Studies on Pathogenesis of Reflux Pancreatitis

Motoo KANAO, Masayuki NISHIKI, Sumiyoshi TAKASUGI, Hideki KAWANISHI and Haruo EZAKI
The Second Department of Surgery, Hiroshima University School of Medicine, 1-2-3, Kasumi, Minami-ku, Hiroshima 734, Japan
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ABSTRACT

The behaviors of proteases were examined in the pancreatic juice pooled into a closed duodenal loop and it was confirmed that the activation of proteases occurred quickly after the operation. Three kinds of pancreatic proteases, trypsin, kallikrein and elastase were purified from the pancreatic juice by successive isoelectric focusing, Soya-bean trypsin inhibitor-Sepharose 4B affinity and Sephadex G-100 column chromatographies. Apparent molecular weights of the purified trypsin, kallikrein and elastase were 26,000, 31,000 and 25,000, respectively. Their isoelectric points were 5.0, 5.0 and 8.0, and their specific activities were 490 TAMe units, 45 TAMe units and 0.47 elastinolytic units/mg protein, respectively. All of these proteases induced a reflux pancreatitis in mongrel dogs and the reflux pancreatitis by the infusion of trypsin was the severest.

INTRODUCTION

It is well known that pancreatitis is a postoperative complication after biliary and gastric surgery, especially gastric resection with a subsequent Billroth II anastomosis. Although there are many theories on the pathogenesis of the postoperative acute pancreatitis, such as the obstruction hypersecretion theory, duodenal content reflux theory and bile reflux theory, recent concepts have implicated proteases in duodenal content as most important factors in the initiation of the disease. Numerous studies have shown that trypsin, infused into the pancreatic duct, is capable of inciting an inflammatory response\(^{5,11,20}\). Geokas\(^{8}\) has investigated on the effect of elastase and suggested that activated elastase played a major role in producing the vascular changes in the pancreas during pancreatitis. It has also been reported that the kallikrein-kinin systems plays a part in the pathogenesis of the pancreatitis\(^{1,5,16}\). Although the part played by these proteases in the pathogenesis of acute pancreatitis has been much discussed during the past years, their significance have not yet been elucidated. It is thought to be a cause for this that proteases were impure and different origin from animals used in experimental acute pancreatitis models.

In the present study, we first tried to isolate several proteases from the dog duodenal contents operated a closed duodenal loop obstruction and examined biochemically and histologically the inflammatory effects of isolated proteases for the dog pancreas by thier infusing into the pancreatic ducts.

MATERIALS AND METHODS

The following commercial preparations were used:

- \(\rho\)-Tosyl-L-arginine methyl ester (TAME, Sigma), \(\alpha\)-N-Acetyl-L-tyrosine ethyl ester (ATEe, Merk), H-D-Val-Leu-Arg-pNA-HCl (S-2266, Kabi), pyro-Glu-Gly-Arg-pNA-HCl (S-2444, Kabi), Casein nach Hammarsten (Merk), Suc-cinyl-L-Alanyl-L-Alanyl-L-Alanyl-pNA (Suc-(Ala)\(_3\)-pNA, Foundation for Promotion of Protein Research, Institute for Protein Research, Osaka, Japan), Congo-Red elastin (Sigma).

Assay of enzymes and proteins:

The esterolytic activity of samples against
TAME and ATEe was measured by the method of Hestrin as modified by Roberts. The final concentration of TAME and ATEe was 15 mM. One esterolytic unit was defined as the amount of enzyme which hydrolyzes 1 µmol of ester in 1 hr on incubation at 37°C. The caseinolytic activity of the enzyme was measured by Lowry's method as modified by Muramatsu et al. The final concentration of casein solution was 2% and 1 caseinolytic unit (CU) was defined as the amount of enzyme releasing 60 µg of acid-soluble tyrosine in 15 min at 37°C. The trypsin and pancreatic kallikrein were determined by their amidolytic activity by using for the former chromogenic substrate S-2444 and S-2266 for the latter. One chromozyme unit was defined as the amount of enzyme required to hydrolyze 1 µmol of chromozyme for 1 min under standard conditions. The final concentration of chromogenic substrate in the present study was 0.3 mM for S-2444 and 0.15 mM for S-2266, respectively. Elastinolytic activity was determined with the chromogenic substrate Suc-(Ala)₃-pNA, which is specific for elastase as follows; 2.5 ml of the sample and Tris-HCl buffer (0.05 M, pH 8.0) mixtures was added to 0.02 ml of Suc-(Ala)₃-pNA (125 mM). After incubation for predetermined intervals at 37°C, the reaction was stopped with 0.1 ml of 100% acetic acid. The absorbance at 410 nm was measured against a substrate control. Elastinolytic activity was also measured by the method of Shotton with Congo-Red elastin as substrate. One elastinolytic unit (EU) was defined as the amount of enzyme which solubilizes 1 mg of elastin in 30 min at pH 8.8 at 37°C.

