Purification and characterization of kallikrein from plasma of patients with acute pancreatitis

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Summary

1. A kallikrein-like enzyme in plasma of patients with acute pancreatitis was further purified by successive hydroxyapatite/cellulose and Sepharose-4B column chromatography.

2. By these procedures 0·26 mg of purified enzyme with a specific activity of 215 S-2266 chromozyme units/mg of protein was obtained from 10 ml of original plasma.

3. The purified material was homogeneous as ascertained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and had an apparent molecular weight of 31 000 as measured by gel filtration on Sephadex G-200.

4. It was confirmed immunologically that this enzyme was pancreatic kallikrein, which is distinct from plasma kallikrein, and that it could combine with α₂-macroglobulin only in the presence of trypsin.

Key words: kallikrein, α₂-macroglobulin, pancreatitis.

Abbreviation used: SDS, sodium dodecyl sulphate.

Introduction

Many reports have been made concerning the important role of the kallikrein–kinin system in acute pancreatitis. Worthington & Cuschieri (1976) reported that arginine esterolytic activity was significantly elevated in plasma of patients with acute pancreatitis and speculated that one of the plasma esterolytic enzymes might be a kallikrein originating from the pancreas. Discovery of a kallikrein-like enzyme in the plasma α₁-macroglobulin fraction of patients with acute pancreatitis was reported (Sumi, Takasugi & Toki, 1978) and the authors succeeded in isolating the enzyme from α₁-macroglobulin fraction by SDS/Sephadex G-200 gel column chromatography (Takasugi, Toki & Sumi, 1980). Some of the biochemical and biological properties of the enzymes were compared with those of human plasma kallikrein and it was strongly suggested that, in view of its molecular weight and ability to be absorbed on DEAE-cellulose, it might be human pancreatic kallikrein, which is different from human plasma kallikrein. In the present study an attempt was made to clarify whether or not this enzyme is pancreatic kallikrein by using successive column chromatography for purification and double immunodiffusion for immunological characterization. Furthermore the interaction between purified pancreatic kallikrein and α₁-macroglobulin was investigated.

Materials and methods

Materials

The following commercial preparations were used: d-Val-Leu-Arg p-nitroanilide hydrochloride (S-2266, Kabi Diagnostica Co. Ltd), Glu-Gly-Arg p-nitroanilide (S-2444, Daiichi Pure Chemical Co.), p-tosyl-L-arginine methyl ester (Sigma), Sephadex G-200 (Pharmacia), hydroxyapatite (Seikagaku Kogyo Co. Ltd), cellulose Mikrokristallin (Merck), Sepharose-4B (Pharmacia), aprotinin (Trasylol) (Bayer), soya
bean trypsin inhibitor (Sigma), ethyl-p-(6-guanidino-\(\text{hexanoyloxy}\))-benzene methane sulfonate (FOY) (Research Laboratories of Ono Pharmaceutical Co., Osaka) and p-tosyl-L-lysine chloromethyl ketone (7-amino-1-chloro-3-L-tosylamidoheptan-2-one, TLCK) (Sigma).

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

Human plasma kallikrein was obtained as described in a previous report (Toki & Yamura, 1979). Human plasma kallikrein (1 mg) was found to possess 213 chromozyme units (defined below). Human urinary and pancreatic kallikrein were kindly supplied by Green Cross Co. Ltd, Osaka. The specific activity of human urinary and pancreatic kallikrein was 262 and 197 chromozyme units/mg of protein respectively. Human plasma \(\alpha_2\)-macroglobulin was purified by the method as described previously (Toki, Ishihara & Yamura, 1977). The concentration of protein in \(\alpha_2\)-macroglobulin was 0.3 mg/ml and its specific activity was 15.5 units/mg. One unit was expressed as the amount of inhibitor required to inhibit completely 1.0 casein unit of trypsin, which was defined as the amount releasing 60 \(\mu\text{g}\) of acid-soluble tyrosine in 15 min on incubation at 37°C.

**Assay of enzyme and protein**

In the present study the kallikrein-like and trypsin-like enzymes were determined by their amidolytic activity by using for the former chromogenic substrate S-2266 which is specific for glandular kallikrein and substrate S-2444 for the latter. One chromozyme unit was defined as the amount of enzyme required to hydrolyse 1 \(\mu\text{mol}\) of chromozyme/min under standard conditions. The final concentration of chromogenic substrate in the present study was 0.15 mmol/l for S-2266 and 0.3 mmol/l for S-2444 respectively. The esterolytic activity of trypsin-like enzyme was also determined by Hestrin’s (1949) method. The inhibitory activities of various trypsin inhibitors on the trypsin-like enzyme were determined from the residual esterolytic and amidolytic activities of the enzyme after incubation with a mixture of enzyme and various amounts of inhibitors for predetermined periods at 37°C. Protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) by using bovine serum albumin (Merck) as the standard.

