ACTA CHEMICA SCANDINAVICA 24 (1970) 569–579

Influence of an Enzyme from *Aspergillus oryzae*, Protease I, on Some Components of the Fibrinolytic System

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Purified plasminogen treated with a proteolytic enzyme preparation isolated from *Aspergillus oryzae*, protease I, is not converted to plasmin. The plasminogen was degraded. The degradation products could be activated by urokinase. No stable complex was formed between protease and plasminogen.

In human plasma treated with protease I, there was no demonstrable activation of plasminogen to plasmin. Such a plasma would, however, release more plasmin when activated with urokinase or streptokinase in comparison with an untreated plasma.

In serum or plasma concentrations below the level for the natural inhibition, protease I forms a stable complex with a protein, probably α_2-macroglobulin. This complex is partly precipitated if plasma is diluted 20 times but not if plasma is diluted 14 times. The complex shows a potent esterolytic activity and a certain proteolytic activity. The proteolytic activity of the complex is not influenced by Trasylol ® but by acidification. The complex is also formed *in vivo* after intravenous injection of protease I to dogs. The complex is not formed if euglobulin is treated with the enzyme.

Several authors have confirmed the thrombolytic efficacy of a proteolytic enzyme, protease I, from *Aspergillus oryzae*. However, opinions on the mechanism of action of the enzyme in causing thrombolysis have varied. Bergkvist and Svärd [1] found that protease I would not activate plasminogen to plasmin in cats. de Nicola *et al.*[2] found an increase in both plasminogen and plasmin after intravenous injections to humans. The present study has been performed to elucidate the behaviour of protease I and some plasma constituents on adding the enzyme to plasma in concentrations below the natural inhibitor levels for the enzyme.[3]

MATERIAL AND METHODS

Protease I ("Astra 1652"; lot 25055, 18056 and 17047) was prepared as described previously.[4] Plasminogen, Grade A Kabi (human, lyophilized). Plasmin, Grade B Kabi (human, lyophilized). Urokinase, Leo Pharmaceutical Products, 5500 Ploug units/mg.

Streptokinase, Kabikinase ®, Kabi. Trasylol ®, Bayer, solution for intravenous use, 5000 Kalikrein ® inhibiting units/ml. Blood plasma was prepared from outdated human ACD blood. Pooled human blood serum was used if not stated otherwise.

The gel filtrations were carried out on columns of Sephadex G 100 or G 200 (Pharmacia Fine Chemicals). Before each filtration, the gel was equilibrated with the same buffer as that used for elution. The buffer commonly used in the gel filtrations contained 0.05 mole TRIS, 0.02 mole lysine, and 0.10 mole NaCl per litre water, the pH being adjusted to 9.0 or 8.0 with conc. HCl. In some experiments, 1.2 % or 0.9 % NaCl solutions were used instead of buffer. The proteins were pumped into the bottom of the column and ascending elution with buffer by means of a peristaltic pump was accomplished.

Proteolytic activity was assayed as described by Bergkvist.[4] Esterase activity was determined using TAEE (*p*-Toluenesulfonyl-L-Arginine Ethyl Ester) as substrate and titrating the acid released in a pH-stat [5] (Radiometer Titrator and Titrigraph).

The activation of plasminogen to plasmin was accomplished by adding 55 Ploug units of urokinase followed by incubation for 30 min at 37°C. In some cases streptokinase was used instead of urokinase.

