RESEARCH PAPER

Islets 1:3, 185-190; November/December 2009; © 2009 Landes Bioscience

A new collagenase blend increases the number of islets isolated from mouse pancreas

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Key words: pancreas, islet, mouse, liberase, collagenase, isolation, diabetes, HPLC

Abbreviations: CMRL, connaught medical research laboratories; Ct, cycle threshold; DMEM, Dulbecco's modified eagle medium; DTZ, dithizone; FCS, fetal calf serum; HMBS, hydroxymethylbilane synthase; HPLC, high performance liquid chromatography; IEQ, islet equivalent; mAU, milli-absorbance unit; NMRI, naval medical research institute; WST-1, water-soluble-tetrazolium-1

Diabetes is a predominant metabolic disorder in the industrialized nations. Since pancreatic islets play a key role in type I and type II diabetes, the isolation of islets from pancreatic tissues represents an important step in diabetes research. However, to date, only a small fraction of all islets, resident within any given pancreas, are harvested by using currently available enzyme blends. This holds true for islet isolation from both the mouse and the human pancreas. In the present study, the newly developed Liberase TL Research Grade was compared to the widely used Liberase RI to investigate the effect of increased collagenase purity on islet yield. The study shows that reducing the degradation products of collagenases during Liberase™ production significantly increases the number of islets isolated from the mouse pancreas by 28%, and, therefore, is expected to lower the numbers of mice and resulting costs for diabetes research accordingly. Furthermore, this study also points to a possibility to increase the number and mass of islets isolated from human pancreases, for which only a limited donor pool exists.

Introduction

Islet isolation procedures employ collagenases, which are proteases used to release islets from the acinar tissue.[1] Crucial criteria for obtaining maximum numbers of viable and functionally intact islets include collagenase composition and purity, and the duration of the enzymatic digestion of the pancreas.[2-4] Collagenases I and II, purified from *Clostridium histolyticum*, exhibit differences in their amino acid sequences and secondary structures, but show very similar catalytic domains. Collagenase I attacks residues near the ends of triple helical collagen domains, whereas collagenase II initially digests at more interior sites.[5] For the isolation of islets from rat pancreas, a clear synergistic effect of collagenases I and II has been demonstrated.[6] In addition, neutral protease activity has been shown to enhance collagenase-induced dissociation of the pancreas.[7]

In the present study, the effect of increased collagenase purity on islet yield was investigated. In particular, the second generation Liberase™ TL Research Grade was compared to the widely used Liberase™ RI. As the ratio of collagenase I to II and the amount of thermolysin are already optimized for pancreatic islet isolation, the main difference between these two enzyme blends is the improved enzyme production and purification process. In particular, the purity and intactness of the collagenase components appears to be critical for their functionality, as degradation of collagenase I correlates with decreased islet release.[8]

Regarding the duration of enzymatic digestion, prolonged isolation stress is responsible for triggering a cascade of events resulting in reduced yield and functional impairment of islets.[9] In addition, longer exposure times to proteases negatively influence islet cell viability by activating apoptotic pathways.

Optimizing collagenase purification should therefore result in a higher number of healthy islets. This may have significant implications for the mouse costs required for diabetes research in the short run, as well as success in human islet isolation and transplantation in the long run.

Results

Chromatographical analysis of liberases. The production procedure of Liberase™ TL Research Grade was optimized by

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Submitted: 05/12/09; Revised: 07/17/09; Accepted: 07/17/09
Previously published online: www.landesbioscience.com/journals/islets/article/9556