**Electrophoretic technique**

SDS/polyacrylamide-gel electrophoresis was carried out by the method of Weber & Osborn (1969) by using 7.5% (w/v) gel with 1% SDS and urea (8 mol/l). Electrophoresis was performed at 8 mA/gel for 3 h. Gels were stained for 2 h with 2% (w/v) Coomassie brilliant blue and destained electrophoretically.

**Estimation of molecular weight**

The molecular weight of the purified kallikrein-like enzyme was estimated by the method of Andrews (1965). Sephadex G-200 (2.5 cm x 50 cm) was equilibrated with Tris/HCl buffer (0.05 mol/l), pH 7.5, containing KCl (0.2 mol/l) and elution was performed at 4°C at a flow rate of about 15 ml/h.

**Preparation of antiserum**

Purified kallikrein-like enzyme (2 mg) was dissolved in 1.0 ml of sodium chloride solution (150 mmol/l), mixed with an equal volume of complete Freund’s adjuvant and injected into toe pads of a rabbit. After 4 weeks the rabbit received, as a booster dose, the same amount of enzyme injected subcutaneously into the back. After 2 weeks a blood sample was drawn and incubated at 37°C for 3 h and at 4°C for 18 h before serum separation by centrifugation at 2400 g.

**Immunodiffusion**

Immunodiffusion was carried out by the method of Ouchterlony (1958) at room temperature for 24 h. Agar gel (Behringwerke) was prepared at a concentration of 1% in veronal buffer (\(I = 0.05, \text{pH 8.6}\)) containing 0.05% sodium azide.

**Results**

**Purification of the kallikrein-like enzyme**

As described previously (Takasugi et al., 1980), the kallikrein-like enzyme was isolated from \(\alpha_2\)-macroglobulin fraction by SDS/Sephadex G-200 gel chromatography and eluted in the adsorbed fraction by DEAE-cellulose column chromatography. In the present study the kallikrein-like enzyme was further purified by successive column chromatography on hydroxyapatite/cellulose and Sepharose-4B.

**Step 1. Hydroxyapatite/cellulose column chromatography**

Hydroxyapatite/cellulose was prepared by mixing hydroxyapatite and cellulose Mikro-
Purification of kallikrein

201

Kristallin at equal volumes in phosphate buffer (0·01 mol/l) at pH 7·4. The kallikrein-like enzyme fractions (total volume 35 ml, total activity 75·3 chromozyme units) obtained from the DEAE-cellulose column were dialysed overnight against phosphate buffer (0·01 mol/l) at pH 7·4 at 4°C. The dialysed solution was chromatographed on a hydroxyapatite/cellulose column (2·0 cm × 8·0 cm) that had been equilibrated with phosphate buffer (0·01 mol/l) at pH 7·4. The kallikrein-like enzyme was eluted with a linear gradient formed from 100 ml of equilibration buffer and 100 ml of phosphate buffer (0·1 mol/l) at pH 7·4, at a flow rate of 30 ml/h (Fig. 1). One main peak with kallikrein-like activity was eluted in fraction nos. 41–56 which were combined, concentrated to 5 ml by ultrafiltration and dialysed against phosphate buffer (0·05 mol/l) at pH 7·4.

**Step 2. Sepharose-4B gel filtration**

The partially purified kallikrein-like enzyme obtained from step 1 (about 5 ml) was subjected to gel filtration on Sepharose-4B (2·0 cm × 65 cm) which had been equilibrated with phosphate buffer (0·05 mol/l) at pH 7·4. The column was eluted with the same buffer at a flow rate of 15 ml/h and fractions of 5 ml were collected. The protein was eluted as a single peak and the kallikrein-like enzyme fractions were pooled and concentrated to 0·1 vol. by ultrafiltration. The overall purification and yield of kallikrein-like enzyme are summarized in Table 1. A final yield of 0·26 mg of purified preparation was obtained from 10 ml of the original plasma. The specific activity of the final product was 215 S-2266 chromozyme units/mg of protein.

**Homogeneity**

The final preparation of the kallikrein-like enzyme gave a single band in SDS/polyacrylamide-gel electrophoresis (Fig. 2).