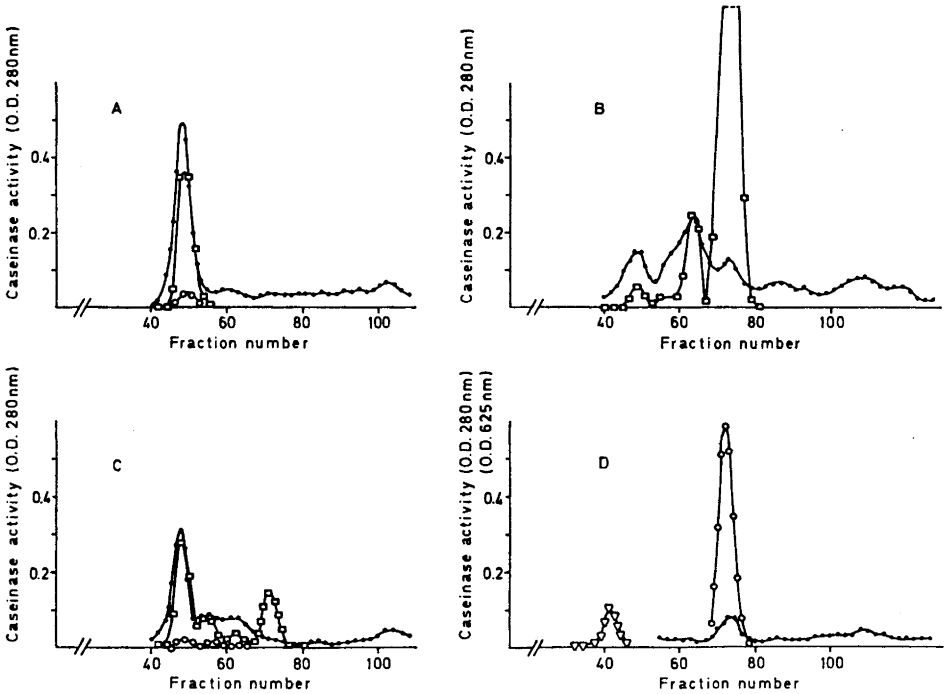

Fig. 1. Gel filtration of plasminogen with various amounts of protease I on a 1 × 116 cm column of Sephadex G 100. The proteins were eluted with Tris buffer, pH 9.0. Flow rate 1.7 ml/h, fraction volume 0.86 ml. Optical density at 280 nm ●; Optical density at 625 nm ▽; Caseinase activity ○; Caseinase activity after activation with urokinase □.

A. 30 mg plasminogen dissolved in 0.7 ml water.
B. 30 mg plasminogen dissolved in 0.7 ml water containing 0.44 mg protease I (lot 25055). In this and the following experiment, incubation was performed for 30 min in an ice bath before the filtration in order to avoid excessive degradation.
C. 30 mg plasminogen dissolved in 0.7 ml water containing 0.10 mg protease I (lot 17047).
D. 2 mg dyed dextran (M.V. 2 000 000) and 0.44 mg protease I (lot 25055) dissolved in 0.7 ml water.

In order to destroy the plasmin inhibiting substances in plasma or serum, a modification of the method described by Hedner and Nilsson [6] was used. The plasma or serum was acidified to pH 2 with 4 M HCl and neutralized with 4 M NaOH 15 min later. These high concentrations of acid and base were used in order to avoid a large increase of the sample volume. When a complete inhibition of plasmin activity was necessary, 250 Kalikrein ® inhibiting units of Trasylol ® per sample followed by incubation for 20 min at 37°C proved sufficient.

The euglobulin fractions were prepared either by diluting plasma or serum 14 times with water and adjusting the pH to 5.9 according to Bergkvist,[1] or by diluting 20 times and adjusting to pH 5.3 as described by de Nicola.[2]

The radioiodination of protease I was accomplished with ^{125}I according to Rosa *et al.*[7] After iodination, the protein solution was desalted by gel filtration. The proteolytically active material was compared with normal protease I by gel electrophoresis and immunodiffusion tests. No significant differences between normal and radioiodinated protease I were detected.

The *in vivo* experiments were carried out on Beagle dogs, weighing 10—15 kg. The enzyme was administered in a physiological saline solution by intravenous infusion. Blood samples were withdrawn at different times before, during and after the infusion.