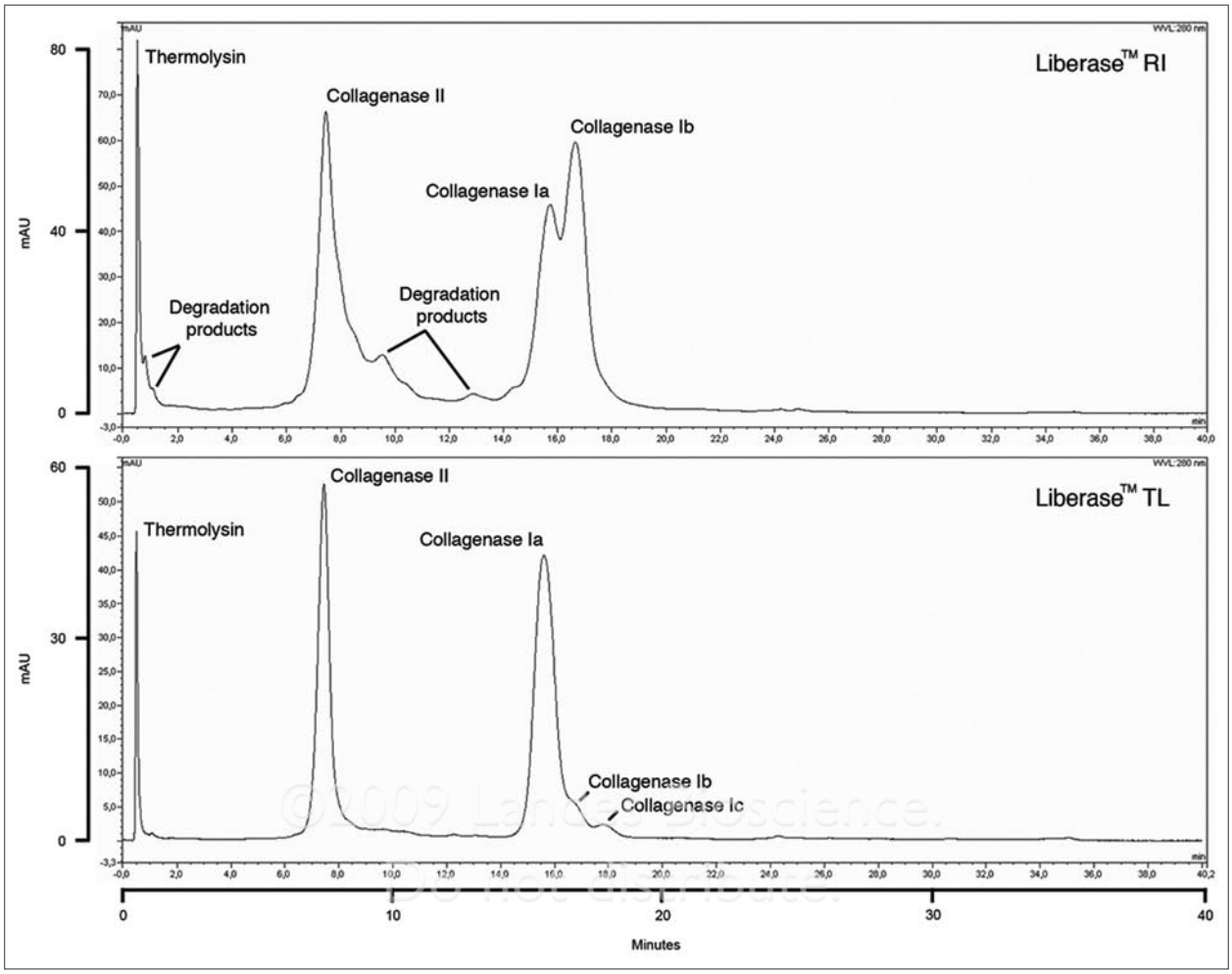

Figure 1. Anion-exchange HPLC chromatograms of Liberase RI and Liberase TL Research Grade. Both collagenase enzyme blends were separated on Mono-Q columns and eluted in Tris-$CaCl_2$-NaCl.

adjusting the chromatographic steps after isolation of collagenase I and II. More specifically, the matrix characteristics of the chromatographic column were changed (Patent pending).

