ACTA CHEMICA SCANDINAVICA 17 (1963) 2239–2249

The Proteolytic Enzymes of *Aspergillus oryzae*

IV. On the Inhibition of the Enzymes by Serum

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The mode of action of serum inhibitors on protease I and II is described. Evidences are presented for the existence in human serum of two inhibitors for protease I, one acting rapidly and reversibly, the other acting more slowly and at a rate that depends on the temperature. Two immediate inhibitors of protease II are also demonstrated, one acting irreversibly and the other reversibly and temperature dependently. The inhibitors were separated by continuous electrophoresis. A number of normal animal sera were assayed for their content of the different inhibitors.

It has been known for more than half a century that normal serum is able to neutralize or destroy the proteolytic activity of many enzymes. The importance of inhibitors has been realized in evaluating the function of the enzymes, but the nature of the serum inhibitors is poorly understood and the number of components present is still a matter of controversy. Hitherto, proteolytic inhibition has usually been studied with trypsin, but more recently, there has been interest in the inhibition of other proteolytic enzymes, *e.g.* plasmin and chymotrypsin.

Three different proteolytic enzymes produced by *Aspergillus oryzae* have been isolated, purified and characterized.[1,2] Whilst investigating their properties, it was observed that most animal sera contained powerful inhibitors of these enzymes. Two of the proteases, referred to as protease I and II, were highly active at physiological pH. The present study deals with the nature and properties of the factors responsible for the inhibition of these enzymes.

EXPERIMENTAL

Protease I and II. The production, isolation and purification of the enzymes, as well as their characterization, have been described in previous papers of this series.[1,2]

Caseinolytic assay. The method used for the quantitative estimation of the different products has been described earlier.[3] One caseinolytic unit (C.U.) was taken as the amount of enzyme producing an increase in optical density at 280 mμ of 1.000 in 30 min under specified conditions.

Assay of inhibition by serum. The serum inhibition was estimated by a simple fibrinolytic method. The enzyme in TRIS buffer of pH 7.4 was mixed with serum to give 0.7 ml of a mixture of known composition. After incubation for the desired time and at a defined temperature, 0.2 ml of 2.50 % of human fibrinogen in TRIS buffer and 0.1 ml of a thrombin solution (containing 100 NIH units per ml) were added. The lysis time of the clot formed was determined by incubation at 37°C. The end point of the lysis was taken when all air bubbles trapped in the meshwork of the clot rose to the surface. Controls were run with mixtures containing buffer in place of serum. The amount of active enzyme could be estimated from a dose response curve obtained by plotting enzyme concentration in C.U. against lysis time of a clot containing the same amount of fibrin as in the assay of inhibition. The difference between the proteolytic activity in the presence of buffer and the activity in the presence of serum represented the units of protease inhibited by the amount of serum used.

Continuous electrophoresis. The method used was essentially identical to that described earlier.[1] The instrument used was the Beckman/Spinco model C P. The separation of human serum was carried out in veronal buffer of pH 8.5 having an ionic strength of 0.025. Schleicher and Schüll No. 2668 filter paper was used, cut to provide 32 drip points at the bottom of the curtain. The feed rate was about 0.2 ml serum per hour.

The protein concentration in the different fractions was estimated by the modified biuret-Folin reaction described by Lowry *et al.*[4] Aliquots of 0.1 ml were used for these determinations. The estimations of the immediate and slow inhibitors were carried out with 1 ml of each fraction.

RESULTS

Most of the methods which have been used for measuring the proteolytic activity can be used for determining the antiproteolytic activity of serum. Casein has, however, been used most frequently as the substrate for the primary assay and the quantitative estimation of the different products. Protease I,

