Isolation and characterization of a pancreatic elastase from plasma of patients with acute pancreatitis

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Summary

1. An elastase-like enzyme in plasma of patients with acute pancreatitis was purified by DEAE-cellulose column chromatography and polyacrylamide-gel disc electrophoresis.

2. In this way 0.24 mg of purified enzyme with a specific activity of 3.94 succinyl-L-alanyl-L-alanyl-L-alanyl-p-nitroanilide units/mg of protein was obtained from 10 ml of plasma.

3. The purified material was homogeneous as ascertained by sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis and had an apparent molecular weight of 24 000 as measured by gel filtration on Sephadex G-100.

4. This enzyme hydrolysed denatured casein and Congo Red-elastin as well as succinyl-L-alanyl-L-alanyl-L-alanyl-p-nitroanilide. Its amidolytic activity was inhibited by soya bean trypsin inhibitor, but not by aprotinin.

5. Although the enzyme was immunologically similar to elastase 2, its kinetic properties and substrate specificity were apparently different.

6. We propose that an elastase-like enzyme, probably different from elastase 1 or elastase 2, is liberated from the pancreas into blood during acute pancreatitis and becomes combined with α1-macroglobulin.

Key words: elastase, α1-macroglobulin, pancreatitis.

Introduction

Pancreatic elastase is an endopeptidase which digests a wide variety of protein substrates. From a pathological point of view it is the most important enzyme among the principal pancreatic endopeptidases because of its ability to digest elastin, the elastic fibrous protein of connective tissue [1]. Recent studies on acute experimental pancreatitis in dogs have demonstrated the role of elastase as the key enzyme responsible for the destruction of vessel walls, for elastolysis, thrombosis and interstitial haemorrhage [2]. These studies also suggest that elastase plays an important role in inducing vascular injury, which results in acute haemorrhagic pancreatitis in man. In 1976, Largman et al. [3] isolated and purified from activated human pancreas tissue extract two types of elastase which they designated as elastase 1 and elastase 2. Although Geokas [4] demonstrated by radioimmunoassay that elastase 2 was present in normal human blood, there are few reports of the purification of pancreatic elastase from plasma of patients with acute pancreatitis. We previously observed that proteolytic enzymes were liberated from the pancreas into the blood during acute pancreatitis and that almost all of these combined with α1-macroglobulin [5, 6]. These enzymes, one of which was pancreatic kallikrein, could be
separated from \( \alpha_2 \)-macroglobulin by chromatography on Sephadex G-200 in the presence of sodium dodecyl sulphate (SDS). In the present study attempts were made to characterize the other proteolytic enzymes bound to \( \alpha_2 \)-macroglobulin in plasma from patients with acute pancreatitis. We have succeeded in purifying one of these proteolytic enzymes, which is tentatively identified as a pancreatic elastase.

**Materials and methods**

**Materials**

The following commercial preparations were used: pig pancreatic elastase (Sigma), \( \alpha-N \)-acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt; Merck), casein (Merck), Congo Red–elastin (Sigma), Sephadex G-100 (Pharmacia), DEAE-cellulose (Brown), succinyl-L-alanyl-L-alanyl-L-alanyl-p-nitroanilide ISuc-(Ala)\(_3\)-pNA I (Foundation for Promotion of Protein Research, Institute for Protein Research, Osaka), aprotinin (Trasylol) (Bayer), and soya bean trypsin inhibitor (Sigma).

**Enzyme and protein assays**

The esterolytic activity of samples against Ac-Tyr-OEt was measured by the method of Hestrin [7] as modified by Roberts [8]. The final concentration of Ac-Tyr-OEt used was 15 mmol/l.

The caseinolytic activity of the enzyme was measured by the method described previously [9]. One caseinolytic unit was defined as the amount of enzyme releasing 60 \( \mu \)g of acid-soluble tyrosine in 15 min at 37°C.

