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STUDIES ON THE ACTIVATION OF CANINE TRYPSINOGENS IN VITRO

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Summary

The activation of canine anionic and cationic trypsinogen by enterokinase, trypsin, thrombin, plasmin and extracts from canine granulocytes were studied in vitro. Enterokinase activates both trypsinogens about 1000 times faster than trypsin. The enterokinase-catalyzed activation is not inhibited by the main serum protease inhibitors, α-macroglobulin and α_1-antitrypsin. α-Macroglobulin cannot inhibit the activation of the trypsinogens by trypsin but this reaction is completely inhibited by α_1-antitrypsin. The results are discussed in relation to the pathogenesis of acute pancreatitis.

Introduction

Pancreatic proteases are stored in the acinar cells and secreted into the pancreatic juice as inactive zymogens [1]. It has long been postulated that activation of zymogens in the pancreatic tissue plays an important role in the pathogenesis of acute pancreatitis [2,3]. The natural activator of all zymogens is trypsin and a possible trigger mechanism for acute pancreatitis may be premature activation of trypsinogen within or around the pancreatic gland [4,5]. Different mechanisms for the activation of trypsinogen in this respect have been studied earlier, but no conclusive results have been obtained. Autoactivation or activation of trypsinogen by the enzymes of the blood-clotting or fibrinolytic systems have been proposed [4].

This report describes in vitro studies on the activation of anionic and cationic canine trypsinogen by different proteolytic enzymes, i.e. porcine enterokinase, canine trypsin, canine plasmin, bovine thrombin and extracts from canine granulocytes. In addition the influence of the dominating serum protease inhibitors α-macroglobulin and α_1-antitrypsin on the activation of trypsinogen is studied.

Materials and Methods

Sephadex G-25, G-100, G-200, CNBr-activated Sepharose and polyacrylamide gradient gels PAA 4/30 were purchased from Pharmacia Fine Chemicals Inc., Uppsala, Sweden. Agarose batch 60030 was obtained from Miles-Seravac, Maidenhead, U.K. and bovine serum albumin and porcine enterokinase (batch 11A) from Miles Laboratories Ltd., U.K. $Na^{125}I$ was purchased from Radiochemical Centre, Amersham, England. Monospecific antisera against canine α-macroglobulins and α_1-antitrypsin were available in the laboratory. Human plasmin and a specific chromogenic enzyme substrate for plasmin S-2251 were products from KABI Diagnostica Stockholm, Sweden. Urokinase was obtained from Lövens, Ballerup, Denmark. Benzoyl-DL-arginine-*p*-nitroanilide-HCl was obtained from Fluka AG, Switzerland and Agarose-L-lysine Lot No. LY 1 from Miles-Yeda Ltd., Israel.

Trypsinogen preparations. Anionic and cationic canine trypsinogens were purified as described elsewhere [6]. The purified trypsinogens were labelled with ^{125}I in a 0.028 M succinate buffer containing 0.001 M $CaCl_2$ at pH 5.6, using a lactoperoxidase method [7]. Free and bound iodine were separated by gel filtration on a Sephadex G-25 column (0.9 × 10 cm) equilibrated and developed with the succinate buffer mentioned above. The effluent was collected in tubes with the same succinate buffer containing bovine serum albumin 20 g/l giving the final samples a protein concentration of 2 g/l. The specific activity was about 5 μCi/g protein.

The commercial enterokinase preparation contained 85 enterokinase units/mg. The specific activity for pure enterokinase is 44 000 enterokinase units/mg and the molecular weight for porcine enterokinase is around 195 000 [8]. In the activation experiments, we used a concentration of the crude enterokinase preparation of about 40 μg/ml corresponding to a concentration of pure enterokinase of about 0.08 μg/ml ($0.4 \cdot 10^{-9}$ M).