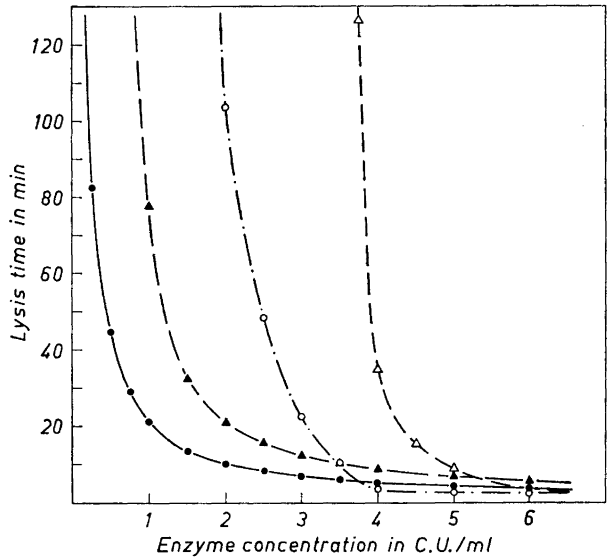

Fig. 1. Clot lysis in the presence of human serum by protease I (●); protease II (▲); chymotrypsin (○); and trypsin (△). The lysis time for clots made from 0.50 % human fibrinogen and 0.2 ml of human serum per ml was estimated at pH 7.4 and 37°C.

protease II, trypsin * and chymotrypsin ** have approximately equal activity against casein at pH 7.4, namely, 15 C.U. per mg.

The antiproteolytic activity of serum is of primary importance in using proteolytic enzymes for thrombolytic therapy. Therefore the principal method used in the present investigation was based on the lysis of a standard clot with and without the addition of serum. The inhibitory effect of normal human serum on the fibrinolytic activity of protease I, protease II, trypsin and chymotrypsin was demonstrated. Varying amounts of the proteases were added to a constant amount of serum and the decay of proteolytic activity was estimated. Examination of the curves obtained by plotting enzyme concentration in C.U. against lysis times reveals remarkable differences in activity of the different proteolytic enzymes in the presence of serum (Fig. 1). It can be seen that the inhibitory effect on trypsin and chymotrypsin is far greater than that on protease I and II. The quantities of the different enzymes which lysed the clot in 20 min under the experimental conditions, were 1.1 units of protease I, 2.1 units of protease II, 3.2 units of chymotrypsin, and 4.3 units of trypsin. In a previous paper [3] it has been reported that protease I shows a more pronounced fibrinolytic activity than a caseinolytically equivalent amount of protease II and trypsin, hence the fact that it is fibrinolytically most active in the presence of serum does not necessarily mean that this enzyme is least inhibited by serum. The digestion of casein by the proteases

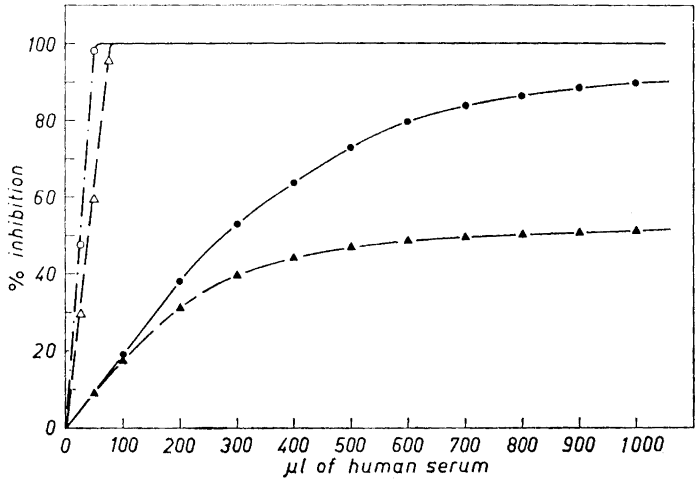

Fig. 2. Inhibition of casein digestion by varying amounts of human serum. Caseinase activity was determined by the rate of hydrolysis of 1.5 % casein at pH 7.4 after incubation for 30 min at 37°C. The final volume of the reaction mixture was 6 ml and 1.0 C.U. of each protease was used in all estimations. Increase in optical density of the trichloroacetic acid filtrate was used as a measure of protease activity. Per cent inhibition was calculated from the difference in activity between the partially inhibited enzyme and the same amount of enzyme incubated with buffer alone. The enzymes are represented by the same symbols as in Fig. 1.

* Trypure Novo.
** Chymar.

has also been investigated, as can be seen from Fig. 2. It is obvious that also by this method trypsin and chymotrypsin are completely inhibited by a much smaller amount of serum than are protease I and II. Protease I is inhibited more than protease II under these conditions, but this is compensated for by its higher fibrinolytic activity, as can be seen from Fig. 1.