Here, the purity of both collagenase I and II in Liberase™ TL Research Grade was analyzed by using HPLC, and then compared to Liberase™ RI (**Fig. 1**). The first peak, eluting from the column, represents the protease thermolysin. The collagenase II peak, eluting after 7.4 min, showed significantly lower amounts of degradation products in Liberase™ TL Research Grade compared to Liberase™ RI (**Fig. 1**).

For collagenase I, three fractions can be distinguished.[10] The peak eluting at 15.6 to 15.7 min corresponds to collagenase Ia, which is the intact fraction. Collagenase Ib and Ic peaks, eluting at 16.5 and 17.7 min, respectively, are the degradation products of collagenase I (**Fig. 1**).

Based on this HPLC profile, the intact fraction of collagenase I represent less than 50% of the whole collagenase I in Liberase™ RI, whereas more than 80% of the collagenase I is intact in Liberase™ TL Research Grade (**Fig. 1**). Similarly, collagenase II does not show any signs of degradation in Liberase™ TL Research Grade (**Fig. 1**).

Islet yield and purity. The digestion times of pancreases perfused with Liberase™ RI and Liberase™ TL Research Grade were optimized for both enzymes on the basis of islet yield as 22 min and 18 min, respectively, showing that the digestion time could be reduced by about 20% (**Fig. 2**).

Furthermore, both preparations showed high purity with the optimized times after overnight culture, as judged by DTZ-staining of islets (**Fig. 3A and B**).

More importantly, in comparison to Liberase™ RI, both the total IEQ (38% increase, $p = 0.030$, **Fig. 3C**) and the total islet number (28% increase, $p = 0.043$, **Fig. 3D**) per pancreas were significantly higher for pancreases perfused with Liberase™ TL Research Grade. The size distribution of islets indicated that the most obvious difference between the groups is for islets with a diameter ranging from 200 to 250 μm ($p = 0.005$) (**Fig. 3E**). In addition to this, the total number of islets larger than 150 μm is also significantly higher for Liberase™ TL Research Grade (RI: 88 ± 20; TL: 152 ± 23; $p = 0.002$).

These results show that Liberase™ TL Research Grade, harboring intact collagenases, allows for shorter digestion times to be used and, at the same time, gives a better islet yield.

