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On the Inhibition of a Fibrinolytic Enzyme from *Aspergillus oryzae* by Serum

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By using a viscosimetric method with gelatin as substrate, inhibitors in serum active against a fibrinolytic enzyme from *Aspergillus oryzae* — protease I — have been studied. After electrophoretic separation of serum, it has been established that the inhibiting effect is exercised by two main fractions. Of these, one also has an inhibiting effect on trypsin and can be regarded as being identical with α_1-antitrypsin on the grounds of electrophoresis experiments. The other component of serum also has an inhibiting effect on plasmin. Complex formation between protease I and the inhibitors takes place immediately. The two inhibitors have been determined separately by heating serum to 60°C whereupon the inhibitor, which is also effective against trypsin, is inactivated. Serums from 17 healthy subjects have been investigated with regard to total, heat labile, and heat stable inhibitor content. A hypothesis for the thrombolytic effect of protease I *in vivo* is discussed.

During work on a factor from *Aspergillus oryzae* having fibrinolytic activity, Stefanini *et al.*[1] established that serum and plasma exercised an inhibiting effect on this activity. After a more intensive study, Bergkvist [2–4] was able to show that the fibrinolytic activity was due to proteolytic enzymes, the most interesting of which — protease I — becoming the object of continued study. It was also shown by Bergkvist that this enzyme was inhibited by serum and that serum contains two inhibitors of protease I. One has an effect which sets in rapidly and is an α_2-globulin while the other reacts slowly with the enzyme and is comprised of an α_1-globulin. The latter inhibitor also has an inhibiting effect on trypsin.

Bergkvist [5] also devised a method for the quantitative determination of the amount of inhibitor in serum using the lysis time of a standardized clot as an expression of the activity. The difference between the activity in the presence of serum and the activity in the presence of buffer is a measure of the inhibiting effect of the serum. Another method for the determination of the amount of inhibitor of this enzyme in whole blood has been proposed by Roschlau.[6] According to this, a blood sample from the test subject is applied

to a series of tubes containing thrombin and increasing amounts of the enzyme. The smallest amount of enzyme which is required for lysis of the clot formed constitutes a measure of the inhibiting effect of the blood.

On studying the inhibitors in serum of another proteolytic enzyme — plasmin — Shulman [7] used ^{131}I-fibrin as substrate and Norman [8] used casein. The former author also studied the effect of serum on trypsin and chymotrypsin. Trypsin inhibitors have been studied previously by Hussey and Northrop [9] with gelatin as substrate, the trypsin activity being measured viscosimetrically. The viscosimetric method for determination of enzyme activity has been further developed by Hultin.[10-12]

In the present study, the method described by Hultin has been of use. The investigation comprises partly a determination of the total amount of inhibitor of protease I in human serum and partly identification and characterization of the inhibitors of protease I together with a quantitative determination of each of the main components of human serum having activity against protease I.

MATERIALS AND METHODS

Enzymes: Protease I. The enzyme (Astra) was prepared according to Bergkvist.[1,2] The activity, measured by means of a caseinolytic method according to Bergkvist, was 17.0±1.5 CU per mg. *Trypsin.* The enzyme (Koch-Light) was twice crystallised, salt free and lyophilized and had a caseinolytic activity of 11.1 CU per mg. *Plasmin.* Fibrinolysin (Koch-Light) from bovine blood having a caseinolytic activity of 1.6 CU per mg. *Streptokinase.* Varidase® (Lederle) containing 100 000 units streptokinase and 25 000 units streptodornase per 25.5 mg.

Viscosimetric method. Ostwald viscosimeters having an outflow time for distilled water of 21—23 sec have been used.

