ACTA CHEMICA SCANDINAVICA 17 (1963) 2230–2238

# The Proteolytic Enzymes of *Aspergillus oryzae*

## III. A Comparison of the Fibrinolytic and Fibrinogenolytic Effects of the Enzymes

ROLF BERGKVIST

*Research Laboratories of AB Astra, Södertälje, Sweden*

The fibrinogenolytic and fibrinolytic activity of protease I and II are studied by different methods and in some cases, by comparison with trypsin.

Protease I is shown to possess stronger fibrinolytic activity than protease II, and there is no big difference in the susceptibility of fibrin and fibrinogen to the action of protease I. Evidence is also presented indicating that protease II proteolyzes fibrinogen at a much greater rate than fibrin.

Current advances in the field of biochemistry and related sciences have firmly established the important role played by the proteolytic enzymes in biological phenomena. Though such enzymes were originally applied directly to diseased areas to effect a local enzymic debridement or solubilization of collections of proteinaceous material, enzyme therapy is now being extended systematically to broaden the scope of its applicability. Proteolytic enzymes have also been used therapeutically as antiinflammatory agents. Empirical observations rather than scientific data dominate, however, and this use of the enzymes remains controversial.

In the search for an ideal agent for intravascular dissolution of thrombi abundant experimental evidence has been presented to document the finding that the intravenous infusion of proteases, under appropriate conditions resolves thrombolytic arterial or venous lesions produced in animals. The differing experimental conditions used have hitherto tended to obscure the real significance of the findings. The evidence in favor of proteolytic enzymes acting *in vivo* are in most cases superseded by secondary effects of sufficient grave character to render their therapeutic use impracticable. Plasmin has appeared to be the only promising one of all proteolytic enzymes tested. It has been reported that plasmin preparations show *in vivo* fibrinolytic activity at non-toxic dose level.

In the search for other and therapeutically useful proteolytic enzymes, we have studied the proteolytic enzymes of moulds. Three different proteases secreted by *Aspergillus oryzae* have been isolated, purified and characterized.[1,2] These enzymes are capable of digesting such varied substrates as gelatine, casein, denatured hemoglobin, certain synthetic esters, fibrinogen and, most important of all, fibrin. Two of these proteases, referred to as protease I and II, are highly active at neutral hydrogen ion concentration.

The present studies were undertaken to obtain some information on the effect of these enzymes on fibrinogen compared with their effect on fibrin, which is the major insoluble constituent of both thrombus and inflammatory exudates.

## EXPERIMENTAL

*Protease I and II.* The production of these enzymes as well as methods for their isolation, purification and characterization have been described in earlier publications.[1,2] Protease I acts on casein within a pH range of 5 to 9.5, optimal at pH 8.2, while protease II is active within a pH range of 4.5 to 9.5 and is optimally active at pH 6.8.

*Caseinolytic assays.* Direct caseinolytic activity was quantitatively determined by a modification [1] of Kunitz's method. In this assay, the test sample was placed in a test tube and diluted to 3.0 ml with 0.2 M phosphate buffer of pH 7.4. The solution was incubated at 37°C for 15 min, 3 ml of a 3 % casein solution adjusted to pH 7.4 was added, mixed, and the reaction allowed to proceed at 37°C. A 2 ml aliquot was taken out immediately (zero time) and another was removed 30 min later. Each aliquot was added to 3.0 ml of 10 % trichloroacetic acid. After standing for at least 30 min at room temperature, the mixture was centrifuged and the clear supernatant liquid decanted and filtered. The optical densities of the filtrates were read at 280 m$\mu$. The difference in optical density from zero to 30 min was used as a measure of the proteolytic activity. One caseinolytic unit (C.U.) was arbitrarily taken as the amount of enzyme producing an increase in optical density at 280 m$\mu$ of 1.000 in 30 min under the specified conditions. A linear relationship was found to exist between the amount of enzyme used and the absorption of the trichloroacetic acid filtrate, up to an extinction of 0.8, and all assays were carried out within this range.

*Fibrinolytic assays.* The fibrinolytic activity was estimated by three different methods.