Protein concentration was determined by the Lowry method with bovine serum albumin (Merk) used as the standard.

**Electrophoresis:**

Isoelectric focusing was performed by the method of Versterberg and Svensson using carrier ampholyte of pH 3.5-10.0. The column (LKB 8100-02, 440 ml) was maintained at 3-4°C and the potential at 900 V for 36 hr.

Soya-bean-trypsin inhibitor (SBTI)-Sepharose 4B affinity column chromatography:

SBTI-Sepharose 4B was prepared from 25 ml of Sepharose 4B (Pharmacia) activated by the treatment with cyanogen bromide as described by Cuatrecasas, and coupled with 100 mg of SBTI (Sigma) in 0.1 M borate buffer containing 0.05 M CaCl₂, pH 9.3. The sepharose slurry was washed with 0.2 M Na₂CO₃ containing 0.5 M NaCl followed by 0.1 M borate buffer, pH 9.0 containing 0.5 M NaCl. The sample solution (80 ml) was passed through a SBTI-Sepharose 4B column (2.0 x 8.0 cm) equilibrated with 0.1 M Tris-HCl buffer, pH 8.0. The column was washed with the excess amounts of the equilibration buffer containing 2 M NaCl until absorbance at 280 nm was lower than 0.02. Elution of absorbed protein was achieved with 0.01 N HCl containing 2 M NaCl, and 5 ml fractions were collected.

**Gel filtration:**

A 2.4 x 70 cm column of Sephadex G-100 (Pharmacia) was equilibrated with 0.1 M phosphate buffer, pH 7.4. Eight ml of the sample solution was applied to the column which was run at a flow rate of 33 ml/hr, and 5 ml fractions were collected.

**Estimation of molecular weight (mol. wt.):**

The mol. wt. of the various proteases was estimated by the method of Andrews using Sephadex G-100.

Operation method of a closed duodenal loop obstruction in the mongrel dog:

A closed duodenal loop obstruction was prepared using the male mongrel dogs weighing approximately 10 kg by a modified method of Pfeffer et al. according to a previous report. In the present study, a balloon catheter (4G, Termo, Japan) was settled into a closed duodenal loop to collect the pancreatic juice.

Preparation of reflex pancreatitis:

Healthy mongrel dogs were anesthetized with GOF intratracheal intubation method (O₂; 2 L/min, N₂O; 4L/min, Furothan; 2%), and under sterile conditions, laparotomy was performed through upper midline incision. The lesser pancreatic duct was ligated, and main duct was canulated through a small duodenectomy using a No. 18-gauge polyethylene catheter. The canula was secured in place with a silk ligature, following which 6 ml of the test solution were infused under 40 cm H₂O pressure. Then the canula was removed and the duct was ligated.

**Microscopic examination:**

The pancreas was removed out at 3 hr after the operation. A small tissue segment of apparently inflammed pancreas was cut out and
fixed in 10% formalin solution for 24 hr. The tissue segment was embedded in paraffin and 5 μm thick histological sections were prepared. The sections were stained with hematoxylin–eosin.

RESULTS

Behaviors of the proteases in the pancreatic juice after the operation of a closed duodenal loop obstruction:

To examine the behaviors of the proteases in the pancreatic juice, aliquots of 5 ml of the pancreatic juice were drawn from a balloon catheter at various time intervals after the operation, and TAMe esterolytic and Congo-Red elastinolytic activities were measured in each sample of pancreatic juice. As shown in Fig. 1, both TAMe esterolytic and Congo-Red elastinolytic activities reached maximum levels immediately after the operation, continued for about 3 hr, and then decreased gradually until their activities became lower than 10% of maximum level. Purification of various proteases from the pancreatic juice:

The isolation of various proteases from the pancreatic juice collected within 3 hr after the operation was achieved by isoelectric focusing, SBTI-Sepharose 4B affinity and Sephadex G-100 gel column chromatographies.

Step 1: Isoelectric focusing of the pancreatic juice.

After electrophoresis of the pancreatic juice (4 ml) for 36 hr at 300 V, fractions of 4 ml were collected. Esterolytic, amidolytic, elastinolytic and caseinolytic activities in each fraction were measured after the dialysis against 0.1 M Tris-HCl buffer, pH 8.0 overnight. As shown in Fig. 2, the pancreatic juice was fractinated into 4 proteolytic enzyme peaks after isoelectric focusing. The first peak fractions with pH about 5.1 possessed TAMe esterolytic, S-2444, S-2266 amidolytic and caseinolytic activities, suggesting that these fractions may contain trypsin and pancreatic kallikrein. The second
peak fractions with pH about 7.5 possessed ATEe esterolytic and caseinolytic activities, suggesting chymotrypsin. The third with pH about 8.0 and the fourth with pH about 9.0 peak fractions possessed about the same Suc-(Ala)₃-pNA amidolytic, Congo-Red elastinolytic and caseinolytic activities, suggesting elastase.

