BBA 63381

Roles of collagenases and other proteolytic enzymes in the dispersal of animal tissues

Crude or partially purified bacterial collagenase preparations are effective in dispersing various animal tissues for culturing[1–5] or metabolic studies[6–8]. However, the role of collagenase proper in such reactions is obscure, since crude collagenase is a mixture of a number of hydrolytic enzymes[9]. Furthermore, recent work in several laboratories has indicated (*cf.* ref. 10) that "collagenase", once considered to be a single enzyme (clostridiopeptidase A, EC 3.4.4.19), is actually a mixture of two types of several collagenases contaminated by a significant amount of a proteolytic enzyme that attacks casein. Recently, I was successful[10] in separating six collagenases, of which three enzymes designated as A-α, B-α, and B-β were inactive against casein. It was then demonstrated[10] that undenatured so-called insoluble collagen was digested rapidly and completely by a mixture of the purified collagenases A-α and B-α, but only very slowly by collagenase A-α alone, and practically not at all by collagenase B-α alone.

Accordingly, effects of "collagenase" on animal tissues were reinvestigated using the newly purified collagenases. The results in the present communication indicate (a) that the mixture of the purified collagenases A-α and B-α, unlike the crude preparation, did not disperse any rat tissue tested; (b) that the enzymes nevertheless solubilized collagen from fresh tissue; and (c) that a combined action of trypsin (and/or chymotrypsin) and the purified collagenases was required to disperse fresh rat tail tendon, adipose tissue, and cardiac muscle.

Collagenases A-α and B-α were purified from a crude collagenase preparation of *Clostridium histolyticum* as described previously[10]. Crystalline trypsin (EC 3.4.4.4), α-chymotrypsin (EC 3.4.4.5), and soybean trypsin inhibitor were purchased from Nutritional Biochemicals Co. Fresh tissues were obtained from male rats of the Sprague–Dawley strain weighing about 200 g. Protein in tail tendon was dissolved in hot 1 M NaOH (5 min at 95°), and assayed by the method of LOWRY *et al.*[11]. Hydroxyproline was assayed as described by MIYADA AND TAPPEL[12], after hydrolysis of the protein (6 M HCl, 18 h). Perfusion of rat heart was carried out as described by MORGAN *et al.*[13]. Yields of dispersed cells were estimated from the content of lactate dehydrogenase (EC 1.1.1.27) in washed cell preparations as described by TAKEDA *et al.*[14].

Preliminary tests indicated that a mixture of the purified collagenases would not disperse any rat tissue even including tail tendon which is readily digestible with a crude collagenase preparation. However, results of chemical analyses (Fig. 1) indicated that the enzymes could solubilize most of the collagen (represented by hydroxyproline) in fresh tail tendon, and that the cord-like appearance of the tendon was retained by the undigested residue which comprised only about 10% of the total protein. This residue, which was not solubilized by the mixture of the purified collagenases even after a long (8 h) incubation, was digestible with trypsin. The data (Fig. 1) also show that, upon treatment of the intact tissue initially with trypsin, approx. 10% of the protein was released. Again, the cord-like appearance was not destroyed, but the tissue treated with trypsin for 1 h (Fig. 1) was then digested rapidly and completely by the mixture of the purified collagenases.

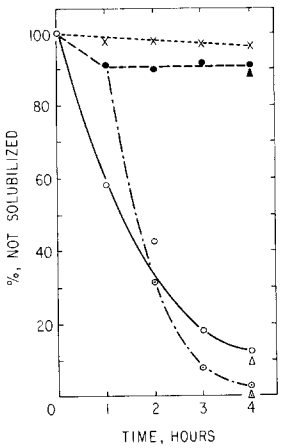

Fig. 1. Enzymatic digestion of tendon. Fresh rat tail tendon (10 mg) was incubated with either trypsin (1 mg) or collagenases (50 μg A-α *plus* 100 μg B-α) in 1 ml of 10 mM veronal buffer containing 0.1 mM $CaCl_2$ (pH 8.0) at 37°. After the incubation, insoluble materials were sedimented by centrifugation, washed, and subjected to analyses. Protein was determined on samples incubated with buffer alone (X), trypsin (●), collagenases (○), or trypsin followed by collagenases (⊙). Hydroxyproline was assayed on samples incubated with trypsin (▲), collagenase (△), or trypsin followed by collagenases (◬). Each point was the mean value of duplicate experiments.