Crude enterokinase was coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions and this immobilized enterokinase was used in the preparation of anionic and cationic canine trypsin. 8 mg of respective trypsinogen was dissolved in 8 ml of 0.028 M succinate buffer, containing 0.001 M $CaCl_2$ at pH 5.6 and was recycled over night at 4°C on a 1.5 × 4 cm column with Sepharose conjugated enterokinase equilibrated with the same buffer. The activation was tested using benzoyl-DL-arginine-*p*-nitroanilide-HCl as substrate [9] and by agarose gel electrophoresis at pH 8.6 [10], where the trypsins display different migration rates compared to the corresponding trypsinogens due to a loss of positively charged groups during activation [11]. After activation, the trypsins were dialyzed against 0.001 M HCl and lyophilized. In the activation experiments, anionic and cationic trypsin were used in concentrations between 2.5—25 μg/ml ($0.1—1 \cdot 10^{-6}$ M).

Canine plasminogen was purified from dog plasma by affinity chromatography on agarose conjugated lysine according to the manufacturer's instructions. The plasminogen containing fractions were pooled and dialyzed against 0.04 M Tris-HCl buffer (pH 7.4), containing 0.02 M $CaCl_2$ and stored at —20°C up to one month before use. Plasminogen was activated by urokinase (100 I.U./ml) and after activation the plasmin concentration was measured

enzymatically using a low molecular synthetic substrate for plasmin (S-2251) and human plasmin as standard according to the manufacter's instructions. The plasminogen pool containg 6 casein units of plasmin after activation corresponding to about 0.9 mg plasminogen/ml. In the activation experiments, plasmin was used in concentrations between 15—150 μg/ml ($1.8—18 \cdot 10^{-6}$ M).

Pure bovine thrombin was kindly provided by Dr. Björn Dahlbäck, Department of Clinical Chemistry, Malmö General Hospital, Malmö, Sweden. The specific activity was 2500 NIH units/mg protein. In the activation experiments, bovine thrombin was used in concentrations between 25—250 μg/ml ($0.65—6.5 \cdot 10^{-6}$ M).

Extracts from granules of canine granulocytes were prepared according to Ohlsson et al. [12]. The concentration of granulocyte elastase in the extract, determined immunochemically, was 50 μg/ml. In the activation experiments, granular extracts were used in amounts giving a concentration of granulocyte elastase between 2—10 μg/ml.

The two dominating serum protease inhibitors α-macroglobulin (M_r 750 000) and α_1-antitrypsin (M_r 55 000) [13] were separated using gel filtration of 5 ml of fresh canine serum on a Sephadex G-200 column 2.6 × 40 cm equilibrated and developed in 0.1 M Tris-HCl containing 0.01 M $CaCl_2$ at pH 7.4. α-Macroglobulin and α_1-antitrypsin concentrations were determined by electroimmunoassay [14] using a pool of normal canine serum as standard, and the peak fractions, containing α-macroglobulin and α_1-antitrypsin, respectively, were pooled separately and called α-macroglobulin preparation and α_1-antitrypsin preparation. They were stored at 4°C up to one week before use. The concentration of α-macroglobulin was about 30% of the concentration of normal dog serum corresponding to approx. 0.4 mg/ml and the concentration of α_1-antitrypsin was about 40% of the concentration of normal dog serum corresponding to approx. 0.5 mg/ml.

Activation studies. 50 μl of ^{125}I-labelled trypsinogen (4 μg/ml) in 0.028 M succinate buffer containing 0.001 M $CaCl_2$ at pH 5.6 were mixed with 150 μl 0.15 M NaCl containing 2 g/l bovine serum albumin and 10 μl 0.5 M Tris-HCl buffer containing 0.5 M $CaCl_2$ at pH 7.4. The different proteases were added in 50-μl aliquots in 0.1 M Tris-HCl buffer containing 0.01 M $CaCl_2$ at pH 7.4. The final trypsinogen concentration was approx. 1 mg/l ($0.4 \cdot 10^{-7}$ M).

