Journal of Clinical Laboratory Analysis 8:407–411 (1994)

Isolation of Porcine Pancreatic Islets: Low Trypsin Activity During the Isolation Procedure Guarantees Reproducible High Islet Yields

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During the past few years, interest in xenotransplantation of porcine islets of Langerhans for the future therapy of type I diabetes has increased markedly. Therefore, we established a semiautomated digestion method for isolating islets from the porcine pancreas. However, although the isolation technique was standardized and collagenase of controlled quality was used, we were unable to attain high islet yields with a satisfactory degree of reproducibility. One hypothesis was that varying degrees of interference by donor pancreatic enzymes were responsible for this failure. The aim of this study was to examine the kinetics of four types of enzymatic activity during the isolation procedure, as well as their effects on islet yield: collagenase, trypsin, neutral protease, and clostripain. Our results indicate that while exogenous collagenase activity decreases slightly during the isolation procedure, the activity of the pancreas enzymes neutral protease and trypsin increases. In some cases, trypsin activity increases very strongly. A strong increase in trypsin activity correlates with poor islet yield, whereas low trypsin activity always correlates with high islet yield. Addition of the protease inhibitor Pefabloc to the isolation medium results in low trypsin activity and reproducible high islet yields.
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Key words: islets of Langerhans, xenogeneic transplantation, swine, enzyme activation, enzyme inhibitors

INTRODUCTION

During the past few years, interest in clinial transplantation of islets of Langerhans to treat type I diabetes has increased markedly. The worldwide donor shortage motivates the search for alternative donor strategies. Because of comparable organ size, physiological similarity, and unlimited availability, the pig may serve as a suitable donor species for xenotransplantation of pancreatic islets. However, the particular fragility of the porcine islets (compared to those of humans and other mammalian species) is one basic problem in isolating functioning islets from the porcine pancreas. We established a standardized technique based on the semiautomated digestion method described by Ricordi and co-workers (1). This technique enabled us to isolate large amounts of islets from the porcine pancreas but, although the isolation technique we used was standardized and the collagenase was of controlled quality, we were not satisfied, because the yields varied considerably (2). Our hypothesis was that failures were caused by individual variations in the pancreatic enzymes. Therefore, the aim of this study was to examine the kinetics of various types of enzymatic activity during the isolation procedure, as well as their effects on islet yield. We investigated the enzymatic activity of both the pancreatic enzymes neutral protease and trypsin and the collagenase preparation used for tissue disintegration.

MATERIALS AND METHODS

Animals

Islet isolations were performed with pancreata from female pigs. The pigs were always >2 years old. These so-called retired breeders had a body weight of 200–250 kg. All organs were harvested at local slaughterhouses.

Organ Preparation

Only the splenic portion of the pancreas was prepared and removed from the donor organ. Immediately after harvesting, the organs were cannulated with a self-made catheter consisting of a 23G × 1-inch needle and a polystyrol tube (inner diameter 0.58 mm, outer diameter 0.96 mm; NeoLab, Heidelberg, Germany). The organs were stored and transported in cold (4°C) Eurocollins solution (Fresenius, Bad Homburg, Germany). Warm ischemia time was 25 min, and cold ischemia time was within the range of 1–3 hr.

Received March 15, 1994; accepted April 21, 1994.