A) *Test tube method.* This test was carried out as follows. 0.1 ml of the assay material was mixed with 0.1 ml of a thrombin solution (containing 100 NIH units per ml) and 0.8 ml of a 0.32 % solution of human fibrinogen in TRIS buffer of pH 7.4 at 37°C. The end point was taken when all bubbles trapped in the clot rapidly rose to the surface.

B) *Opacity method.* In this method the use of a spectrophotometer permitted the estimation not only of the end point but also provided a continuous record of the clot formation and lysis.

0.1 ml of the assay material was mixed with 0.1 ml of a thrombin solution (containing 100 NIH units per ml) and 3.0 ml of a 0.5 % solution of human fibrinogen in TRIS buffer of pH 7.4. The components were mixed in a cuvette having a 10 mm light path and the incubation was carried out at 25°C. The clotting time was estimated as the time from start to maximum opacity of the mixture, and the lysis time as the time interval between the beginning of opacity decrease and the point of return to the baseline.

C) *Fibrin plate method.* This method can be used for the estimation of either fibrinolytic activity in combination with plasminogen activation or of fibrinolytic or proteolytic activity alone, after denaturation of the plasminogen originally present in the fibrinogen.

(a) Unheated fibrin plates: Fibrin plates were prepared by the method of Astrup and Müllertz.[3] 9 ml of a 0.3 % human fibrinogen solution in phosphate or TRIS buffer of pH 7.4, with ionic strength 0.15, was clotted with 1 ml of thrombin solution (25 NIH units per ml) in a 10 cm diameter Petri dish. The tests were conducted by placing 0.025 ml of the samples on the fibrin layer. Controls using buffer only were prepared in a similar way. Plates were incubated at 37°C for 18 h and the results observed. The areas of the dissolved zones were taken as a measure of the fibrinolytic activity.

(b) Heated fibrin plates: Fibrin plates were prepared as described above. After the fibrinogen was clotted and the fibrin layer observed to be firm, the plates were heated at 85°C for 30 min. This procedure denatured the contaminating plasminogen and rendered the plates insusceptible to the action of plasminogen activators, but also slightly changed their sensitivity to proteolysis. The tests were carried out as described above.

*Fibrinogenolytic assay.* Some of the methods for the estimation of fibrinolytic activity gave information about the fibrinogenolytic activity too.

The fibrinogenolytic activity could also be estimated by the incubation at 25°C of a 0.5 % human fibrinogen solution in TRIS buffer of pH 7.4 containing 1.5 C.U. per ml of protease I or II. Residual clottable fibrinogen was indicated by adding aliquots of 0.5 ml of the incubation mixture to 0.1 ml of thrombin solution (containing 50 NIH units per ml). The time interval from the start to the point at which no clot was formed was taken as the fibrinogenolysis time.

*Estimation of the release of TCA-soluble products.* The release of TCA-soluble products by digestion of fibrinogen and fibrin with protease I and II was estimated as follows.

To a series of tubes containing protease mixed with TRIS buffer of pH 7.4 was added a solution of human fibrinogen in the same buffer until a final concentration of 0.5 % was obtained. The mixture had a total volume of 1 ml. Three ml of 10 % TCA was added to one tube immediately after mixing and then to the remaining tubes after incubation for different periods at 37°C. The degradation of fibrinogen was followed by quantitative estimations of the digestion products by means of ultraviolet spectrophotometry, after removal of the TCA-precipitates by centrifugation. In a second series, the fibrinogen was clotted by addition of thrombin. The fibrin degradation also was followed by estimation of the increase in absorbancy of the TCA-supernatants.

## RESULTS

Different preparations of the proteolytic enzymes showed small variations in activity. For that reason and for the purpose of standardization, caseinolytic activity was used as a means of quantitating the different products. The results obtained with this substrate were more reproducible than those obtained with the different methods using fibrin or fibrinogen as substrate, hence the caseinolytic method was adopted as the primary assay in all our studies. The activity of protease I and II was compared with that of crystalline trypsin, and all three proteases had an activity of about 15 C.U. per mg.