In preliminary experiments it was observed that the inhibition by serum varied with temperature. This temperature dependence was further investigated by the incubation of serum and protease at different temperatures and the estimation of the residual activity. Fig. 3 illustrates the inhibition after

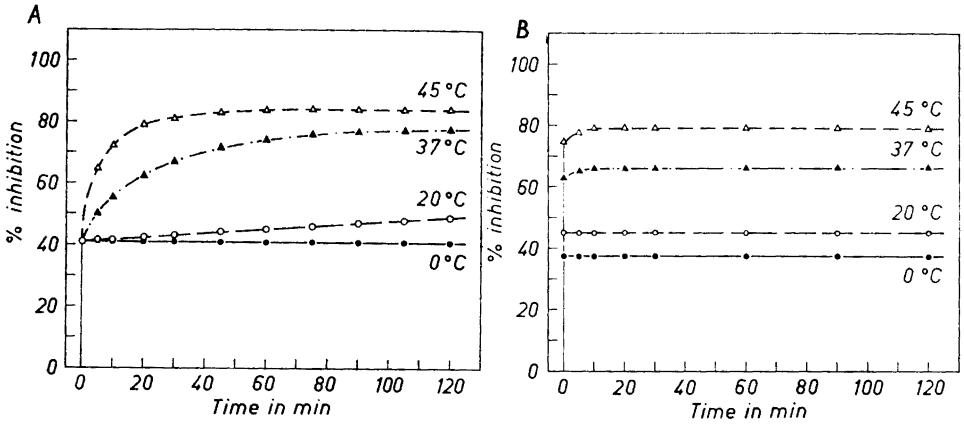

Fig. 3. Per cent inhibition by serum of A) protease I and B) protease II at different temperatures. The inhibition of the fibrinolytic activity of 2.0 C.U. of each enzyme was estimated. At zero time, 0.2 ml of human serum was added to each sample, and the residual activity was estimated after different times of incubation.

incubation for different times and at different temperatures, expressed in per cent of the activity estimated in the absence of serum. The rate of inhibition of protease I at various temperatures upon addition of human serum indicates a two-stage reaction. There is an immediate inhibition independent of the temperature, followed by a slower inhibition which proceeds over the entire period measured. The second reaction is highly temperature dependent, being negligible at 0°C, and increasing rapidly with rise in temperature. On the basis of these experiments it seems that there are two substances in human serum which have different modes of inhibitory action on protease I. These substances are referred to henceforth as the "rapid" inhibitor and the "slow" inhibitor. The results in Fig. 3 indicate furthermore that the inhibition of protease II is practically immediate and temperature dependent, but it is not possible to establish if one or several inhibitors are responsible for this effect.

Since the action of the "slow" inhibitor of protease I was suspended by the addition of substrate, the inhibition of protease I shown in Fig. 1 must be due to the action of the "rapid" inhibitor only, while the same figure shows the total inhibition of protease II. A linear relationship exists between the reciprocal of the lysis time and the concentration of protease I (Fig. 4 A). Though