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Islet Isolation

The isolation procedure was based on the semiautomated digestion method described by Ricordi et al. (1) and was further modified in our laboratory. Peripancreatic fat, lymph nodes, and vessels were dissected from the gland and discarded. Weight of the organs ranged from 15.0 to 99.7 g (45.0 ± 23.4 g). The collagenase solution containing Hank's buffered salt solution (HBSS) (Gibco/BRL, Eggenstein, Germany), 25 mM Hepes (Boehringer, Mannheim, Germany) and 1.8 PZ U/ml collagenase (cat. no. 17448, Serva, Heidelberg, Germany) was adjusted to pH 7.6, prewarmed to 28°C, and then injected into the pancreas via the catheter. After infusion the organ was loaded into a stainless steel chamber with a screen (mesh 420 μm) and five Teflon/steel beads (diameter 2 cm). The chamber was connected to a circulation system including a reservoir and a heating circuit (45°C). After the organ was loaded into the chamber, the system was filled with collagenase solution and recirculation was started (flow rate 70 ml/min). The chamber was gently shaken by hand for 10 sec/min. Every other minute, a sample was taken, stained with dithizone (3), and screened microscopically. During the procedure, the temperature and pH of the solution were constantly recorded. When a significant number of well-digested islets were observed (after 21.5 ± 5.42 min), recirculation was interrupted, and the elution of the digested tissue was started. The elution was performed with HBSS, supplemented with 25 mM Hepes and 5% fetal calf serum (FCS) (Conco, Wiesbaden, Germany), and precooled to 4°C, pH 7.4. Shaking and monitoring were continued during the elution phase. When no more islets were observed (after 17.0 ± 5.68 min), the elution was terminated. The eluted digested tissue was centrifuged (270*g*, 4 min, 4°C, 2×). After sedimentation, islet samples were taken, stained with dithizone, and counted, and the number of islets per gram of organ and the islet equivalent were calculated. The viability of the islets was determined by staining them with fluorescein diacetate and propidium iodide (FDA/PI).

Enzymatic Activity

During the isolation procedure, samples of the collagenase solution were collected from the recirculation system and filtered (0.2-μm filter), and the activity of selected enzymes (collagenase, neutral protease, trypsin, and clostripain) was measured by means of photometric assay. Collagenase activity was measured by two different assays using artificial substrates. The first assay was performed using PZ (4-[phenylazobenzyl-oxycarbonyl]-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine; Serva, Heidelberg, Germany) as substrate (4) and the second using Falgpa (N-[3-(2-furylacryloyl)]-L-leucylglycyl-L-prolyl-L-arginine; Boehringer, Mannheim, Germany) as substrate. Neutral protease activity was detected with the Azocolltest. Trypsin activity was evaluated using BAEE (Nα-benzoyl-L-arginine ethyl ester·HCl; Serva, Heidelberg, Germany) as substrate. Clostripain activity was also measured with BAEE as substrate, but using a different protocol. All activity tests were performed following the standard protocols of Boehringer Mannheim. In order to inhibit proteolytic enzymes, the collagenase solution was supplemented with 1.0 mM Pefabloc (4-(2-aminoethyl)benzenesulfonyl fluoride, hydrochloride; Boehringer, Mannheim, Germany). The inhibition control was performed with porcine trypsin (Sigma, Deisenhofen, Germany).

RESULTS

Kinetics of the Examined Enzymes During the Isolation Procedure

Twelve isolations were performed using the standardized procedure. Samples of the collagenase solution were collected before injection into the gland and at the end of the recirculation period. The actual time depended on the results of screening of well-digested islets (after 21.5 ± 5.4 min). The kinetics developed as follows (Fig. 1): Collagenase activity decreased from 1.82 ± 0.22 to 1.22 ± 0.19 PZ-U/ml or from 1.55 ± 0.30 to 0.97 ± 0.32 Falgpa U/ml. Clostripain activity was unaffected: 0.48 ± 0.08 BAEE U/ml before injection and 0.42 ± 0.07 BAEE U/ml after recirculation (not shown). Neutral protease increased from 55.2 ± 6.04 to 169 ± 50.7 Azocoll U/ml. Unexpectedly, trypsin activity showed two different kinds of kinetics. In eight cases, it increased slightly from 0.22 ± 0.14 to 0.57 ± 0.31 BAEE U/ml, and in four other cases it increased very strongly from 0.50 ± 0.08 to 3.34 ± 0.27 BAEE-U/ml.

For a representative documentation of the kinetics of the four enzymes during the isolation procedure, a single isolation was performed with a preset recirculation (=digestion) time of 30 min, independent of the results of screening of well-digested islets. Samples of the collagenase solution were taken before injection into the gland, immediately after (0 min) and 4, 7, 10, 15, 20, and 30 min after starting the recirculation. Collagenase, neutral protease, trypsin, and clostripain activities of the samples were measured (Fig. 1). Collagenase activity decreased from 1.73 to 1.04 PZ U/ml, or from 1.91 to 0.51 Falgpa U/ml. Clostripain activity was unaffected: 0.57 BAEE U/ml before injection and 0.50 BAEE U/ml after 30 min (not shown). Neutral protease activity was 58.9 Azocoll U/ml before injection and increased to 188 Azocoll U/ml after 30 min, and trypsin activity increased from 0.51 to 3.65 BAEE U/ml. The results can be summarized as follows: during the isolation procedure, the collagenase activity decreased slightly and the activity of the pancreatic enzymes neutral protease and trypsin increased; in some cases, trypsin activity increased very strongly.

