Cardayré and Raines 1994). The side chain of Asp83 is important for stabilizing the transition state during the cleavage of uridine-containing substrates; this residue has no effect on the kinetics of cytidine cleavage (del Cardayré and Raines 1995).

Composition

The shape of the protein resembles a kidney, with the active site residues laying in the cleft (Richardson 1981, and Raines 1998). The secondary structure contains long four-stranded anti-parallel beta-sheets and three short alpha-helices (Raines 1998). RNase A contains four disulfide bonds, which are critical to the stability of the native enzyme. Two of these disulfide bonds lie between an alpha-helix and a beta-sheet and contribute more to the thermal stability than do the other two (Klink *et al.* 2000). RNase B is a glycoprotein containing at Asn34 a single oligosaccharide composed of six residues of mannose and two residues of N-acetylglucosamine (Tarentino *et al.* 1970).

IUB

- 3.1.27.5

CAS

- 9001-99-4

Protein Accession Number

- P61823

CATH: Classification (v. 3.3.0)

Class:

- Alpha-Beta

Architecture:

- Roll

Topology:

- P-30 Protein

Molecular Weight

- RNase B: 14.700 $\pm$ 0.3 (Plummer and Hirs 1963)

Extinction Coefficient

- 8,640 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{280}$ = 5.77 (Theoretical, RNase B)

Active Residue

- Histidine (H12, H119)
- Lysine (K41)

Activators

- Sodium chloride (Weickmann *et al.* 1981)
- Sulfate (Moosavi-Movahedi *et al.* 2006)

Inhibitors

- Heavy metal ions
- Ribonuclease inhibitor (RI), a 50 kDa protein that constitutes $\leq$ 0.01% of the protein in the cytosol of mammalian cells (Takahashi 1967)
- Uridine-vanadate complexes (Lindquist *et al.* 1973)

Applications

- RNA removal during DNA isolation
- RNA sequence analysis
- RNase protection assays
- RNA quantification or mapping
- Purifying plasmid DNA
- Genomic DNA isolation
- Molecular weight marker

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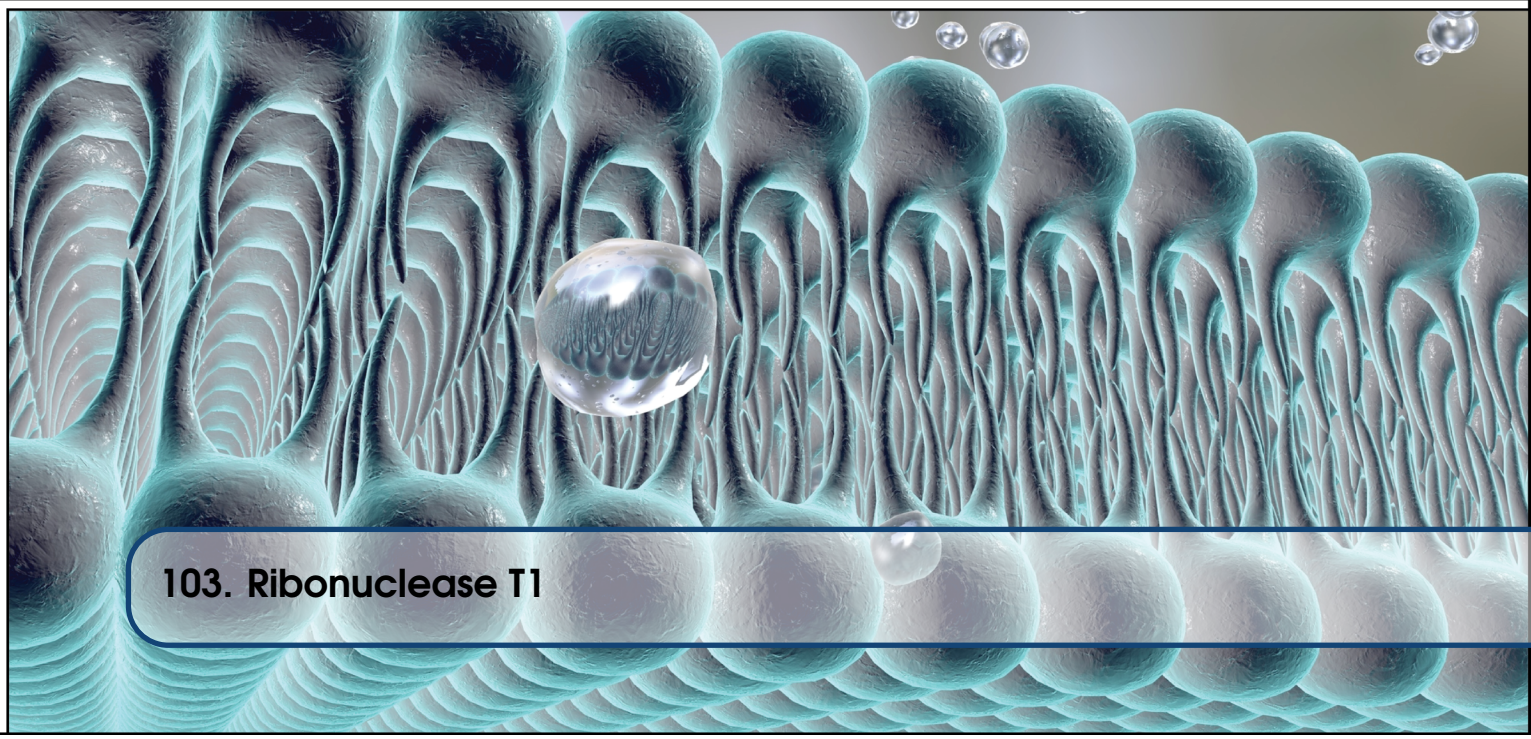

103. Ribonuclease T1

Ribonuclease T1 is a low molecular weight, highly specific, monomeric endoribonuclease. That from the fungus *A. oryzae* is the leading member of the family of microbial enzymes that cleave single stranded RNA by a reaction mechanism involving formation of intermediate 2',3' cyclic phosphates.

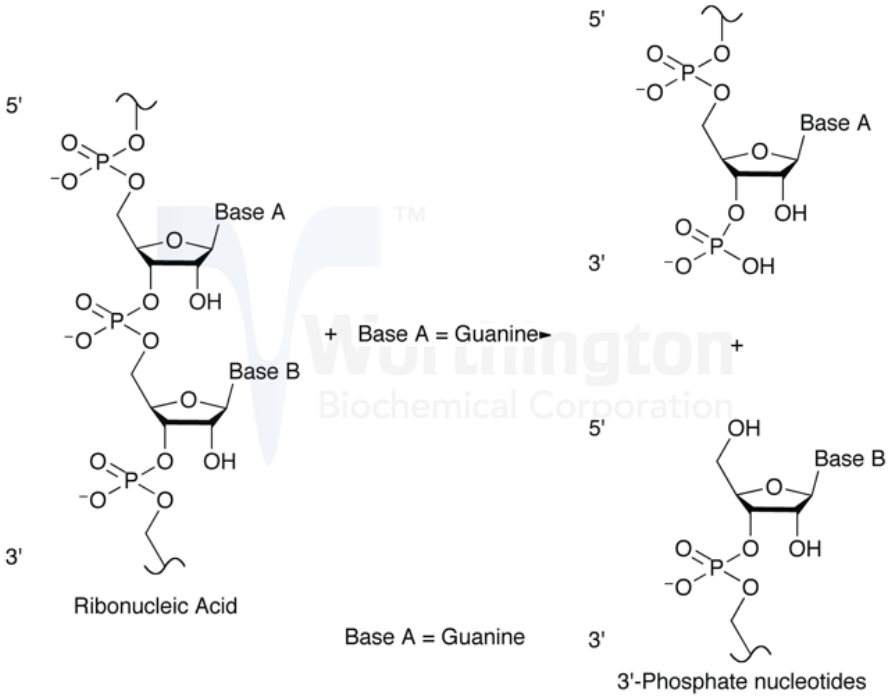

Figure 103.1: Enzymatic Reaction - Ribonuclease T1

History

In 1957, Sato and Egami first discovered ribonuclease T1 in a commercial enzyme mixture from *A. oryzae* called Takadiastase. The primary structure was determined (Takahashi 1971a, b) and revised and confirmed in 1985 (Takahashi 1985).

In the late 1980s, the enzyme was well characterized by NMR spectroscopy, chemical modification, and x-ray crystallography (Hoffman and Rüterjans 1988, Arnie *et al.* 1988, Koepke *et al.* 1989). Although the gene encoding RNase T1 had not yet been cloned, DNA corresponding to the full amino acid sequence of mature RNase T1 was synthesized and expressed in *Escherichia coli* as a fusion protein (Ikehara *et al.* 1986, and Quaas *et al.* 1988). Using chemically synthesized DNA, researchers also modified the active sites to investigate the relationship between structure and function (Nishikawa *et al.* 1986, and Hakoshima *et al.* 1988). The gene (rntA) was cloned and sequenced by Fuji *et al.* in 1995.

RNase T1 continues to be used in RNA mapping experiments, aiding in the determination of RNA secondary structure (Quarrier *et al.* 2010) and identification of a given RNA molecule's origin (Matthiesen and Kirpekar 2009). RNase T1 has been used as a model protein for structural studies (Moors *et al.* 2009, and Yoshida *et al.* 2006).

Molecular Characteristics

RNase T1 is composed of 104 amino acid residues cross-linked by two disulfide bridges. A conserved disulfide bond exists between Cys-6 and Cys-103 (Yoshida 2001). The sequences of RNase N1

(from *Neurospora crassa*), and U1 (from *Ustilago sphaerogena*) are highly homologous to RNase T1 (Takahashi 1985). The nucleotide sequence of the rntA gene contains an open reading frame of 393 bp with one intervening intron (114 bp). The intron is present within the region corresponding to the signal sequence. A sequence related to the TATA box (TATAA) is present at nucleotides 121 to 117, and a CAAT box is present at nucleotides 176 to 173 (Fujii *et al.* 1995).

Specificity

RNase T1 is highly specific in its mode of attack. The enzyme first cleaves RNA (or deaminated RNA) between 3'-guanylic residues (or inosine 3'-phosphate and xanthosine 3'-phosphate) and the 5'-hydroxy residues of adjacent nucleotides with the formation of the corresponding intermediate 2',3'-cyclic phosphates (Takahashi and Moore 1971). This step is reversible and is much faster than the second step. In the second step, the intermediate is hydrolyzed to a 3'-phosphate (Yoshida 2001). All members of the RNase T1 family are guanyl specific or preferential, except RNase U2. It is believed that the highly conserved region from Tyr-42 to Glu-46 is primarily responsible for guanine binding (Yoshida 2001).

Composition

RNase T1 is a low molecular weight, stable, monomeric protein. The architecture consists of four antiparallel beta strands over an alpha helix (Yoshida 2001).

IUB

- 3.1.27.3

CAS

- 9026-12-4

Protein Accession Number

- P00651

CATH: Classification (v. 3.3.0)

Class:
- Alpha Beta

Architecture:
- Roll

Topology:
- Nuclear Transport Factor 2; Chain A

Molecular Weight

- 11 kDa (Egami *et al.* 1964)
- 11.1 kDa (Theoretical)

Optimal pH

- 7.5

Isoelectric Point

- 2.9 (Egami *et al.* 1964)
- 4.12 (Theoretical)

Extinction Coefficient

- 17,690 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$
- $E^{1\%}_{278}$ = 2.9 (Egami *et al.* 1964)

Active Residue

- Histidine (H40 and H92)
- Glutamic acid (E58)
- Arginine (R77)

Activators

- Histidine
- EDTA

Inhibitors

- Ag^{+}, Zn^{2+}, Cu^{2+}, and Hg^{2+} at 1 x 10^{-3} M (strongly inhibitory)

Applications

- Cleavage enzyme for the determination of structure, nearest neighbor frequencies, and sequence of RNA (Miura and Egami 1960, Rushizky and Sober 1962, Miura 1964b, Holley *et al.* 1965, Madison and Kung 1967, and Neelon *et al.* 1967)
- Preparation of nucleoside 2',3'-cyclic phosphates
- Synthesis of oligonucleotides
- Removal of RNA from DNA preparations (Saito and Miura 1963)
- Removal of poly(A) sequences from mRNA in the presence of oligo(dT)
- Removal of mRNA during single-strand cDNA synthesis
- RNA fingerprinting
- Oligodeoxyribonucleotide-directed cleavage of RNA

Assay Information

Method

- The method used at Worthington is essentially that of Egami et al. (1964) based upon the release of acid soluble oligonucleotides following the digestion of yeast RNA. One unit releases acid

soluble oligonucleotides equivalent to one A_{260} in the reaction mixture at 37°C and pH 7.5 for 15 minutes.

Reagents

- 0.2 M Tris · HCl buffer, pH 7.5: Dissolve 2.4 g Tris (MW 121.14) in 95 ml reagent grade water and adjust pH to 7.5. Bring to a final volume of 100 ml with reagent grade water.
- 0.02 M EDTA: Dissolve 745 mg into a final volume of 90 ml reagent grade water. Adjust pH to 7.5 with NaOH and bring to a final volume of 100ml.
- RNA Soluton: Prepare a fresh solution of 12 $\frac{mg}{ml}$ RNA (Roche catalog #109223; if not available consult supervisor) by suspending in approximately 80% of final volume of reagent grade water. Slowly add 1N NaOH so pH approaches 8.0. pH may drop as RNA dissolves. Repeat pH adjustment until RNA is completely dissolved and pH remains steady at 8.0 ± 0.1. QS to final volume wth reagent grade water. Check the A_{260} of the RNA using a 350x dilution to ensure the RNA is at or near 12 $\frac{mg}{ml}$. The A_{260} of a 350x dilution should be 0.85 ±0.05.
- Note: A_{260} (dilution factor) (40 $\frac{\mu g}{ml}$) = $\frac{mg}{ml}$ RNA
- Perchloric acid/uranyl acetate solution: Dilute 22 ml 70% perchloric acid to 100 ml with reagent grade water. Add 750 mg uranyl acetate and dissolve.

Enzyme

- For protein determination, read A_{280}, diluting as needed in reagent grade water and reading vs a 2.8 M ammonium sulfate/reagent grade water blank.

Procedure

Buffer mixture: Prepare according to number of reactions, at the following proportion:
0.25 ml 0.2 M Tris, pH 7.5
0.10 ml 0.02 M EDTA, pH 7.5
0.30 ml deionized water
For 40 sample tubes, prepare buffer mix of:
10 ml 0.2 M Tris, pH 7.5
4 ml 0.02 M EDTA, pH 7.5
12 ml deionized water

Pipette into tubes as follows:
0.65 ml buffer mix 0.10 ml diluted enzyme sample

Include at least 2 tubes with reagent grade water instead of sample as blanks. Incubate tubes in a 37°C water bath for 5 minutes. At timed intervals add 0.25 ml RNA solution to all tubes. Incubate exactly 15 minutes then stop reaction by ading 0.25 ml perchloric acid solution. Centrifuge at 2500 RPM for 5 minutes. Withdraw 0.20ml clear supenatant to tubes containing 4.80 ml reagent grade water. Mix and read A_{260} versus water.

Calculation

- $\frac{\text{units}}{\text{ml}} = \frac{(A_{260}\text{- blank}) \text{ x } 312 \text{ x dilution}}{0.1}$
- Specific Activity = $\frac{\frac{\text{units}}{\text{ml}}}{\frac{\text{mg}}{\text{ml}}}$

Notes

- This assay should be run with dilutions yielding results of A_{260} sample between 0.25 and 0.15 after subtracting blank.