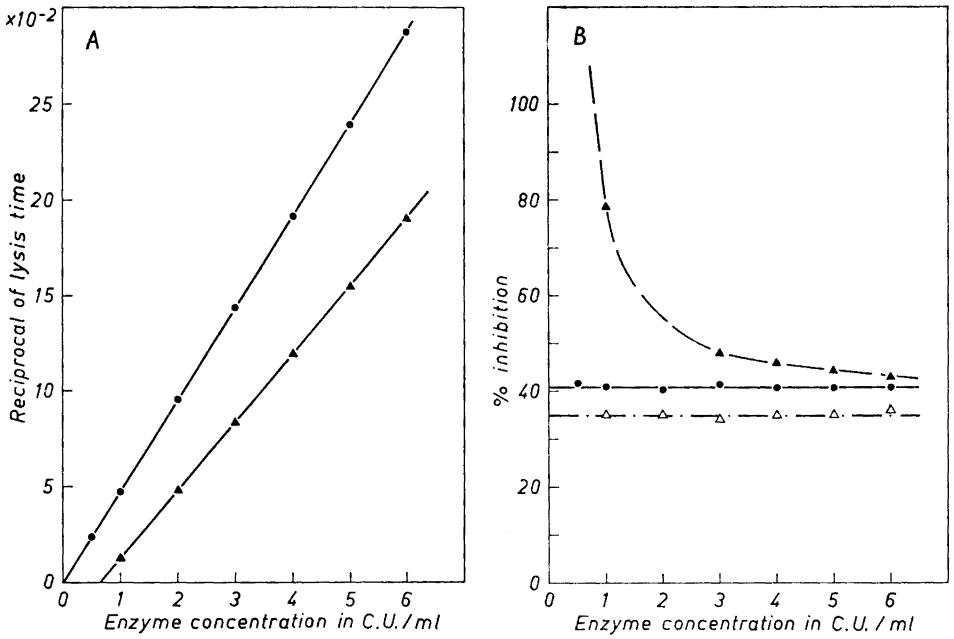

Fig. 4. A) Reciprocal of lysis time as a function of enzyme concentration, protease I (●); protease II (▲).
B) Per cent inhibition as a function of enzyme concentration, protease I (●); protease II (▲); inhibition of protease II by the reversible inhibitor (△), obtained by correcting for the irreversible inhibition. The experimental conditions were the same as in Fig. 1.

the same relationship was also obtained with protease II, this line was displaced from the origin, indicating the presence of an irreversible inhibitor of protease II in the serum. The enzyme added initially is inhibited, and it is only after enough has been added to combine with the whole of this inhibitor that further amounts of enzyme will remain active. The relationships obtained indicate further the presence of a second inhibitor of protease II, which combines reversibly with the enzyme, exactly as the "rapid" inhibitor of protease I. The two inhibitors of protease II will be referred to henceforth as the "first" inhibitor and the "second" inhibitor. The inhibition of the enzymes could be calculated from the dose response curves estimated without serum. It was found that a constant percentage of protease I was inhibited by fixed amount of serum, independent of the concentration of enzyme used (Fig. 4 B). This fact suggests also a reversible reaction between protease I and the "rapid" inhibitor. In the same way it could be demonstrated that the "second" inhibitor of protease II combines reversibly with the enzyme. In this case, however, correction must be made for the irreversible inhibition.

The reversible inhibition by some of the serum components was also tested in another way. It was shown that the actual inhibition became less with higher concentrations of substrate, indicating that the substrate competes

with the inhibitor for the enzyme. The lysis times for clots containing varying amounts of fibrin and a constant amount of serum and protease were determined. The inhibition of protease I by the "rapid" inhibitor could be estimated directly, and the inhibition by the immediate inhibitors of protease II could be estimated as described above. The curves in Fig. 5 show the inhibition

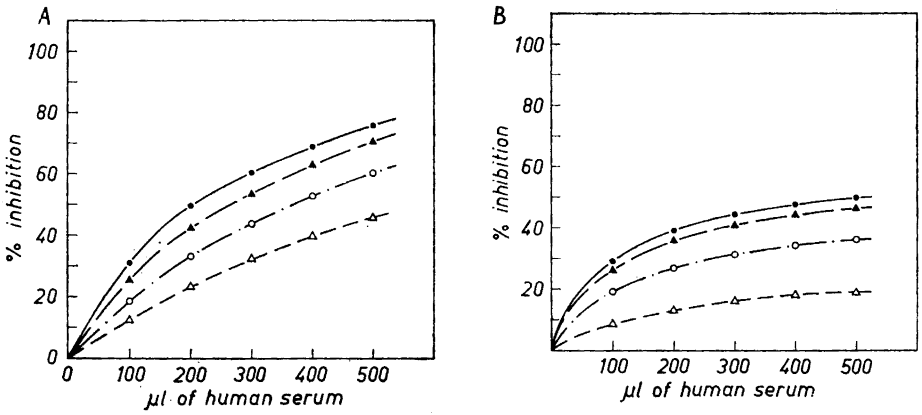

Fig. 5. The effect of different concentrations of fibrin on the inhibition A) of protease I by the "rapid" inhibitor and B) of protease II by the "second" inhibitor. The clots were made from 2 % human fibrinogen (△); 1 % (○); 0.5 % (▲); and 0.25 % (●). The inhibition of 2.0 C.U. of each enzyme by human serum was estimated at pH 7.4 and 37°C. Controls were run with the serum replaced by buffer.