The above results suggested that a tissue dispersible with crude collagenase may also be dispersible by using the purified collagenases A-α and B-α in conjunction with trypsin. In fact, epididymal adipose tissue, which is readily dispersible with crude collagenase[6], was easily dispersed by incubating the tissue with trypsin (and/or chymotrypsin) and a mixture of purified collagenases under the conditions described

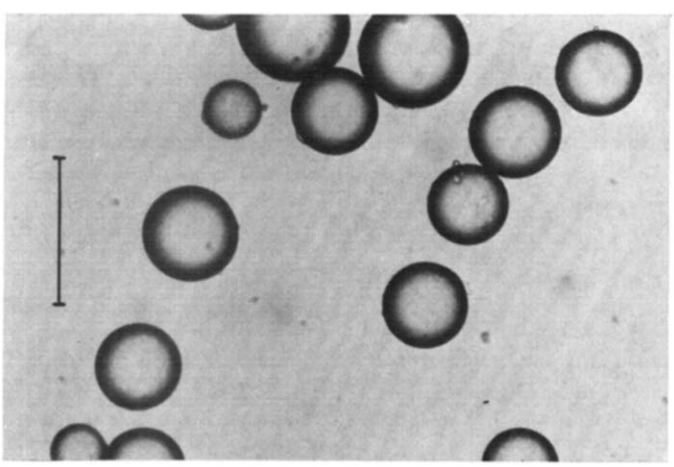

Fig. 2. Photomicrograph of fat cells. The cells were prepared as described below. Fresh rat epididymal adipose tissue (0.1 g) was incubated for 1 h at 37° with trypsin (0.5 mg) *plus* α-chymotrypsin (0.5 mg) in 1 ml of Krebs–Henseleit bicarbonate buffer (pH 7.4). The partially digested tissue was rinsed with buffer containing soybean trypsin inhibitor (0.1 mg/ml) and albumin (2%; bovine serum fraction V). The rinsed tissue was incubated for 1 h at 37° with collagenases (50 μg A-α *plus* 100 μg B-α) in 1 ml buffer containing albumin (2%). The dispersed cells were washed as described by RODBELL[6]. The scale in the figure represents 0.1 mm.

in the legend for Fig. 2. The appearance of free fat cells thus obtained (Fig. 2) was identical with that of the cells dispersed with crude collagenase[6]. The yield of cells was 70–80%.

Using the same combination of enzymes, a well-dispersed cell suspension was obtained also from cardiac muscle tissue (Fig. 3). Dispersion of adult rat hearts has not been reported previously. The microscopic appearance of the cells (Fig. 3) was similar to that of embryonic chick hearts dispersed with crude collagenase[5], *i.e.* 60–80% of the cells were rod-shaped and the rest spherical. The yield of cells was 20–40%. However, most of the cells were completely digested when the perfusion medium (see legend for Fig. 3) contained more than 1 mM $CaCl_2$.

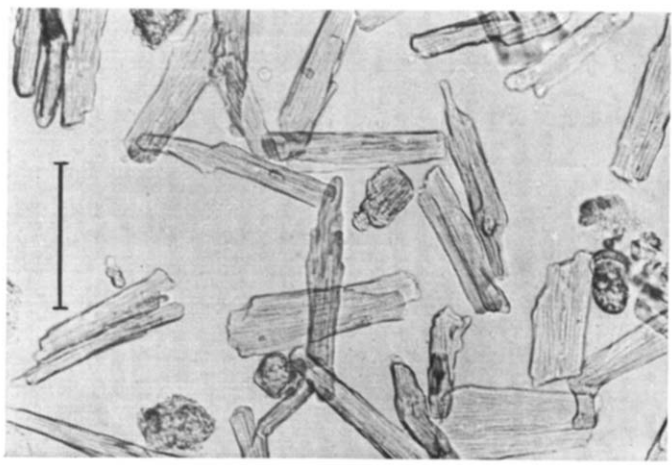

Fig. 3. Photomicrograph of cardiac muscle cells. The cells were prepared as described below. A rat heart was perfused for 30 min at 37° with trypsin (5 mg) and α-chymotrypsin (5 mg) in 25 ml Krebs–Henseleit bicarbonate buffer (pH 7.4) containing glucose (5 mM) but a very low concentration of $CaCl_2$ (10 μM). The partially digested right ventricular tissue (0.1 g) was cut, washed, and incubated with collagenases as described above for adipose tissue, but in the presence of glucose (5 mM). The scale in the figure represents 0.1 mm.