Step 2: SBTI-Sepharose 4B affinity column chromatography of the 1st peak fractions after the isoelectric focusing.

To separate the pancreatic kallikrein from the trypsin, 80 ml of the 1st peak fractions after the isoelectric focusing were passed through a SBTI-Sepharose 4B column, and elution of absorbed proteins was achieved with 0.01 N HCl containing 2 M NaCl. The SBTI-Sepharose 4B affinity column pattern of the 1st peak fractions is shown in Fig. 3. Unabsorbed fractions possessed the S-2266 amidolytic and a little TAME esterolytic activities, and the adsorbed and eluted fractions possessed the S-2444 amidolytic, TAME esterolytic and caseinolytic activities. Furthermore, unadsorbed fractions could generate kinin from human plasma kininogen prepared by the method of Eisen et al. These results indicate that the unadsorbed fractions contain the pancreatic kallikrein and the absorbed and eluted fractions contain the trypsin, and that SBTI-Sepharose 4B affinity column chromatography can separate the pancreatic kallikrein from the trypsin.

Step 3: Sephadex G-100 column chromatography of the 3rd peak fractions after the isoelectric focusing.

To further purify the elastase, 80 ml of the 3rd peak fractions after the isoelectric focusing was concentrated to 8 ml by the ultrafiltration and applied on Sephadex G-100 column (2.4 × 70 cm). As shown in Fig. 4, Suc-(Ala)₃-pNA amidolytic activity was eluted as a single protein peak, and its mol. wt. was calculated to be approximately 25,000.

By these procedures, trypsin (pI; 5.0, specific activity; 490 TAME units/mg protein, mol. wt.; 26,000), pancreatic kallikrein (pI; 5.0, specific activity; 45 TAME units/mg protein, mol. wt.; 31,000) and elastase (pI; 8.0, specific activity; 0.47 EU/mg protein, mol. wt.; 25,000) were isolated respectively from 8 ml of the pancreatic juice.

The effects of protease on the pancreatic tissue:

Trypsin. The adsorbed- and eluted fractions possessed the S-2444 amidolytic, TAME esterolytic and caseinolytic activities.
after the SBTI-Sepharose 4B affinity column chromatography were collected, dialyzed against physiologic saline at 4°C overnight, and then concentrated to 3 ml by the ultrafiltration. Six ml of the trypsin solution (11.3 mg of protein, 5530 total TAME units) purified like this was infused into the pancreatic duct. Immediately after the infusion, massive interstitial edema and extensive haemorrhage occurred in the head and the corps of the pancreas. The edema and haemorrhage gradually became severe, and massive necrotic change developed after 9 hr of the infusion (Fig. 5). Intraperitoneal cavity contained the medium volumes of bloody ascites. Microscopically, the picture of the massive interstitial liquefaction necrosis with severe extravasation of red cells and abundant leucocyte infiltration was shown particularly in the central regions of the lobules of the pancreas (Fig. 6).

Pancreatic kallikrein. The unadsorbed fractions after the SBTI-Sepharose 4B affinity chromatography were collected, dialyzed against the physiologic saline, and then concentrated to 3 ml by the ultrafiltration. Six ml of the pancreatic kallikrein solution (31 mg of protein, 1,400 TAME units) was infused into the pancreatic duct. Almost the same macroscopic changes as the trypsin infusion were recognized in the pancreas, but the severity was milder than that of the trypsin infusion. Histological findings of the pancreas after 9 hr of the infusion showed the focal necrosis of the interstitial and the fatty tissue (Fig. 7).

Pancreatic elastase. Suc-(Ala)₉-pNA amidolytic enzyme fractions after the Sephadex G-100 gel chromatography were collected and concentrated to 3 ml by the ultrafiltration. Six ml of the pancreatic elastase solution (32 mg of protein, 15 elastinolytic units) was infused into the pancreatic duct. After the intrapancreatic-infusion of the pancreatic elastase, the macro- and microscopic findings were similar to those of the trypsin and pancreatic kallikrein pancreatitis. However, their severities were milder than those of the trypsin and kallikrein pancreatitis, and elastic fiber damage of the vessels were extensive (Fig. 8).

Physiologic saline. Six ml of physiologic saline solution was infused into the pancreatic duct under the same conditions as a control.
experiment. Slightly edematous changes were recognized in the head of the pancreas immediately after the infusion, but recovered to normal appearance after 9 hr of the infusion.