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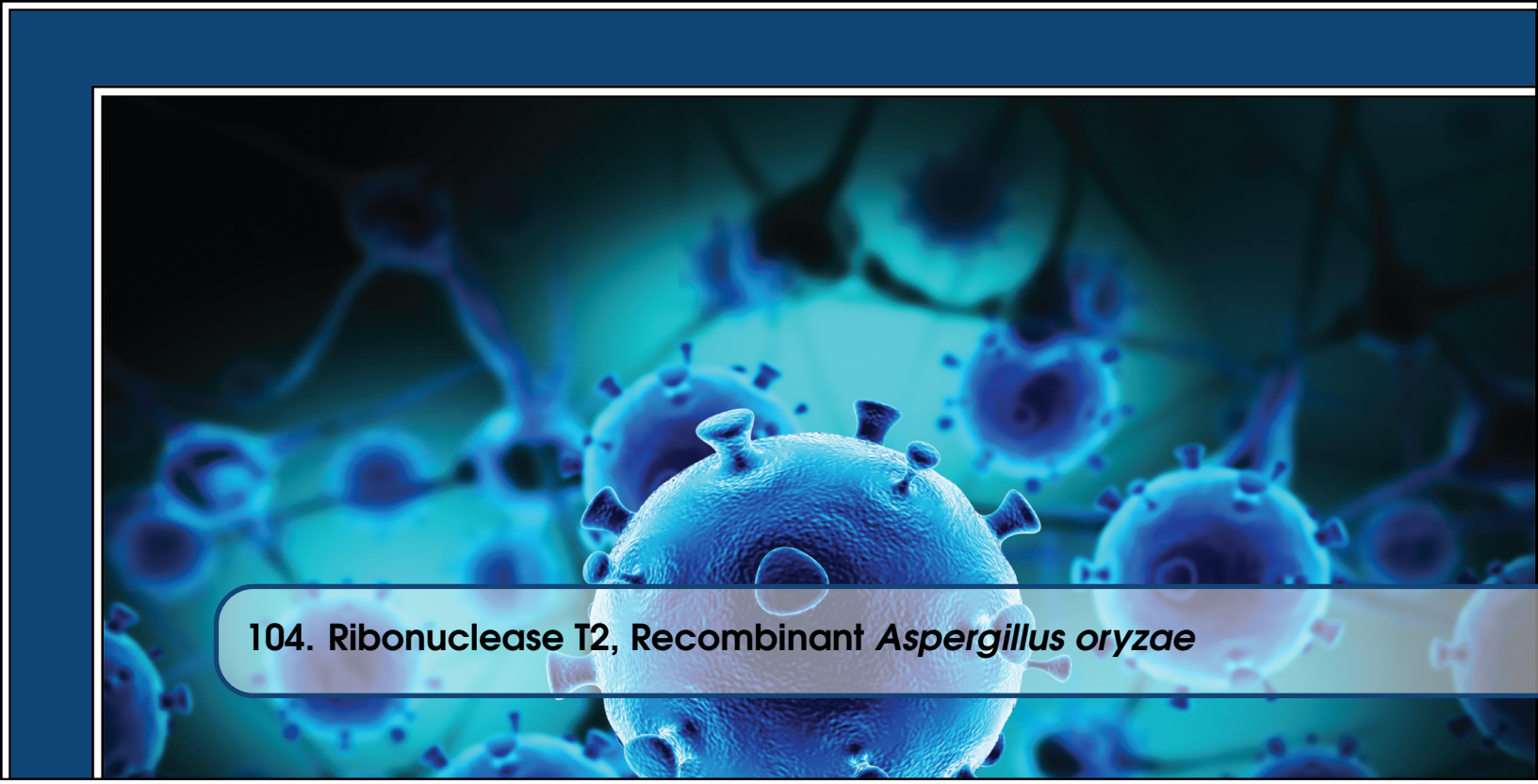

104. Ribonuclease T2, Recombinant *Aspergillus oryzae*

Aspergillus oryzae Ribonuclease T2 is a member of the RNase T2 family of endonucleases that are present in a wide variety of microbial, plant and animal species. In contrast to *Aspergillus oryzae* Ribonuclease T1, which is an exclusively guanylic-acid specific endonuclease, all RNase T2-like enzymes are essentially base non-specific. However, RNase T2 endonucleases from different species can show slight base preferences. The fungal enzymes, including Aspargillus oryzae RNaseT2, show slight base preference in the following order: A > G > C, U. RNase T2 cleaves between the 3'-phosphate residue of one base and the 5'-OH residue of the adjacent nucleotide forming a 2', 3'-cyclic phosphate intermediate followed by the generation of oligonucleotides with 3'-phosphate residues. RNase T2 has a molecular weight of 36 kDa and 12-15% of its mass is composed of carbohydrate.

IUB

- 3.1.27.1

CAS

- 37278-25-4

Optimal pH

- 4.5

Isoelectric Point

- 5

Activators

- EDTA will stimulate activity, especially in the presence of divalent cations.

Inhibitors

- Strongly inhibited by Cu^{2+}, Zn^{2+} and Hg^{2+}
- Ca^{2+}, Mg^{2+} and heparin (to a lesser degree)
- Mononucleotides and RNase T2 digestion products (competitively)

Applications

- 3' analysis of RNA and RNase protection assays

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105. Ribonuclease, E-Rase™ RNase Blend

E-Rase™ is an optimized blend of purified ribonucleases with differing base cleavage specificities designed to achieve more complete digestion of target RNA in applications requiring its removal such as in plasmid purification, ribonuclease protection assays, RT-PCR, etc. Bovine pancreatic RNase A (RPDF) cleaves after U and C whereas *Aspergillus oryzae* RNase T1 (RT1C) preferentially cleaves RNA after G. The combination of these enzymes digests RNA more efficiently than either one alone. E-Rase™ contains 0.5 $\frac{\text{mg}}{\text{ml}}$ RPDF and 100,000 $\frac{\text{units}}{\text{ml}}$ of RT1C and is prepared in 50% glycerol for convenient storage and use.

Unit Definition: One unit is defined as the amount of E-Rase™ required to completely digest 10μg of yeast RNA in 20μl 10mM Tris $\cdot$ HCl, pH 7.5 containing 15mM NaCl in 30 minutes at 37°C.

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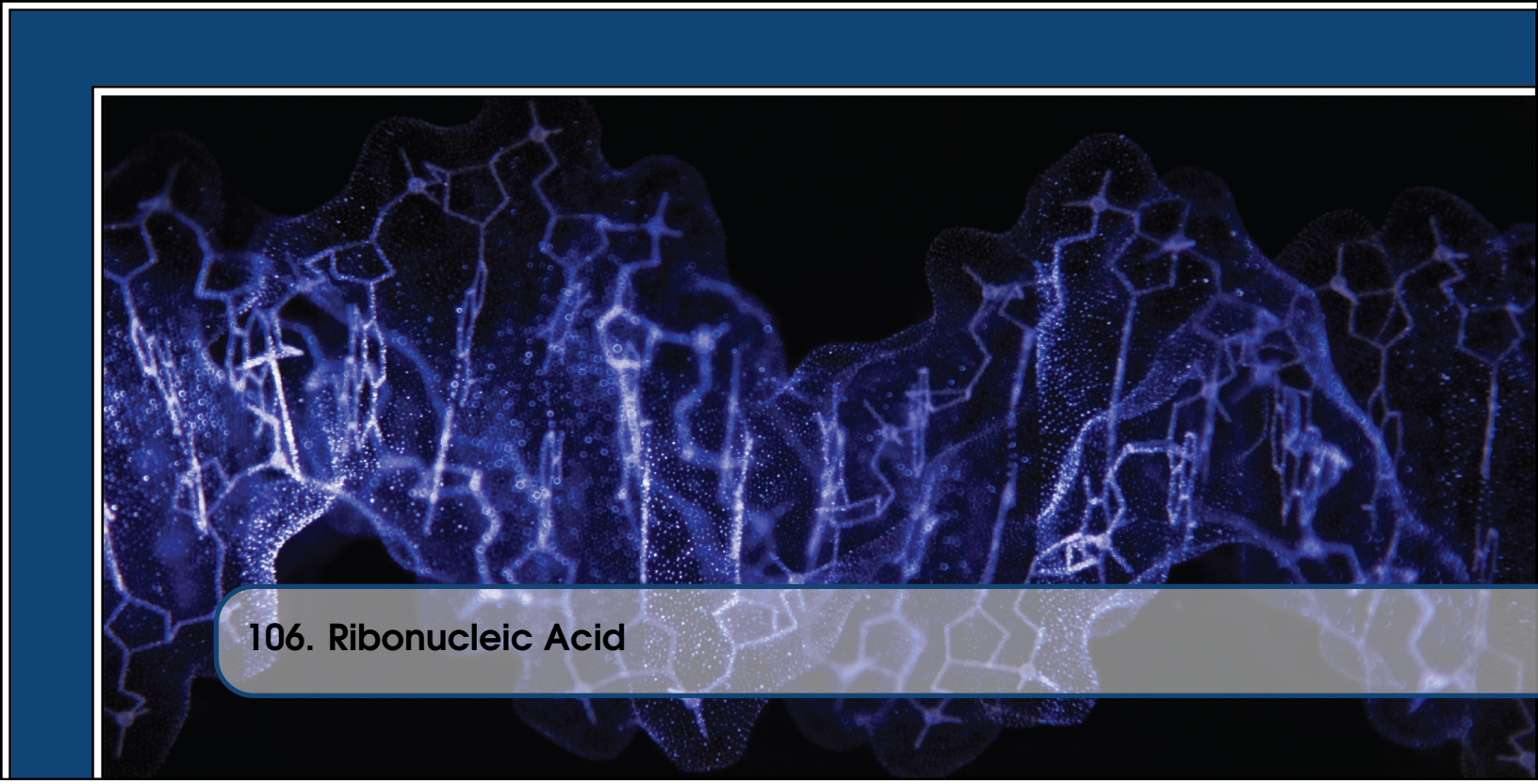

106. Ribonucleic Acid

Ribonucleic acids are long-chain polymers of nucleotides linked through 3',5'-phosphodiester bonds. Constituent bases are derivatives of purine and pyrimidines, the sugar is D-ribose. The RNA nucleotide sequence, which is genetic information transferred from DNA, acts as a template in sequencing amino acids in protein synthesis. See Chapters 11 - The Transcription of RNA Upon DNA Templates and 12 - Involvement of RNA in Protein Synthesis in J.D. Watson's Molecular Biology of the Gene, 3rd Edition, (W.A. Benjamin, Inc., Menlo Park, California 1975). Ribonucleate core consists of the limit polynucleotides remaining after exhaustive ribonuclease hydrolysis of yeast sodium nucleate.

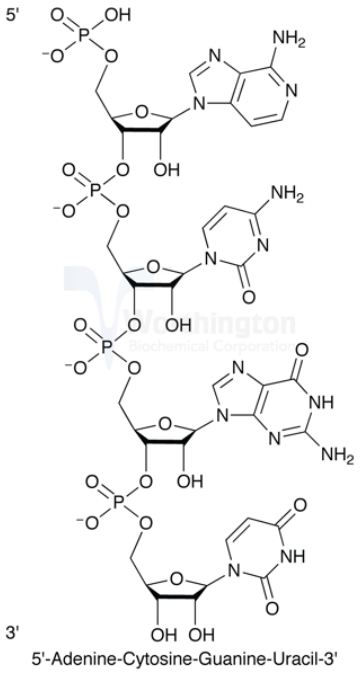

Figure 106.1: Chemical Structure - Ribonucleic Acid

CAS

- 63231-32-0

Assay Information

Method

- Determine suitability as substrate for RNase by using as substrate in RNase assay. Standardization of ribonuclease activity has been difficult due to varying rates at which reactions occur as well as to the significant differences in nucleotide patterns in RNA isolated from biological sources. Crook et al. (1960) have published an assay using a synthetic substrate, cytidine 2', 3'-phosphate. Zimmerman and Sandeen (1965) described a sensitive assay using polycytidylic acid.
- The method of Kalnitsky et al. (1959) is used at Worthington. The rate of hydrolysis of yeast RNA at pH 5.0 is determined by measuring the amount of acid soluble oligonucleotide released under defined conditions. One unit causes an increase in absorbance of 1.0 at A_{260} at 37°C and pH 5.0 under the specified conditions.

Reagents

- 0.10 M Sodium acetate buffer, pH 5.0: Dissolve 5.75 ml of glacial acetic acid in 900 ml reagent grade water. Adjust pH to 5.0 with 5 N NaOH and bring to a final volume of 1000 ml with reagent grade water.
- 25% Perchloric acid containing 0.75% Uranyl acetate: Dissolve 50 ml of 70% perchloric acid ($HClO_4$) in 90 ml reagent grade water. Add 750 mg uranyl acetate (MW 424.15) and stir to

dissolve.

- RNA Solution: Dissolve 100 mg Worthington Ribonucleic Acid (RNA) in 10 ml 0.1 M sodium acetate buffer, pH 5.0 Stir gently to dissolve to prevent denaturation. Record A_{260} at 1:50 dilution in 0.10M sodium acetate pH 5.0 to determine $\frac{\text{mg RNA}}{\text{ml}}$. $\frac{\text{mg RNA}}{\text{ml}}$ = A_{260} x dilution x 0.04
- Dilute to 2.5 $\frac{\text{mg}}{\text{ml}}$ RNA in 0.10M sodium acetate, pH 5.0 for assay. Incubate at 37°C for 5-10 minutes prior to assay. Prepare a solution of the RNA being tested, and another solution of a different lot of RNA for use as Control.
- RNAse
- Prepare stock solutions at 1 $\frac{\text{mg}}{\text{ml}}$ in reagent grade water.
- Immediately prior to assay, dilute further to 2, 4, and 6 $\frac{\mu\text{g}}{\text{ml}}$ in 0.10 M sodium acetate, pH 5.0.

Procedure

Determine % Native RNA: Dissolve 10 mg RNA in 10 ml 0.015% NaCl. Dilute 1:50 in NaCl and pipette 3 ml into cuvette and read at 260 nm and 320 nm. Add 0.2 ml 5N NaOH (Fresh, stored in plastic). Read at 260 and 320 nm. Stand one hour at 37°C. Read at 260 and 320 nm. Determine % Native: (Contact supervisor if less than 60%.).

A_{320} is a check for extraneous background.

Calculation of % Native RNA:

% Native RNA = 100 x 3 x [(Final A_{260} x $\frac{3.2}{3}$) - Initial A_{260}] x 0.045 x 50

RNase Blank Assay: (Can be set up with RNase assay) Set up a blank for sample RNA and a blank for test RNA. Pipet one ml of 0.10 M sodium acetate buffer pH 5.0 into centrifuge tubes. Equilibrate to37°C. Add one ml of RNA Solution (2.5 $\frac{\text{mg}}{\text{ml}}$). Let set one hour at 37°C. Then add one ml of uranyl acetate-perchloric acid solution. Transfer to an ice bath and cool for 5 minutes. Clarify by centrifugation and dilute 0.1ml of clear supernatatnt to 3.0ml with reagent grade water. Read A_{260} vs blank. Blank should not increase more than 50% in one hour at 37°C.

RNase Assay: Set up one set of tubes for Sample and one for Control. Include a Blank with the Sample set of tubes, and a Blank with the Control set of tubes. Pipette one ml of respective enzyme dilution into centrifuge tubes; pipette one ml of 0.10 M sodium acetate buffer pH 5.0 into Blank tubes. Incubate all tubes at 37°C for 58 minutes. At timed intervals, add one ml of RNA Solution (2.5 $\frac{\text{mg}}{\text{ml}}$) to all tubes (Samples, Controls, and Blanks). Incubate each tube exactly 4 minutes and stop reaction by the addition of one ml of uranyl acetate-perchloric acid solution. Transfer to an ice bath and cool for 5 minutes. Clarify by centrifugation and dilute 0.1 ml of clear supernatant to 3.0 ml with reagent grade water. Read A_{260} versus blank.

Calculation

- $\frac{\text{units}}{\text{mg}}$ dw = (A_{260} - blank) x 30 x dilution x standardization factor
- Standardization Factor = $\frac{\text{Assigned Value}}{\text{Observed Value}}$

Notes

- Because the RNA used as a substrate is derived from a natural source, lot to lot variation occurs. To overcome this, a ribonuclease standard is run and values are adjusted to the standard. Example: If the value assigned to the standard is 2500 $\frac{\text{units}}{\text{mg}}$ and the value obtained for the standard is 2000 $\frac{\text{units}}{\text{mg}}$, then a factor of 1.25 is to be applied to the assay values.