RESULTS

Effects on purified plasminogen and plasmin. Fig. 1 gives the results of gel filtration experiments on mixtures of protease I and plasminogen. It is seen that plasminogen is degraded by protease I. The position of protease in the elution pattern can be seen in Fig. 1 B, C, and D. The degraded plasminogen is situated between unchanged plasminogen and protease. The cleaved products can still be activated to plasmin by urokinase (Fig. 1 C). A small amount of plasmin is destroyed by protease. After adjusting for the salt content of the plasminogen preparation used, the amount of plasmin activity is 0.90 casein units for untreated plasminogen (Fig. 1 A), for plasminogen treated with protease in a ratio of 17/1 plasminogen to protease 0.85 casein units (Fig. 1 C),

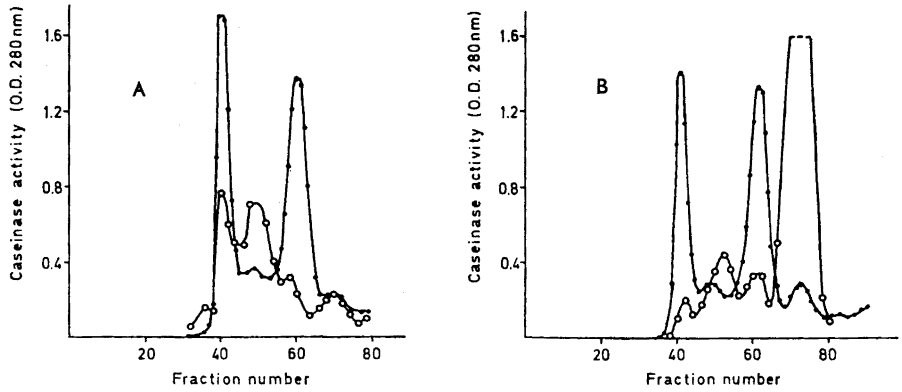

Fig. 2. Gel filtration of plasmin with protease I on a 1 × 116 cm column of Sephadex G 100. The proteins were eluted with Tris buffer, pH 9.0. Flow rate 1.7 ml/h, fraction volume 0.86 ml. Optical density at 280 nm ●; Caseinase activity ○.

A. 45 mg plasmin dissolved in 0.7 ml water.

B. 45 mg plasmin dissolved in 0.7 mg water containing 0.68 mg protease I (lot 18056). The mixture was incubated for 30 min at 0°C before gel filtration.

and for plasminogen to protease 7/1 0.74 casein units (Fig. 1 B). There does not seem to be any activation of plasminogen to plasmin by the enzyme (Fig. 1 A and 1 C).

The plasmin used contained several caseinolytically active components (Fig. 2 A). By treating plasmin with protease I, the plasmin activity was partly diminished (Fig. 2 B).

Effects on activation of plasminogen in plasma. In euglobulin fractions precipitated from plasma treated with protease I, more proteolytic activity is found after activation with urokinase than in untreated plasma according to the data in Fig. 3. The activity cannot be ascribed to protease I — which may partly be precipitated in the euglobulin fractions — as the activity is inhibited by Trasylol®. Protease I is not, however, inhibited by that agent but by acidification to pH 2 and subsequent neutralization.

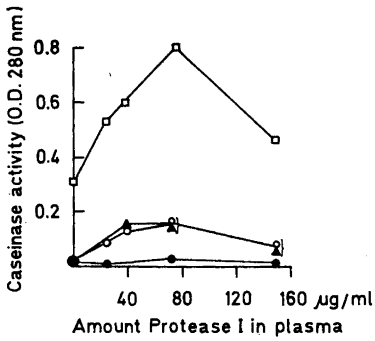

Fig. 3. Five 30 ml portions of human plasma were incubated with different amounts of protease I (lot 25055) for 30 min at 37°C. Each incubation mixture was then diluted to 600 ml (20 times) with water and acidified to pH 5.3 with acetic acid. The euglobulin precipitate formed from each portion was dissolved in 15 ml phosphate buffer, 0.2 M, pH 7.4. Caseinase activity in 3 ml samples ○; Caseinase activity in 3 ml samples after acidification to pH 2 and neutralization ●; Caseinase activity in 3 ml samples after activation with urokinase □; Caseinase activity in 3 ml samples after activation with urokinase and treatment with Trasylol® ▲.