Substrate. 35 g gelatin U.S.P. granular (Fisher Scientific Comp.) and 0.05 g of the Na salt of ethylmercurythiosalicylic acid (Merthiolate, Lilly) were dissolved in 400 ml TRIS buffer, 0.05 M and pH 7.4, at 35.5°C. After storing this solution for *ca.* 24 h, 0.67 g sodium oxalate (according to Sörensen, *p.a.*, Kebo) was added followed by TRIS buffer to a volume of 500 ml. After storing for *ca.* 15 h, the precipitate of calcium oxalate formed was removed by centrifuging. The pH was adjusted to 7.4 by addition of dilute alkali. The clear gelatin solution was dispensed in 50 ml flasks which were stored in a refrigerator at +4°C. After dilution of the gelatin solution (3 ml solution +1 ml TRIS buffer), the outflow time in the viscosimeters described above was 110—130 sec.

Procedure. 1 ml samples of 0.05 M TRIS buffer containing increasing amounts of proteolytic enzyme were treated with 3 ml of substrate. All solutions were thermostated at 35.5°C. After careful mixing, 3 ml of the reaction mixture was transferred to a viscosimeter at the same temperature. The outflow time was recorded at different times after commencement of the reaction.

Calculation of enzyme activity. In principle, the method of calculation described by Hultin has been used in determining the proteolytic activity. The activity has been determined according to the formula:

$$A = \frac{(1/\eta_{sp})\tau_t - (1/\eta_{sp})\tau_0}{\tau_t - \tau_0} \times 10^3$$

A=enzymatic activity; $(1/\eta_{sp})\tau_t$=reciprocal of the specific viscosity at the time of measurement in sec; τ_t=time of reaction at the start of measurement plus half the outflow time; $(1/\eta_{sp})\tau_0$=reciprocal of the specific viscosity of the substrate and TRIS buffer. On determining inhibitor, serum or plasma is substituted for 0.1 ml buffer.

The inhibiting effect of serum. 0.1 ml human serum was incubated at 35.5°C for 5 min with 0.9 ml TRIS buffer containing 5—10 μg protease I more than the assumed amount of inhibitor in the sample. The proteolytic activity remaining was determined and the

inhibitor level in the sample was worked out by subtracting the proteolytic activity remaining from the amount added.

Electrophoresis investigations. Spinco continuous flow paper electrophoresis instruments, model CP, were used. A solution of 20.2 g TRIS and 1.53 g boric acid per litre having pH 9.3 was used as buffer. Serum was applied at a rate of 0.3 ml per h, the duration being 4 h. The current strength was 45 mA.

Before determination of the amount of inhibitor in the various fractions, the pH was adjusted to 7.4 with 1 N HCl.

RESULTS

Viscosimetric investigations. The changes in viscosity which take place on treating the given substrate with protease I in amounts of 2.5—20.0 μg during the first 700 sec after incubation are illustrated in Fig. 1. The viscosity was expressed as $1/\eta_{sp}$ on every occasion of measurement. As seen, the relation between viscosity and time (τ) is linear for amounts of enzyme up to 10 μg and for reaction times up to 600 sec. Calculation of the enzymatic activity according to the given formula shows that a linear relation exists between the activity and the amount of enzyme, provided that the amount of enzyme does not exceed 10 μg and that the reaction time is less than 600 sec.

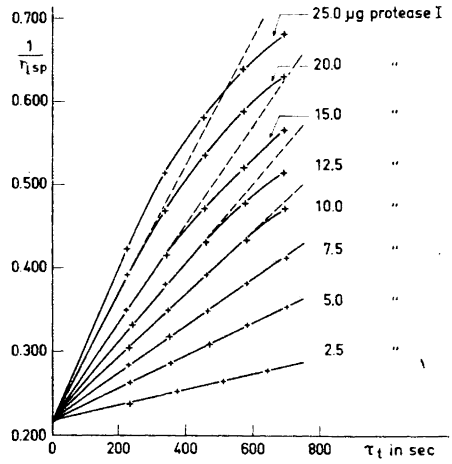

Fig. 1. Change in the reciprocal of the specific viscosity ($1/\eta_{sp}$) with time (τ_t) on treating the substrate with different amounts of protease I.