**Estimation of molecular weight**

The approximate molecular weight of the kallikrein-like enzyme was estimated by gel filtration on a Sephadex G-200 column by the method of Andrews (1965). The molecular weight of the kallikrein-like enzyme was found to be 31,000 on the basis of a linear relationship between the elution volumes of marker proteins and the logarithms of their molecular weights.

**Immunological identification**

The identity of the kallikrein-like enzyme was investigated by a double immunodiffusion method by using human plasma, urinary or pancreatic kallikrein. As shown in Fig. 3, rabbit antiserum against the kallikrein-like enzyme formed a single immunoprecipitin line with

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**Table 1. Summary of the purification of pancreatic kallikrein**

<table>
<thead>
<tr>
<th></th>
<th>Total activity (chromozyme units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Recovery in total activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>375*</td>
<td>740</td>
<td>0·51</td>
<td>100</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>227*</td>
<td>130</td>
<td>1·75</td>
<td>60·5</td>
</tr>
<tr>
<td>SDS/Sephadex G-200</td>
<td>177*</td>
<td>22·7</td>
<td>7·80</td>
<td>47·2</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>75·3</td>
<td>1·89</td>
<td>39·84</td>
<td>20·0</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>68·5</td>
<td>0·34</td>
<td>201·47</td>
<td>18·3</td>
</tr>
<tr>
<td>Sepharose-4B</td>
<td>56·1</td>
<td>0·26</td>
<td>215·76</td>
<td>15·0</td>
</tr>
</tbody>
</table>

* Calculated from 7-tosyl-L-arginine methyl ester hydrolytic activity in the sample.
human pancreatic or urinary kallikrein, but failed to react with human plasma kallikrein. These results indicate that the kallikrein-like enzyme is immunologically the same as pancreatic or urinary kallikrein and is distinct from plasma kallikrein.

Preparation of the trypsin-like enzyme

As described previously (Takasugi et al., 1980) some proteolytic enzymes other than pancreatic kallikrein were isolated from α₂-macroglobulin by SDS/Sephadex G-200 column chromatography. DEAE-cellulose column chromatography also showed that the kallikrein-like enzyme fractions were contaminated with a trypsin-like enzyme because they possessed caseinolytic activity. In the present study, to examine the effect of the trypsin-like enzyme on interaction between α₂-macroglobulin and pancreatic kallikrein, the trypsin-like enzyme was partially purified. As shown in Fig. 1, S-2444 amidolytic activity was eluted in adsorbed fractions (fraction nos. 35–39), which were different from the fractions with S-2266 amidolytic activity. This indicates that the trypsin-like enzyme can be isolated from pancreatic kallikrein by hydroxypapatite column chromatography. The trypsin-like enzyme fractions were collected and concentrated to 0·1 vol. by ultrafiltration. By these procedures 0·072 mg of partially purified enzyme with a specific activity of 111 S-2444 chromozyme units/mg of protein was obtained from 10 ml of the original plasma. Although its purity is not yet confirmed, its molecular weight was about 26 000 by the gel filtration method. Furthermore this enzyme was confirmed to be trypsin from the inhibitory spectra of some trypsin inhibitors for the esterolytic and amidolytic activities of this enzyme (Table 2).

Interaction between purified α₂-macroglobulin and purified pancreatic kallikrein

The interaction between α₂-macroglobulin and pancreatic kallikrein was investigated by mixing 5 ml of purified α₂-macroglobulin (containing 1·5 mg of protein, 23·3 units) with 2 ml of purified pancreatic kallikrein (0·087 mg of protein, 18·7 S-2266 units) which was incubated for 1 h at 25°C, and then chromatographed on Sephadex G-200 (2·5 cm × 50 cm). As shown in Fig. 4(a), the amidolytic activity of pancreatic kallikrein was eluted in fractions different from α₂-macroglobulin fractions.