All reaction mixtures were incubated at 37°C, 20-μl aliquot samples were withdrawn at variable intervals and further reaction prevented by freezing the samples at −70°C. After thawing the samples, 20 μl of dog serum was immediately added to every sample and incubated for 10—20 min at room temperature. Trypsin present in the test samples was complexed with the protease inhibitors contained in the added canine serum and could be separated from free unactivated trypsinogens using preparative electrophoresis on: (1) Pharmacia's polyacrylamide gradient slab gel electrophoresis (PAA 4/30) at pH 8.5 according to the manufacturer's instructions for anionic trypsinogen. 10-μl samples were applied, the electrophoresis was run for 4 h at 12.5 V/cm and the gel was cut in 0.5-cm slices and the radioactivity of each slice was counted. (2) For cationic trypsinogen agarose slab gel electrophoresis at pH 8.6 [10] was used. 25-μl samples were applied, the albumin front was allowed to migrate 5.0

cm and the gel was cut in 0.5-cm slices and the radioactivity of each slice was counted. Trypsinogen activation was expressed as the amount of radioactivity obtained as trypsin protease inhibitor complexes in per cent of the total amount of radioactivity recovered after electrophoresis.

To ensure the presence of free and unreacted protease inhibitors in the samples after the addition of the canine serum, the samples were also subjected to crossed immunoelectrophoresis using antibodies against α-macroglobulin and α_1-antitrypsin [15].

When the inhibitory effect of the serum protease inhibitors on the activation of trypsinogen by anionic and cationic trypsin was tested, the albumin containing saline in the incubation mixture was exchanged for whole serum or the different protease inhibitor preparations. The final concentrations of α-macroglobulin after the addition of serum was approx. 0.6 mg/ml ($0.8 \cdot 10^{-6}$ M) and after the addition of the α-macroglobulin preparation around 0.2 mg/ml ($0.25 \cdot 10^{-6}$ M). The final concentration of α_1-antitrypsin was approx. 0.75 mg/ml ($15 \cdot 10^{-6}$ M) after the addition of serum and 0.25 mg/ml ($5 \cdot 10^{-6}$ M) after the addition of the α_1-antitrypsin preparation.

Results

Gel filtration profiles before and after activation of cationic trypsinogen in serum by porcine enterokinase are shown in Fig. 1. After activation, approx.

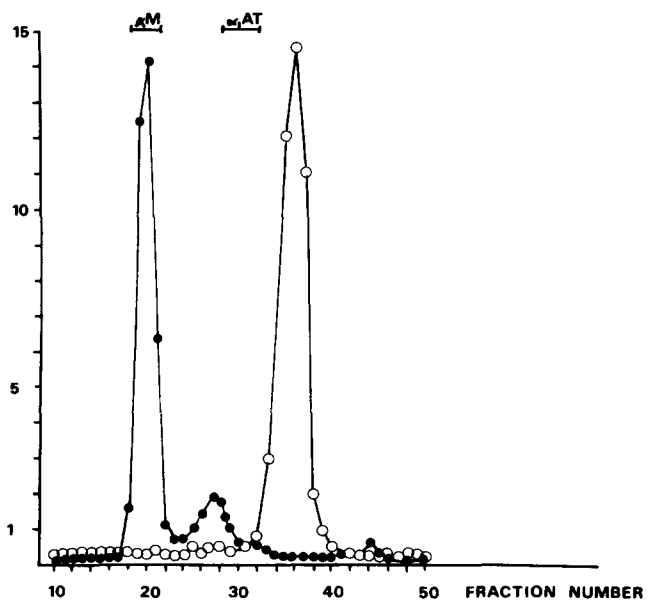

Fig. 1. Partition of ^{125}I cationic trypsinogen in serum before (○———○) and after (●———●) activation by enterokinase. Gel filtration was performed on a Sephadex G-100 column 0.9 × 50 cm equilibrated with 0.01 M Tris-HCl buffer containing 0.12 M NaCl, 0.005 M EDTA and bovine serum albumin 2 g/l at pH 7.4. Flow rate 2 ml/h. Fraction volume 0.4 ml. The bars indicate the elution volumes for α-macroglobulin (αM) and α_1-antitrypsin (α_1-AT).

80% of the radioactivity was demonstrated in the void volume after gel filtration using a Sephadex G-100 column, representing trypsin in complex with α-macroglobulin, while 20% eluted in a volume corresponding to the molecular size of trypsin in complex with α_1-antitrypsin. The elution profile after activation of anionic trypsinogen in serum was identical.