Amidolytic activity was determined with the chromogenic substrate Suc-(Ala)\(_3\)-pNA, which is specific for elastase [10]. A portion (1 ml) of each fraction was added to test tubes containing 1-5 ml of Tris buffer (pH 8-0) and 0-02 ml of Suc-(Ala)\(_3\)-pNA (125 mmol/l). After incubation for 5 min at 37°C, the reaction was stopped with 0-1 ml of acetic acid. The absorbance at 410 nm was measured against a substrate control. A 125 mmol/l solution of the resulting Suc-(Ala)\(_3\)-pNA gave an absorbance of 8-3 at 410 nm after complete hydrolysis with pig elastase. One amidolytic unit was defined as the amount of enzyme which releases 1 \( \mu \)mol of \( p \)-nitroaniline/min at 37°C.

Elastolytic activity was determined by the method of Shotton [11] with Congo Red–elastin as substrate. One elastolytic unit 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 [11] with bovine serum albumin (Merck) used as the standard.

**Estimation of effects of various inhibitors on the elastase-like enzyme**

To measure the effects of various inhibitors on caseinolytic or Suc-(Ala)\(_3\)-pNA amidolytic activity, 0-05 or 0-1 ml of enzyme solution was mixed with various amounts of inhibitors. After incubation for 15 min at 37°C, 0-25 ml of 8% (w/v) casein or 0-02 ml of Suc-(Ala)\(_3\)-pNA (125 mmol/l) was added to the test tube, which was then incubated for 30 or 6 min at 37°C respectively. The concentration of enzyme solution was adjusted to 0-1 mg/ml before use.

**Electrophoretic technique**

SDS/polyacrylamide-gel electrophoresis was carried out by the method of Weber & Osborn [12] with 7-5% (w/v) gel with 1% SDS and 8 mol of urea/l. Electrophoresis was performed at 8 mA/gel for 3 h. Gels were stained for 2 h at room temperature with 2% Coomassie Brilliant Blue dissolved in methanol/acetic acid/water (5 : 1 : 4, by vol.) and destained electrophoretically with methanol/acetic acid/water (25 : 7 : 68, by vol.).

**Immunological experiments**

Double-diffusion analysis (Ouchterlony method [13]) was performed at room temperature for 24 h in 1% agarose (Behringwerke) in veronal buffer (\( I = 0-05 \), pH 8-6) containing 0-05% \( \mathrm{NaN}_3 \).

**Molecular weight determination**

The molecular weight of the purified elastase-like enzyme was determined by the method of Andrews [14]. Sephadex G-100 (column 2-5 cm x 60 cm) was equilibrated with phosphate buffer (0-1 mol/l, pH 7-4) and eluted at 4°C at a flow rate of about 15 ml/h. Elastase-like enzyme fractions were detected by the amidolytic activity in each fraction. The molecular weight of elastase-like enzyme was also determined by SDS/polyacrylamide-gel electrophoresis. Bovine serum albumin (molecular weight 67 000), ovalbumin (45 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100) and \( \alpha \)-lactalbumin (14 400) were used as marker proteins.

**Preparation of elastase 1 and elastase 2**

Elastase 1 and elastase 2 were prepared by the following procedures from pancreatic tissue
obtained at autopsy; acetone treatment, acid precipitation, \((\text{NH}_4)_2\text{SO}_4\) fractionation and CM-Sephadex C-50 column chromatography as described by Largman et al. [3]. By these procedures, fourfold purification of elastase 1 and 13-fold purification of elastase 2 were achieved, and their specific activities were 3-4 and 8-2 Congo Red–elastin units/mg of protein respectively.

**Preparation of human plasma \(\alpha_2\)-macroglobulin**

Human plasma \(\alpha_2\)-macroglobulin was purified as described previously [3]. The concentration of protein in the \(\alpha_2\)-macroglobulin fraction was 3 mg/ml and its specific activity was 15.5 units/mg of protein. One unit was defined as the amount of inhibitor required to inhibit completely 1-0 caseinolytic unit of trypsin.