Effect of trypsin on interaction between α₂-macroglobulin and pancreatic kallikrein

To examine the effect of trypsin on the complex-formation between α₂-macroglobulin and pancreatic kallikrein 1·5 ml of trypsin (0·043 mg of protein, 4·8 S-2444 chromozyme units) was added to test tubes containing 5 ml of purified α₂-macroglobulin and 2 ml of purified plasma kallikrein. Purified kallikrein (approx. 50 f.1g) was subjected to electrophoresis on a polyacrylamide-gel column (0·5 em × 7 em). The diluted sample consisted of urea (8 mol/l), 1% SDS and phosphate buffer (0·01 mol/l) at pH 7·2.
Purification of kallikrein

TABLE 2. Inhibitory spectrum of trypsin-like enzyme

KIU, Kallikrein inhibitory unit. Values express the amount of inhibitor that is required to completely inhibit 0·1 mg of enzyme. To measure the inhibitory effect of various inhibitors on p-tosyl-L-arginine methyl ester esterolytic or S-2444 amidolytic activity of trypsin-like enzyme, 0·5 or 0·1 ml of enzyme solution was mixed with various amounts of inhibitor respectively. After the incubation for 15 min at 37°C 0·5 or 0·1 ml of p-tosyl-L-arginine methyl ester (20 mmol/l) or 0·1 ml of S-2444 (3 mmol/l) was added and the mixture incubated for 1 h or 10 min respectively.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>Aprotinin (KIU)</th>
<th>Soya bean trypsin inhibitor (μg)</th>
<th>Ethyl-p-(6-guanidino-2-hexanoyloxy)benzenemethanesulphonate (mmol/l)</th>
<th>p-Tosyl-L-lysine chloromethyl ketone (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Tosyl-L-arginine methyl ester</td>
<td>Aprotinin</td>
<td>42·5</td>
<td>90</td>
<td>2·25</td>
<td>3·5</td>
</tr>
<tr>
<td>S-2444</td>
<td>Aprotinin</td>
<td>50·0</td>
<td>95</td>
<td>2·50</td>
<td>4·0</td>
</tr>
</tbody>
</table>

FIG. 4. Interaction between purified a2-macroglobulin and purified pancreatic kallikrein. See the text for details of column chromatography. The measurement of kallikrein and trypsin activities were carried out as described in Fig. 1. (a) Column chromatography of a2-macroglobulin/pancreatic kallikrein mixture. (b) Column chromatography of a2-macroglobulin/pancreatic kallikrein/trypsin mixture.

pancreatic kallikrein. After incubation for 1 h at 25°C, the reaction mixture was chromatographed on Sephadex G-200 (2·5 cm × 50 cm). As shown in Fig. 4(b), purified pancreatic kallikrein together with trypsin was eluted in a2-macroglobulin fractions. This result shows that 2-8 μmol of pancreatic kallikrein can be combined with 2 μmol of a2-macroglobulin in the presence of 1-6 μmol of trypsin. The stoichiometry of this interaction was investigated on addition of various concentrations of trypsin to test tubes containing 2 μmol of a2-macroglobulin and 10 μmol of pancreatic kallikrein. After incubation for 1 h at 25°C, 8 ml of the reaction mixture was chromatographed on a Sephadex G-200 column (2·5 cm × 50 cm). The amount of pancreatic kallikrein that combined with a2-macroglobulin was calculated from the S-2266 amidolytic activity in the a2-macroglobulin fractions. The amount of pancreatic kallikrein bound to 2 μmol of a2-macroglobulin was increased in proportion to the increase in the amount of trypsin and reached a plateau with a concentration of 2 μmol. The amount of pancreatic kallikrein bound to a2-macroglobulin at the plateau was 4 μmol. This result shows that the binding capacity of a2-macroglobulin for trypsin and pancreatic kallikrein approaches saturation at 1 and 2 mol/mol of a2-macroglobulin respectively.

Effect of preincubation of a2-macroglobulin/trypsin mixture on complex-formation between a2-macroglobulin and pancreatic kallikrein

To elucidate further the complexing mechanism between a2-macroglobulin and pancreatic kallikrein the effect of preincubation of a2-macroglobulin/trypsin mixture on complex-formation between a2-macroglobulin and pancreatic kallikrein was examined. Trypsin (2 μmol) was added to 2 μmol of a2-macroglobulin. After incubation of the a2-macroglobulin/trypsin mixture at 25°C for various time intervals, 4 μmol of pancreatic kallikrein was added and incubated at 25°C for 1 h. Then 8 ml of the reaction mixture was chromatographed on a Sephadex G-200 column (2·5 cm × 50 cm). As shown in Fig. 5, the binding capacity of a2-macroglobulin for pancreatic kallikrein was rapidly diminished by the preincubation.