A fibrinolytic assay, that is, an assay depending on the dissolution of a fibrin clot, is of course the most suitable for the evaluation of fibrinolytically active preparations. The simplest method for estimation of the fibrinolytic effect of an enzyme solution consists in determining the time in which a standard fibrin clot in a test tube will dissolve. Completely formed fibrin to which a protease is added is markedly less susceptible to proteolysis than is fibrinogen. In order to demonstrate a possible relative specificity of the enzyme for fibrin, the clot must be formed in the presence of the protease. Examination of the curves obtained by plotting enzyme concentration against lysis time reveals remarkable differences in activity of protease I and II against fibrin, as can be seen from Fig. 1. The amount of protease I required for the lysis of a clot in 10 min is 1.25 C.U., while 3.35 C.U. of protease II are necessary for obtaining the same effect. Consequently, protease I shows a much higher fibrinolytic activity than protease II though the enzymes have about the same caseinolytic activity per weight.

The lysis of clotted fibrinogen is produced either directly by proteolytic enzymes that split the fibrin molecule, or indirectly by activators of plasmi-

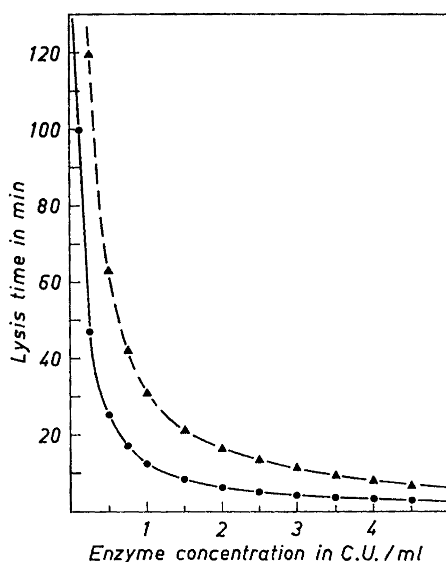


*Fig. 1.* Dose response curves of protease I (●) and of protease II (▲). The lysis time for clots made from 0.25 % human fibrinogen was estimated at pH 7.4 and 37°C.

nogen that convert plasminogen in the substrate into plasmin, with a subsequent splitting of fibrin. Both proteolytic enzymes and activators can be estimated by the fibrin plate method. The fibrin used as substrate contains plasminogen as a contamination. This can, however, be denatured by heat treatment. The plate method has a very high sensitivity and only very small amounts of the enzymes are necessary. The areas of the dissolved zones obtained were taken as a measure of the enzymatic activity (products of two perpendicular diameters in $mm^2$). The results were plotted on double logarithmic

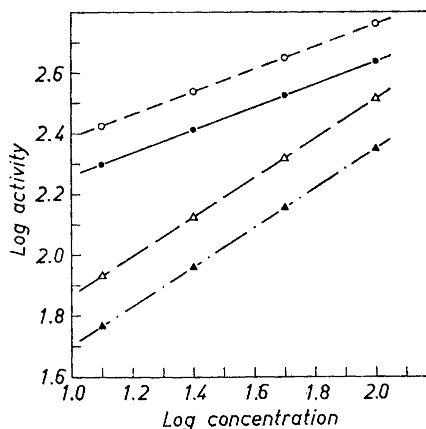


*Fig. 2.* Determination of fibrinolytic activity of protease I and II on normal and heated fibrin plates. Abscissa: logarithm of the percent concentration of the stock solution. Ordinate: logarithm of the activity. The stock solutions contained 2.0 C.U. per ml of protease I and II, respectively, and 0.025 ml of the different solutions were used for the estimations. The plates were incubated at 32°C for 18 h with protease I (●), and protease II (▲) on normal plates; protease I (O), and protease II (△) on heated plates.

paper, taking the enzyme concentration as abscissa and the product of the diameters as ordinate (Fig. 2). The quantities of protease I and II necessary for obtaining a dissolved zone with a diameter of 15 mm on a normal fibrin plate were $8.5 \times 10^{-3}$ C.U. and $50 \times 10^{-3}$ C.U., respectively, which clearly demonstrates the higher fibrinolytic activity of protease I. An increase in the lysis on heated plates compared with the effect on normal fibrin plates could also be demonstrated with both protease I and II. The same results have been obtained when using other highly purified proteolytic enzymes not possessing activator properties with respect to plasminogen. This striking discrepancy between the sensitivity of normal and heated plates can be explained by the well-known fact that denatured proteins undergo easier degradation by proteolytic enzymes than normal ones. The results show that none of the proteases is able to activate plasminogen. Studies of the effect of the enzymes on purified human plasminogen were also in accordance with these findings.