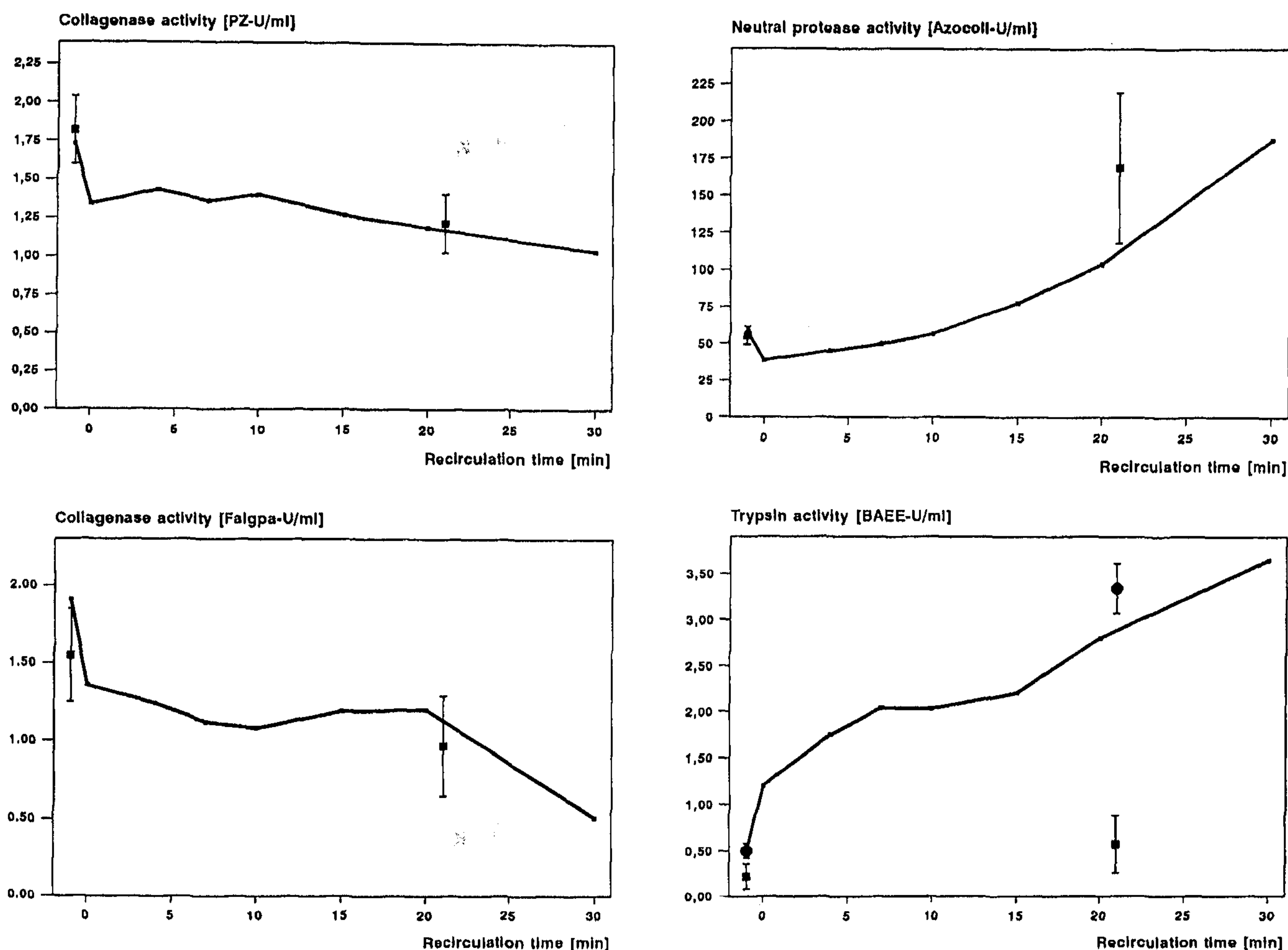

Fig. 1. Enzyme kinetics during the isolation procedure. Results of the tests of collagenase (with PZ and Falgpa as substrate), neutral protease and trypsin activity. The squares illustrate the results of 12 experiments when samples of the collagenase solution were taken only before injection into the gland and at the end of recirculation (after 21.5 ± 5.42 min). Trypsin activity showed two different kinds of kinetics: a slight increase (■, mean ± sa, n=8) or a strong increase (●, mean ± sa, n=4). The lines represent the results of the representative experiment when samples of the collagenase solution were taken before injection into the gland, immediately after (0 min) and 4, 7, 10, 15, 20, and 30 min after starting the recirculation.

Relationship Between Islet Yield and Trypsin Activity

Great variations in the islet yield, of 0–12,491 islets/g of organ after the above isolations, forced us to look for a correlation between enzymatic activity and islet yield. These experiments showed that trypsin activity definitely influences the islet yield (Fig. 2): a poor islet yield (507 ± 739 islets/g of organ) correlates with a strong increase in pancreatic trypsin activity (>1.50 BAEE U/ml), whereas low trypsin activity (≤1.50 BAEE U/ml) correlates with a high islet yield (6,795 ± 3,697 islets/g of organ). Neutral protease activity has no, or only weak, influence on the islet yield.

Reproducibly High Islet Yield Following Inhibition of Trypsin and Neutral Protease Activity by Pefabloc

The negative influence of high trypsin activity on the islet yield led us to try to inhibit the trypsin activity. A trypsin inhibitor used in islet isolations had to fulfill several conditions: considering the susceptibility of the islets, the inhibitor had to be nontoxic; it should not interfere with the activity tests and, furthermore, the inhibition had to be irreversible. An inhibitor with these properties is Pefabloc. We performed three isolations with collagenase solution supplemented with Pefabloc (Fig. 2). There was only a slight increase in neutral protease (from 57.6 ± 3.90 to 62.3 ± 11.4 Azocoll U/ml) and trypsin activity (from 0.17 ± 0.09 to 0.35 ± 0.15 BAEE U/ml) during these isolations, and the islet yield was very satisfactory (8,395 ± 3,553 islets/g of organ).

DISCUSSION

Each of the four enzymes tested showed characteristic kinetics during the islet isolation procedure. The slight decrease in collagenase activity may be caused by two effects: (a) binding of collagenase to the donor tissue, which was separated