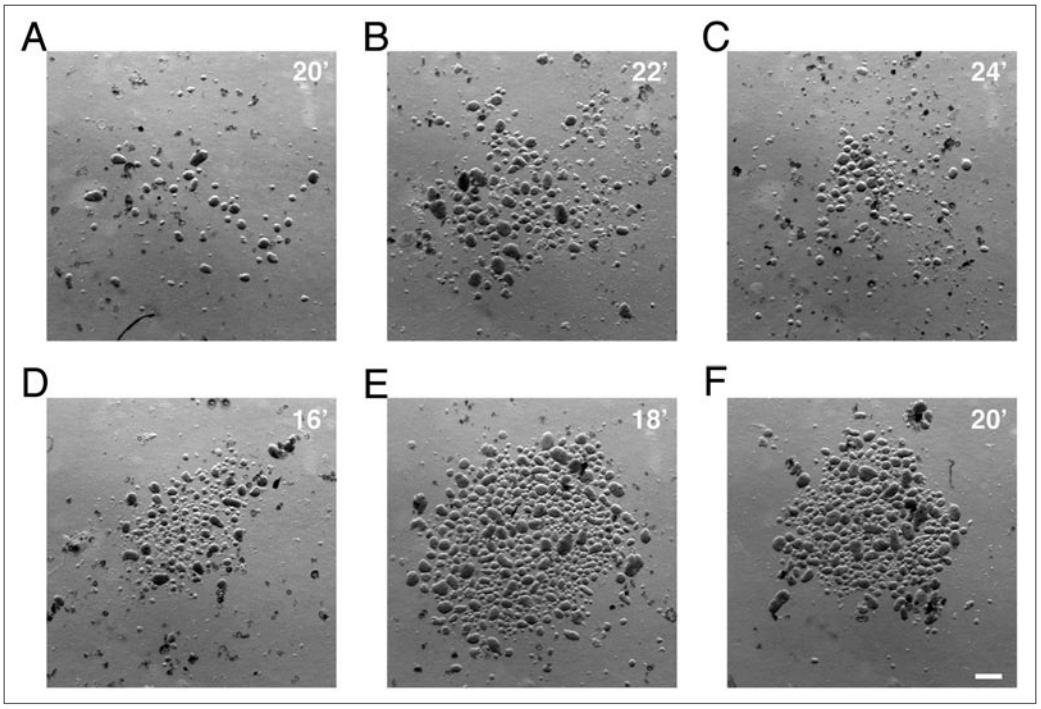

Figure 2. Digestion time optimization for each enzyme. Pictures of the isolated islets after digestion with Liberase RI (A–C) and Liberase TL Research Grade (D–F) for the designated time points are shown. Scale bar represents 500 μm.

Islet cell viability and function. Islet cell viability, as determined by the WST-1 assay, showed a tendency towards higher cell viability in islets isolated with Liberase™ TL Research Grade compared to Liberase™ RI (**Fig. 3F**). However, glucose-stimulated insulin secretion and islet specific gene expression appeared similar in islets isolated with Liberase™ TL Research Grade and Liberase™ RI (**Fig. 3G and H**).

Discussion

Pancreatic islets play key roles in both type I and type II diabetes mellitus and, therefore, it is important to optimize islet isolation. A poor islet isolation efficiency arises not only from the procedure itself, but is also due to the properties of the collagenases used to digest the pancreatic tissue. Various attempts have been reported for improving the quality of the collagenase preparations.[2,4,11] As previously described, the thermolysin concentration, the ratio of collagenase I to II, and the purity of the enzyme blends are three of the most significant parameters for achieving an optimal islet isolation.[6,7] Here, we show that an enzyme blend, which harbors more intact and less degraded collagenase I and II, significantly increases the number and total mass of islets isolated from the mouse pancreas, while preserving their function and viability.

Collagenase II was shown to be important for rat pancreatic islet isolation,[6,12] and a major role for collagenase I has been demonstrated for human islet isolation.[8] More importantly, in the latter study, collagenase Ib was shown to reduce islet yields, and a correlation between degraded collagenase and reduced islet viability has been previously demonstrated.[3] In line with these findings, we show that decreased collagenase degradation results in an increase in islet yield.

Interestingly, we could show that the increased islet yield, obtained with a Liberase™ that harbors intact collagenases, mainly arises from islets with a diameter larger than 150 μm. As the mean murine islet diameter is ≈77 μm, islets having a diameter larger than 150 μm are classified as large islets.[13] As larger islets have more contacts to the extracellular matrix, an efficient digestion of this matrix may be particularly important for their isolations. In addition, a shorter digestion time reduces a non-specific digestion of islets by thermolysin.

Reduction of digestion time is also crucial, because reduced exposure to the enzymes alleviates the isolation stress imposed on the islets, which could otherwise impair islet viability and islet function.[9] Additionally, we show that reducing the digestion time does not lead to a larger contamination of islets with exocrine tissue. This is important, since exocrine tissue secretes digestive, proteolytic enzymes that compromise islet integrity and viability.[14]

A major challenge for human islet transplantations is the limited pool of pancreas donors. However, a better islet isolation procedure, using an improved blend of collagenases, could contribute to obtaining more islets for human islet transplantation. Therefore, our data and previous reports on the role of collagenases in human islet isolation point to a possibility of improving human islet isolation with clinical applications.