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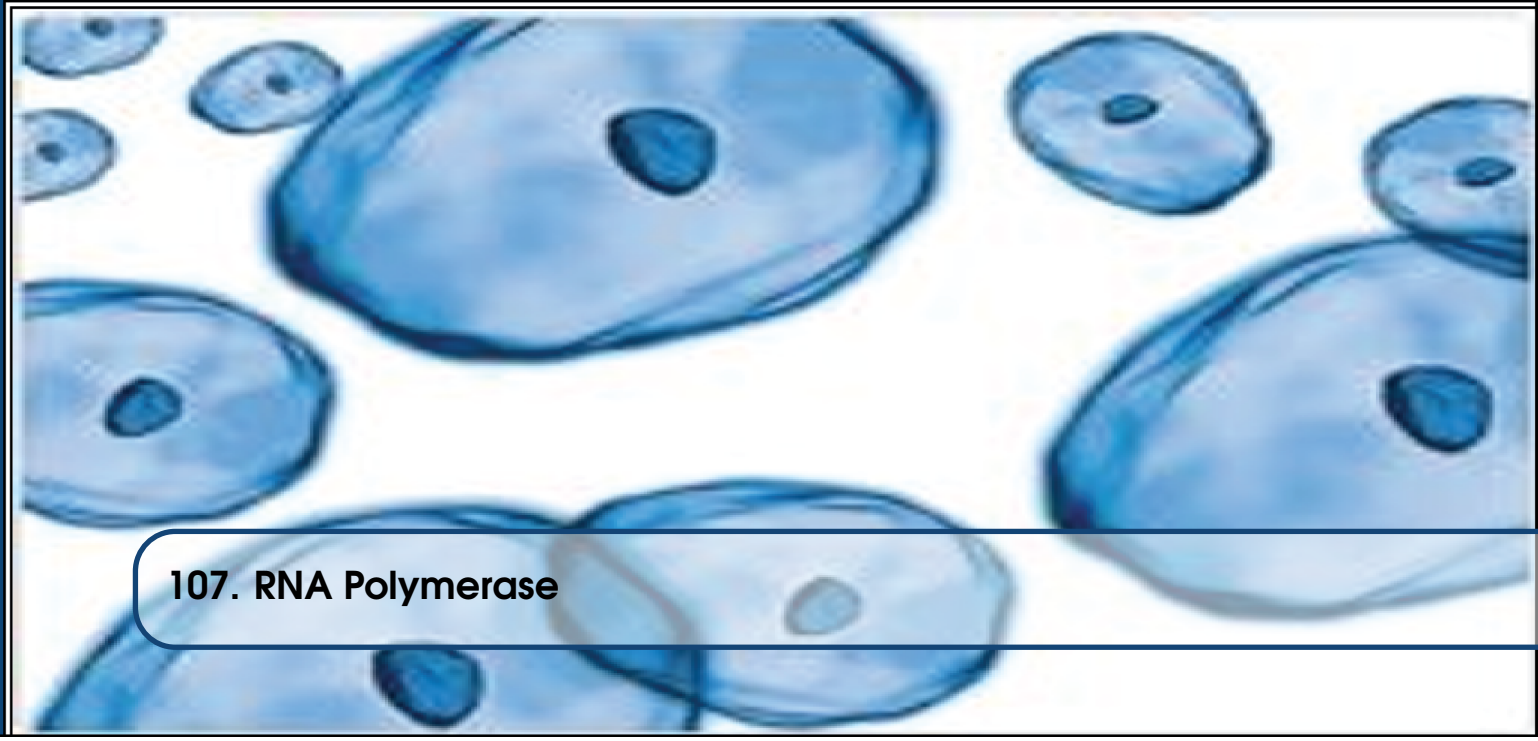

107. RNA Polymerase

Nucleoside-Triphosphate: RNA Nucleotidyltransferase (DNA-Directed)

Escherichia coli DNA-dependent RNA polymerase catalyzes the initiation (site selection), elongation and termination of polyribonucleotide chains, using ribonucleoside triphosphates as substrates and DNA as template.

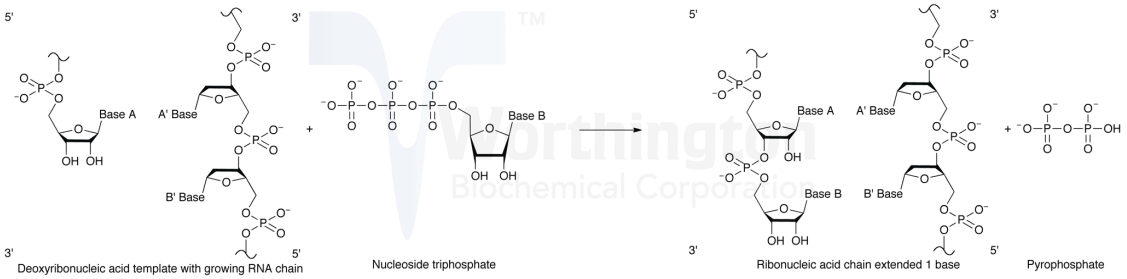

Figure 107.1: Enzymatic Reaction - RNA Polymerase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Escherichia coli*

Composition

The enzyme has a complex subunit structure with two configurations designated RNA polymerase holoenzyme and core RNA polymerase exhibiting enzymatic activity. The holoenzyme has the subunit composition $\sigma 2'\beta\beta\alpha$,and can be resolved into two components: the core enzyme $\sigma 2'\beta\beta$ and the sigma factor. The holoenzyme appears to be involved in the synthesis of most cellular RNA.

IUB

- 2.7.7.6

Molecular Weight

- The enzyme consists of four major subunits designated β',β, α, and σ with molecular weights in that order: 160, 150, 86, 40 Kda.

Activators

- *Escherichia coli* RNA polymerase has absolute requirements for divalent metal ions such as Mg^{++}, Mn^{++}, or Co^{++}.

Inhibitors

- Rifampicin, streptovaricins, streptolydigin, and sulfydryl reagents are among the inhibitors of *Escherichia coli* RNA polymerase.

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108. Superoxide Dismutase

Superoxide: Superoxide Oxidoreductase

It protects oxygen-metabolizing cells against harmful effects of superoxide free-radicals (Petkau *et al.* 1975; Fridovich 1972, 1973; Lavelle *et al.* 1973; Paschen and Weser 1973). It has been reviewed by Malmström *et al.* (1975).

McCord (1974) found that SOD protects hyaluronate against depolymerization by free-radicals and indicated that exogenous SOD might have an anti-inflammatory effect (Salin and McCord 1975). The O^{2-} ion, which has been considered important in aging, lipid peroxidation and the peroxidative hemolysis of red blood cells (Fee and Teitelbaum 1972), is formed by the univalent reduction of O_2 during various enzymatic reactions or by ionizing radiation. (See also Fee *et al.* 1975). There is also superoxide radical formation during leukocyte phagocytosis (Allen *et al.* 1974; DeChatelet *et al.* 1974). See also Dionisi *et al.* (1975). Winterbourn *et al.* (1975) indicate that SOD deficiency might lead to Heinz body hemolytic anemia. Fridovich (1986) reports on the biological effects of the superoxide radical.

Superoxide dismutase is widespread in nature. Gregory *et al.* (1974) indicate it to be present in all oxygen-metabolizing cells. Hewitt and Morris (1975) have found it in anaerobic bacteria. It has been purified from diverse sources such as: fungi (Rapp *et al.* 1973); green pea (Sawda *et al.* 1972); Streptococcus mutans (Vance *et al.* 1972); wheat germ (Beauchamp and Fridovich 1973); *Escherichia coli* (Gregory *et al.* 1973); *Saccharomyces cerevisiae* (Goscin and Fridovich 1972) and *Neurospora crassa* (Misra and Fridovich 1972).

Three superoxide dismutases are characterized by different metal content. A blue-green Cu(II)-Zn(II) enzyme comes from human and bovine erythrocytes, a wine-red Mn(III) protein is found in *Escherichia*

coli, and in chicken, and rat (Peeters-Joris *et al*. 1975) liver mitochondria (Tyler 1975) and a yellow Fe(III) enzyme from *Escherichia coli* (Villafranca *et al*. 1974). It is of interest that the chicken liver cytosomal enzyme is the copper-zinc type (Weisiger and Fridovich 1973). Peeters-Joris *et al*. (1975) show SOD activity in many organs of the rat. Gregory *et al*. (1973) have reported on intra-cellular sites and functions.

Bovine erythrocyte SOD, to which the following data apply, has been extensively studied. It is identical to the enzyme from human erythrocytes and from beef heart (Bannister *et al*. 1971; Keele *et al*. 1971 and Nyman 1960). Superoxide dismutase (SOD) catalyzes the destruction of the O^{2-} free radical.

$$2O^{2-} + 2H^{+} \rightarrow O_2 + H_2O_2 \qquad (108.1)$$

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Bovine Erythrocytes

Composition

Superoxide dismutase consists of two subunits of identical molecular weight joined by a disulfide bond. The molecular weight is 32,500 (Keele *et al*. 1971). There are two Cu(II) and two Zn(II) atoms per molecule (Bannister *et al*. 1971). Crystal studies have been reported by Richardson *et al*. (1972) and Lieberman and Fee (1973). The amino acid sequence was determined by Steinman, Naik, Abernethy and Hill (1974), Abernethy *et al*. (1974) and Evans *et al*. (1974); the subunit tertiary structure by Richardson *et al*. (1975). Rotilio *et al*. (1972, 1973, 1974) have reported on the roles of copper and zinc. According to Forman and Fridovich (1973) zinc has a structural, stabilizing role, while Cu^{2+} is directly involved in the catalytic activity. See also Calabrese *et al*. (1991). Both zinc and copper have been removed to yield the apoenzyme (Carrico and Deutsch 1970). Fee and co-workers have published studies on the metal binding sites (Fee 1973; Fee and DiCorleto 1973; Fee and Gaber 1972). and enzymatic activity (Fee *et al*. 1973). Beem *et al*. (1974) report on the replacement of zinc by cobalt, mercury, and cadmium. Forman *et al*. (1973) and Rigo *et al*. (1975) indicate histidine to be involved at the active site. Nuclear magnetic resonance studies have been reported by Stokes *et al*. (1973), Lieberman and Fee (1973) and Villafranca *et al*. (1974). Superoxide dismutase activity and kinetics have been reported by Halliwell (1975), Rigo *et al*. (1975), Fielden *et al*. (1974), Goda *et al*. (1974), Michelson (1974), Hodgeson and Fridovich (1973), Rotilio (1973), and Grunow and Schöpp (1989).

IUB

- 1.15.1.1

CAS

- 9054-89-1

Molecular Weight

- 32,500 (Keele *et al*. 1971)

Isoelectric Point

- 4.95 (Bannister *et al*. 1971)

Extinction Coefficient

- The protein is peculiar in that it does not show an absorption maximum at 280 nm (McCord and Fridovich 1969; Bannister *et al*. 1971). According to Symonyan and Nalbandyan (1972) $\frac{A_{259}}{A_{680}} = 30$.

Inhibitors

- Cyanide inhibits cupro-zinc SOD but has no effect on the mangano enzyme of chicken liver mitochondria (Beauchamp , in Weisiger and Fridovich 1973). SOD is inactivated by H_2O_2 (Symonyan and Nalbandyan 1972, Fielden *et al*. 1973) and may be protected by catalase (Bray *et al*. 1974) with which it is usually associated. Hartz *et al*. (1973) found that in some tissue including cerebral cortex and thyroid, SOD is present but not catalase.

Stability/Storage

- SOD is an unusually stable enzyme although its apoenzyme is very unstable. (Forman and Fridovich 1973). Worthington SOD retains its activity for up to a year at 5°C.

Assay Information

Activity of SOD has been determined in two ways:

1. Inhibition by the enzyme of an O^{2-} dependent reaction.

2. Pulse radiolytic methods (Rigo *et al*. 1975) See: Beauchamp and Fridovich (1971); Misra and Fridovich (1972); Tyler (1975).

Method

- The method employed at Worthington is essentially that of Winterbourn et al. (1975) and is based on the ability of superoxide dismutase to inhibit the reduction of nitro-blue tetrazolium by superoxide. One unit is defined as that amount of enzyme causing half the maximum inhibition of NBT reduction. The reaction velocity will depend largely on somewhat variable assay conditions such as light intensity and reaction temperature. Calibration of the method in individual laboratories is recommended.

Reagents

- 0.067 M Potassium phosphate buffer, pH 7.8
- 0.1 M Ethylene diamine tetraacetic acid (EDTA) containing 0.3 mM sodium cyanide
- 0.12 mM Riboflavin (store cold in a dark bottle)
- 1.5 mM Nitroblue tetrazolium (NBT) (store cold)

Enzyme

- Prepare stock solution at one $\frac{\text{mg}}{\text{ml}}$.

Procedure

Pipette into a series of tubes:

- EDTA/Cyanide - 0.2 ml
- NBT - 0.1 ml
- Enzyme - *
- Phosphate buffer - q.s. to 3.0 ml

Include several tubes with no enzyme as controls.

* A series of samples ranging from 0.1-10 micrograms is recommended. A tube containing approximately 100 micrograms will generally produce maximum inhibition. Place the tubes in a light box providing uniform light intensity. (A foil-lined box approximately 4' long x 8 x 6 with an internally mounted 40 W fluorescent bulb has been used successfully). Incubate the tubes for 5-8 minutes to achieve a standard temperature. At zero time and at timed intervals add 0.05 ml riboflavin. Incubate all tubes in the light box for 12 minutes and at timed intervals read A_{560}. Determine percent inhibition of NBT reduction. Plot percent inhibition versus amount of enzyme in test. Determine the amount of enzyme resulting in one half of maximum inhibition.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{1000}{\mu\text{g enzyme resulting in } \frac{1}{2} \text{ maximum inhibition}}$

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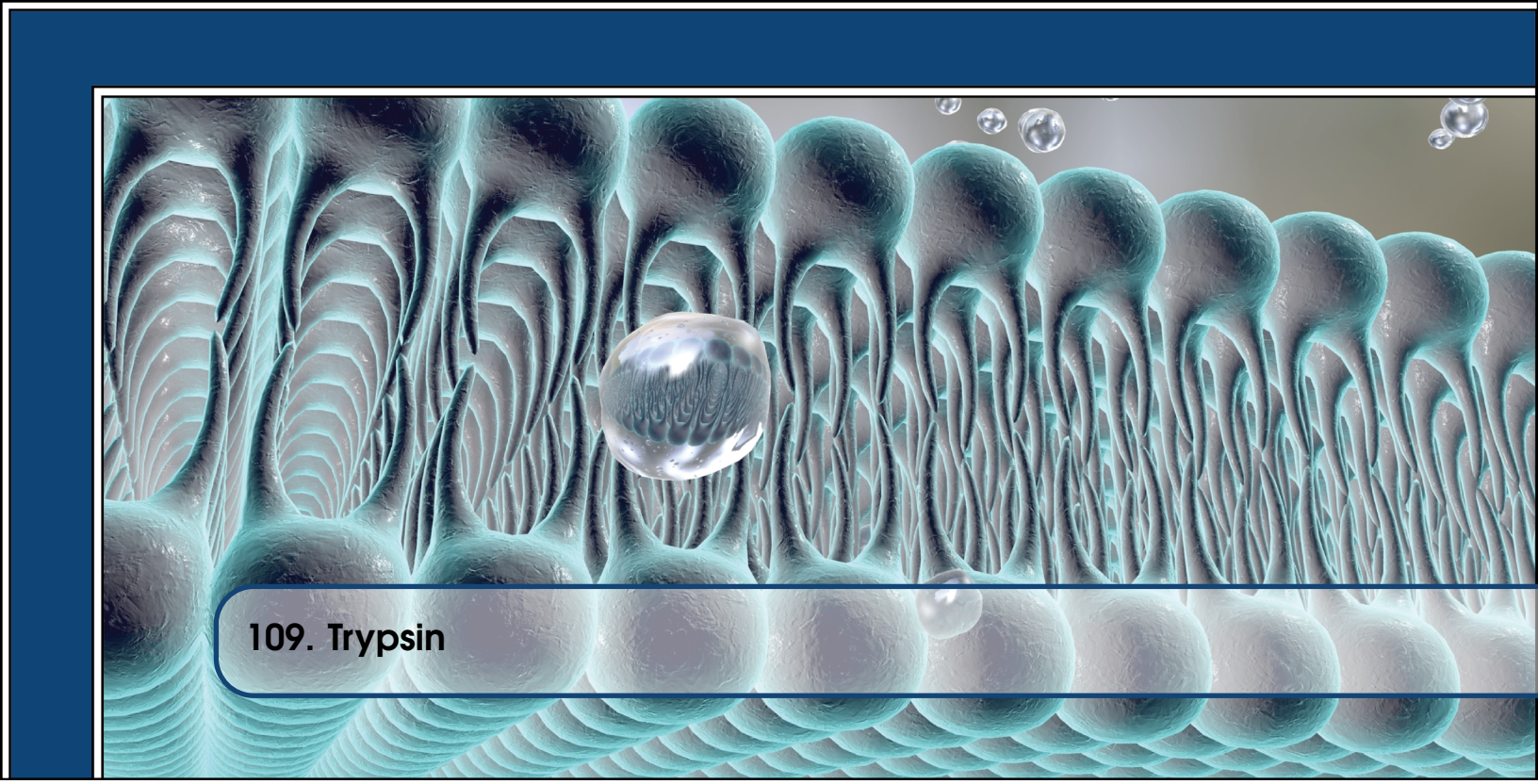

109. Trypsin

Trypsin is a pancreatic serine protease with substrate specificity based upon positively charged lysine and arginine side chains (Brown and Wold 1973). The enzyme in excreted by the pancreas and takes part in the digestion of food proteins and other biological processes. Trypsin is a medium-sized globular protein and is produced as an inactive proenzyme, trypsinogen (Chen *et al.* 2009).