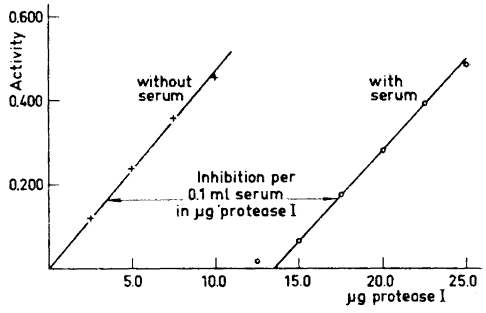

Fig. 2. Inhibiting effect of protease I by human serum.

Determination of the total amount of inhibitor in human serum. Increasing amounts of protease I in 0.9 ml TRIS buffer and 0.1 ml serum were incubated at 35.5°C for 30 min. Afterwards, the activity remaining was determined. It is apparent from Fig. 2 that the relation between the activity remaining and the amount of enzyme is linear. On comparing with those values obtained

with enzyme only, it is seen that a parallel displacement of the reaction path has occurred in the presence of serum. The difference reflects the inhibition exercised by serum on protease I, expressed in μg protease per 0.1 ml serum.

In order to study the rate of formation of enzyme-inhibitor complex, serum was incubated with protease I for various periods of time as described above. It is apparent from Fig. 3 that complex formation took place very rapidly under the experimental conditions used. The enzyme inhibition already reached its maximum after 60 sec.

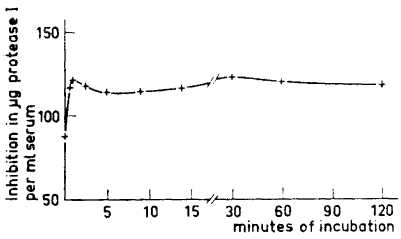

Fig. 3. The influence of the incubation time on the inhibiting effect of protease I by human serum.

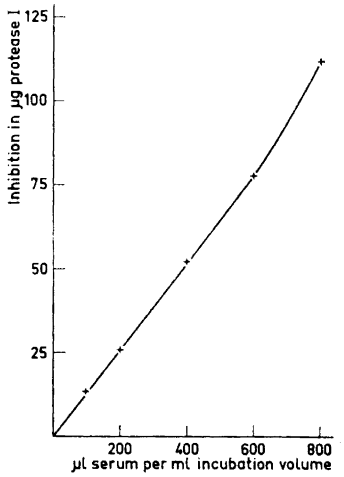

Fig. 4. Inhibition of protease I by increasing amounts of human serum per ml incubation volume after heating at 35.5°C for 5 min.

In order to study whether the extensive dilution (0.1 ml → 1 ml) of the serum used affects the formation of the enzyme-inhibitor complex, the concentration of serum in the incubation mixture was increased. Consequently, it could be established that after incubation of protease I and increasing amounts of serum for 5 min, the inhibition of the enzyme was proportional to the amount of serum up to 0.6 ml serum per 1.0 ml incubation volume (Fig. 4).

Electrophoretic investigations. According to Bergkvist,[5] there are two different inhibitors present in serum having an effect directed against protease I. In order to investigate whether the value for the amount of inhibitor determined by means of the viscosimetric method represents a total value for these two inhibitors, the serum was fractionated by means of electrophoresis. It is apparent from Fig. 5, where the separation of the serum portions into different fractions is illustrated by the extinction at 280 mμ, that two main inhibitors of protease I can be detected. These have already formed a complex with the enzyme after an incubation time of 5 min. It is seen that the rate of migration at the pH used was such that one of the components migrated