Materials and Methods

Animals. Pancreatic islets were isolated from age- (7- to 10-weeks) and weight-matched male NMRI mice (Harlan Laboratories

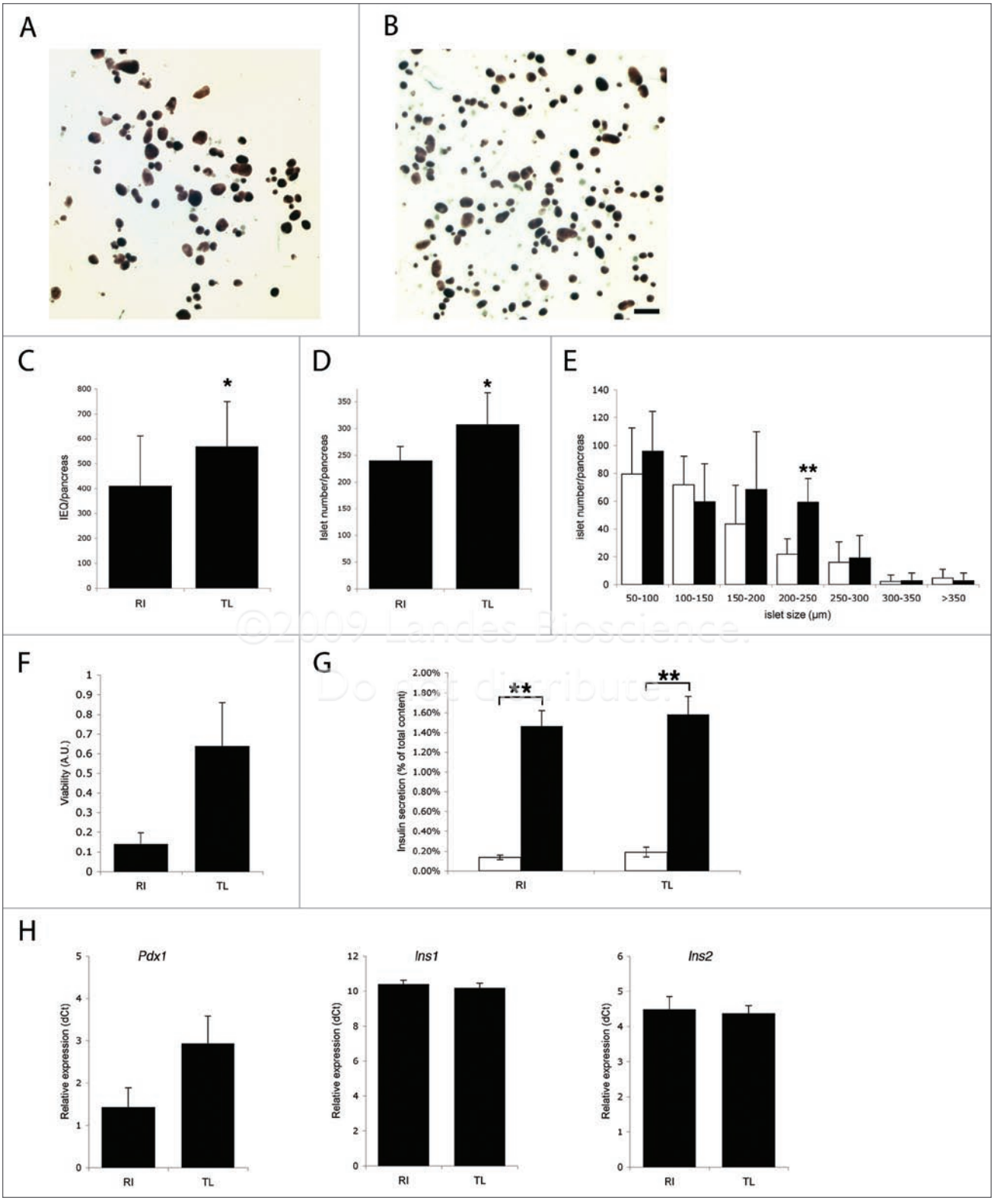

Figure 3. Significant increase in mouse islet yield upon isolation with Liberase™ TL Research Grade. Islet purity for the optimal digestion times, as assessed by DTZ staining after overnight culture for Liberase RI (A) and Liberase™ TL Research Grade (B). Scale bars represent 500 µm. Islet yield as expressed by islet equivalent number (IEQ) (C) and islet number (D) per pancreas with the optimized digestion times for each enzyme. n = 5 with two pancreas perfusions per enzyme in each isolation, *$p < 0.05$. (E) Graph showing the size distribution of the isolated islets (n = 5), **$p < 0.01$. (F) Islet viability was assessed using the WST-1 assay (values expressed as arbitrary units (A.U.); n = 4), (G) Insulin secretion from islets at 2 mM glucose (white bars) and 25 mM glucose (black bars) is shown. The secreted insulin is normalized to total insulin content. The data from five separate experiments (each consisting of 2 replicates) were pooled. **$p < 0.01$, unpaired t test with unequal variance. (H) Quantitative RT-PCR analysis for *Pdx1*, *Ins1* and *Ins2* gene expression in islets (n = 4; unpaired t test with unequal variance). Values are expressed as means ± SEM.

GmbH, Venray, Netherlands). All animal experiments were performed in compliance with the German Animal Welfare Act following the 'Principles of laboratory animal care' (NIH publication no. 85-23, revised 1985).