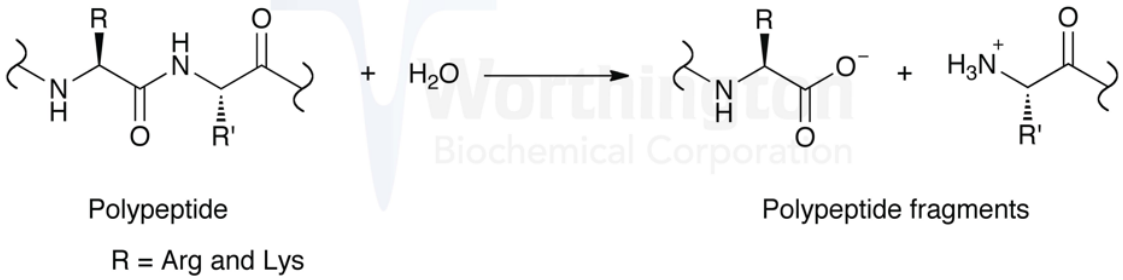

Figure 109.1: Enzymatic Reaction - Trypsin

History

In 1876, trypsin was first named by Kuhne who described the proteolytic activity of this pancreatic enzyme. He compared trypsin and pepsin, discovering the differentiating factor to be the optimal pH. In 1931, Northrop and Kunitz purified trypsin by crystallization shortly after first purifying pepsin in 1930.

In 1974 the three dimensional structure was determined, which served as a prototype for the serine endopeptidase S1 family to which trypsin belongs.

In the late 1980s, and early 1990s, site directed mutagenesis with recombinant trypsin determined the role of particular amino acid residues (Sprang *et al*. 1987, McGrath *et al*. 1989, Corey and Craik 1992, and Corey *et al*. 1992).

In the late 1990s trypsin's role in hereditary pancreatitis was investigated, and it was determined that a mutation at Arg117His is responsible for preventing autolysis thereby causing pancreatitis.

Today, trypsin continues to be used in the development of cell and tissue culture protocols (Soleimani *et al*. 2009, Banumathi *et al*. 2009, and Yang *et al*. 2009), as well as protein identification through peptide sequencing techniques (Manz *et al*. 2004 and Schuchert *et al*. 2009). In the medical field, the role of trypsin in pancreatic diseases, including cystic fibrosis (Tzetis *et al*. 2007, and Li *et al*. 2009) and chronic pancreatitis (Chen *et al*. 2009), has been the subject of current research, and trypsin has been used to model the decomposition of articular cartilage in osteoarthritis (Wang *et al*. 2008).

Molecular Characteristics

Bovine pancreas expresses two forms of trypsin, the dominant cationic and minor anionic forms. These protein sequences share 72% identity, while their coding regions share 78% identity.

Each of these proteins are further processed into alternate forms. Catalytic trypsin contains a flexible "autolysis loop" (residues G145-V157) (Schroeder and Shaw 1968, and Bartunik *et al*. 1989), and autolysis of the dominant, single-chain form B-trypsin at K148-S149 within this loop leads to the formation of A-trypsin. Further autolysis at K193-D194 leads to the formation of Psi-trypsin (Fehlhammer and Bode 1975).

Both the cationic and anionic trypsin proteins are expressed as trypsinogen proenzymes, with a 15-residue signal peptide (M1-A15) and an 8-residue propeptide (F16-K23). The three-dimensional fold of all known trypsins is highly conserved. In addition, the catalytic triad and regions flanking the catalytic triad are highly conserved (Hartley 1970).

Specificity

Trypsin cleaves peptides on the C-terminal side of lysine and arginine amino acid residues. If a proline residue is on the carboxyl side of the cleavage site, the cleavage will not occur. If an acidic residue is on either side of the cleavage site, the rate of hydrolysis has been shown to be slower.

Composition

Trypsinogen may be activated by removal of a terminal hexapeptide to yield single-chain β-trypsin. Subsequent limited autolysis produces other active forms having two or more peptide chains bound by disulfide bonds. The predominant forms are α-trypsin, having two peptide chains and β-, a single chain. Different activity and thermal stability are shown by α- and β-trypsin.

Other structural features include surface loops at amino acids 185-193, which influence specificity, despite not making direct contact with the substrate. A high affinity Ca^{2+} binding site is required for stability, and when not present, autolysis occurs. The autolysis loop (located at amino acids 143-151) is very flexible in both trypsin and trypsinogen. Cleavage at the lysine yields the alpha form which retains some catalytic activity. The protein has six completely conserved disulfide bonds (Halfon and Craik 1998).

IUB

- 3.4.21.4

CAS

- 9002-07-7

Protein Accession Number

- P00760

CATH: Classification (v. 3.3.0)

Class:
- Mainly Beta

Architecture:
- Beta Barrel

Topology:
- Thrombin, subunit H

Molecular Weight

- 23.3 kDa

Optimal pH

- 7.5-8.5 (Koutsopoulos *et al.* 2007)

Isoelectric Point

- Trypsinogen: pH 9.3 (Walsh and Neurath 1964)
- Trypsin: pH 10.5 (Cunningham 1954)

Active Residue

- Histidine (H63)
- Aspartic acid (D107)
- Serine (S200)

Activators

- The rate of trypsinogen conversion is enhanced by using lanthanide in place of calcium ions (Gomez *et al*. 1974)

Inhibitors

- Pancreatic-, soybean-, lima bean-, and egg white- trypsin inhibitors (see section on Trypsin Inhibitors)
- DFP
- Aprotinin
- Ag^{+}
- Benzamidine
- EDTA (White and White 1997)

Applications

- Tissue dissociation, especially when combined with other enzymes such as collagenase, and elastase
- Cell harvesting by "trypsinization"
- Mitochondria isolation
- *in vitro* studies of proteins
- Removing monolayers of cells from plastic and glass
- Various hemagglutination procedures
- Sample preparation for flow cytometric DNA analysis
- Tryptic mapping
- Fingerprinting and sequencing work
- Environmental monitoring
- Reduction of cell density in tissue culture
- Subculturing cells
- Cleavage fusion proteins
- Generating glycopeptides from purified glycoproteins (White and White 1997)

Assay Information

Method

- Various assays have been described in the literature. Spencer et al. (1975) report an assay using as substrate an intact protein to which has been attached a fluorescent dye, 1-anilino-8-naphthalenesulfonate (ANS). Stewart (1973) has described a method using chromogenic substrates N-benzoyl-DL-arginine p-nitroanilide (DL-BAPA), N-glutaryl-L-phenylaline p-nitroanilide (L-GPNA). Ford et al. (1973) report on the use of P-nitro-phenyl p-guanidino benzoate (NPGB) to determine active site exposure of immobilized trypsin, a stable acylated enzyme is formed, plus p-nitrophenol spectrophotometrically determined at 410 nm. The assay used in the Worthington laboratory is described below.
- One unit hydrolyzes 1 μmole of p-toluene-sulfonyl-L-arginine methyl ester (TAME) per minute at 25°C, pH 8.2, in the presence of 0.01 M calcium ion.

Reagents

- 0.046 M Tris · HCl buffer, pH 8.1 with 0.0115 M calcium chloride
- 0.01 M TAME (p-toluene-sulfonyl-L-arginine methyl ester)
- 0.001 N HCl

Enzyme

- Dilute to a concentration of 10-20 $\frac{\mu g}{ml}$ in 0.001 N HCl.

Procedure

Spectrophotometer Settings: Wavelength: 247 nm Temperature: 25°C

Incubate in spectrophotometer at 25°C for 3-4 minutes to achieve temperature equilibration and establish a blank rate, if any. Add 0.1 ml diluted enzyme and record A_{247} for 3-4 minutes. Determine ΔA_{247} from initial linear portion of the curve. The reaction remains linear to an A_{247} of about 0.320. The reaction should be linear for at least three minutes. If this is not so repeat using less enzyme.

Pipette into each cuvette as follows:

- 0.046 M Tris · HCl buffer, pH 8.1 - 2.6 ml
- 0.01 M TAME - 0.3 ml

Calculation

- $$\frac{\text{Units}}{\text{mgP}} = \frac{\frac{\Delta A_{247}}{\text{min}} \text{ x } 1000 \text{ x } 3}{540 \text{ x mg Trypsin in reaction mixture}}$$

where 540 is the extinction coefficient of p-toluenesulfonyl-L-arginine at 247 nm

Notes

- 1 TAME Unit = 19.2 USP/NF Units = 57.5 BAEE Units.

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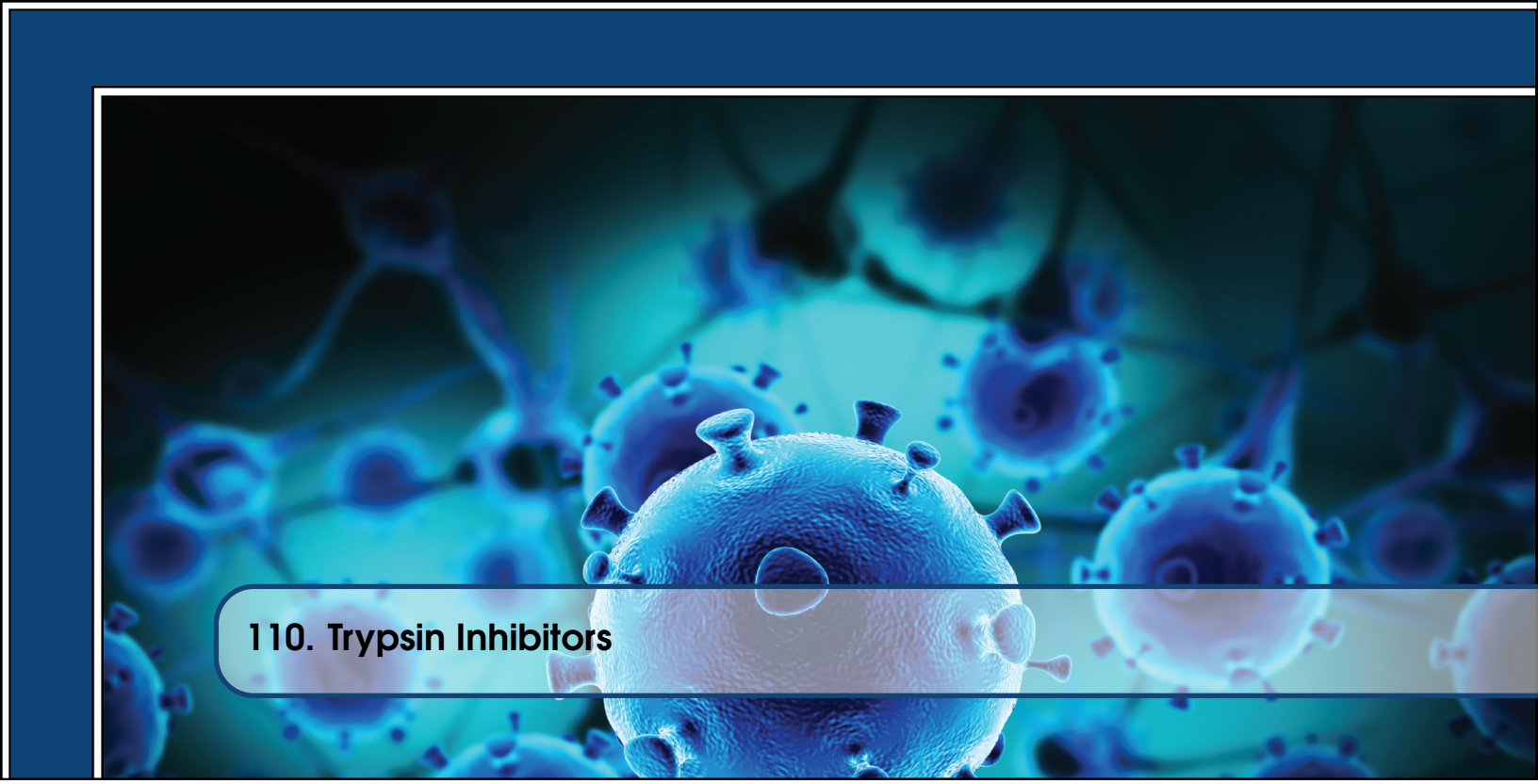

110. Trypsin Inhibitors

Two bovine pancreatic trypsin inhibitors have been isolated. Both are produced by the acinar cells and provide security against accidental trypsinogen activation and consequential unbridled proteolysis. The secretory trypsin inhibitor isolated by Kazal *et al.* (1948) is secreted with the zymogens into the pancreatic juice. It has been reported on by Sealock and Laskowski (1973), Schweitz *et al.* 1973), Greene and Giordano (1969), Cerwinsky *et al.* (1967) and Greene *et al.* (1966). It differs from the Kunitz inhibitor by forming a much less stable complex: the inhibitor is eventually digested by the trypsin. The Kazal inhibitor is active on thrombin clotting activity but does not inhibit thrombin esterase activity nor does it act upon chymotrypsin, kallikrein, plasmin, urokinase (Burch *et al.* 1967) or human trypsin (Feeney *et al.* 1969). The intracellular basic trypsin inhibitor of Kunitz was first crystallized by Kunitz and Northrop in 1936. It has been shown by Anderer and Hornle (1966) to be identical with the kallikrein inhibitor of bovine lungs and parotid gland. Chauvet and Archer (1972) report on a preparation from bovine ovary. Basic pancreatic trypsin inhibitor (BPTI) forms a very stable 1:1 complex with bovine trypsin between pH 3 and 10 (Avineri-Goldman *et al.* 1967; Cole and Parthasarathy 1972), and also human trypsins (Figarela *et al.* 1974). The dissociation constant at pH 8.0 has been reported as 6×10^{-14} Vincent and Lazdunski 1972). Chymotrypsin is also inhibited by BPTI (Blow *et al.* 1972) but is bound less strongly. Chauvet and Archer (1975) indicate that Lys-15 is involved in the reaction with chymotrypsin. See also Engel *et al.* (1974). Imhoff and Keil-Dlouhá (1971) indicate that the "chymotryptic" active site participates in the reaction with trypsin. The inhibitor-chymotrypsin complex has been crystallized (Ruhlmann *et al.* 1971). Plasmin is inhibited by BPTI. Summaria *et al.* (1975) have reported on the plasmin-inhibitor complex. Reddy and Markus (1973) have shown that BPTI forms a ternary complex with plasminogen and streptokinase that is inactive. Spilberg and Osterland (1970) indicate that it inhibits the proteolytic activity of polymorphonuclear lysosomal lysates. Sardesai and Thal (1966) report that abnormal increase in plasma proteases of pancreatitis can be reduced to normal ranges but that the normal proteolytic activity of plasma is not inhibited. The esterolytic, proteolytic and elastolytic activities of porcine elastase are not inhibited by BPTI (Gertler and Feinstein

1971). BPTI is a single polypeptide chain of 58 amino acids, including six cysteines forming three disulfide bridges (Huber *et al.* 1971), and having a molecular weight of 6,500. It may exist under physiological conditions as a dimer (Trautschold *et al.* 1967). Structure, conformation and binding studies have been reported by Creighton (1975), Geratz *et al.* (1975), Gelin and Karplus (1975), Quast *et al.* (1975), Pershina and Hvidt (1974), Vincent *et al.* (9174), Wang and Kassell (1974), Karplus *et al.* (1973), Blow *et al.* (1972), Wilson and Laskowski (1971). Huber *et al.* (1971), Liu *et al.* (1971), Chauvet *et al.* (1966), Edelhoch and Steiner (1965), Kassell and Laskowski (1965), and Gallagher *et al.* (1992). The inhibitor has been reviewed by Trautschold *et al.* (1967). Kallikreins are proteases that specifically release from kininogen an α2-globulin fraction of the serum, and biologically active polypeptides such as bradykinin and kallikin (kinin 9 and kinin 10 respectively) (Kato and Suzuki 1970; Colman *et al.* 1969; Trautschold *et al.* 1966). The kinins are very active, even in nanogram quantities, causing smooth muscle stimulation, peripheral vasodilation, enhancement of capillary permeability and pain (Lim *et al.* 1969), symptoms manifest in hemorrhagic or surgical shock, pancreatitis, etc. The physiologic and pathologic roles of kinins have been reviewed by Kellermayer and Graham (1968). Much information on kallikrein and hypotensive peptides may be found in the Proceedings of the International Symposium on Hypotensive Peptides (October, 1965, Florence, Italy) edited by Erdos *et al.* (1966). Spillerg and Osterland (1970) observed an anti-inflammatory effect of BPTI in induced acute arthritis in rabbits.

BPTI is reported to be stable in acid or neutral milieu. Its isoelectric point is 10.5 (Trautschold *et al.* 1967). Activity of inhibitors is usually expressed in terms of substrate inhibited. In terms of a kallikrein inhibitor one K-I unit is that quantity which inactivates 2 kallikrein units being biologically determined. Trautschold indicates there to be 6000-7000 KI units per milligram of crystalline or chromatographically purified inhibitor.