Estimations of fibrinolysis by methods employing subjective measurements, such as the visual observation of the disappearance of the clotted fibrin, will give results varying from one laboratory to another. To obviate this difficulty, methods have been evolved in which the lytic activity is followed by the use of an instrument.[4,5] A modification of this method was also used in our study of the properties of protease I and II in comparison with trypsin.

A fibrin clot was prepared by polymerization of human fibrinogen by the action of thrombin, and the resulting opacity was estimated at 600 m$\mu$. The opacity rose rapidly to a maximum within a few minutes during the clotting period and then gradually subsided, if a fibrinolytic agent was present. The maximum opacity depended on the concentration of fibrinogen and thrombin, on the molarity of the buffer, and on the pH. It has been recognized for a long time that there is a linear correlation between the fibrinogen concentration and the degree of opacity after clotting. Therefore, the method could be used for the estimation of both the fibrinogenolytic and the fibrinolytic activity of an enzyme. Delay in the addition of thrombin to a mixture of fibrinogen and a protease resulted in a decrease in the maximum opacity of the clot, depending on the fibrinogenolytic activity of the enzyme. The fibrinolytic activity of the enzyme could be estimated from the lysis time, measured by the interval between the beginning of opacity decrease and the point of return to the baseline.

Typical results obtained with protease I and II are shown in Fig. 3. The measurements were carried out at 25°C to reduce the fibrinogenolytic activity to a conveniently measurable velocity. It is clear that an appreciable fibrinogenolytic activity of protease I occurred even after a delay of 7.5 min in the time of thrombin addition to a fibrinogen-protease I mixture. In spite of this, the enzyme showed a strong fibrinolytic activity and the clot was lysed in less than 50 min. The lysis time estimated for an amount of protease II showing the same caseinolytic activity was about 150 min, showing that protease II was not so active against fibrin. On the other hand, the evidences presented indicate clearly that fibrinogen is proteolyzed much more rapidly by protease II than by protease I. A preincubation time of 2.5 min before the addition of thrombin resulted in a substantial reduction of the fibrinogen content of the clot, and no clot could be formed after a preincubation of 5 min. It has also

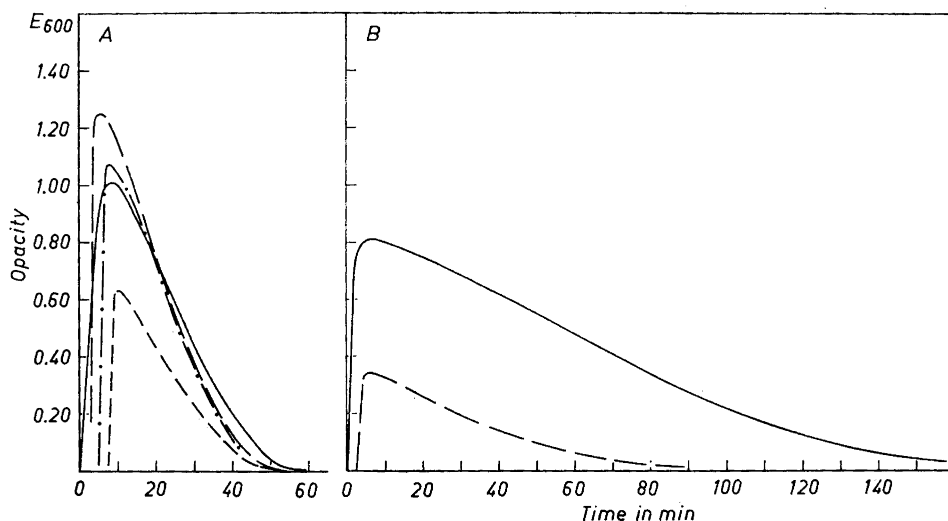


*Fig. 3.* Clot formation by thrombin and lysis by A) protease I and B) protease II. Thrombin was added after a delay of 0 min ——; 2.5 min —— ——; 5 min ——·——; 7.5 min — — —. The incubation mixtures had a final concentration of 0.5 % human fibrinogen and 1.5 C.U. of protease I and II per ml, respectively.