**Preparation of \(\alpha_2\)-macroglobulin–elastase 2 complex**

\(\alpha_2\)-Macroglobulin–elastase 2 complex was prepared by mixing 5 ml of purified \(\alpha_2\)-macroglobulin (containing 15 mg of protein, 233 units) with 2 ml of purified elastase 2 (0.5 mg of protein, 4-1 units) which was incubated for 1 h at 25°C, and then chromatographed on Sephadex G-200 (column 2-5 cm x 50 cm). \(\alpha_2\)-Macroglobulin fractions were combined and concentrated to 5 ml by ultrafiltration (PM 10 membrane; Amicon Corp.). Complete complex formation between \(\alpha_2\)-macroglobulin and elastase 2 was confirmed by the elution of only one protein peak, in the void volume, from a Sephadex G-200 column.

**Preparation of antisera**

Purified elastase-like enzyme (0.5 mg of protein) was dissolved in 1.0 ml of sodium chloride solution (154 mmol/l: saline), mixed with an equal volume of complete Freund’s adjuvant and injected into the toe pads of a rabbit. After 4 weeks, the same amount of elastase-like enzyme was injected subcutaneously into the rabbit’s back as a booster dose. Four weeks later, a blood sample was drawn from the rabbit and incubated at 37°C for 3 h and at 4°C for 18 h before serum separation by centrifugation at 2500 g.

**Results**

**Purification of the elastase-like enzyme**

As described previously [5, 6], plasma samples were collected from 10 patients with a serum amylase level of more than 1000 Somogyi units/dl. \(\alpha_2\)-Macroglobulin fractions were obtained from Sephadex G-200 chromatography and further chromatographed on a SDS/Sephadex G-200 column. Fractions with Ac-Tyr-OEt esterolytic activity (total volume 60 ml, total activity 2-27 chromozyme units) obtained from the latter column were pooled and dialysed at 4°C overnight against phosphate buffer (0.01 mol/l) at pH 7.4, containing NaCl (0.05 mol/l). The dialysed solution was chromatographed on a DEAE-cellulose column (2.0 cm x 8.0 cm) that had been equilibrated with phosphate buffer (0.01 mol/l) containing NaCl (0.05 mol/l) at pH 7.4. After washing with 100 ml of this buffer, the column was eluted with a linear gradient formed from 150 ml of equilibration buffer and 150 ml of phosphate buffer (0.01 mol/l) at pH 7.4 containing NaCl (0.3 mol/l), at a flow rate of 25 ml/h. As shown in Fig. 1, two types of proteolytic enzymes with both Ac-Tyr-OEt esterolytic and caseinolytic activities were eluted in the non-adsorbed and adsorbed fractions respectively. Since one of these might be elastase, we examined the amidolytic activity of each fraction by using Suc-(Ala)_2-pNA, a specific substrate for elastase; amidolytic activity was detected in the non-adsorbed fraction. These elastase-like enzyme fractions (fraction nos. 4–13, total volume 50 ml) from the DEAE-cellulose column were pooled and concentrated to 1 ml by ultrafiltration (PM 10 membrane; Amicon Corp.) and further purified by polyacrylamide-gel electrophoresis as described by Davis [16]. The concentrated elastase-like enzyme (100 \(\mu\)g of protein) was run on each polyacrylamide gel. One gel was stained and the others were sliced into 2 mm segments, each of which was eluted overnight at 4°C with 0-9 ml of Tris/HCl buffer at pH 8-8. A major protein band was found in segment 24 and three minor ones in segments 6, 22–23 and 26–27. The eluate from each gel segment was tested for Ac-Tyr-OEt esterolytic, caseinolytic and amidolytic activities. As shown in Fig. 2, caseinolytic activity was present in both segments 6 and 24. Conversely, Ac-Tyr-OEt esterolytic activity was present in segment 6 and amidolytic activity was mainly found in segment 24. By polyacrylamide-gel electrophoresis an elastase-like enzyme was isolated from some other protein components, especially a chymotrypsin-like enzyme with Ac-Tyr-OEt esterolytic and caseinolytic activities. Ten sliced gel band segments (segment 24) of the elastase-like enzyme were collected, eluted with 9 ml of Tris/HCl buffer at pH 8-8 and freeze-dried. The elastase-like en-
FIG. 1. DEAE-cellulose column chromatography of SDS-treated plasma. The Ac-Tyr-OEt esterolytic activity (○), caseinolytic activity (●) and amidolytic activity (△) of each fraction (5 ml) were measured as described in the text. The broken line indicates the salt gradient in the eluant, and the solid line the protein content ($A_{280}$).