Soybean Trypsin Inhibitor (Kunitz)

Soybean trypsin inhibitor (SBTI) first crystallized by Kunitz (1945) is one of several such inhibitors found in soybeans. (Fratalli 1969; Millar *et al.* 1969; Fratalli and Steiner 1968; Birk *et al.* 1967). The best known preparation is that of Kunitz. Steiner and Fratalli (1969) have reviewed the Kunitz and Bowman-Birk inhibitors. A protein (or polypeptide) proteinase inhibitor probably has peptide bonds compatible with the protease reactive site. Finkenstadt and Laskowski (1965 and 1967) and Ozawa and Laskowski (1966) indicate that a single Arg-Ile bond is cleaved by trypsin; a covalent bimolecular complex of inhibitor and trypsin results. On dissociation either virginal or modified inhibitor appears; see Hixson and Laskowski (1970a), Isheda *et al.* (1970) and Niekamp *et al.* (1969).

Ovomucoid

Ovomucoids are the glycoprotein protease-inhibitors of avian egg white. There are several protease inhibitors in egg white. One acts upon ficin and papain (Fossum and Whitaker 1968); another, ovoinhibitor (Matsushima 1958), is a significant contaminant of crude ovomucoid preparations and acts upon bovine trypsin and chymotrypsin as well as porcine elastase and fungal proteinase (Feinstein and Gertler 1972; Gertler and Feinstein 1971; Liu *et al.* 1971; Tomimatsu *et al.* 1966). Chicken ovomucoid inhibits bovine trypsin mole for mole but does not inhibit human trypsin (Travis 1971; Feeney *et al.* 1969). It is electrophoretically heterogenous (Beeley and McCairns 1972; Beeley 1971; Melamed 1967; Bier *et al.* 1953). Both Bier and Beeley reported three major and two minor components with

similar trypsin inhibiting activity but differing galactose, sialic acid and N-acetylglucosamine moieties. Ovomucoid conformation studies have been done by Murthy *et al.* (1973), Beeley (1972) and Donovan 1967). The arginyl residue for trypsin binding has been indicated by Liu *et al.* (1968). It does not contain tryptophan.

Lima Bean Trypsin Inhibitor

Lima bean trypsin inhibitor (LBI), which inhibits bovine as well as human trypsin (Feeney *et al.* 1969) and plasmin (Lewis and Ferguson 1953), acts upon both trypsin and chymotrypsin by forming equimolar complexes. The binding sites are distinct and independent (Krahn and Stevens 1972, 1971). The trypsin susceptible binding site is a lys-ser peptide bond (Krahn and Stevens 1972). The site of chymotrypsin action is a leu-ser bond (Krahn and Stevens 1970). As in other protease-inhibitors the "complexing" involves a reversible hydrolysis of the peptide bond (Krahn and Stevens 1973). See also Stevens and Doskoch (1973).

Lima bean trypsin inhibitors may be chromatographically separated into as many as six variants (Haynes and Feeney 1967). Jones *et al.* (1963) characterized four. All have similar but not identical amino acid composition, contain six or seven disulfide bonds and lack methionine and tryptophan. Molecular weights vary between 8,000-10,000. The complete amino acid sequence of component IV has been reported by Tan and Stevens (1971a and b). Krahn and Stevens (1972) report finding variations in activity of the four variants particularly with chymotrypsin, while essentially identical with respect to their trypsin inhibitory activity.

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Soybean Trypsin Inhibitor (Kunitz), Ovomucoid, and Lima Bean Trypsin Inhibitor

Specificity

Soybean Trypsin Inhibitor: Soybean inhibitor inhibits trypsin mole-for-mole and to a lesser extent chymotrypsin. (Bidlingmeyer *et al.* 1972). Lanchantin *et al.* (1969) report soybean inhibitor to form a one-to-one complex with beef or human thrombin thus blocking its specific proteolytic capacity to activate prothrombin. Nanninga and Guest (1964) report plasmin to be inhibited. STI has been reported to inhibit leukocytic proteases. (Lieberman and Gawad 1971), but not the esterolytic, proteolytic or elastolytic activities of porcine elastase (Gertler and Feinstein 1971).

Ovomucoid: Feeney *et al.* (1967) have shown that although ovomucoid of different species show different inhibiting specificities, their physical properties differ only slightly. Thus, Rhodes *et al.* (1960) have shown that golden pheasant ovomucoid inhibits only chymotrypsin whereas that from turkey inhibits both trypsin and chymotrypsin simultaneously and duck ovomucoid inhibits two moles of trypsin and one of chymotrypsin simultaneously.

Composition

Soybean Trypsin Inhibitor: The Kunitz soybean inhibitor consists of a single polypeptide chain crosslinked by two disulfide bridges (Steiner 1965). Structural studies of the inhibitor and active site

have been reported, Ellis *et al.* (1975), Woodward and Ellis (1975), Koide *et al.* (1974), Koide and Ikenaka (1973), Bidlingmeyer *et al.* (1972), Ikenaka *et al.* (1971), Papaioannou and Liener (1970), Hixson and Laskowski (1970), Kato and Tominager (1970), and Wu and Scherage (1962). Donovan and Beardslee (1975) have reported on the thermal denaturation of inhibitor complexes.

CAS

- 9035-81-1

Molecular Weight

- Soybean Trypsin Inhibitor: 21,500 $\pm$ 800 (Wu and Scheraga 1972a)
- Ovomucoid: The molecular weight is approximately 28,000 (Feeney *et al.* 1963). Davis *et al.* (1971) report it as 27,300 and Waheed and Salahuddin (1975) as 28,500 $\pm$ 3,500 for a distinct homogeneous variant.

Optimal pH

- Soybean Trypsin Inhibitor: 7

Isoelectric Point

- Soybean Trypsin Inhibitor: 4.5 (Kunitz 1947)

Assay Information

Method

- The ability of the various trypsin inhibitors to prevent trypsin hydrolysis of benzoyl-L-arginine ethyl ester is measured spectrophotometrically. No unit of activity is currently assigned. The activity of the inhibitors is expressed as the amount of twice crystallized trypsin (TRL) inhibited by one milligram of inhibitor.

Reagents

- Trypsin reagent: 0.25 mM benzoyl-L-arginine ethyl ester in 0.067 M phosphate buffer, pH 7.0
- 0.001 N Hydrochloric acid
- 0.5 M Sodium potassium phosphate buffer, pH 6.5
- Trypsin solution: Prepare trypsin (Worthington Code: TRL) at 0.5 $\frac{\text{mg}}{\text{ml}}$ in 0.001 N HCl.

Inhibitors - Dissolve various inhibitors as follows:

- Lima bean inhibitor - 1 $\frac{\text{mg}}{\text{ml}}$ in 1% NaCl
- Ovomucoid - 1 $\frac{\text{mg}}{\text{ml}}$ in 0.001 N HCl
- Pancreatic inhibitor - 1 $\frac{\text{mg}}{\text{ml}}$ in 0.001 N HCl
- Soybean inhibitor - 1 $\frac{\text{mg}}{\text{ml}}$ in 0.01 M phosphate buffer, pH 6.5 with 0.15M NaCl

Procedure

Pipette the following into a series of test tubes:

- Trypsin solution - 1.0 ml
- 0.5 M Phosphate buffer - 2.0 ml
- Inhibitor - *
- Reagent grade water - q.s. to 10 ml

Include 2 test tubes with no inhibitor as controls.

* The following amounts are recommended:

Lima bean inhibitor: 50 - 300 micrograms

Ovomucoid and purified soybean inhibitor: 100 - 500 micrograms

Pancreatic inhibitor: 20 - 500 micrograms

Crude soybean inhibitor : 200 - 1000 micrograms

Calculation

- $$\frac{\text{mg TRL inhibited}}{\text{mg inhibitor}} = \frac{\left(\frac{\Delta A_{253}}{\text{min}}_{\text{Control}} - \frac{\Delta A_{253}}{\text{min}}_{\text{Sample}}\right) \times 5.0}{\frac{\Delta A_{253}}{\text{min}}_{\text{Control}} \times \mu\text{g inhibitor in reaction mixture}}$$

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111. Trypsinogen

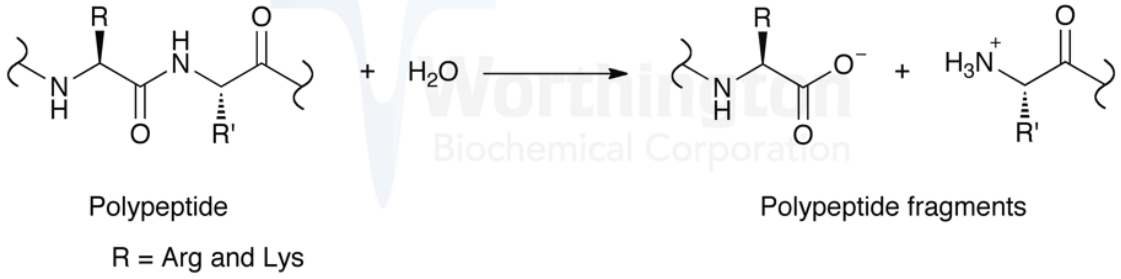

Figure 111.1: Enzymatic Reaction - Trypsinogen

Composition

Trypsinogen has been shown to have certain intrinsic activity. See Knights and Light (1974) for further references. It is usually considered, however, as the inactive precursor of trypsin which may be activated by removal of a terminal hexapeptide to yield single-chain β-trypsin. Subsequent limited autolysis produces other active forms having two or more peptide chains bound by disulfide bonds. The predominant forms are α-trypsin, having two peptide chains and β-, a single chain. Different activity and thermal stability are shown by α- and β-trypsin.

CAS

- 9002-08-8

Molecular Weight

- Trypsinogen: 24,000 (Walsh and Neurath 1964)

Isoelectric Point

- Trypsinogen: pH 9.3 (Walsh and Neurath 1964)

Extinction Coefficient

- 45,250 $\frac{1}{\text{cm}}\frac{1}{\text{M}}$

Activators

- The rate of trypsinogen conversion is enhanced by using lanthanide in place of calcium ions (Gomez *et al*. 1974).

Stabilizers

- Calcium ion retards trypsin autolysis and promotes activation of trypsinogen. Sipos and Merkel (1970) have reported on the calcium-trypsin complex. See also Griffiths and Brechner (1973).

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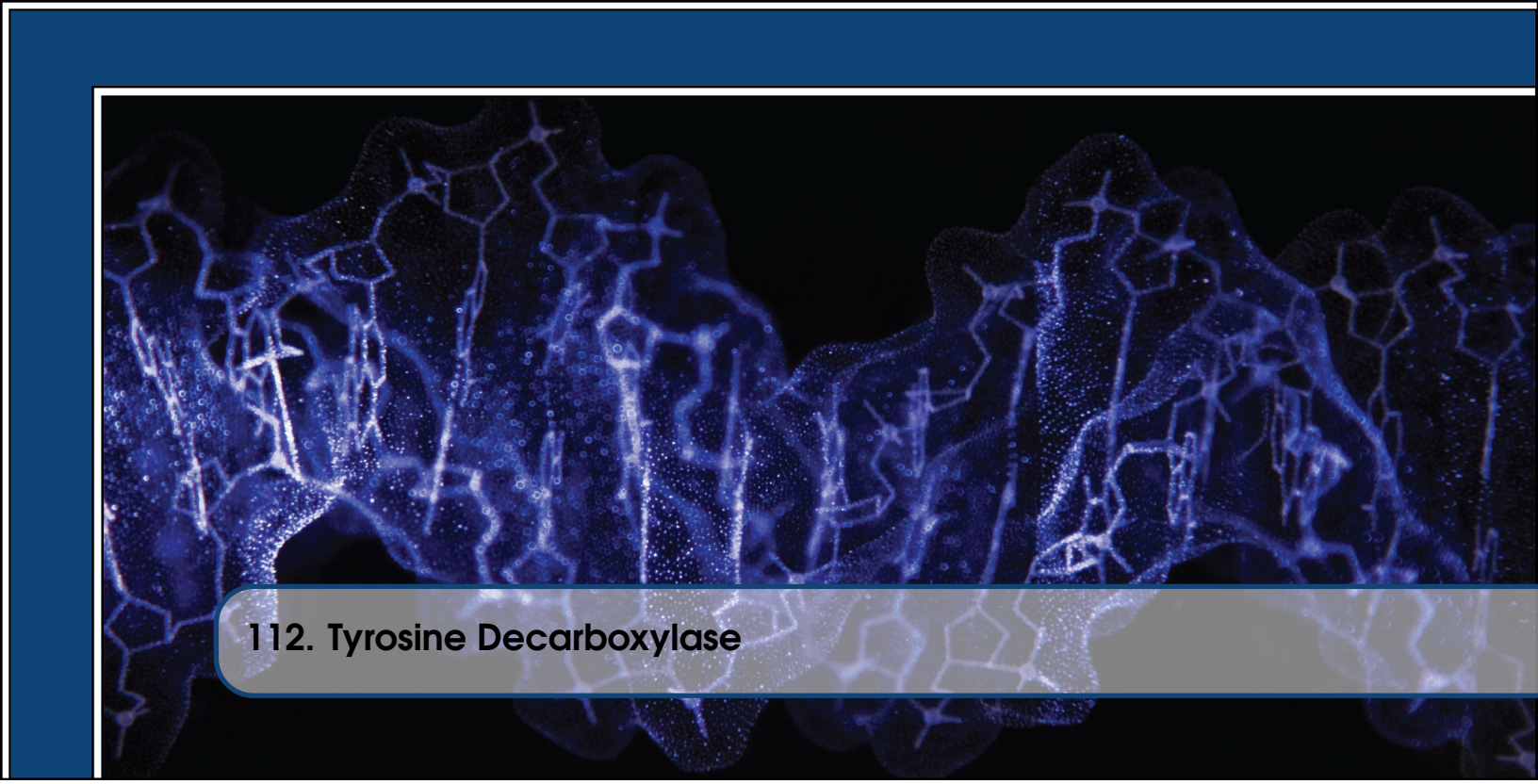

112. Tyrosine Decarboxylase

L-Tyrosine Carboxy-lyase

Pyridoxal 5'-phosphate is a necessary coenzyme. By utilizing the apoenzyme (TYDAPO), prepared from cells grown on a vitamin B6 deficient medium, pyridoxal phosphate may be determined. See Umbreit *et al.* (1945) and Gunsalus and Smith (1957). Decarboxylation of radio-labelled tyrosine has permitted the determination of much smaller amounts of pyridoxal phosphate (Maruyama and Coursin 1968; Sundaresan and Coursin 1970). Acetone-dried cells were used as the source in a relatively simple extraction and purification of the apoenzyme.

The holoenzyme may be used to determine tyrosine, phenylalanine and dihydroxyphenylalanine either manometrically or colorimetrically. Tashian (1957) has reported a determination of tyrosine in urine based on this principle. Plasma phenylalanine can also be determined using this enzyme. The original method was described by Udenfriend and Cooper (1953), and modifications have been published by Hsia (1959) and Knox and Messinger (1958). Poisner (1960) determined serum phenylalanine levels in schizophrenics using Hsia's method and obtained a significant elevation over normals.

The amino acid decarboxylases have been reviewed by Boeker and Snell (1972). See also Snell and DiMari (1970). Tyrosine decarboxylase catalyzes the removal of the carboxyl group from tyrosine to produce tyramine and carbon dioxide:

$$HO\Phi CH_2CHNH_2CO_2 \rightarrow HO\Phi CH_2CH_2NH_2 + CO_2 \qquad (112.1)$$

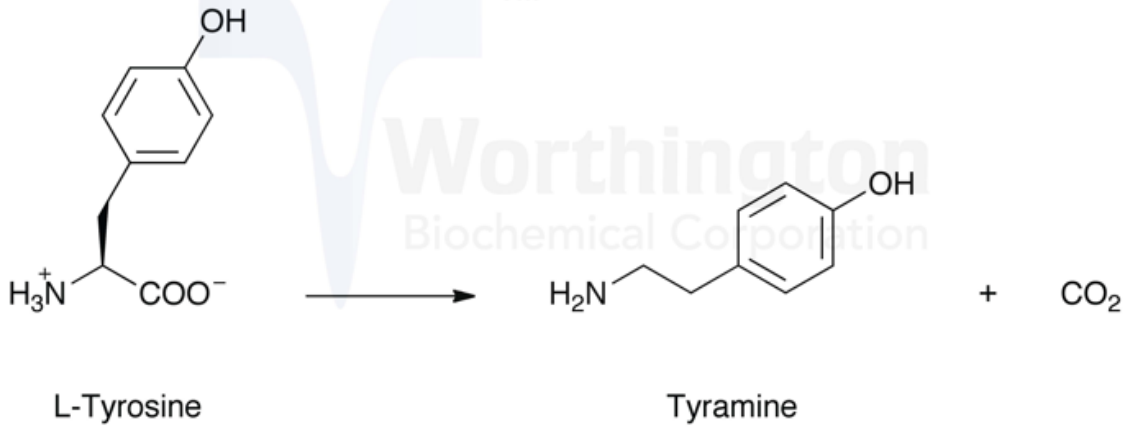

Figure 112.1: Enzymatic Reaction - Tyrosine Decarboxylase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- *Streptococcus faecalis*

Specificity

L-phenylalanine and dihydroxyphenylalanine are acted upon at a much slower rate than L-tyrosine.