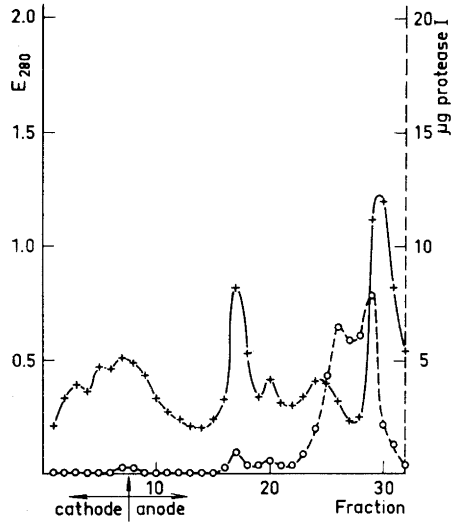

Fig. 5. Electrophoretic separation of human serum with regard to inhibitors of protease I.
+ The extinction at 280 mμ after dilution of fractions 1 → 3 with TRIS-boric acid buffer. ○ Inhibiting effect on protease I per 0.9 ml fraction.

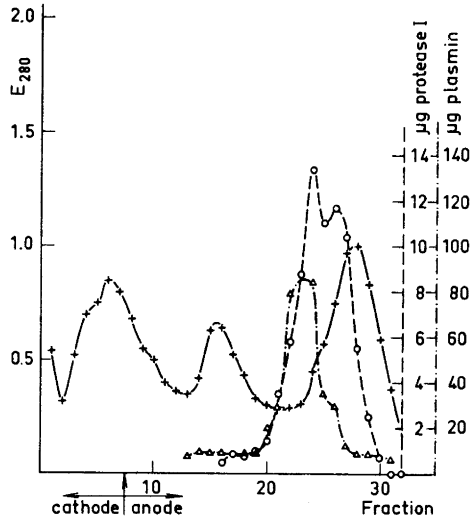

Fig. 6. Electrophoretic separation of human serum with regard to inhibitors of protease I and plasmin.
+ Extinction at 280 mμ after dilution of fractions 1 → 3 with TRIS-boric acid buffer. ○ Inhibiting effect on protease I per 0.9 ml fraction. △ Inhibiting effect on plasmin per 0.9 ml fraction.

immediately after the albumin while the other migrated somewhat more slowly.

To elucidate the properties of the inhibitors separated by means of electrophoresis, the serum was investigated with regard to plasmin inhibitors according to the same method and with the same incubation time as in the experiments with protease I. It is evident from Fig. 6 that a main component is present which has an effect directed against plasmin. This has the same rate of migration as one of the inhibitors directed against protease I. It could also be established that preincubation of plasmin with each of the fractions at 35.5°C for 30 min gave the same pattern of inhibitors in the different fractions as an incubation of 5 min.

The change in inhibitors of protease I was also investigated in test serum, which was activated with streptokinase, by adding 3.15 mg Varidase® dissolved in 0.75 ml 0.05 M TRIS buffer at pH 7.4 to 15 ml serum and incubating this mixture at 35.5°C for $2\frac{1}{2}$ h before the electrophoresis experiment. On analysing the inhibitors of protease I, it could be established, as is apparent from Fig. 7, that the rapidly migrating inhibitor still remained, but not the inhibitor directed against plasmin.

The fractions obtained by means of electrophoresis were also investigated with regard to their inhibiting effect on trypsin by means of the same viscosimetric method as that used in the experiments with protease I. As is apparent

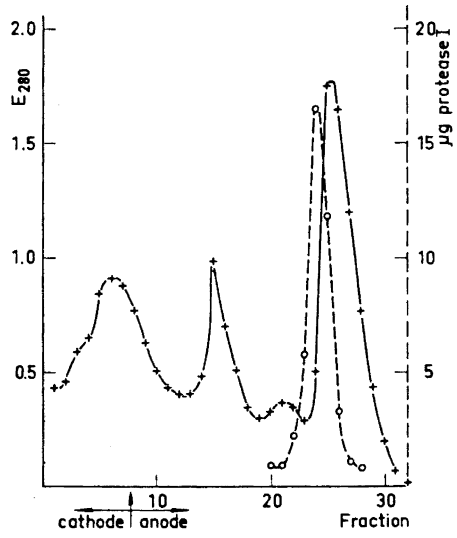

Fig. 7. Electrophoretic separation of human serum treated with streptokinase. + Extinction at 280 mμ after dilution of fractions 1 → 3 with TRIS-boric acid buffer. O Inhibiting effect on protease I per 0.9 ml fraction.