FIG. 2. Polyacrylamide-gel electrophoresis of an elastase-like enzyme (100 µg of protein). The Ac-Tyr-OEt esterolytic (○), caseinolytic (●) and amidolytic (△) activities in the eluate are shown. M, Bromothymol blue marker.

zyme obtained from these procedures was homogeneous as shown by a single band of protein on SDS/polyacrylamide-gel electrophoresis. The overall purification and yield of elastase-like enzyme are summarized in Table 1. A final yield of 0.24 mg of the purified preparation was obtained from 10 ml of original plasma. The specific activity of the final product was 3.94 Suc-(Ala)$_3$-pNA units/mg of protein.

Estimation of molecular weight

The approximate molecular weight of the elastase-like enzyme after polyacrylamide-gel
Substrate specificity studies

To clarify the identity of this enzyme, the kinetics of the amidolytic activity, and the caseinolytic and elastolytic activities of the purified enzyme were determined and compared with those of pig elastase, human elastase 1 and elastase 2. The kinetic parameters for hydrolysis of Suc-(Ala)₃pNA were evaluated as described by Del Mar et al. 17}. The kinetic parameters $K_m$ and $k_c$ in this study for pig elastase, elastase 1, elastase 2 and elastase-like enzyme are presented in Table 2. The elastase-like enzyme, as well as pig elastase, hydrolyses this substrate much more rapidly than do elastase 1 and elastase 2. Its elastolytic activity with Congo Red–elastin and its caseinolytic activity were less than those of porcine elastase, but were remarkably higher than those of elastase 1 and elastase 2.

Inhibition of the elastase-like enzyme by various proteinase inhibitors

The inhibitory effects of aprotinin and soya bean trypsin inhibitor on the caseinolytic and amidolytic activities of the elastase-like enzyme were studied. As shown in Table 3, only the amidolytic activity could be inhibited by soya bean trypsin inhibitor.

**Immunological identity of elastase-like enzyme with elastase 2**

From the substrate specificity studies, the elastase-like enzyme in this study was thought to be different from the elastase 1 and elastase 2 described by Largman et al. [3]. Therefore, to compare further the enzyme with elastase 1 and elastase 2, its immunological characterization was investigated. As shown in Fig. 3, the antiserum to the elastase-like enzyme formed a single immunoprecipitin line with the purified enzyme and elastase 2, but not with elastase 1.

### Table 1. Summary of purification of the elastase-like enzyme

<table>
<thead>
<tr>
<th>N.D., Not determined.</th>
<th>Total activity (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>N.D.</td>
<td>740</td>
<td>--</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>N.D.</td>
<td>130</td>
<td>--</td>
</tr>
<tr>
<td>SDS/Sephadex G-200</td>
<td>2.27</td>
<td>15</td>
<td>0.15</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>1.29</td>
<td>1.3</td>
<td>1.00</td>
</tr>
<tr>
<td>Polyacrylamide-gel electrophoresis</td>
<td>0.95</td>
<td>0.24</td>
<td>3.95</td>
</tr>
</tbody>
</table>

* Suc-(Ala)₃pNA units.