IUB

- 4.1.1.25

CAS

- 9002-09-9

Optimal pH

- 5.5 (Boeker and Snell 1972)

Inhibitors

- Heavy metals (Ag^+, Cu^{2+}, Fe^{2+}, Fe^{3+}, Pb^{2+}) KCN, NHOH, N_2H_4, and $SO_4 =$

Stabilizers

- Glycerol 10-25%, mercaptoethanol (5 mM) and L-tyrosine (0.5 mM) (Chabner and Livingston 1970; Sundaresan and Coursin 1970).

Assay Information

TYD Enzyme

Method

- The assay used here is based on that of Gunsalus and Smith (Methods in Enzymology, III, (Colowick, S., and Kaplan, N., eds.), Academic Press, NY, 963, 1957). One unit yields one micromole of CO_2 per minute from L-tyrosine at 37°C and pH 5.5 under the specified conditions.

112.0.1 Reagents

- 0.8 M Sodium acetate, pH 5.5
- 0.074 M Sodium acetate, pH 5.5
- 0.0065 M L-tyrosine in reagent grade water. Dissolve by warming to 80°C while stirring. Equilibrate to 37°C. (If solution cools crystals may form).

112.0.2 Enzyme

- Dissolve enzyme in 0.074 M sodium acetate, pH 5.5 at a concentration of 2 $\frac{\text{mg}}{\text{ml}}$. Mix to uniformity immediately before withdrawing aliquots.

Procedure

Into the main well of the Warburg flasks pipette the following:

- 0.8 M Sodium acetate, pH 5.5 - 0.2 ml
- 0.0065 M L-tyrosine at 37°C - 2.3 ml

Pipette 0.5 ml of the enzyme suspension into the side arm of the flasks. Include one flask containing no enzyme as a blank and a flask containing no enzyme to serve as a blank and a flask containing 3.0 ml of reagent grade water to serve as a thermal barometer. Attach flasks to manometer. After 10 minutes of equilibration, close the manometers, tip in and mix the substrate and replace the flasks in the bath. Determine the micromoles of CO_2 released at approximately 5 minute intervals for 30 minutes. Plot micromoles CO_2 released versus reaction time and determine micromoles released per minute from the linear portion of the curve.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\text{microliters } CO_2 \text{ released}}{\text{min}}}{\text{22.4 x mg enzyme in reaction mixture}}$

Assay Information

TYD-APO

Reagents

- 0.8 M Sodium acetate, pH 5.5: Add 11.45 ml glacial acetic acid (MW 60.05, specific gravity 1.05) to 200ml reagent grade water. Adjust pH to 5.5 with 5N NaOH and bring to a final voulme of 250 ml with reagent grade water.

- 0.222 M Sodium acetate, pH 5.5: Dilute 10 ml of 0.8 M Sodium acetate, pH 5.5, with 26ml of reagent grade water.
- 0.0065 M L-tyrosine in reagent grade water. Dissolve by warming to 80°C while stirring. Equilibrate to 37°C. (If solution cools, crystals may form. Reheat to dissolve.)
- Pyridoxal Phosphate(B-6) Standard, freshly prepared.
- B-6 Solution #1: Dissolve 5mg in 100 ml reagent grade water, to obtain a concentration of 50 $\frac{\mu g}{ml}$
- B-6 Solution #2: Dilute B-6 Solution #1 further 500x immediately before use to obtain a concentration of 100 $\frac{ng}{ml}$.

Enzyme

- Suspend at 5 $\frac{mg}{ml}$ in reagent grade water. Mix to uniformity before withdrawing aliquots.

Procedure

Set up pyridoxal phosphate curve in test tubes to give 0, 5 ng, 10 ng, 15 ng and 5 μg curve in Warburg flasks. Each Warburg flask will contain 1 mg of enzyme.
Test Tubes, Warburg Flasks
Mix well. Transfer 0.5 ml from each tube to the side arm of a corresponding Warburg flask. Into each

Tube	**Freshly mixed 5 $\frac{mg}{ml}$ enzyme**	**0.222 M sodium acetate**	**reagent grade water**	**pyridoxal phosphate std 0.0005%**	**pyridoxal phosphate std 0.005%**
0 ng	0.4 ml	0.3 ml	0.3 ml	—	—
5 ng	0.4 ml	0.3 ml	0.2 ml	0.1 ml	—
10 ng	0.4 ml	0.3 ml	0.1 ml	0.2 ml	—
15 ng	0.4 ml	0.3 ml	—	0.3 ml	—
5 ug	0.4 ml	0.3 ml	0.1 ml	—	0.2 ml

main well of the Warburg flasks pipette the following:

0.8 M Sodium acetate, pH 5.5 - 0.2 ml

0.0065 M L-tyrosine at 37°C - 2.3 ml

Include one flask containing no enzyme as a blank and a flask containing 3.0 ml of reagent grade water to serve as a thermal barometer. Attach flasks to manometer. After 10 minutes of equilibration close the manometers. Re-equilibrate 10 minutes with manometers closed, and read to establish zero time point. Tip in and mix the substrate and replace the flasks in the bath. Determine the micromoles of CO_2 released at approximately 5 minute intervals for 30 minutes. Plot micromoles CO_2 released versus reaction volume for each tube 0 - 15 ng and unknown samples.

Calculation

- $\frac{\text{Units}}{\text{mg}} = \frac{\frac{\text{microliters } CO_2 \text{ released}}{\text{min}}}{\text{22.4 x mg enzyme in reaction mixture}}$

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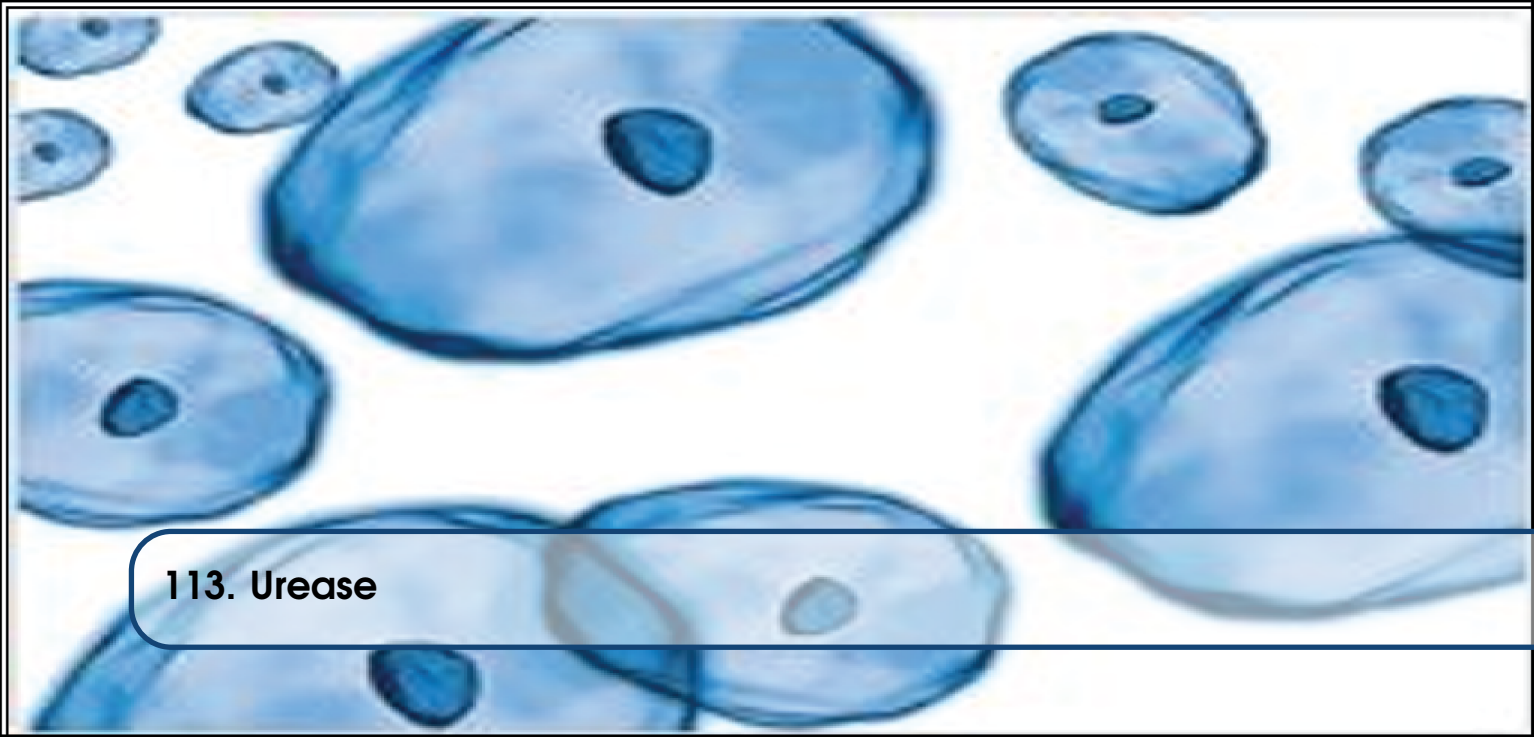

113. Urease

Urea amidohydrolase

Jespersen (1975) reports that ammonium carbamate is produced in citrate and Tris buffer.

Urease occurs in many bacteria, several species of yeast and a number of higher plants. Varner (1960) has reviewed it. Two of the best sources are: Jack beans (*Canavlia ensiformis*) from which it has been crystallized and thoroughly studied, and *Bacillus pasteurii*.

The enzyme is important in assaying for urea. See Guilbault and Montalvo (1970). Its immobilization has been reported: *et al*. (1974), James and Pring (1975), Messing (1974), Nakamoto *et al*. (1975), Sundaram (1973) and Tran-Minh and Broun (1975).

$$(NH_2)_2CO + 3H_2O \rightarrow CO_2 + 2NH_4OH$$

$$\text{Urea} + H_2O + 2H^+ \overset{Urease}{\rightarrow} 2NH_4^+ + CO_2 \qquad (113.1)$$

$$2NH_4^+ + 2\alpha\text{-Ketoglutarate} + 2\text{NADH} \overset{GLDH}{\rightarrow} 2\text{ Glutamate} + 2NAD^+ + 2H_2O$$

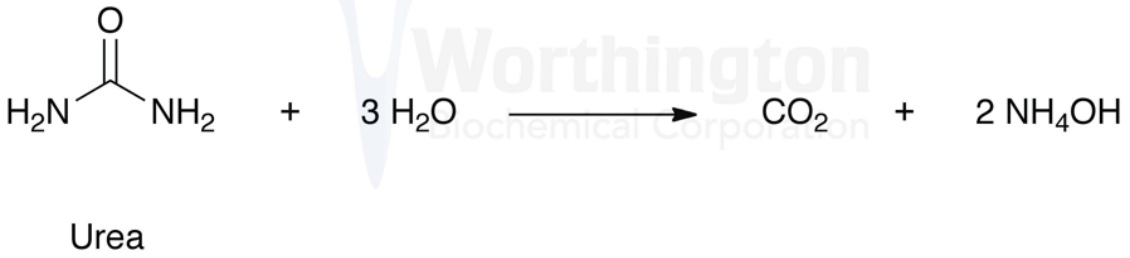

Figure 113.1: Enzymatic Reaction - Urease

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Jack Bean

Specificity

Urease is specific for urea and hydroxyurea (Fishbein and Carbone 1965). See also Sundaram and Laidler (1970).

Composition

Monomeric (a)urease can polymerize to form six unit polymers of about three million daltons. (Fishbein *et al*. 1970; Fishbein and Nagarajan 1972a). Andrews and Reithel (1970) report on the sulfhydryl groups. Contaxis and Reithel (1971) indicate the molecule can be split in half with no loss of activity. See also Contaxis and Reithel (1972), Fishbein and Nagarajan (1972b), Lynn (1970), and Bailey and Boulter (1969).

IUB

- 3.5.1.5

CAS

- 9002-13-5

Molecular Weight

- 480,000 (Fishbein *et al*. 1970)

Optimal pH

- 7.4 (Cesareo and Langton, 1992)

Inhibitors

- Heavy metals. NH_4^+ ions formed. See also Fishbein and Carbone (1965). Sodium and potassium ions are inhibitors (Cesareo and Langton, 1992).

Stabilizers

- EDTA in concentrations of 1 x 10^{-3} M. 50% glycerol solutions protect urease crystalline suspension for several months at 4°C.

Constants

- K_m: 1.3mM in Tris · HCl (Cesareo and Langton, 1992)

Assay Information

Method

- Worthington has adopted an assay method where the hydrolysis of urea is measured by coupling ammonia production to a glutamate dehydrogenase reaction (See Reaction). One unit results in the oxidation of one micromole of NADH per minute at 25°C and pH 7.6 under the specified conditions. In addition to increased sensitivity, the assay method possesses the advantage that it can be manipulated to permit quantitation of urea.
- Urea + H_2O + $2H^+ \overset{Urease}{\rightarrow} 2NH_4^+ + CO_2$
- $2NH_4^+$ + 2α-Ketoglutarate + 2NADH $\overset{GLKH}{\rightarrow}$ 2 Glutamate + $2NAD^+ + 2H_2O$

Reagents

- 0.1 M Potassium phosphate buffer, pH 7.6
- 0.023 M Adenosine-5'-diphosphate (ADP) in phosphate buffer
- 0.0072 M NADH in phosphate buffer
- 0.026 M a-Ketoglutarate in phosphate buffer
- 1.8 M Urea in phosphate buffer
- Glutamate Dehydrogenase: Dilute to approximately 500 $\frac{\text{units}}{\text{ml}}$ in 50% glycerol or phosphate buffer. Store cold during use.

Enzyme

- Dissolve enzyme at one $\frac{\text{mg}}{\text{ml}}$ in 0.1 M phosphate buffer, pH 7.6. Immediately prior to use, dilute further in buffer to obtain a rate of 0.02-0.04 $\frac{\Delta A}{\text{min}}$.

Procedure

Spectrophotometer Settings: Wavelength: 340 nm Temperature: 25°C

Pipette into each cuvette as follows:

- 0.10 M Phosphate buffer, pH 7.6 - 2.4 ml
- 0.023 M ADP - 0.1 ml
- 0.0072 M NADH - 0.1 ml
- 0.026 M α-Ketoglutarate - 0.1 ml

- 1.8 M Urea - 0.1 ml
- GLDH (500 $\frac{\text{units}}{\text{ml}}$) - 0.1 ml

Incubate in spectrophotometer at 25°C for 5-10 minutes to achieve temperature equilibration and establish blank rate, if any. A slight change in absorbance may be observed due to trace ammonia in reagents. Upon obtaining a zero change in absorbance, add 0.1 ml appropriately diluted enzyme. Record decrease in A_{340} for 8-10 minutes. Determine $\frac{\Delta A_{340}}{\text{min}}$ from the linear portion of the curve. A slight lag may occur.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{340}}{\text{min}}}{6.22 \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$$

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114. Uricase

Urate: O_2 Oxidoreductase

This reaction represents the termination of purine catabolism in all mammals excluding man, higher apes and the Dalmatian dog.

The enzyme has been reviewed by Mahler (1963).

Uricase is very important for the determination of uric acid in biological fluids. Not only is the reaction specific, it may be monitored at 292 nm, 340 nm or colorimetrically by coupled chromogenic response. Non-enzymatic methods are interfered with by turbidity or the presence of aspirin, ascorbic acid, glutathione, paracetanol and many antibiotics. See Itiaba *et al.* (1975); Kuan *et al.* (1975); Pesce *et al.* (1974); G*ö*kicke and G*ö*kicke (1973); Kabasakalian *et al.* (1973); Lum and Gambino (1973); Steele (1970) and Troy and Purdy (1970). Uricase (urate oxidase) catalyzes the following overall reaction:

$$\text{Urate} + O_2 + H_2O \rightarrow \text{Allantoin} + H_2O_2 + CO_2 \qquad (114.1)$$

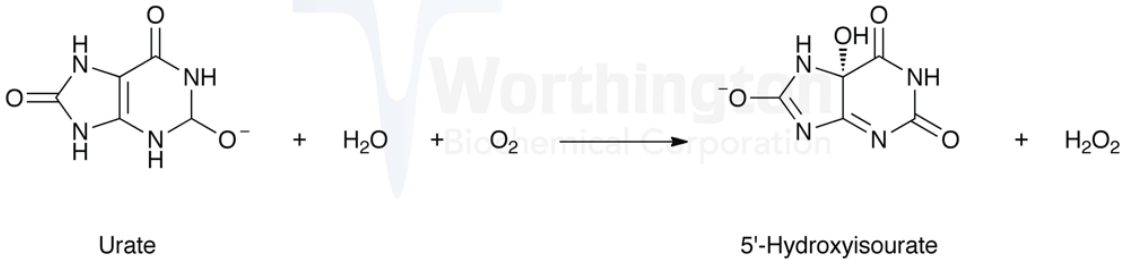

Figure 114.1: Enzymatic Reaction - Uricase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Hog Liver

Specificity

The enzyme is highly specific for uric acid. (See Mahler 1963).