Due to differences in molecular size, the anionic trypsinogen could be separated from the larger anionic trypsin-protease inhibitor complexes also by gradient gel electrophoresis (Fig. 2). The free and unactivated anionic trypsinogen readily penetrated the gel, while the α_1-antitrypsin-trypsin complexes were

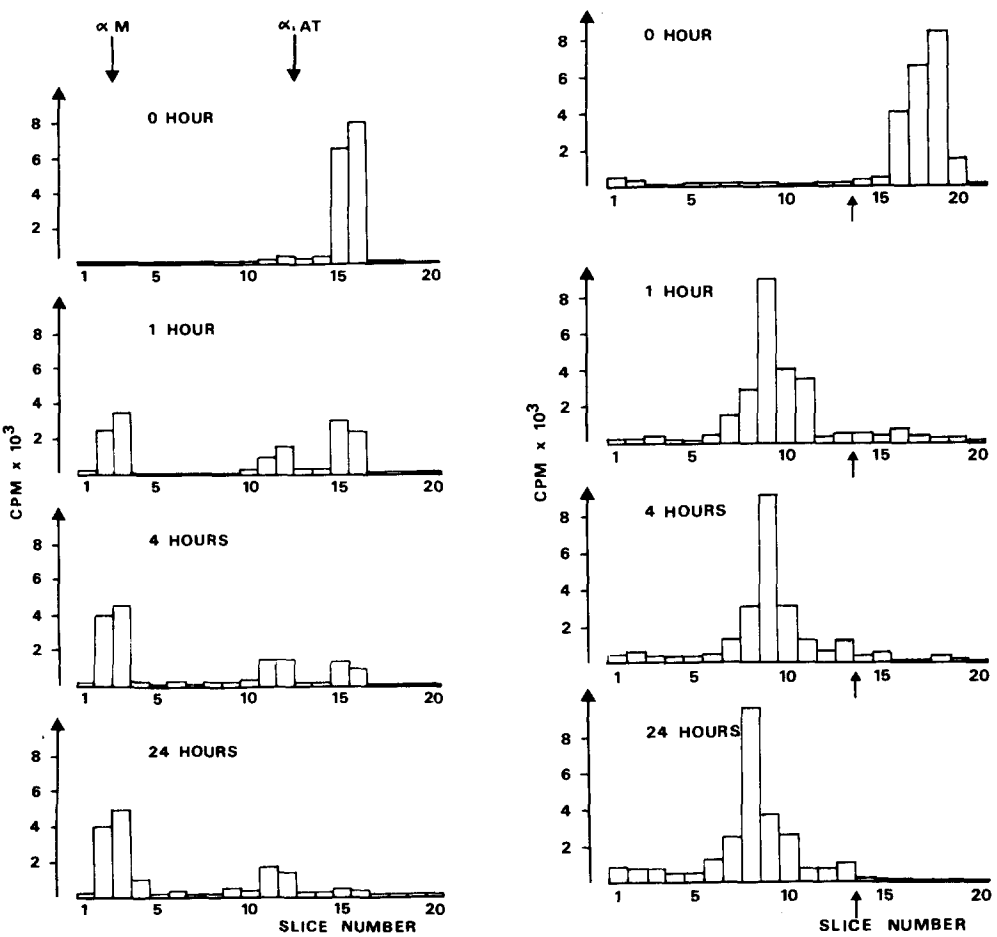

Fig. 2. Activation of anionic trypsinogen ($0.4 \cdot 10^{-7}$ M) by enterokinase ($0.4 \cdot 10^{-9}$ M), analyzed by preparative polyacrylamide gradient gel electrophoresis. Anode to the right. The unactivated trypsinogen readily penetrates the gel, while the activated trypsin complexes with the serum protease inhibitors are retarded. Arrows indicate migration rates for the major serum protease inhibitors, α-macroglobulin (αM) and α_1-antitrypsin (α_1-AT).