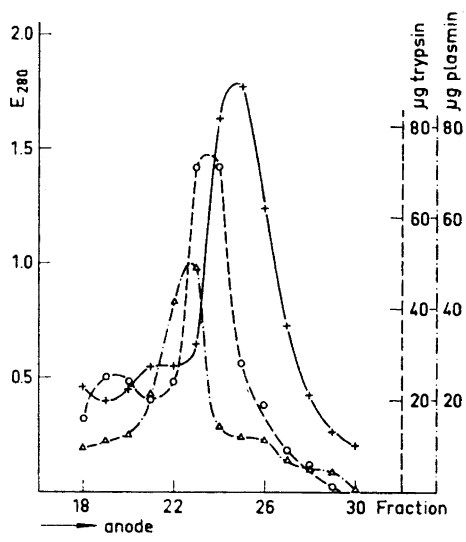

Fig. 8. The rate of migration of trypsin and plasmin inhibitors in relation to that of albumin on electrophoretic separation of human serum. + The albumin fraction according to extinction measurement at 280 mμ. O Inhibiting effect on trypsin per 0.9 ml fraction. △ Inhibiting effect on plasmin per 0.9 ml fraction.

from Fig. 8, the component which migrates most rapidly has the main trypsin-inhibiting effect. By concurrent analysis of the plasmin-inhibiting effect, it could be established that this activity was exerted by a factor which migrated somewhat more slowly than the trypsin inhibitor and that the position in relation to albumin was the same for the trypsin inhibitor as for the more rapidly migrating protease I inhibitor.

Investigations on the heat stability of the inhibitors of protease I present in serum. According to Shulman,[13] inactivation of the trypsin inhibitor in serum takes place on heating to 60°C. After heating for *ca.* 20 min, practically all the inhibitor has been inactivated. Since protease I is inhibited by a trypsin inhibitor as well as by a plasmin inhibitor, attempts were made to analyse how great a part of the total amount of inhibitor can be attributed to one or the other of these inhibitors by using Shulman's heating method. Consequently, serum was heated at 58, 59, 60, 61 and 62°C for different periods of time whereupon the inhibition remaining was analysed.

In the beginning of the heat treatment, a part of the total inhibitor amount was inactivated rapidly, but after this initial inactivation, the inhibiting effect of serum changed slowly. For this slow change, which is illustrated by Fig. 9 with three different serums, a linear relation exists between the time