Composition

The enzyme is composed of four subunits of 32,000 M.W. There is one copper atom per molecule (125,000 M.W.) (Pitts *et al.* 1974).

IUB

- 1.7.3.3

CAS

- 9002-12-4

Molecular Weight

- 125,000 (Pitts *et al.* 1974)

Optimal pH

- 9.0 (Mahler 1963)

Isoelectric Point

- 6.3 (Mahler 1963)

Extinction Coefficient

- $E^{1\%}_{278}$ = 11.3 (Mahler 1963)

Inhibitors

- Various purine analogues of urate (Bergmann *et al.* 1963; Baum *et al.* 1956), cyanide and other copper chelating agents. Fridovich (1965) reports that urate in alkaline solution may be converted to a potent inhibitor, oxonate.

Stability/Storage

- Purified uricase in 10% saturated ammonium sulfate is stable for a year at 5°C.

Assay Information

Method

- The reaction velocity is determined by measuring the decrease in absorbance at 290 nm resulting from the oxidation of uric acid to allantoin. One unit oxidizes one micromole of uric acid per minute at 25°C and pH 8.5 under the specified conditions.

Reagents

- 0.1 M Sodium borate buffer, pH 8.5
- 0.12 mM Uric acid. Dissolve 60 mg lithium carbonate in 15 ml water and filter. Prepare fresh solution by dissolving 100 mg uric acid in filtrate. Heating to 50 - 60°C may be necessary to effect solution. Cool and bring to a volume of 100 ml with reagent grade water. Dilute 1/100 with 0.1 M borate, pH 8.5.
- Note: Prior to use, the 0.1 M sodium borate buffer and the 0.12 mM uric acid solution should be oxygenated by bubbling O_2 through the solutions for 10-15 minutes. Reoxygenate every 20 minutes.

Enzyme

- Dissolve at one $\frac{\text{mg}}{\text{ml}}$ in cold (5°C) 0.1 M sodium borate buffer, pH 8.5.

Procedure

Spectrophotometer Settings: Wavelength: 290 nm Temperature: 25°C

Pipette into a cuvette as follows:

- Borate buffer - 0.5 ml
- 0.12 mM Uric acid - 2.0 ml

Incubate in spectrophotometer for 4-5 minutes to achieve temperature equilibration and to establish blank rate, if any. At zero time add 0.5 ml of enzyme and record decrease in A_{290} for 6-7 minutes. Calculate ΔA_{265} from initial linear portion of curve.

Calculation

- $$\frac{\text{Units}}{\text{mg}} = \frac{\frac{\Delta A_{290}}{\text{min}}}{12.2 \text{ x } \frac{\text{mg enzyme}}{\text{ml reaction mixture}}}$$

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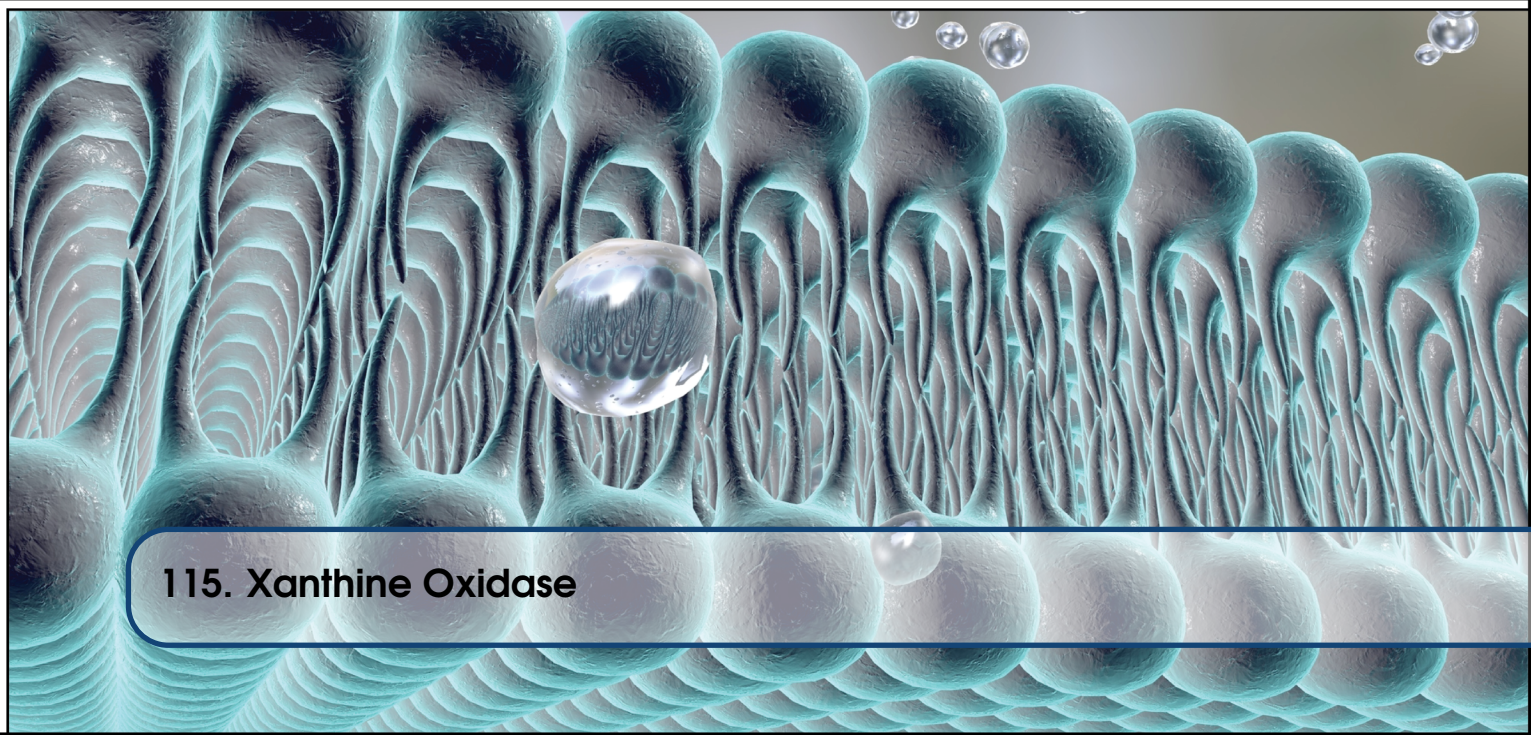

115. Xanthine Oxidase

Xanthine: O Oxidoreductase

Characteristics of Xanthine Oxidase from Milk:

Xanthine oxidase, being a unimolecular, multicomponent electron transport systm, has been the target of extensive study employing electron paramagnetic resonance spectroscopy (Bray *et al*. 1964; Ehrenberg and Bray 1965; Handler *et al*. 1964; Nakamura and Yamazaki 1969). The enzyme has been reported to have anti-tumor effect in mice (Haddow *et al*. 1958) and to participate in the release of iron from hepatic ferritin stores in the plasma (Mazur *et al*. 1958). Xanthine oxidase catalyzes the oxidation of xanthine to uric acid:

$$\text{Xanthine} + O_2 + 2H_2O \rightarrow \text{Urate} + 2H_2O_2 \qquad (115.1)$$

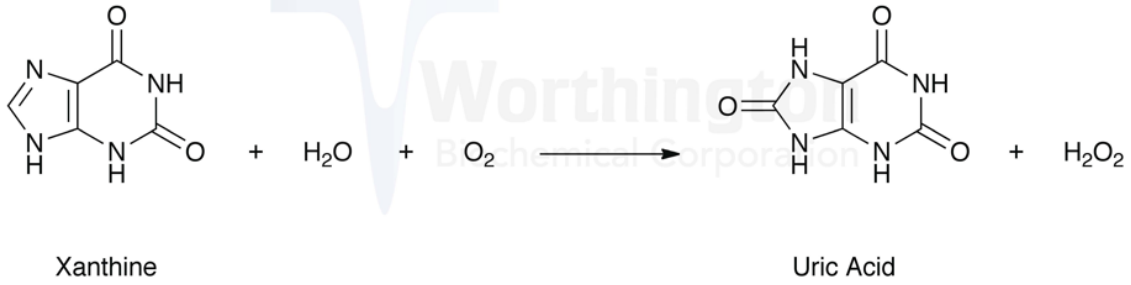

Figure 115.1: Enzymatic Reaction - Xanthine Oxidase

Source for Characteristics

The enzyme characteristics described herein come from the following sources:

- Milk

Specificity

The enzyme has a broad specificity catalyzing the reduction of O_2, cytochrome c, NO^{-3}, $Fe(CN)_6^{-3}$ and various quinones and dyes by aldehydes and purines (Nakamura and Yamazaki 1969).

Composition

Each protein molecule contains 2 moles FAD, 2 gram-atoms Mo, and 8 gram-atoms Fe. The amino acid composition has been determined (Hart *et al.* 1970).

IUB

- 1.1.3.22

Molecular Weight

- 275,000 (Hart *et al.* 1970)

Optimal pH

- 4.6 (Westerfeld *et al.* 1959)

Extinction Coefficient

- $E^{1\%}_{280}$ = 11.26 (Massey *et al.* 1969)

Inhibitors

- Metal ions, urea, purine 6-aldehyde, 2-amino-4-hydroxypteridine 6-aldehyde (Westerfeld *et al.* 1959)

Stability/Storage

- Ammonium sulfate suspensions of the enzyme are stable for weeks when refrigerated, and for several days at room temperature.

Stabilizers

- Salicylate, cysteine, histamine, and versenate act as stabilizers.

Assay Information

Method

- The rate of formation of urate from hypoxanthine is determined by measuring increased absorbance at 290 nm.
- A unit of activity is that forming one micromole of urate per minute at 25°C.

Reagents

- Buffer: 0.05 M Phosphate, pH 7.5
- Substrate: 10 mg hypoxanthine in 500 ml reagent grade water

Enzyme

- Dilute stock suspension with 0.05 M phosphate buffer, pH 7.5, to contain 0.1 to 0.2 $\frac{\text{units}}{\text{ml}}$.

Procedure

Into cuvettes pipette the following:

Description	Test	Control
Buffer	1.9 ml	1.9 ml
Reagent grade water	—-	1.0 ml
Enzyme	0.1 ml	0.1 ml
Substrate (at zero time)	1.0 ml	—-

Record increase in absorbance and determine ΔA_{290} from the linear portion of the curve. The rate is proportional to enzyme concentration within limits of 0.01 to 0.02 units per test.

Calculation

- $\frac{\text{Units}}{\text{mgP}} = \frac{\frac{\Delta A}{\text{min}} \text{ x } 1000}{1.22 \text{ x } 10^4 \text{ x } \frac{\text{mg}}{\text{ml}} \text{ reaction mixture}}$
- $\frac{\text{Units}}{\text{ml}} = \frac{\frac{\Delta A}{\text{min}} \text{ x } 1000 \text{ x } 3 \text{ ml x dilution}}{1.22 \text{ x } 10^4 \text{ x } 0.1 \text{ ml}}$

The molar absorbancy of uric acid = $1.22 \text{ x } 10^4 \frac{1}{\text{cm}}$ (Westerfeld *et al.* 1959)

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116. How to Cite Worthington Literature

How to Cite Worthington Literature

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Worthington Enzyme Manual

- **Print version example (Papain):** Papain, *in Worthington Enzyme Manual*, (Worthington, C., et al), 280 (1993).
- **Online version example (Papain):** Worthington Enzyme Manual. Worthington, C., et al. (2011). Worthington Biochemical Corporation. Date of Access (http://www.worthington-biochem.com/pap/default.html).

Worthington Biochemical Tissue Dissociation Guide

- **Using the Hepatocyte Isolation System:** Hepatocyte Isolation System, *in Worthington Biochemical Corporation Tissue Dissociation Guide*, (Santangelo, C., Ed.), 13 (2008).
- **Online version example:** Worthington Biochemical Online Tissue Dissociation Guide. Santangelo, C. 2011. Worthington Biochemical Corporation. Date of Access (http://www.worthington-biochem.com/tissuedissociation/basic.html).

117. Miscellaneous Information

The following lists and tables appear in various publications. They are reproduced here for convenience and in easy-to-read formats.

Nucleic Acid Data

- Average weight of a DNA basepair (sodium salt) = 650 daltons
- Molecular weight of double-stranded DNA molecule: MW = bp x 650 daltons
- Moles of ends of a double-stranded DNA molecule: Moles = 2 x G / MW
- Moles of ends generated be restriction endonuclease cleavage: Moles = 2 x M x S (circular DNA molecule)
- Moles of ends generated be restriction endonuclease cleavage: Moles = 2 x M x S +2 x M (linear DNA molecule)

  where...
  bp = number of base pairs
  G = grams of DNA
  MW = molecular weight expressed in daltons
  M = moles of DNA
  S = number of sites

- 1.0 A_{260} unit double-stranded DNA = 50 μg/ml = 0.15 mM (in nucleotides)
- 1.0 A_{260} unit single-stranded DNA = 33 μg/ml = 0.10 mM (in nucleotides)
- 1.0 A_{260} unit single-stranded RNA = 40 μg/ml = 0.11 mM (in nucleotides)
- 1.0 kB DNA = coding capacity for 333 amino acids 37,000 dalton protein
- 10,000 dalton protein $\approx$ 270 bp DNA

- 50,000 dalton protein ≈ 1.35 kb DNA

Table 117.1: Lengths and Molecular Weights of Common Nucleic Acids

Nucleic Acid	Number of Nucleotides	Molecular Weight
λ DNA	48,502 (dsDNA)	3.0 x 10^7
pBR322 DNA	4,363 (dsDNA)	2.8 x 10^6
28S rRNA	4,800	1.6 x 10^6
23S rRNA	3,700	1.2 x 10^6
18S rRNA	1,900	6.1 x 10^5
16S rRNA	1,700	5.5 x 10^5
5S rRNA	120	3.6 x 10^4
tRNA (*E. Coli*)	75	2.5 x 10^4

Table 117.2: Nucleic Acid Equivalencies - 1

1 μg of	number of pmol	number of molecules
1000 bp DNA	1.52	9.1 x 10^{11}
pUC18/19 DNA (2686 bp)	0.57	3.4 x 10^{11}
pBR322 DNA (4361 bp)	0.35	2.1 x 10^{11}
M13mp18/19 (7250 bp)	0.21	1.3 x 10^{11}
λ DNA (48502 bp)	0.03	1.8 x 10^{11}

Table 117.3: Nucleic Acid Equivalencies - 2

1 pmol of	number of grams
1000 bp DNA	0.66
pUC18/19 DNA (2686 bp)	1.77
pBR322 DNA (4361 bp)	2.88
M13mp18/19 (7250 bp)	4.78
λ DNA (48502 bp)	32.01

Table 117.4: Nucleotides

Compound	Molecular Weight	λ Max (pH 7.0)	Absorbance at λ Max 1 M solution (pH 7.0)
ATP	507.2	259	15,400
CTP	483.2	271	9,000
GTP	523.2	253	13,700
UTP	484.2	262	10,000
dATP	491.2	259	15,200
dCTP	467.2	271	9,300
dGTP	507.2	253	13,700
dTTP	482.2	267	9,600

Table 117.5: Abbreviations

Abbreviation	Description
bp	Base Pair(s)
kb	kilo Base Pair(s)
ds	Double-Stranded (DNA)
da	Dalton (unit of molecular mass)
M	molarity (number of moles per liter of solution)
ss	Single-Stranded (DNA)

Table 117.6: Agarose Gel Resolution

% Gel	Optimum Resolution for Linear DNA (kbp)
0.5	30 to 1.0
0.7	12 to 0.8
1	10 to 0.5
1.2	7 to 0.4
1.5	3 to 0.2