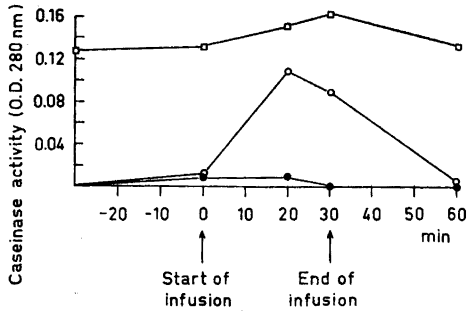

Fig. 4. Protease I (lot 17047) was administered to a dog by intravenous infusion. The enzyme preparation was dissolved in physiological saline to a concentration of 0.01 %. The infusion time was 30 min and the dose of the enzyme was 1 mg per kg body weight. Blood samples (10 ml) were withdrawn at different times before, during and after the infusion and citrate plasma was prepared. Caseinase activity in 2 ml samples ○; Caseinase activity in 1 ml samples after acidification to pH 2 and neutralization ●; Caseinase activity in 1 ml samples after acidification to pH 2, neutralization and subsequent activation with urokinase □.

A similar pattern is obtained if plasma is used instead of euglobulin, if fibrin plates are used instead of casein as substrate for determination of plasmin activity, or if streptokinase is used for the activation of plasminogen. It has also been possible to demonstrate a facilitated plasminogen activation in the dog after intravenous injection of protease I. An example is given in Fig. 4.

Effects on plasma and serum. Gel filtrations of serum, plasma, and euglobulin precipitated from serum or plasma were performed. A typical experiment with euglobulin fraction is recorded in Fig. 5 A. The position of plasminogen in the elution sequence is illustrated. Plasmin activity was inhibited by Trasylol®.

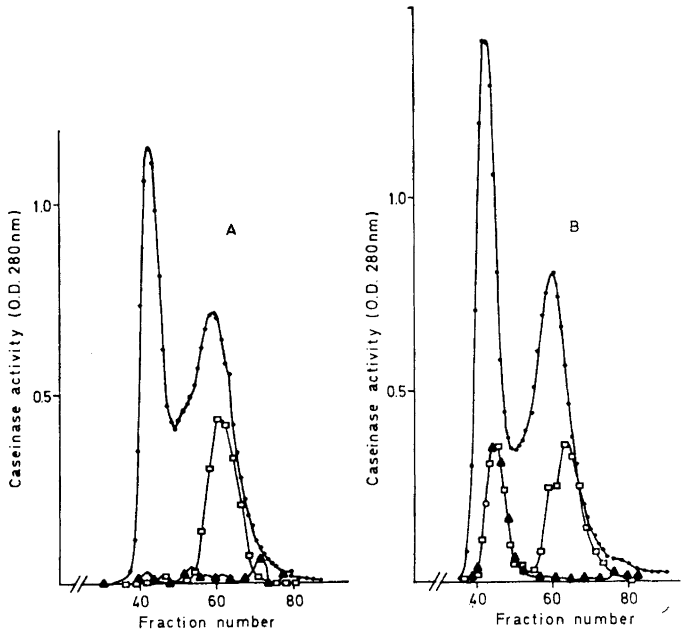

Fig. 5. Gel filtration of euglobulin on a 2 × 110 cm column of Sephadex G 200. The proteins were eluted with Tris buffer, pH 8.0. Flow rate 7.2 ml/h, fraction volume 3.6 ml. Optical density at 280 nm ●; Caseinase activity after activation with urokinase □; Caseinase activity after treatment with urokinase and Trasylol ® ▲.