Discussion

It has been reported that the release of a kallikrein-like enzyme into blood plays an impor-
Conversely, Vahtera & Hamberg (1976) reported that pancreatic or urinary kallikrein could not combine with \( \alpha_2 \)-macroglobulin. As confirmed in previous reports (Sumi et al., 1978; Takasugi et al., 1980) and in the present study almost all of the pancreatic kallikrein, which was released from the pancreas into blood, also combined with \( \alpha_2 \)-macroglobulin. If the findings of Vahtera & Hamberg (1976) are correct, how should we interpret the phenomenon of pancreatic kallikrein being combined with \( \alpha_2 \)-macroglobulin observed in the present study? Vahtera & Hamberg (1976) also reported that, if pancreatic or urinary kallikrein could combine with \( \alpha_2 \)-macroglobulin, the complex-formation might be due to contamination with another proteinase in the kallikrein preparation. In an effort to resolve this question and clarify further the relationship between proteinase and \( \alpha_2 \)-macroglobulin in acute pancreatitis the interaction between purified \( \alpha_2 \)-macroglobulin and purified pancreatic kallikrein from plasma of patients was investigated. As shown in Fig. 4(a), the purified pancreatic kallikrein could not combine with \( \alpha_2 \)-macroglobulin. Conversely, as suggested by Vahtera & Hamberg (1976), the addition of trypsin to the \( \alpha_2 \)-macroglobulin and pancreatic kallikrein mixture prepared from plasma of a patient with acute pancreatitis gave rise to the formation of a complex between \( \alpha_2 \)-macroglobulin and pancreatic kallikrein (Fig. 4b). These results indicate that the presence of trypsin is required for complex-formation between \( \alpha_2 \)-macroglobulin and pancreatic kallikrein. In an effort to elucidate the importance of trypsin in the complexing mechanism between \( \alpha_2 \)-macroglobulin and pancreatic kallikrein the stoichiometry of this interaction was investigated. The binding capacity of \( \alpha_2 \)-macroglobulin for trypsin approached saturation at a molar ratio of 1:1. In 1966 Ganrot found that
the maximum molar binding capacity of α₂-
macroglobulin for trypsin was 1.3–1.7 mol. The lower trypsin-binding capacity in this study may be
due to the different trypsin preparations used. There is another possibility that the complex-formation of α₂-macroglobulin and trypsin may be affected by the simultaneous complex-formation between α₂-macroglobulin and pancreatic kallikrein. The most important and interesting problem is why pancreatic kalli-
krein can combine with α₂-macroglobulin in a 2 : 1 molar ratio in the presence of trypsin, despite the failure of complex-formation in the absence of trypsin. It has been found by many workers (Barrett & Starkey, 1973; Harpel, 1973; Roberts, Riesen & Hall, 1974) that alteration in the structure of α₂-macroglobulin (that is, peptide bond cleavage of the subunit chain in α₂-
macroglobulin by the proteinase) is necessary for such complex-formation. The complexing mech-
nanism of α₂-macroglobulin for trypsin in this study is well understood from these studies. Although the number of enzyme-combining sites of α₂-macroglobulin is still debatable (Barrett & Starkey, 1973), it is difficult to confirm a molar ratio of 1 : 2 for complex-formation of α₂-
macroglobulin and pancreatic kallikrein from the stoichiometry in the present study.

Conversely, in 1975 Ohlsson proposed that the molecular size and type of the enzyme first bound may perhaps decide the conformational change induced in α₂-macroglobulin in such a way as to permit or to inhibit the binding reaction of a second enzyme molecule. We consider that the binding of pancreatic kallikrein to α₂-
macroglobulin in the presence of trypsin may also be due to a conformational change of α₂-
macroglobulin induced after alteration of the subunit chain structure in α₂-macroglobulin by trypsin, which may bring about the exposure of the two binding sites for pancreatic kallikrein on the surface of one molecule of α₂-macroglobulin. We also speculate that the binding site of pancreatic kallikrein for α₂-macroglobulin is different from its active site because α₂-
macroglobulin-bound pancreatic kallikrein itself possesses esterolytic and kinin-forming activities (Sumi et al., 1978) and that a weak bond, such as a hydrogen or hydrophobic bond, may be formed between α₂-macroglobulin and pancreatic kalli-
krein since pancreatic kallikrein can be separated from α₂-macroglobulin by SDS/ SephadeX G-200 column chromatography (Takasugi et al., 1980).

It is of particular interest that after preincubation at 25°C for more than 5 min α₂-macroglobulin/trypsin mixture does not bind pancreatic kalli-
krein (Fig. 5). This result suggests that the secondary molecular conformational change in α₂-
macroglobulin for the complex-formation between α₂-macroglobulin and pancreatic kalli-
krein may be a temporary and reversible pheno-
menon.

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