Amino Acids / The Genetic Code

Table 117.7: Abbreviations, Molecular Weights and Codons for Amino Acids

Amino Acid	3-Letter Abbreviation	1-Letter Symbol	Molecular Weight	Codon
Alanine	Ala	A	89	GCA GCC GCG GCU
Arginine	Arg	R	174	CGA CGC CGG CGU or AGA AGG
Asparagine	Asn	N	132	AAC AAU
Aspartic Acid	Asp	D	133	GAC GAU
Asparagine or Aspartic Acid	Asx	B	—	—
Cysteine	Cys	C	121	UGC UGU
Glutamine	Gln	Q	146	CAA CAG
Glutamic Acid	Glu	E	147	GAA GAG
Glutamine or Glutamic Acid	Glx	Z	—	—
Glycine	Gly	G	75	GGA GGC GGG GGU
Histidine	His	H	155	CAC CAU
Isoleucine	Ile	I	131	AUA AUC AUU
Leucine	Leu	L	131	CUA CUC CUG CUU or UUA UUG
Lysine	Lys	K	146	AAA AAG
Methionine	Met	M	149	AUG
Phenylalanine	Phe	F	165	UUC UUU

Proline	Pro	P	115	CCA CCC CCG CCU
Serine	Ser	S	105	UCA UCC UCG UCU or AGC AGU
Threonine	Thr	T	119	ACA ACC ACG ACU
Tryptophan	Trp	W	204	UGG
Tyrosine	Tyr	Y	181	UAC UAU
Valine	Val	V	117	GUA GUC GUG GUU

Table 117.8: Standard Genetic Code

1st Base	2nd Base U		2nd Base C		2nd Base A		2nd Base G		3rd Base
U	UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys	**U**
	UUC		UCC		UAC		UGC		**C**
	UUA		UCA		UAA	Stop	UGA	Stop	**A**
	UUG		UCG		UAG	Stop	UGG	Trp	**G**
C	CUU	Leu	CCU	Pro	CAU	His	CGU	Arg	**U**
	CUC		CCC		CAC		CGC		**C**
	CUA		CCA		CAA	Gln	CGA		**A**
	CUG		CCG		CAG		CGG		**G**
A	AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser	**U**
	AUC		ACC		AAC		AGC		**C**
	AUA		ACA		AAA	Lys	AGA	Arg	**A**
	AUG	Met	ACG		AAG		AGG		**G**
G	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly	**U**
	GUC		GCC		GAC		GGC		**C**
	GUA		GCA		GAA	Glu	GGA		**A**
	GUG		GCG		GAG		GGG		**G**

Enzyme/Protein Classification Table

Table 117.9: IUB, CAS, Protein Acc #

Enzyme/Biochemical	IUB	CAS	Protein Acc. #
Actin		51005-14-2	P68135
Adenosine Deaminase	3.5.4.4	9026-93-1	P56658
Albumin, Nuclease-Free		9048-46-8	P02769
Alcohol Dehydrogenase	1.1.1.1	9031-72-5	P00330
Aldolase	4.1.2.13	9024-52-6	P00883
Amino Acid Oxidase, D-	1.4.3.3	9000-88-8	P00371
Amino Acid Oxidase, L-	1.4.3.2	9000-89-9	O93364
Amylase, Alpha	3.2.1.1	9000-90-2	P00690
Amylase, Beta	3.2.1.2	9000-91-3	P10537
Arginase	3.5.3.1	9000-96-8	Q2KJ64
Asparaginase	3.5.1.1	9015-98-3	P00805
Aspartate Aminotransferase	2.6.1.1	9000-97-9	P00503
Avidin		1405-69-2	P02701
Beta Agarase	3.2.1.81	37288-57-6	P13734
Carbonic Anhydrase	4.2.1.1	9001-03-0	P00921
Carboxypeptidase A	3.4.17.1	11075-17-5	P00730
Carboxypeptidase B	3.4.17.2	9025-24-5	P09955
Carboxypeptidase Y	3.4.16.5	9046-67-7	P00729
Casein, Alpha		9000-71-9	P02662
Catalase	1.11.1.6	9001-05-2	P00432
Cellulase	3.2.1.4	9012-54-8	Endoglucanase I: P07981 Endoglucanase II: P07982 Endoglucanase IV: O14405 Endoglucanase V: P43317 Endoglucanase VII: Q7Z9M7 beta-1,4-glucanase: O00095 Cellobiohydrolase I: P62694 Cellobiohydrolase II: P07987 beta-Glucosidase I: AAA18473 beta-Glucosidase II: O93785
Cholesterol Esterase	3.1.1.13	9026-00-0	NP_001116606
Cholinesterase, Acetyl	3.1.1.7		
Cholinesterase, Butyryl	3.1.1.8	9001-08-5	
Chymotrypsin	3.4.21.1	2594868	P00766
Chymotrypsinogen A		9035-75-0	P00766
Clostripain (Endoproteinase-Arg-C)	3.4.22.8	9028-00-6	P09870
Collagen		9007-34-5	P02453 (alpha-1(I) chain) P02465 (alpha-2(I) chain)
Collagenase	3.4.24.3	9001-12-1	Q9X721 (ColG) Q46085 (ColH)
Concanavalin A		11028-71-0	
Creatine Kinase	2.7.3.2		
Cytochrome C Oxidase	1.9.3.1	9001-16-5	

Deoxyribonuclease I	3.1.21.1	9003-98-9	P00639
Deoxyribonuclease II	3.1.22.1	9025-64-3	O62855
DNA		9007-49-2	
Dextranase	3.2.1.11	9025-70-1	CAB91097
Diaphorase	1.6.99.1	9001-68-7	Q97E86
DNA Ligase, T4	6.5.1.1	9015-85-4	P00970
DNA Polymerase I	2.7.7.7	9012-90-2	P00582
DNA Polymerase, Taq	2.7.7.7	9012-90-2	P19821
DNA Polymerase, T4	2.7.7.7	9012-90-2	P04415
Elastase	3.4.21.36	9004-06-2	P00772
Galactose Oxidase	1.1.3.9	9028-79-9	P0CS93
Galactosidase, Beta	3.2.1.23	9031-11-2	
Glucose Oxidase	1.1.3.4		
Glucose-6-Phosphate Dehydrogenase	1.1.1.49	9001-40-5	P11411
Glucosidase, Beta	3.2.1.21		
Glucuronidase, Beta	3.2.1.31		
Glutamate Decarboxylase	4.1.1.15	9024-58-2	P69908
Glyceraldehyde-3-Phosphate Dehydrogenase	1.2.1.12		
Glycerol Dehydrogenase	1.1.1.6		
Glycerol Kinase	2.7.1.30		
Hemoglobin		9008-02-0	
Hexokinase	2.7.1.1	9001-51-8	
Histones		37244-51-2	
Hyaluronic Acid		9004-61-9	
Hyaluronidase	3.2.1.35	37326-33-3	
Hydroxysteroid Dehydrogenase	1.1.1.50 1.1.1.51	9028-56-2 9015-81-0	
Lactate Dehydrogenase	1.1.1.27	9001-60-9	
Lactate Dehydrogenase, L-	1.1.2.3		
Lactoperoxidase	1.11.1.7	9003-99-0	
Leucine Aminopeptidase	3.4.11.1	9001-61-0	P28839 P00727
Lipase	3.1.1.3		
Lysozyme	3.2.1.17	9001-63-2	P00698
Malate Dehydrogenase	1.1.1.37		
Maltase	3.2.1.20		
Mucin		84195-52-8	
Myoglobin		11080-17-4	P02192
NADase (DPNase)	3.2.2.5		
Neuraminidase	3.2.1.18	9001-67-6	
Neutral Protease (Dispase)	3.4.24.28	9001-92-7	P29148
Nitrate Reductase	1.9.6.1	9029-42-9	
Nuclease, Micrococcal	3.1.31.1	9013-53-0	
Nuclease, S1	3.1.30.1	37288-25-8	
Nucleohistone		37244-51-2	
Ovalbumin		9006-59-1	P01012

Oxalate Decarboxylase	4.1.1.2		
Papain	3.4.22.2	9001-73-4	P00784
Papain, Chymo	3.4.22.6	9001-09-6	P14080
Pectinase	4.2.2.10	9033-35-6	
Pepsin	3.4.23.1	9001-75-6	P00791
Peroxidase	1.11.1.7	9003-99-0	
Phosphatase, Acid	3.1.3.2	9001-77-8	
Phosphatase, Alkaline	3.1.3.1	9001-78-9	
Phosphodiesterase I	3.1.4.1	9025-82-5	
Phosphodiesterase II	3.1.16.1	9068-54-6	
Phosphoenolpyruvate Carboxylase	4.1.1.31		
Phosphoglucomutase	5.4.2.2		
Phospholipase A2	3.1.1.4	9001-84-7	
Phospholipase C	3.1.4.3		
Plasma Amine Oxidase	1.4.3.21	9001-53-0	
Pokeweed Antiviral Toxin		63231-57-2	
Polyphenol Oxidase	1.14.18.1	9002-10-2	
Protease	3.4.21.19	66676-43-5	
Proteinase K	3.4.21.64	39450-01-6	
Pyruvate Kinase	2.7.1.40		
Reverse Transcriptase	2.7.7.49	9068-38-6	
Ribonuclease A	3.1.27.5	9001-99-4	P61823
Ribonuclease B	3.1.27.5	9001-99-4	P61823
Ribonuclease T1	3.1.27.3	2603057	P00651
Ribonuclease T2	3.1.27.1	37278-25-4	
Ribonucleic Acid		63231-32-0	
RNA Polymerase	2.7.7.6		
Superoxide Dismutase	1.15.1.1	9054-89-1	
Trypsin	3.4.21.4	2594141	P00760
Trypsin Inhibitors		9035-81-1	
Trypsinogen		9002-08-8	
Tyrosine Decarboxylase	4.1.1.25	9002-09-9	
Urease	3.5.1.5	9002-13-5	
Uricase	1.7.3.3	9002-12-4	
Xanthine Oxidase	1.1.3.22		

Table 117.10: Class. Sorted by IUB #

Enzyme/Biochemical	IUB	CAS	Protein Acc. #
Alcohol Dehydrogenase	1.1.1.1	9031-72-5	P00330
Glycerol Dehydrogenase	1.1.1.6		
Lactate Dehydrogenase	1.1.1.27	9001-60-9	
Malate Dehydrogenase	1.1.1.37		
Glucose-6-Phosphate Dehydrogenase	1.1.1.49	9001-40-5	P11411
Hydroxysteroid Dehydrogenase	1.1.1.50 1.1.1.51	9028-56-2 9015-81-0	
Lactate Dehydrogenase, L-	1.1.2.3		
Glucose Oxidase	1.1.3.4		
Galactose Oxidase	1.1.3.9	9028-79-9	P0CS93
Xanthine Oxidase	1.1.3.22		
Glyceraldehyde-3-Phosphate Dehydrogenase	1.2.1.12		
Amino Acid Oxidase, L-	1.4.3.2	9000-89-9	O93364
Amino Acid Oxidase, D-	1.4.3.3	9000-88-8	P00371
Plasma Amine Oxidase	1.4.3.21	9001-53-0	
Diaphorase	1.6.99.1	9001-68-7	Q97E86
Uricase	1.7.3.3	9002-12-4	
Cytochrome C Oxidase	1.9.3.1	9001-16-5	
Nitrate Reductase	1.9.6.1	9029-42-9	
Catalase	1.11.1.6	9001-05-2	P00432
Lactoperoxidase	1.11.1.7	9003-99-0	
Peroxidase	1.11.1.7	9003-99-0	
Polyphenol Oxidase	1.14.18.1	9002-10-2	
Superoxide Dismutase	1.15.1.1	9054-89-1	
Aspartate Aminotransferase	2.6.1.1	9000-97-9	P00503
Hexokinase	2.7.1.1	9001-51-8	
Glycerol Kinase	2.7.1.30		
Pyruvate Kinase	2.7.1.40		
Creatine Kinase	2.7.3.2		
RNA Polymerase	2.7.7.6		
DNA Polymerase I	2.7.7.7	9012-90-2	P00582
DNA Polymerase, Taq	2.7.7.7	9012-90-2	P19821
DNA Polymerase, T4	2.7.7.7	9012-90-2	P04415
Reverse Transcriptase	2.7.7.49	9068-38-6	
Lipase	3.1.1.3		
Phospholipase A2	3.1.1.4	9001-84-7	
Cholinesterase, Acetyl	3.1.1.7		
Cholinesterase, Butyryl	3.1.1.8	9001-08-5	
Cholesterol Esterase	3.1.1.13	9026-00-0	NP_001116606
Phosphatase, Alkaline	3.1.3.1	9001-78-9	
Phosphatase, Acid	3.1.3.2	9001-77-8	
Phosphodiesterase I	3.1.4.1	9025-82-5	
Phospholipase C	3.1.4.3		
Phosphodiesterase II	3.1.16.1	9068-54-6	

Deoxyribonuclease I	3.1.21.1	9003-98-9	P00639
Deoxyribonuclease II	3.1.22.1	9025-64-3	O62855
Ribonuclease T2	3.1.27.1	37278-25-4	
Ribonuclease T1	3.1.27.3	12/4/26	P00651
Ribonuclease A	3.1.27.5	9001-99-4	P61823
Ribonuclease B	3.1.27.5	9001-99-4	P61823
Nuclease, S1	3.1.30.1	37288-25-8	
Nuclease, Micrococcal	3.1.31.1	9013-53-0	
Amylase, Alpha	3.2.1.1	9000-90-2	P00690
Amylase, Beta	3.2.1.2	9000-91-3	P10537
Cellulase	3.2.1.4	9012-54-8	Endoglucanase I: P07981 Endoglucanase II: P07982 Endoglucanase IV: O14405 Endoglucanase V: P43317 Endoglucanase VII: Q7Z9M7 beta-1,4-glucanase: O00095 Cellobiohydrolase I: P62694 Cellobiohydrolase II: P07987 beta-Glucosidase I: AAA18473 beta-Glucosidase II: O93785
Dextranase	3.2.1.11	9025-70-1	CAB91097
Lysozyme	3.2.1.17	9001-63-2	P00698
Neuraminidase	3.2.1.18	9001-67-6	
Maltase	3.2.1.20		
Glucosidase, Beta	3.2.1.21		
Galactosidase, Beta	3.2.1.23	9031-11-2	
Glucuronidase, Beta	3.2.1.31		
Hyaluronidase	3.2.1.35	37326-33-3	
Beta Agarase	3.2.1.81	37288-57-6	P13734
NADase (DPNase)	3.2.2.5		
Leucine Aminopeptidase	3.4.11.1	9001-61-0	P28839 P00727
Carboxypeptidase Y	3.4.16.5	9046-67-7	P00729
Carboxypeptidase A	3.4.17.1	11075-17-5	P00730
Carboxypeptidase B	3.4.17.2	9025-24-5	P09955
Chymotrypsin	3.4.21.1	7/3/04	P00766
Trypsin	3.4.21.4	7/7/02	P00760
Protease	3.4.21.19	66676-43-5	
Elastase	3.4.21.36	9004-06-2	P00772
Proteinase K	3.4.21.64	39450-01-6	
Papain	3.4.22.2	9001-73-4	P00784
Papain, Chymo	3.4.22.6	9001-09-6	P14080
Clostripain (Endoproteinase-Arg-C)	3.4.22.8	9028-00-6	P09870
Pepsin	3.4.23.1	9001-75-6	P00791
Collagenase	3.4.24.3	9001-12-1	Q9X721 (ColG) Q46085 (ColH)
Neutral Protease (Dispase)	3.4.24.28	9001-92-7	P29148
Asparaginase	3.5.1.1	9015-98-3	P00805

Urease	3.5.1.5	9002-13-5	
Arginase	3.5.3.1	9000-96-8	Q2KJ64
Adenosine Deaminase	3.5.4.4	9026-93-1	P56658
Oxalate Decarboxylase	4.1.1.2		
Glutamate Decarboxylase	4.1.1.15	9024-58-2	P69908
Tyrosine Decarboxylase	4.1.1.25	9002-09-9	
Phosphoenolpyruvate Carboxylase	4.1.1.31		
Aldolase	4.1.2.13	9024-52-6	P00883
Carbonic Anhydrase	4.2.1.1	9001-03-0	P00921
Pectinase	4.2.2.10	9033-35-6	
Phosphoglucomutase	5.4.2.2		
DNA Ligase, T4	6.5.1.1	9015-85-4	P00970
Actin		51005-14-2	P68135
Albumin, Nuclease-Free		9048-46-8	P02769
Avidin		1405-69-2	P02701
Casein, Alpha		9000-71-9	P02662
Chymotrypsinogen A		9035-75-0	P00766
Collagen		9007-34-5	P02453 (alpha-1(I) chain) P02465 (alpha-2(I) chain)
Concanavalin A		11028-71-0	
Deoxyribonucleic Acid and Related Products		9007-49-2	
Hemoglobin		9008-02-0	
Histones		37244-51-2	
Hyaluronic Acid		9004-61-9	
Mucin		84195-52-8	
Myoglobin		11080-17-4	P02192
Nucleohistone		37244-51-2	
Ovalbumin		9006-59-1	P01012
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Trypsinogen		9002-08-8	

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