Fig. 3. Activation of cationic trypsinogen ($0.4 \cdot 10^{-7}$ M) by enterokinase ($0.4 \cdot 10^{-9}$ M), analyzed by preparative agarose gel electrophoresis. Arrow indicates application point. Anode to the left. The cationic trypsinogen migrates towards the cathode, while activated cationic trypsin forms complexes with the serum protease inhibitors and migrates towards the anode.

slightly retarded, whereas the α-macroglobulin complexes barely entered the gel. Due to differences in electric charge, the positively charged cationic trypsinogen could be separated from the negatively charged cationic trypsin-protease inhibitor complexes using agarose gel electrophoresis (Fig. 3). The free and unactivated cationic trypsinogen migrates towards the cathode (right), while the trypsin-protease inhibitor complexes migrates towards the anode (left). The α-macroglobulin trypsin and the α_1-antitrypsin-trypsin complexes were, however, not separated by this method.

Cationic trypsinogen was completely activated by enterokinase within 1 h, while only 60% of the anionic trypsinogen was activated during the same time (Figs. 2 and 3). The activation rate of the trypsinogens by enterokinase was somewhat decreased in the presence of whole serum, the α-macroglobulin preparation and the α_1-antitrypsin preparation according to Fig. 4.

Both trypsinogens could be activated by trypsin (Fig. 5), however, to achieve equal activation rates for the two trypsinogens, approx. 10 times more trypsin was required for activating anionic trypsinogen than for cationic trypsinogen. No difference in the ability of the two trypsins to activate the trypsinogens was observed. If whole serum was added to the incubation mixtures, the subsequent activation rate decreased to approx. 50% of the original according to Fig. 6. If the α-macroglobulin preparation was added, there was a decrease of the activation rate similar to that, when serum was added, while the α_1-antitrypsin preparation completely prevented activation of both trypsinogens by both trypsins.

The albumin concentration normally used in the incubation mixture had slight influence on the activation rate of trypsinogens by trypsin (Fig. 7). When the albumin concentration was increased by a factor 4, the initial activation rate was decreased by a factor of 2.

There was no autoactivation of the two trypsinogens in these concentrations (1 mg/l) during incubation for up to 48 h at 37° C. Bovine thrombin in con-

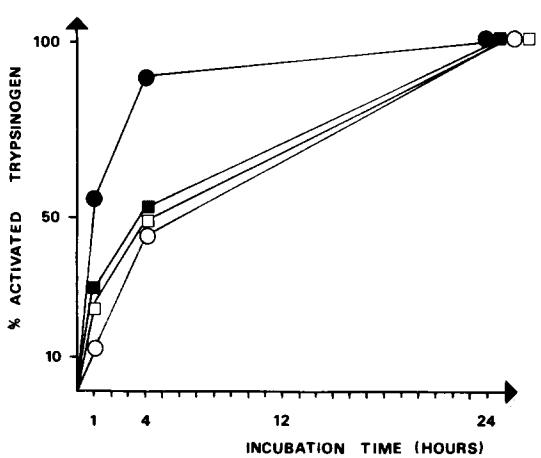

Fig. 4. Activation of anionic trypsinogen ($0.4 \cdot 10^{-7}$ M) by enterokinase ($0.4 \cdot 10^{-9}$ M) in NaCl 0.15 M containing bovine serum albumin 2 g/l (●———●), whole serum (■———■), α-macroglobulin preparation ($0.25 \cdot 10^{-6}$ M) (○———○) and α_1-antitrypsin preparation ($5 \cdot 10^{-6}$ M) (□———□).

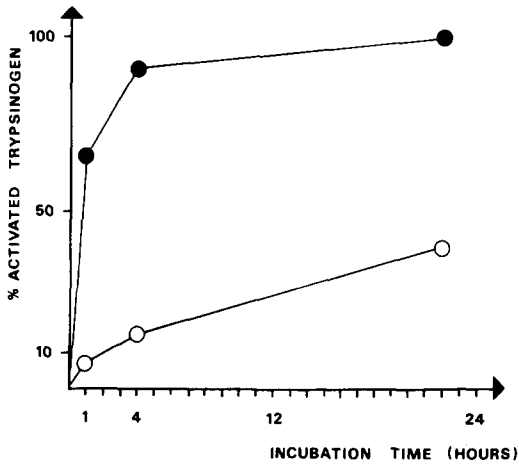

Fig. 5. Activation of anionic (○———○) and cationic (●———●) trypsinogen ($0.4 \cdot 10^{-7}$ mol/l) by anionic trypsin ($0.25 \cdot 10^{-6}$ mol/l).

centrations up to 250 μg/ml did not activate any of the trypsinogens even after 24 h of incubation at +37°C.