Enzyme blend composition. Two different purified enzyme blends were obtained from Roche Applied Science (Penzberg, Germany). Both mixtures, Liberase™ RI and Liberase™ TL Research Grade, have the same ratio of collagenase I and collagenase II and are blended with equal amounts of thermolysin. Collagenases I and II were isolated from *Clostridium histolyticum* and thermolysin was derived from *Bacillus thermoproteolyticus*. Chromatographical purification was optimized for the second generation Liberase™ TL Research Grade to remove by-products and proteases, such as trypsin and clostripain, from the collagenase fractions. Combined collagenolytic activity of the collagenase I and II isoforms was measured using the Wuensch assay employing the enzymatic cleavage of a synthetic substrate. The resulting lipophilic fragment is separated by solvent extraction and quantified by an increase in absorbance at 320 nm as previously described.[15] Further information on the properties of both enzyme blends is shown in **Table 1**.

HPLC. Anion exchange HPLC was performed on an Agilent 1100 Series HPLC System (Agilent Technologies Inc., Santa Clara, CA) using Mono-Q 5/50GL columns (GE Healthcare, Uppsala, Sweden) at 25°C. The mobile phase consisted of Tris-$CaCl_2$ buffer (pH 7.5) with a Tris-$CaCl_2$-NaCl gradient. Separated protein fractions were detected at 280 nm. The collagenase fraction was analyzed by the area under the curve method using the Chromeleon 6.8 Chromatography Software (Dionex Corporation, Sunnyvale, CA).