of protease I by the "rapid" inhibitor and protease II by the "second" inhibitor in the presence of varying amounts of fibrin. They suggest clearly competition between the serum components and the fibrin substrate for the active site of the enzymes, and support the conclusion that the reactions are reversible. The fact that the inhibition of the enzymes by a fixed amount of serum was less when more substrate was present, was verified in experiments using casein as substrate.

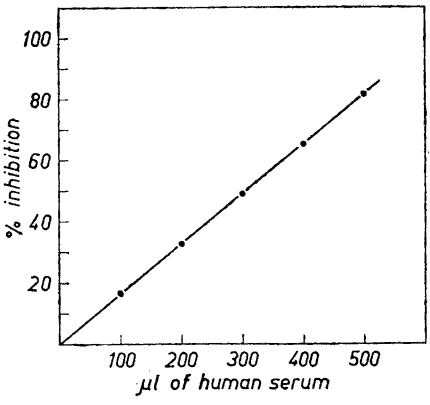

Fig. 6. Inhibition of protease II by the "first" inhibitor. The estimations were carried out under the same conditions as in Fig. 5. The amount of enzyme inhibited by a fixed amount of serum showed no variation with the different concentrations of fibrin used.

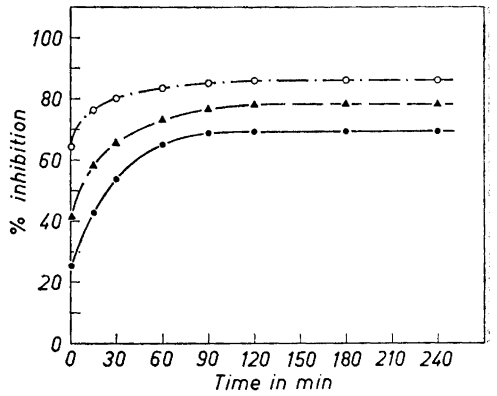

Fig. 7. Inhibition at 37°C of 2.0 C.U. of protease I by varying amounts of human serum. The residual activity was estimated after incubation for different times with 100 μl of serum (●); 200 μl (▲); and 400 μl (○).

The experimental results of the inhibition of protease II by the "first" inhibitor are presented in Fig. 6, which shows that the amount of enzyme inhibited by the "first" inhibitor was directly proportional to the amount of serum present, throughout the entire range of inhibition. The amount of enzyme inhibited by a fixed quantity of serum showed no variation, although the amounts of fibrin used for the estimations varied considerably. The inhibition of protease II by the "first" inhibitor, therefore, appears to be an irreversible reaction.

The rate of inactivation of protease I by the "slow" inhibitor was followed at 37°C using varying amounts of serum, and the results are shown in Fig. 7. The initial inhibition represents the effect of the "rapid" inhibitor and the remainder curve represents the progressive action of the "slow" inhibitor. It is apparent that the rate of inactivation by the "slow" inhibitor depends on the concentration of serum. When this is increased, the rate of inactivation as indicated by the initial slopes of the curves is increased. Furthermore the reaction seems to go almost to completion, that is, until one component is used up. The possibility still exists, however, that the "slow" inhibition of protease I is enzyme catalyzed.

The evidence presented above for the existence of two types of inhibitors each for protease I and II was further confirmed by the separation and estimation of two inhibitors for each enzyme. Human serum was fractionated by continuous flow paper electrophoresis, and each fraction separated was examined with respect to its level of antiproteolytic activity. Both the "rapid" and the "slow" inhibitor of protease I as well as the "first" and the "second" inhibitor of protease II were identified. The inhibitors of each enzyme were clearly separated and they were found in different fractions. Typical curves of the separation of proteins and of the inhibitors of the enzymes protease I, protease II, trypsin and chymotrypsin are shown in Fig. 8. In these experiments the α_1-globulins were not completely separated from the albumin but appeared as a shoulder on the anodal side of the albumin peak. The two types of inhibitors of protease I, however, were unequivocally separated; some fractions contained only "rapid" inhibitor and others only "slow" inhibitor. It was found that the "rapid" inhibitor of protease I migrates with the α_2-globulins