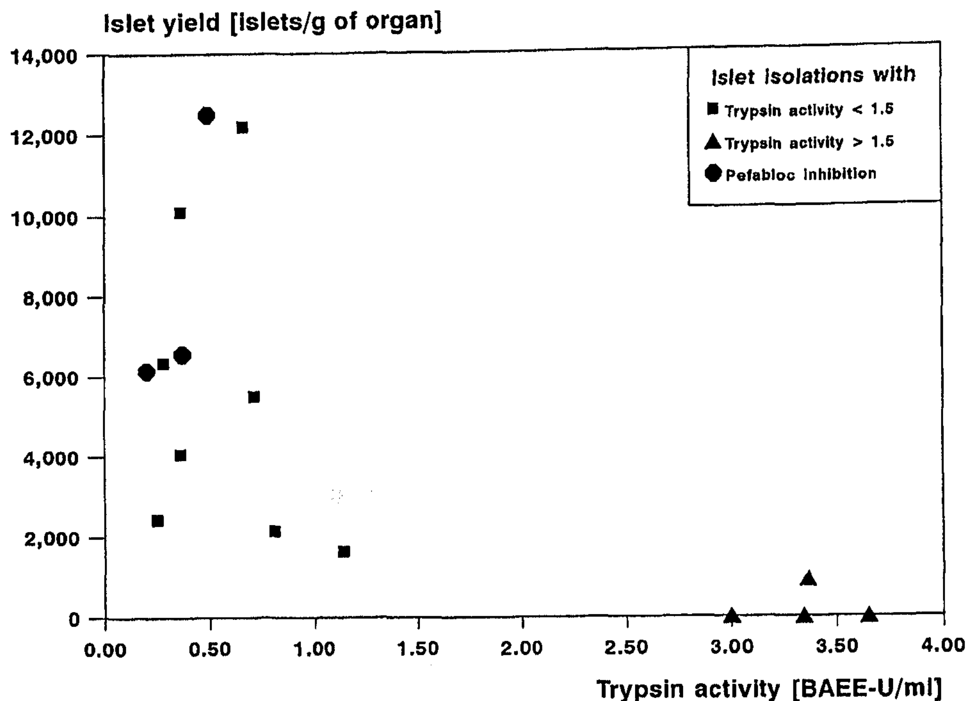

Fig. 2. Correlation of trypsin activity and islet yield. Results of trypsin activity tests and islet yields. Three groups of isolations are shown: ■, isolations with trypsin activity <1.5 BAEE U/ml (n=8); ▲, isolations with trypsin activity >1.5 BAEE U/ml (n=4); ●, isolations with trypsin inhibition by Pefabloc (n=3).

from the samples before the start of the activity test; and (b) proteolytic damage to collagenase molecules. These effects should be considered when determining a suitable collagenase concentration. A concentration of 1.80 PZ U/mL collagenase/ml worked very well in the present study.

The major problem faced in isolating islet from both the porcine and the human pancreas is to achieve reproducibly high islet yields. In previous studies, the influence of such parameters as pig age (2,6), pig race (2,7), and pH of the isolation medium (2) on the islet yield was clearly shown. We standardized the isolation technique accordingly and used collagenase of controlled quality, and particularly controlled proteolytic activity, but we still did not achieve satisfactorily reproducible high islet yields. That forced us to look into the basics, namely the enzymatic digestion process itself.

The established principle behind the enzymatic isolation technique is the specific disintegration of the collagen-containing connective tissue by collagenase. Wolters et al. (5) showed an increase in proteolytic activity during the isolation procedure when isolating islets from various donor species. In this study, we confirmed these findings for porcine islets. We extended them by determining that trypsin in particular was released during the isolation procedure, presumably caused by the disintegration of the exocrine tissue. Trypsin disintegrates a large number of proteins and thus accelerates the process. The combination of exogenous collagenous and endogenous trypsin may easily initiate a nonspecific, fast, and uncontrollable tissue disintegration process.

The present experiments show for the first time that trypsin activity >1.50 BAEE U/ml damages the tissue in a way that obviously clearly prevents the release of high islet yields. Furthermore, the experiments show individual variations in trypsin activity. The failure of some islet isolations was apparently caused by the high trypsin activity of some porcine pancreata. We speculate that such factors as pig age (2,6), pig race (2,7), nutrition (8), and retrieval procedure (9) may directly influence the trypsin content of the pancreas. Indirectly, via trypsin activity during the isolation procedure, they may also affect the islet yield. The proteolytic activity of the collagenase preparation may have an additional effect, because of the activation of trypsinogen by trypsin and other proteolytic enzymes (10).

If high trypsin activity is the cause of the failure of some isolations, prophylactic inhibition of this activity should result in reproducibly high yields. Therefore, Wolters et al. (5) used a collagenase solution supplemented with 10% bovine serum albumin (BSA) as protease inhibitor and described a positive effect (+48%) on rat islet yield. In our opinion, however, BSA has three disadvantages: it interferes with the activity tests, it obviously inhibits collagenase (data not shown), and it is only a competitive inhibitor. In this study, we showed

that Pefabloc is a much more suitable trypsin inhibitor. It does not interfere with the activity tests, it does not inhibit collagenase, and the inhibition of trypsin is irreversible. All isolations performed with Pefabloc resulted in low trypsin activity and a high islet yield. However, the role of residual trypsin activity awaits further investigation. In order to obtain a complete and well-timed disintegration of the connective tissue, a small but well-controlled degree of proteolytic activity may be necessary.

The similarity of the technical problems encountered in isolating porcine and human islets and the physiological similarity of the porcine and human pancreas will probably allow us to transfer the above knowledge from porcine to human islet isolation. Prophylactic trypsin inhibition with Pefabloc during islet isolation may have a comparably positive effect on human islets.

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