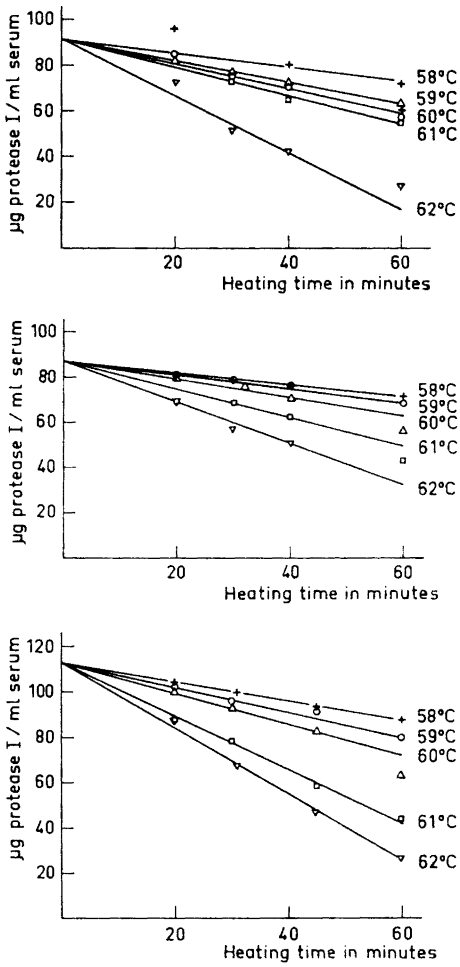

Fig. 9. The change in the inhibitor amount in serum from 3 different subjects after heating at 58, 59, 60, 61 and 62°C for different times.

of heating and the inactivation at the temperatures 59 and 60°C. On extrapolating to zero time, the value for heat stable inhibitor is obtained. The difference between the total inhibitor and the heat stable inhibitor contents can be regarded as a measure of the amount of heat labile inhibitor which exerts an effect on protease I. Qualitative experiments show that the heat labile inhibitor is the one also having an anti-trypsin effect.

Determination of the total, heat labile and heat stable amounts of inhibitor in healthy subjects. Serum from 17 healthy subjects was investigated with regard to the content of amount of total inhibitor. Subsequently, a differentiation of the two main inhibitors was undertaken by determining the heat stable and heat labile inhibitor after heating at 60°C for different periods of time. The results are accounted for in Fig. 10 A and B and in Table 1. As is apparent

Table 1. Protease I-inhibitors of 17 healthy persons.

	Inhibitor value corresponding to μg PI/ml serum		
Case	Total	Heat labile	Heat stable
A.M.	131	78	53
P.S.	106	43	63
A.H.	139	73	66
G.L.	149	72	77
O.M.	158	80	78
A.O.	156	67	89
L.L.	162	73	89
P.E.	153	62	91
R.S.	154	62	92
A.–G.D.	148	51	97
L.S.	152	38	114
K.O.	167	44	123
H.S.	202	69	133
B.E.	120	54	66
S.L.	116	48	68
J.C.	141	60	81
T.R.	135	48	87
$\bar{X} \pm$S.D.	146 ± 22	60 ± 13	86 ± 22
Range	106–202	38–80	53–133

from the figures, the slow change in the inhibitors took place at the same rate in 13 of the serums which were investigated. However, in 4 of the cases, this inactivation took place more slowly (Fig. 10 B). The amount of inhibitor in serum from the different subjects varied, as shown in the table, from 106 to 202 μg protease I per 1.0 ml serum, the average being 146. The values for the heat labile inhibitors varied from 38 to 80, the average being 60 μg protease per 1.0 ml serum, while the heat stable inhibitors varied from 53 to 133 μg protease per 1.0 ml serum, the average value being 86.

DISCUSSION

According to Bergkvist and Svärd [14] a dissolution of experimentally produced vein thrombi takes place on systemic treatment with protease I. These experiments were carried out on cats and rabbits with doses of the enzyme which did not overcome the actual inhibitor capacity of the animals. On carrying out investigations on dogs, Roschlau and Tosoni [15] established that occluding arterial thrombi could be dissolved to an extent of up to 75 % during a three day treatment with the enzyme. During the treatment, the authors considered that the dose of protease was such that inhibitors were