A. 25 ml of human serum was diluted to 500 ml with water and acidified to pH 5.3 with acetic acid. The precipitate formed was dissolved in Tris buffer pH 8.0 and applied to the column.

B. The same amount of serum as above was incubated with 1.87 mg protease I (lot 17047) for 30 min at 37°C. The incubation mixture was then treated as described in Fig. 5 A.

In gel filtrations of serum or euglobulin from plasma or serum treated with varying amounts of protease I, plasminogen was found at the same position as in untreated materials. In addition, a new proteolytically active constituent was found which was eluted before the plasminogen (Fig. 5 B). This peak is active without any activation by streptokinase or urokinase (Fig. 6) and it is not inhibited with Trasylol ® (Fig. 5 B). The activity was destroyed by acidification to pH 2. The new component displays esterase activity (Fig. 6). The mean of the ratio of the amounts of esterolytically and caseinolytically active enzyme in the complex is from three independent gel filtrations 4.0. This suggests that the esterolytic activity is influenced to a lesser extent by the complex formation.

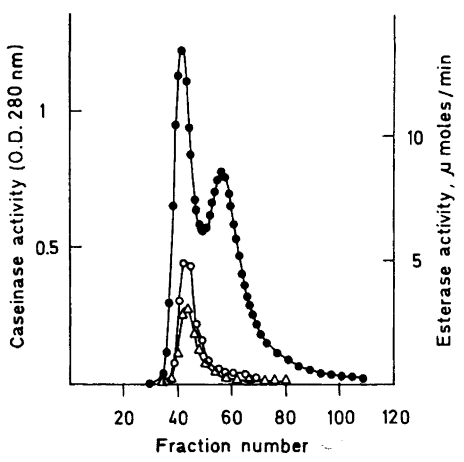

Fig. 6. Gel filtration of euglobulin on Sephadex G 200 in 1.2 % NaCl solution. Dimension of column the same as in Fig. 5. Flow rate was 7.0 ml/h, fraction volume 3.5 ml. The euglobulin was obtained from 25 ml human plasma treated in the same manner as described in Fig. 5. The precipitate was dissolved in 5 ml 1.2 % NaCl solution and applied to the column. Optical density at 280 nm ●; Caseinase activity ○; Esterase activity (TAEE as substrate) △.

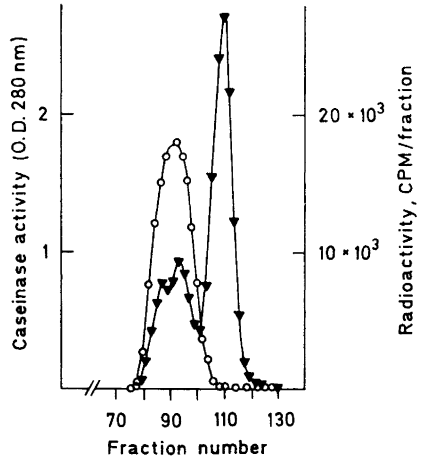

Fig. 7. 2 ml aqueous solution of radioiodinated protease I was filtered through a 2 × 115 cm column of Sephadex G 200. The proteins were eluted with Tris buffer, pH 8.0. Flow rate was 7.0 ml/h, fraction volume 3.5 ml. Caseinase activity ○; Radioactivity ▼.

The conditions for proving the presence of the new active component are to some extent critical. Such a component can be shown to be present in serum or plasma treated with protease and likewise in the euglobulin fraction if plasma or serum is diluted 20 times, but not after dilution 14 times. It is not formed when the euglobulin fraction is directly treated with protease.

In gel filtrations protease I is eluted after plasminogen (cf. Figs. 1, 5 A, and 7). In filtration experiments performed with human serum incubated with different amounts of protease it was possible to demonstrate the presence of free enzyme at a concentration of 0.18 mg/ml, but not in concentrations up to 0.14 mg/ml serum. This value is in accordance with Lindvall [3] who with a different technique found free activity to occur at an average concentration of 0.146 mg enzyme/ml serum.