Dog plasmin in a concentration of 150 μg/ml activated 15% of both trypsinogens after 24 h of incubation at +37°C, however, if serum was added to the incubation mixture, the activation was inhibited.

Extracts from canine granulocytes were used in amounts capable of producing up to 20% complexation of the α_1-antitrypsin of the serum added after incubation. No activation was seen. After gel filtration, variable amounts of radioactivity appeared in a volume larger than that of trypsinogen indicating degradation of the trypsinogens.

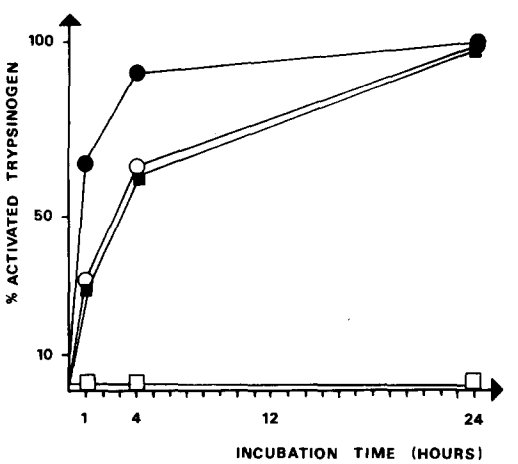

Fig. 6. Activation of cationic trypsinogen ($0.4 \cdot 10^{-7}$ M) by anionic trypsin ($0.25 \cdot 10^{-6}$ M) in the presence of 0.15 M NaCl containing 2 g/l bovine serum albumin (●———●). Serum (■———■), α-macroglobulin preparation ($0.25 \cdot 10^{-6}$ M) (○———○) and α_1-antitrypsin preparation ($5 \cdot 10^{-6}$ M) (□———□).

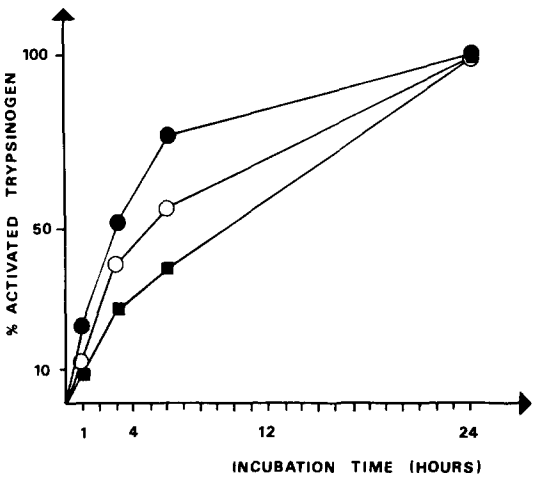

Fig. 7. Activation of cationic trypsinogen ($0.4 \cdot 10^{-7}$ M) by cationic trypsin ($0.25 \cdot 10^{-6}$ M) in the presence of a bovine serum albumin concentration of 4 g/l (●———●), 8 g/l (○———○) and 16 g/l (■———■).

Using the high concentrations mentioned for the different proteolytic enzymes in the activation experiments, the α-macroglobulins of the serum added after the incubation were saturated with enzymes ad judged by crossed electroimmunoassay. However, in all activation experiments, α_1-antitrypsin was never more than 10—20% saturated by proteolytic enzymes. The capacity to bind the activated labelled trypsin was thus never exceeded.

Discussion

These experiments demonstrate that enterokinase and trypsin can activate canine anionic and cationic trypsinogen in vitro. Comparing the amounts of enzymes used to obtain approximately equal activation rates in 1 h, trypsin has to be used in molar concentrations, which are at least 10^3 times higher than those of enterokinase. This is in agreement with earlier studies showing that bovine trypsinogen was activated $2 \cdot 10^3$ times faster by enterokinase than by trypsin [16]. It has been shown earlier that anionic trypsinogen from bovine and procine species are activated more rapidly by enterokinase than the cationic forms are [17]. In the present study with canine trypsinogens, the cationic form seems to be more easily activated.