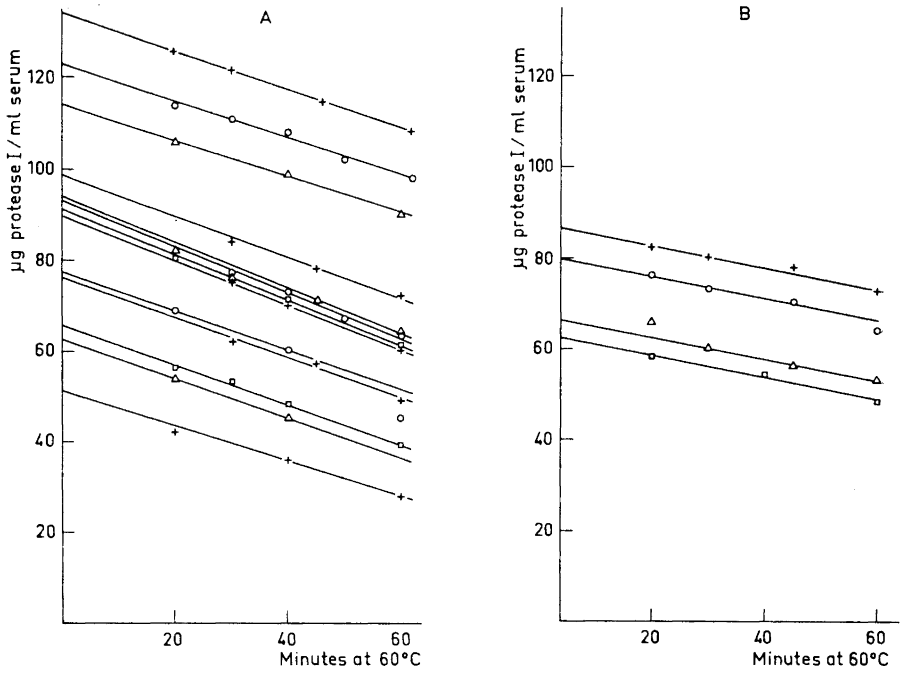

Fig. 10. The change in the inhibitor amount in serum from 17 different subjects after heating at 60°C for different times.

still present in the serum after each treatment. On clinical treatment of eye vein thrombi, favourable results have been obtained by Jürgens.[16] The doses used — 150—200 mg according to Jürgens — ought not to have involved any transgression of the total inhibitor level, in analogy with the results obtained by Jürgens *et al.*[17] on studies concerning the inhibitors in plasma from patients who have been treated with the enzyme.

The mechanism for the *in vivo* thrombolytic effect of protease I was considered by Bergkvist and Svärd [14] to be that the enzyme formed a reversible complex with an inhibitor in the serum. When in contact with fibrin, the protease was competitively removed from the complex, resulting in dissolution of the fibrin. This is analogous to the hypothesis advanced by Norman, according to which plasmin should immediately form a reversible complex with α_2-globulin whereupon the plasmin on contact with fibrin should be able to exert fibrinolytic activity.

By means of electrophoretic separation experiments, it has been established that two main components having an inhibiting effect on protease I occur in serum. These have an effect which sets in immediately and already after a short incubation of serum and protease, the inhibition effect has reached a maximum. On characterizing both these inhibitors, it has proved that each one of them inhibits, in addition, different proteolytic enzymes. The inhibitor

which occurs partly in the albumin fraction inactivates trypsin as well as protease I and ought to be identical with α_1-antitrypsin. The protease inhibitor which migrates somewhat more slowly also inactivates plasmin. The results of the experiments with gelatin as substrate thus show that protease I has an α_1-globulin as inhibitor, having activity against trypsin, and a fraction, having an antiplasmin effect which also migrates in the α_1-fraction under the reaction conditions employed as its main inhibitors in serum.

The immediate inactivation of protease I by serum, which has been established in the present investigations, indicates a rapid complex formation between protease I and the inhibitors. Since one of the inhibitors is common for protease I and plasmin and if the complex between inhibitor and protease I is stronger than that between inhibitor and plasmin, it could be conceived that protease I, because of its affinity for the plasmin inhibitor, deprives this of the opportunity of inhibiting physiologically activated plasmin *in vivo*. Such a mechanism could also explain why a thrombolytic effect is obtained with protease I in doses below the inhibitor level. However, according to this point of view an artificial clot should not be dissolved *in vitro* unless the inhibitor level in serum is exceeded. Experimental results exist showing that large doses are required in this case.[18] The hypothesis for the activity *in vivo* will be the object of further studies.

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