Radioactive protease. The results obtained indicated that the proteolytic activity preceding plasminogen was due to complex formation between protease I and serum proteins. However, when using a rabbit anti-protease serum no immunological reaction specific for protease I could be demonstrated with rabbit serum incubated with protease in concentrations up to 0.2 mg/ml serum.[8] Experiments with ^{125}I-labeled enzyme were therefore made to elucidate the fate and localisation of protease I when added to plasma.

Table 1. Distribution of radioactive protease I in different gel filtration experiments.

Treatment of plasma	Material applied to column	Amount of protease I in fraction 40–60[a] (μg)	Amount of protease I in fraction 40–60[b] (μg)	Amount of protease I in fraction 80–100[b] (μg)
25 ml plasma treated with 75 μg protease I/ml, 30 min, 37°C	The euglobulin fraction dissolved in 5 ml buffer	246	50	
»	»	194	38	
25 ml plasma treated with 40 μg protease I/ml, 30 min, 37°C	»	118	29	
»	»	119	25	0
25 ml plasma treated with 250 μg protease I/ml, 30 min, 37°C	»	83	14	18
Plasma treated with 75 μg protease I/ml, 30 min, 37°C	5 ml incubated plasma	415	35	0
Plasma treated with 250 μg protease I/ml, 30 min, 37°C	»	326	43	828
Dog, which received 1000 μg protease I/kg body weight	5 ml dog plasma	24	4	0

[a] The amount of radioactive protease I in fractions 40 to 60 was calculated:

$$\frac{\sum(\text{cpm in fractions 40-60} - \text{cpm in blank})}{\text{cpm per } \mu g \text{ protease I} \times 0.356}$$

[b] The amount of caseinolytically active protease I was determined as the sum of caseinolytic activity in the fractions divided by the specific caseinolytic activity of protease I.

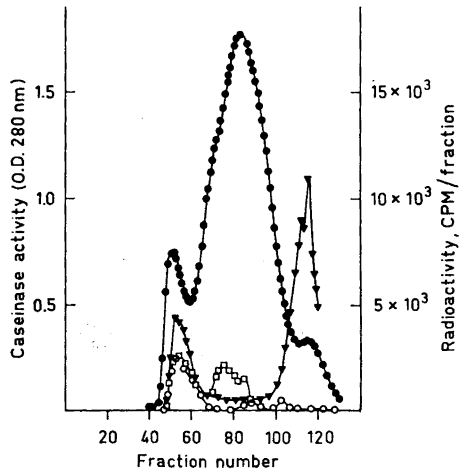

Fig. 8. 5 ml human plasma was incubated with radioiodinated protease I at 37°C for 30 min. The amount of labeled enzyme corresponds to 0.4 mg protease I. The incubation mixture was filtered through a 2×115 cm column of Sephadex G 200. The proteins were eluted with Tris buffer, pH 8.0. Flow rate 6.8 ml/h, fraction volume 3.4 ml. Optical density at 280 nm ●; Caseinase activity ○; Caseinase activity after activation with urokinase □; Radioactivity ▼.

When radioactive protease is added in low final concentrations to plasma, which is subsequently filtered through Sephadex, a peak is obtained which contains proteolytic and radioactive material (Fig. 8 and Table 1). Negligible amounts of radioactivity are accompanied by plasminogen. Radioactive material is also found in the region of low molecular weight material (Fig. 7). When higher amounts of radioactive protease are used, and the plasma filtered similarly, a second proteolytic peak also containing radioactivity appears in addition to the first one. In the elution sequence, this second peak coincides with unlabeled protease I.