### Table 3. Inhibitory spectrum of the elastase-like enzyme

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitors</th>
<th>Aprotinin</th>
<th>Soya bean trypsin inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>No*</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Suc-(Ala)₃pNA</td>
<td>No</td>
<td>140 μg†</td>
<td></td>
</tr>
</tbody>
</table>

* No inhibition with 250 k.i.u. of aprotinin or 500 μg of soya bean trypsin inhibitor.
† Amount of inhibitor required to inhibit completely 0–1 mg of enzyme.

### Table 2. Kinetics for Suc-(Ala)₃pNA as substrate and proteolytic activity of various elastases

<table>
<thead>
<tr>
<th>Property</th>
<th>Pig elastase</th>
<th>Elastase 1</th>
<th>Elastase 2</th>
<th>Elastase-like enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_\text{a}/K_m$ (mmol⁻¹ s⁻¹)</td>
<td>19-3/1-6 = 12-1</td>
<td>0-61/1-5 = 0-41</td>
<td>0-19/14 = 0-013</td>
<td>18-23/1-6 = 11-4</td>
</tr>
<tr>
<td>Activity against:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Congo Red–elastin (units/mg of protein)</td>
<td>87.5</td>
<td>3.4</td>
<td>8.2</td>
<td>63</td>
</tr>
<tr>
<td>Casein (units/mg of protein)</td>
<td>46</td>
<td>1.9</td>
<td>2.1</td>
<td>28</td>
</tr>
<tr>
<td>Ratio of elastinolytic/caseinolytic activities</td>
<td>1.9</td>
<td>1.8</td>
<td>3.9</td>
<td>2.3</td>
</tr>
</tbody>
</table>
column chromatography showed that elastase 2 was separated from $\alpha_2$-macroglobulin and was eluted in fractions immediately succeeding the albumin fractions, which were different from $\alpha_2$-macroglobulin fractions. The substrate specificities for Suc-(Ala)$_3$-pNA, Congo-Red–elastin and casein of this isolated elastase 2 were examined and compared with those of the original elastase 2, and it was confirmed that there was no difference in substrate specificities between the two.

Discussion

Since Geokas et al. [2] proposed that pancreatic elastase plays an important role in the pathogenesis of vascular injury in acute haemorrhagic pancreatitis in man, many reports on its purification and estimation have been published. In 1974 Mallory & Travis [18] isolated a proteolytic enzyme which hydrolyses all of the synthetic elastase substrates and casein, while failing to digest elastin from homogenates of human pancreas tissue, and named it proteinase E. In 1976, Largman et al. [3] reported that there were two proteinases with elastolytic activity in extracts of human pancreas and named them elastase 1 and elastase 2. Their molecular weights were determined to be 29 300 and 25 000 respectively. The kinetic parameters of elastase 1 and elastase 2 were tested against Suc-(Ala)$_3$-pNA, which was designed as a specific substrate for pig elastase, and it was confirmed that $k_e/K_m$ (mmol$^{-1}$ s$^{-1}$) values for elastase 1 and elastase 2 were 0.39 and 0.008 respectively, which were remarkably lower than $k_e/K_m (=11)$ for pig elastase. They proposed that elastase 2 was clearly distinct from proteinase E, but elastase 1 appeared to be similar to proteinase E in terms of amino acid composition, molecular weight and ionic character.