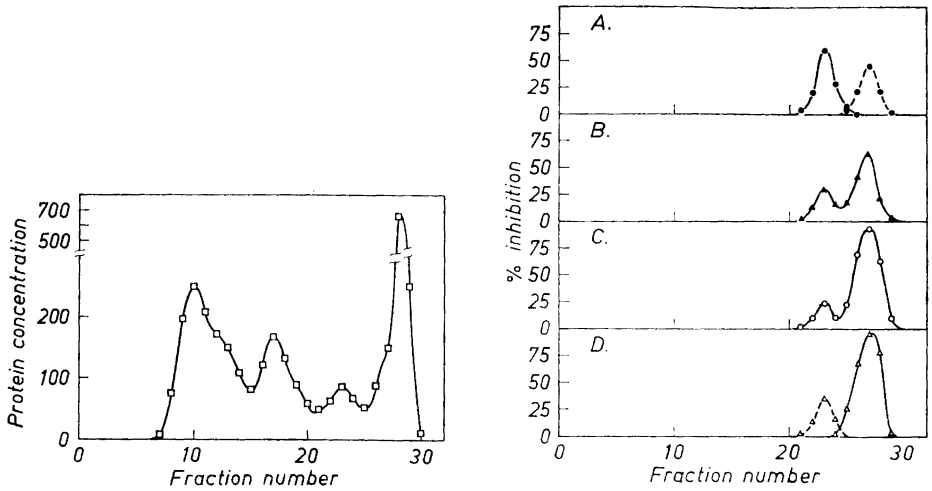

Fig. 8. Separation of antiproteolytic substances in human serum by continuous electrophoresis.

The left part of the figure shows the distribution of the proteins after separation in veronal buffer of pH 8.5 and ionic strength of 0.025. The current was 70 mA and the temperature 20°C. The right part shows the separation of the inhibitors of A) protease I; B) protease II; C) trypsin; and D) chymotrypsin. Though the inhibition could be estimated with either casein or fibrin as substrate the latter gave the best results. The inhibition of 0.5 C.U. of the enzymes with 0.5 ml of the different fractions was estimated directly (solid line) and after preincubation for 60 min (broken line).

and the "slow" inhibitor with the α_1-globulins. The inhibitors of protease II were also clearly separated, and the "first" inhibitor was found among the α_1-globulins and the "second" inhibitor among the α_2-globulins. The inhibitors of trypsin and chymotrypsin were also studied and two inhibitors of each enzyme could be shown to be present in serum. The major portion of the serum trypsin inhibitor migrated with the α_1-globulins, while a small amount migrated with the α_2-globulins. Both inhibitors combined rapidly with trypsin. Two inhibitors of chymotrypsin were also separated, one rapid inhibitor migrating with the α_1-globulin fraction and a smaller amount of a slow inhibitor migrating with the α_2-globulin fraction.

The quantities of inhibitors in the serum of some normal animals have been estimated. It was found that the levels of inhibitors in the serum from the same animal species varied within rather narrow limits. There were, however, great differences between the groups of animals, as can be seen from Table 1. Possibly there are differences not only in the amount of inhibitor in the different sera but also in the number of components. The increase in inhibition by serum during various states of disease has been extensively studied during recent years with different proteolytic enzymes [5,6]; similar variations can be expected also in the amounts of inhibitors of protease I and II.

Table 1. Inhibition of protease I and II by serum from different animal species. The inhibition of 2.0 C.U. of the enzymes by 0.2 ml of serum was estimated directly, and in the case of protease I also after preincubation for 2 h at 37°C. The inhibition was calculated from determinations of the lysis times for clots made from 0.5 % human fibrinogen in a total volume of 1 ml.