The proteolytically active part of the radioactive protease I contains 35.6 % of the radioactivity (Fig. 7). In the gel filtration experiments with treated plasma it is feasible to assume that the first radioactive peak contains the proteolytically active part of protease I. Paying regard to this, one can calculate the amount of protease appearing in the first peak and also the amount of protease, which is proteolytically active. This has been done and the results are given in Table 1.

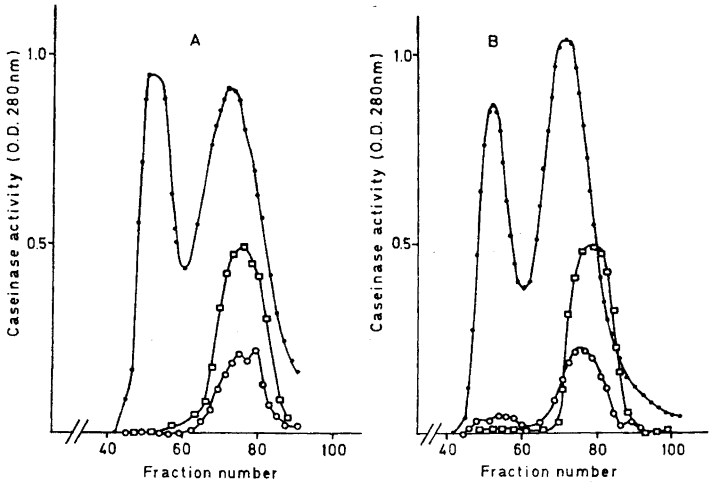

Fig. 9. Gel filtration of euglobulin dissolved in 5 ml Tris buffer, pH 8.0 on Sephadex G 200, dimension of column 2 × 115 cm. The proteins were eluted with the same buffer. Flow rate was 6.8 ml/h, fraction volume 3.4 ml.
Optical density at 280 nm ●; Caseinase activity (5 cm cell) ○; Caseinase activity after activation with urokinase (1 cm cell) □.
A. 25 ml dog plasma was diluted 20 times and acidified to pH 5.3 with acetic acid. The precipitate was dissolved and applied to the column.
B. Protease I (lot 17047) was administered to the same dog as in Fig. 9 A. The enzyme was administered by intravenous infusion during 30 min and the dose was 1 mg per kg body weight. The enzyme preparation was dissolved in physiological NaCl solution to a concentration of 0.01 %. 20 min after the end of the infusion 45 ml blood was withdrawn and citrate plasma was prepared. 25 ml of the plasma was treated exactly as described in the preceding section.

Animal experiments. In order to study the formation of proteolytically active constituents, *in vivo* experiments were also performed with a total of 13 dogs. An increase in the proteolytic activity was observed which had disappeared half an hour after the end of the infusion (Fig. 4). This activity was destroyed by acidification to pH 2 and neutralization. It is seen from Fig. 9 that infusion of protease will produce a small active peak in the beginning of the gel filtration diagram which is absent in a diagram from the same untreated animal. Infusion of radioactive protease will give a radioactive peak in that region where the proteolytic activity will appear (Fig. 10).

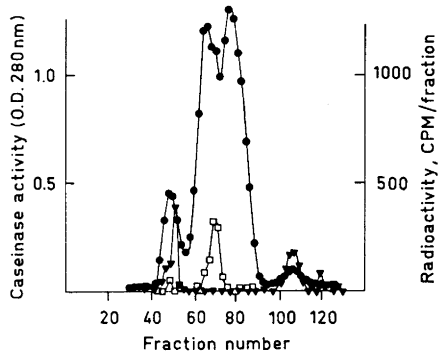

Fig. 10. Radioiodinated protease I was infused in a dog as described in Fig. 9. 20 min after the end of infusion 10 ml blood was withdrawn and citrate plasma was prepared. 5 ml of the plasma was filtered through a 2 × 115 cm column with Sephadex G 200. The proteins were eluted with Tris buffer, pH 8.0. Flow rate 6.6 ml, fraction volume 3.3 ml. Optical density at 280 nm ●; Caseinase activity after activation with urokinase □; Radioactivity ▼.