As part of our work on acute pancreatitis, we have now succeeded in purifying an elastase-like enzyme from the $\alpha_2$-macroglobulin fraction of patients’ plasma. The purified material appeared to be homogeneous on SDS/polyacrylamide-gel electrophoresis and its molecular weight was determined as 24 000 by gel filtration on Sephadex G-100. In 1977 Geokas [4] found that elastase 2 was present in $\alpha_2$-macroglobulin fractions of normal human serum by radioimmunoassay of column fractions after Sephadex G-200 column chromatography, and also that elastase 2, as measured by radioimmunoassay, increased in serum of pancreatic inflammation patients. From their reports and the similarity of molecular weight between the elastase-like enzyme in this study and elastase 2, we presumed that it might be elastase 2. Therefore, to confirm its identity, the substrate specificities were compared with those of pig elastase, elastase 1 and elastase 2. As shown in Table 2, the substrate specificities of this enzyme were more similar to those of pig elastase than to those of elastase 1 and elastase 2. It was of particular interest that this enzyme had almost the same $k_e/K_m$ value for hydrolysis of Suc-(Ala)$_3$-pNA (11.4) as did pig elastase (11.0).

To elucidate further whether or not the origin of this enzyme is elastase 2, its immunological characterization was investigated by double immunodiffusion. Contrary to the results of the substrate specificities, as shown in Fig. 3, it was confirmed to be immunologically identical with elastase 2. Furthermore, as it could not be isolated from the plasma $\alpha_2$-macroglobulin fractions of healthy persons or those who had recovered from acute pancreatitis, it was considered to be an enzyme related to acute pancreatitis and released into blood from the pancreas. How then should we interpret the contradictory results on immunological identity and substrate specificities observed between it and elastase 2? One possibility is that another elastase which possesses similar antigenicity and dissimilar substrate specificities to elastase 2 may be produced in the pancreas and released into blood during acute pancreatitis. Another possibility is that the formation of a complex between

![Diagram](image-url)
elastase 2 and α₂-macroglobulin or the subsequent isolation of the enzyme from α₂-macroglobulin by SDS/Sephadex G-200 column chromatography may bring about a conformational change of elastase 2 and create high amidolytic, caseinolytic and elastolytic activities. In the present study, to confirm whether or not α₂-macroglobulin–SDS/Sephadex G-200 treatment of elastase 2 brings about changes in substrate specificities, α₂-macroglobulin–elastase 2 complex was prepared, from which elastase 2 was isolated by SDS/Sephadex G-200 column chromatography and its substrate specificities were examined. However, there was no change of amidolytic, caseinolytic and elastolytic activities.

Treatment. Hence, we propose that another substrate specificities of elastase pancreatic elastase with the same antigenicity as α₂-macroglobulin-SDS/Sephadex G-200 the pancreas and released into the blood during acute pancreatitis, may combine with α₂-macroglobulin and circulate in the blood stream as α₂-macroglobulin-bound elastase. This also suggests that α₂-macroglobulin plays a more important role than α₁-antitrypsin in binding and inhibiting elastase.

It was of particular interest that the elastase-like enzyme could be isolated from the α₂-macroglobulin fraction of patients’ plasma and that elastase 2 could also be combined with α₂-macroglobulin. Many reports on the interaction between plasma proteinases and plasma proteinase inhibitors have been presented, but the interaction between α₂-macroglobulin and elastase in acute pancreatitis is not understood. Rowley et al. [21] reported that α₁-antitrypsin inhibited the elastase of pancreas and of granulocytes. Ohlsson & Laurell [22] showed that incubation of α₁-antitrypsin–elastase complex with α₂-macroglobulin resulted in a transfer of elastase from the former to the latter inhibitor. Meyer et al. [23] observed that, although elastase could be inhibited by α₁-antitrypsin, α₂-macroglobulin-bound elastase could no longer be inhibited by α₁-antitrypsin. Lestienne & Bieth [24] also proposed that α₂-macroglobulin-bound elastase would be completely resistant to inhibition by α₁-antitrypsin. These reports, and the results from the present study, imply that elastase, which is released from the pancreas into blood during acute pancreatitis, may combine with α₂-macroglobulin and circulate in the blood stream as α₂-macroglobulin-bound elastase. This also suggests that α₂-macroglobulin plays a more important role than α₁-antitrypsin in binding and inhibiting elastase.

References