As in the case of tail tendon, neither adipose tissue nor cardiac muscle was dispersed by either a mixture of trypsin and chymotrypsin alone or a mixture of the purified collagenases alone. Accordingly, it may be postulated that the cells in these tissues are doubly bound by two types of intercalated proteins, one digestible with trypsin (and/or chymotrypsin) and the other with a mixture of the purified collagenases A-α and B-α. It is suggested that a matrix of the same types of proteins may also exist in pituitary rudiment and lung of chick embryos, since SOBEL[2] and GROVER[4] have reported that these tissues were dispersible by the combined action of trypsin and partially purified collagenase preparations, which presumably corresponded to the afore mentioned clostridiopeptidase A.

This work was supported by U.S. Public Health Service Grants RO1 AM 06725-05 and 07462-2 AMP, and carried out with the technical assistance of Mrs. Frances W. Barham. I am indebted to Dr. C. R. Park for his helpful discussions and to Dr. G. H. Smith for help in the preparation of this paper.

Department of Physiology,
Vanderbilt University
School of Medicine,
Nashville, Tenn. (U.S.A.)

TETSURO KONO

1 E. Y. LASFARGUES, *Exptl. Cell. Res.*, 13 (1957) 553.
2 H. SOBEL, *J. Embryol. Exptl. Morphol.*, 6 (1958) 518.
3 R. W. HINZ AND J. T. SYVERTON, *Proc. Soc. Exptl. Biol. Med.*, 101 (1959) 19.
4 J. W. GROVER, *Exptl. Cell Res.*, 26 (1962) 344.
5 D. J. CAVANAUGH, W. O. BERNDT AND T. E. SMITH, *Nature*, 200 (1963) 261.
6 M. RODBELL, *J. Biol. Chem.*, 239 (1964) 375.
7 J. N. FAIN, N. REED AND R. SAPERSTEIN, *J. Biol. Chem.*, 242 (1967) 1887.
8 S. L. HOWELL AND K. W. TAYLOR, *Biochem. J.*, 108 (1968) 17.
9 I. MANDL, *Advan. Enzymol.*, 23 (1961) 163.
10 T. KONO, *Biochemistry*, 7 (1968) 1106.
11 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
12 D. S. MIYADA AND A. L. TAPPEL, *Anal. Chem.*, 28 (1956) 909.
13 H. E. MORGAN, M. J. HENDERSON, D. M. REGEN AND C. R. PARK, *J. Biol. Chem.*, 236 (1961) 253.
14 Y. TAKEDA, A. ICHIHARA, H. TANIOKA AND H. INOUE, *J. Biol. Chem.*, 239 (1964) 3590.

Received September 16th, 1968
Revised manuscript received December 24th, 1968

Biochim. Biophys. Acta, 178 (1969) 397–400

BBA 63385

Differences between the active and the inhibited conformations of the allosteric *N*-acetylglutamate 5-phosphotransferase

The catalytic activity of the first enzyme in the arginine pathway of *Chlamydomonas reinhardti*, ATP:*N*-acetylglutamate 5-phosphotransferase is inhibited by the end product arginine. As reported, the kinetic relationship between acetylglutamate and arginine is competitive, while between ATP and the allosteric modifier it is non-competitive. Urea in low concentrations (less than 1.5 M) suppresses the inhibition caused by arginine without impairing the catalytic activity of the enzyme[1].

These data suggest that phosphotransferase has separate binding sites for the substrates and the inhibitor, and has at least two different conformations. One of these conformations is catalytically active and binds the substrate acetylglutamate, while the other one is inactive, binding the inhibitor arginine. The two functional and structural forms of the enzyme are mutually exclusive.

A sigmoidal kinetic pattern has been found in the inhibition of phosphotransferase, indicating that subunit structure plays some role in the allosteric transition[2]. For this reason the molecular weight of the enzyme has been estimated under different circumstances. The estimation is carried out by Sephadex G-200 gel filtration, according to the method of ANDREWS[3]. For calibration of the column (50 cm × 2.5 cm diameter) β-galactosidase (EC 3.2.1.23) and crystalline D-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) are used. Their elution volumes are 90 ± 2 ml and