Mouse pancreatic islet isolation. Islets were isolated from matched adult male NMRI mouse pancreases using Liberase™ RI or Liberase™ TL Research Grade (Roche, Penzberg, Germany) as previously described.[16] Liberases were dissolved in low glucose DMEM containing 1.8 mM Ca^{2+} (Invitrogen GmbH, Karlsruhe, Germany). Briefly, 2 ml Liberase™ (Roche, Penzberg, Germany) solution (1.081 Wuensch Units/ml) was injected into the bile duct, followed by digestion at 37°C for different time intervals for optimization purposes. Enzyme action was stopped with DMEM containing 15% FCS (PAA Laboratories GmbH, Coelbe, Germany). After vigorous shaking, subsequent washes, filtering (400 µm mesh) and gradient centrifugation (1,100 g for 30 min), islets were collected from the interphase between Histopaque 1077 (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and DMEM. The collected islets were washed twice with DMEM and cultured overnight with CMRL (Invitrogen GmbH, Karlsruhe, Germany) media, supplemented with 15% heat-inactivated FCS, 100 U/ml penicillin with 0.1 mg/ml streptomycin (Invitrogen GmbH, Karlsruhe, Germany), 11 mM glucose, 0.15% $NaHCO_3$ (Invitrogen GmbH, Karlsruhe, Germany) and incubated at 37°C and 5% CO_2. All functional experiments were performed after overnight culture. Islets were photographed using a stereomicroscope after isolation and after overnight culture to assess purity, yield and viability. Islet quality was determined after staining with a zinc-chelating dye (DTZ, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) for 1 min. DTZ solution was prepared by dissolving 100 mg DTZ in 10 ml Dimethylsulfoxide (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and 40 ml phosphate buffered saline and filtered through 0.22 µm filter.

Table 1. Characteristics of enzyme products

Purified enzyme blend	WU/mg total protein	Endotoxin (EU/mg)	Neutral protease (casein units/WU)
Liberase RI	4.7	<50	42.5
Liberase TL Research Grade	5.0	<1	43.6

Islet counting. Duplicate samples of each of the two batches of islet preparations were examined. After DTZ staining, individual islets were grouped according to their diameter and counted using an Olympus CKX41 stereomicroscope. The number of islets in each diameter range was manually counted and converted to IEQ as previously described.[17] An IEQ is defined as an islet with a diameter of 150 µm.

Viability. The viability of islet cells was measured using the WST-1 assay (Roche, Penzberg, Germany). The metabolic activity of the islets was assessed based upon the reduction of the tetrazolium salt WST-1 to formazan by cellular dehydrogenases. Fifty islets in 3 to 5 replicates per experiment were incubated with 1:10 diluted WST-1 reagent for 3 h at 37°C, and the absorbance was measured at 450 nm. The background absorbance was measured at 690 nm.

Real-time reverse-transcriptase-PCR (RT-PCR). Total RNA was isolated using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. cDNA was synthesized from 600 ng of total RNA using Moloney murine leukemia virus reverse transcriptase and Oligo(dT) primer (Promega GmbH, Mannheim, Germany). mRNA levels were monitored by real-time PCR using the SYBR Green I detection method (FullVelocity SYBR green QPCR Master Mix, Stratagene, Amsterdam, Netherlands), following the manufacturer's protocol. Data were analyzed according to the Ct method. Results are expressed as dCt. HMBS was used as an internal control. Primer sequences are given in **Table 2**.

Insulin secretion from pancreatic islets. Insulin secretion measurements were performed with 10 to 15 islets in duplicate

Table 2. Primers used for real-time RT-PCR

Gene name	Forward primer	Reverse primer
Ins1	TCTTCTACACACCCAAGTCCCG	TCCACAATGCCACGCTTCTG
Ins2	TGGGGAGCGTGGCTTCTTC	GCTGGTAGAGGGAGCAGATGC
Pdx1	TCACCTCCACCACCACCTTC	TGTGTAAGCACCTCCTGCC
HMBS	CTGGATGAGCTGCAGGAA	TCCAGGTGCCTCAGAAAAG

as described before.[18] Secreted insulin was normalized to insulin content.

Statistical analysis. All functional experiments were performed at least three times, where n always represents the number of independent isolations performed. Data are expressed as means ±SEM. Unless stated otherwise, differences between groups were calculated by using a paired two-tailed Student's t-test. A p-value of less than 0.05 was considered significant.

Acknowledgements

We would like to thank Udo Eichenlaub for helpful comments and William Paer Hayes for critically reading the manuscript. We also acknowledge the Biomedical Services at the MPI-CBG for their expertise with animal management. The Deutsche Forschungs-gemeinschaft (DFG LA1216) funded this project.

Liberase is a trademark of Roche.

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