**DISCUSSION**

There are a number of defensive mechanism that normally prevent a reflux of duodenal contents, such as sphincter Oddi, the mucous folds at the transmural portion of the ducts and the pressure gradient between the pancreatic duct and the duodenum. These natural protective mechanisms could be destroyed by the organic changes in the papilla, functional disturbances of the sphincter muscle, and an increased pressure in the duodenum. In the present study, the authors utilized a closed duodenal loop obstruction method of Pfeffer et al. to clarify the pathogenesis of postoperative acute pancreatitis. It has been reported that the acute pancreatitis produced in the mongrel dogs by a closed duodenal loop obstruction method is similar to the postoperative acute pancreatitis in man, and that there is a reflux of duodenal content into the pancreatic duct due to overdistention of the blind loop. On the other hand, the pancreas is normally protective against the protease effects of its own digestive enzymes in different ways. It is well known that enzymes are synthesized as inactive zymogens and that there are inhibitors in the pancreas tissue and the pancreatic juice which can inactivate prematurely activated proteases. Many authors have considered that the reflux pancreatitis is induced by a retrograde infusion of the pancreatic proteases in the duodenal contents. However, it has not still been clarified whether or not any proteases are activated in the duodenum. In the present study, the authors first settled a balloon catheter into a closed duodenal loop and succeeded in measuring the behaviors of activated proteases. Fig. 1 shows that the trypsinogen, kallikreinogen and proelastase in the pancreatic juice can be activated quickly after their excretion into duodenum, and suggest that the amount of the protease inhibitors in the pancreatic juice may be less than the amount of proteolytic proenzymes. It was of particular interest that protease activities in the pancreatic juice decreased quickly after 3 hr of the operation. The cause for this, however, is still unclear.

There is one possibility that a large amounts of proteolytic inhibitor might be excreted from the pancreas into the duodenum as a feed-back phenomenon. There is another possibility that the phenomenon may be due to the exhaustion followed by the decrease of proenzymes.

Fig. 1 showed that at least 3 kinds of proteases, trypsin, kallikrein and elastase could be activated in the pancreatic juice in a closed duodenal loop. By the isoelectric focusing, SBTI-Sepharose 4B affinity and Sephadex G-100 column chromatographies, about 6 mg of the trypsin with pI of 5.0, mol. wt. of 26,000 and specific activity of 490 TAME units/mg protein and 16 mg of the pancreatic kallikrein with pI of 5.0, mol. wt. of 31,000 and specific activity of 45 TAME units/mg protein and 16 mg of the pancreatic elastase with pI of 8.0, mol. wt. of 25,000 and specific activity of 0.47 EU/mg protein were purified respectively from 4 ml of original pancreatic juice. As shown in Fig. 2, it was confirmed that 2 kinds of pancreatic elastase which pI were different were present in the pancreatic juice of the mongrel dog. This indicates that the pancreatic elastase of the dog as well as human and porcine may be 2 or 3 kinds of isozyme. In the present study, the pancreatic elastase with pI of 8.0 was purified and used for the retrograde infusion experiment. As shown in Fig. 2, the trypsin was eluted as the same isoelectric point as the pancreatic kallikrein. It was very difficult to separate the trypsin from the pancreatic kallikrein, because their similar mol. wt. and substrate specificities. It has been reported that the SBTI could inhibit the trypsin, but not the pancreatic kallikrein. According to the difference of their inhibitory spectra, the authors first prepared the SBTI-Sepharose 4B, and succeeded in separating completely the two by the SBTI-Sepharose 4B affinity column chromatography. The purifications of these three kinds of proteases from the pancreatic juice of the mongrel dog are the first success in the world.

There are many reports on the pathogenesis of the reflux pancreatitis due to the pancreatic proteases. Geokas induced a reflux pancreatitis in the mongrel dog using the porcine trypsin...
and elastase, and Anderson et al.\(^2\) using the porcine and bovine trypsin. In the present study, the authors first induced a reflux pancreatitis in the mongrel dog using the dog pancreatic proteases. As shown in Fig. 5–8, it was confirmed macro- and microscopically that all of the three kinds of pancreatic proteases could induce a reflux pancreatitis. Although it is very difficult to compare the severity of 3 kinds of the reflux pancreatitis, because of the difference in the enzyme activities infused, we believe that the trypsin must induce the severest reflux pancreatitis in all of the pancreatic proteases, because we infused into the pancreatic ducts almost the same amounts of the proteases isolated from the pancreatic juice collected under the same experimental conditions. We consider that the severe pancreatitis by the infusion of the trypsin may be the phenomena due to co-effect of the trypsin and some proteases activated secondary by the trypsin, such as kallikrein, elastase and phospholipase A.

REFERENCES