DISCUSSION

After treatment of purified plasminogen with protease I and subsequent gel filtration, different plasminogen fractions can be detected. It appears unlikely that the different plasminogen fractions are complexes of protease and intact plasminogen. Judging from their positions in the gel filtration diagrams, the products probably have lower molecular weights than unchanged plasminogen. The cleaved products of plasminogen can still be activated to plasmin. However, in the experiments performed with gel filtration of plasma treated with protease I or euglobulin from such plasma, it was not possible to show the formation of such degraded active plasminogens.

The experiments described provide no evidence for a direct protease-mediated activation of plasminogen to plasmin. An ability of protease I to increase the amount of plasmin which can be released by streptokinase or urokinase has, however, been demonstrated. This property is not unique. Thus Forbes *et al.*[9] have found that the venom from *Echis carinata* increases the yield of plasmin after urokinase activation.

The present investigation provides evidence for the formation of a complex between an unidentified plasma protein and protease I, when the latter is added to plasma or injected to dogs. This complex is at least partly precipitated in the euglobulin fraction. These observations may explain why de Nicola *et al.*[2] recorded increased fibrinolytic activity in the euglobulin fraction of plasma from patients treated with protease I. Since de Nicola *et al.* made no

attempts to characterize the increased fibrinolytic activity, this may have been due to above-mentioned complex. Bergkvist and Svärd [1] postulated such a complex and explained the thrombolytic activity observed *in vivo* by the reversibility of the complex. The complex is fairly stable as it can be separated by gel filtration. Among those proteins which are to be found in the first peak of a gel filtration diagram, the one most likely to form a complex with protease I is α_2-macroglobulin. This protein is known as "immediate plasmin inhibitor" and also inhibits trypsin activity.

The complex between protease I and protein is not formed when protease is added to euglobulins, suggesting that the protein in question is not present in the euglobulin fraction. On the other hand, when the protease has been added to plasma the complex is recovered in the euglobulin precipitate. The complex is partly precipitated when plasma is diluted 20 times and not precipitated when plasma is diluted 14 times.

By forming this complex, protease I loses about 60 to 90 % of its activity with such a high molecular weight substrate as casein, but is influenced to a lesser extent and retains its activity with a low molecular weight substrate, TAEE. This is analogous to the observations of Ganrot,[10] who found similar changes in the activity of plasmin when in complex with its inhibitor α_2-macroglobulin. The general opinion is that α_2-macroglobulin, being an antiplasmin, is not precipitated in the euglobulin fraction. The close analogy between the present findings and those reported for plasmin and α_2-macroglobulin suggests that α_2-macroglobulin is the protein which forms the complex with protease I.

Ganrot [11] gives the amount of α_2-macroglobulin in plasma to be 1.8 mg/ml. The molecular weight is 820 000. This corresponds to 2.2 nmole/ml plasma. The amount of protease I found in the complex after gel filtration of 5 ml plasma treated with 0.075 mg or 0.25 mg per ml protease was 0.415 and 0.326 mg respectively. The molecular weight of protease I is around 30 000.[12] The figures given above result in a concentration of protease I of 2.8 to 2.5 nmole/ml plasma. Therefore if α_2-macroglobulin is involved in the complex formation and if all the α_2-macroglobulin takes part in the reaction, one mole of α_2-macroglobulin will bind one mole of protease I. Ganrot [11] has found that one mole of α_2-macroglobulin will bind one mole of plasmin and two moles of trypsin.

Acknowledgement. Thanks are due to Dr. Bertil Sjögren, former director of research, and Dr. Per Olov Svärd for fruitful discussions and criticism, to Miss Majken Boström and Miss Kerstin Hallman for skillful technical assistance. Technical help is also acknowledged to colleagues at the Toxicology Laboratories in performing the animal experiments.

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Received August 8, 1969.