Previous reports that trypsinogen can be activated by thrombin [18] could not be reproduced by us. Geokas and Rinderknecht [4] have also failed to demonstrate trypsinogen activation by thrombin but have demonstrated that human plasmin could activate bovine trypsinogen. It was necessary, however, to use 600 times more plasmin compared to trypsin (mol) to contain similar activation rates. We were also able to show that canine plasmin could activate canine trypsinogen although at a rate inferior to that of trypsin by a factor of 10^2—10^3. Extracts from canine granulocytes did not activate the trypsinogens, which is in agreement with the fact that no proteases with

'trypsin-like' activity have been reported in granulocytes.

Bovine serum albumin was used in the incubation buffers to minimize wall effects and to increase the recovery of proteins after preparative gel electrophoresis. The influence of the albumin concentration on the trypsin catalyzed trypsinogen activation is probably due to substrate competition and this effect is reasonably more pronounced in vivo, where the protein concentration is higher.

Trypsin bound to α-macroglobulin can hydrolyse low molecular weight esters and amide substrates at rates comparable to those of free trypsin [19]. Low molecular weight peptides, like angiotensin, vasopressin and proinsulin, are also attacked by α-macroglobulin trypsin complexes [20]. Bovine trypsin in complex with human α-macroglobulin has been shown to activate bovine trypsinogen and chymotrypsinogen [19]. These results are confirmed by our observations. In acute pancreatitis, large amounts of zymogens are found in ascitic fluid and serum together with active enzymes in complex with α_1-antitrypsin and α-macroglobulin [21,22]. The α-macroglobulin trypsin complexes are cleared rapidly from serum preventing harmful effects [23]. The elimination of these complexes from ascitic fluid is probably slower and here the low molecular weight specific trypsin inhibitor, produced by pancreas, may play an important role in preventing further zymogen activation by the α-macroglobulin-trypsin complexes. This low molecular weight trypsin inhibitor has been shown to be a powerful inhibitor of α-macroglobulin-trypsin complexes in vitro and in vivo [24,25].

We found no autoactivation of the trypsinogens. This may be due to the low concentration used (1 mg/l), since it has been reported that high concentrations of trypsinogen are required (2—7 mg/ml) before autoactivation occurs [26]. Patients with enterokinase deficiency have been reported [27] and suffer primarly due to maldigestion, since there is no activation of the pancreatic zymogens in the intestines. The process of autoactivation is evidently not able to make up for the loss of enterokinase in the duodenum. Our conclusion is that the biological significance of autoactivation of trypsinogen is questionable and we rather favour the view that the conversion of trypsinogen to active trypsin during acute pancreatitis is initially triggered by an enzymatic process other than autoactivation, leading to a local consumption of protease inhibitors. An activation cascade of the proenzymes is unlikely to occur before the protease inhibitors are consumed. Studies on human pancreatic juice during acute inflammation of the pancreatic gland have been performed and demonstrate a considerable degree of proteolytic activity with an apparent lack of the trypsin inhibiting capacity found in normal pancreatic juice [4,5]. The initial premature activation of trypsinogen must take place in the presence of protease inhibitors and this makes an activation by blood clotting or fibrinolytic enzymes unlikely. The present work shows that enterokinase activation of trypsinogen is not inhibited by the serum protease inhibitors, further enterokinase is not inhibited by the low molecular weight specific trypsin inhibitor in pancreatic juice [16]. Thus enterokinase might be a most dangerous component in case of a backflow of duodenal juice through the pancreatic duct. This may occur as a consequence of increased intraduodenal pressure seen for example in profuse vomiting in alcoholism [28]. Reaching the interstitial

tissues and around the gland, minute amounts of enterokinase may activate the trypsinogens leading to a consumption of the protease inhibitors locally. Then a trypsin catalyzed activation cascade of all the proenzymes may occur with a subsequent autodigestion of the pancreatic gland.

Acknowledgements

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