Animal species	Per cent inhibition		
	Protease I		Protease II
	Direct	After preincub.	Direct
Cat	46	67	48
Dog	35	0	36
Rabbit	64	74	15
Guinea-pig	34	38	34
Rat	73	78	72
Sheep	51	70	82
Pig	66	78	66
Horse	76	80	62
Ox	50	79	66

DISCUSSION

The antiproteolytic activity of serum is still little understood in spite of the extensive work done in this field with different proteolytic enzymes. The results of the work reported here indicate the existence of more than one inhibitor in human serum against protease I and II produced by *Aspergillus oryzae*.

In 1948 Ratnoff [7] postulated that there may be several plasma inhibitors and in 1954 [8] believed that he had demonstrated three such substances. By studies using ^{131}I-fibrinogen as substrate, Shulman [9] concluded that plasmin inhibitors were different entities from trypsin inhibitors. In 1953 Jacobsson [10] separated by zonal electrophoresis human serum inhibitors into two fractions, one migrating with the α_1-globulins and one with the α_2-globulins. α_1-Inhibitor was shown to be strongly antitryptic, whereas the α_2-inhibitor was mainly antifibrinolytic. Other authors have also reported that plasmin and trypsin inhibitors of serum are separate substances. Norman *et al.*[11,12] have reported the presence of one antiplasmin migrating with the α_1-globulins by electrophoresis and another migrating with the α_2-globulins.

It is still impossible to decide whether or not any of these serum factors act specifically on one single enzyme. The possibility that the inhibition of several proteases might be performed by the same substance is supported by the similarity in electrophoretic mobility and other properties of some inhibitors. Some purified inhibitors, *e.g.* soybean inhibitor, have been found to inhibit a number of proteolytic enzymes to different degrees, hence it is possible that some of the inhibitors of protease I and II are identical with inhibitors studied earlier.

A comparison of the inhibition of protease I and plasmin by serum shows the most striking resemblance. One rapid inhibitor migrating with the α_2-globulins and one slow inhibitor migrating with the α_1-globulins by electro-

phoresis have been found for both enzymes. The reactions of serum inhibitors with both protease I and plasmin are profoundly affected by the presence of a protein substrate. The inhibition by the α_1-inhibitor, although not readily reversible, does not progress further in the presence of a substrate, whereas the inhibition by the α_2-inhibitor is readily reversed by an increase in the concentration of a protein substrate. This similarity between plasmin and protease I is of the greatest importance in the evaluation of the usefulness of the latter enzyme as a thrombolytic agent.

The clinical use of plasmin has for a long time been considered objectionable due to the high level of circulating antiplasmins in blood. Nevertheless, plasmin has in several clinical studies proved to be an effective thrombolytic agent.[13,14] The dose of plasmin used in these experiments was insufficient to neutralize all the circulating inhibitors, and its mode of action remained an intriguing question.

It has been shown *in vitro* that a plasmin-antiplasmin complex with no caseinolytic and no plasminogen activator activity may still be fibrinolytic, due to the effective competition of fibrin with the antiplasmin for plasmin. Quite recently it has been shown by Ambrus *et al.*[15] that plasmin-antiplasmin complexes formed *in vitro* which displayed no obvious caseinolytic activity were as effective in dissolving fibrin clots *in vivo* as an equal quantity of plasmin alone. Streptokinase was significantly less effective than streptokinase activated plasmin, alone or complexed with inhibitor. It has also been reported that after an infusion of labelled plasmin into dogs, the plasmin concentration of the blood rapidly decreased.[16] A small amount of enzyme remained circulating for many hours, however, presumably in a reversible complex with an inhibitor. Dissolution of experimental clots occurred long after completion of the plasmin infusion, due to dissociation of this plasmin-inhibitor complex in the presence of a fibrin substrate.

In accordance with these observations, it may be possible that a complex between protease I and the "rapid" inhibitor may serve as a transport from or reservoir of the enzyme in blood, free enzyme being released when fibrin clots are available. The inhibitor complex would thus act both as a transport system, and as a physiological protector of the enzyme, preventing its immediate degradation. Such a mechanism would also protect other plasma proteins from the proteolytic activity of protease I.

It is likewise possible that the complex between the reversible inhibitor of protease II and the enzyme may act in the same way. The first and irreversible inhibition of this enzyme, however, together with its lower fibrinolytic activity render it less interesting for *in vivo* trials.

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Received December 21, 1962.