been established that trypsin attacks fibrinogen with still greater facility than it digests fibrin. The largest amount of this enzyme which could be added to the fibrinogen without making it unclottable corresponded to a caseinolytic activity of 0.2 C.U. No lysis of the clot was observed even after incubation for several hours. It was also observed in these experiments that the clotting time of fibrinogen that had been preincubated with protease I or II was shorter than that of normal fibrinogen.

The fibrinogenolysis was also compared with the fibrinolysis by incubation of the proteases with human fibrinogen at 25°C and testing for residual clottable fibrinogen by adding small aliquots to thrombin solutions. The time at which no clot was formed was taken as the fibrinogenolysis time. In another series, the mixtures of proteases and fibrinogen were clotted and the lysis of the clots was followed at the same temperature. Also in these experiments it could be established that protease I has a more pronounced fibrinolytic activity than protease II, which, however, attacks fibrinogen more rapidly (Fig. 4). The time for clot lysis with protease I was about six times that necessary for making the same amount of fibrinogen unclottable after incubation with the proteolytic enzyme without addition of thrombin. Protease II, however, made fibrinogen unclottable 40 times more rapidly than the clotted fibrinogen was lysed. The effect of the proteases was compared with that of trypsin, which made fibrinogen immediately unclottable in concentrations above 0.1 C.U. per ml of incubation mixture, and no lysis at all could be observed on clots formed in the presence of such small concentrations of trypsin. The clot formation may

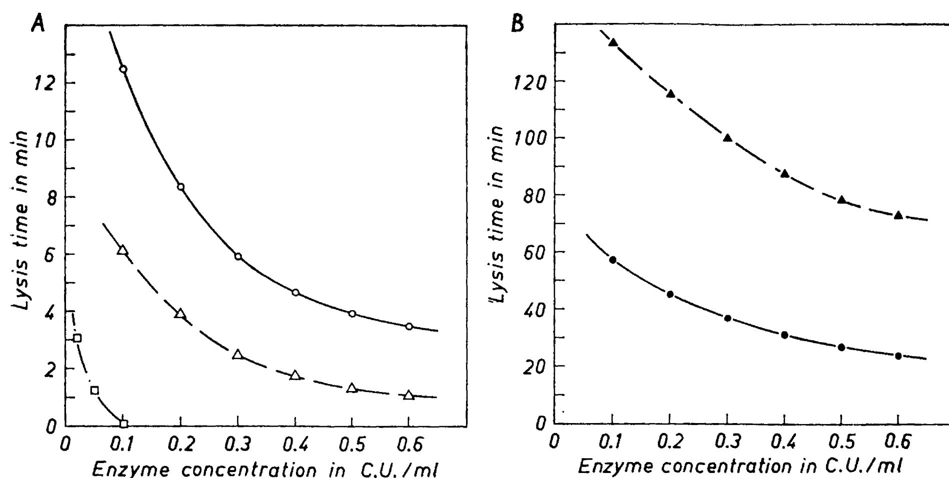


*Fig. 4.* Fibrinogenolytic and fibrinolytic activity of protease I and II. A) The time necessary for making 0.5 % fibrinogen unclottable was estimated with different concentrations of protease I (○), protease II (△) and trypsin (□). B) The lysis times for equivalent amounts of clotted fibrinogen were also estimated with protease I (●), protease II (▲). The incubation temperature was 25°C and pH of the mixtures 7.4.

fail to take place in these systems for reasons other than complete proteolysis of all available fibrinogen. For example, products of hydrolysis may interfere with the initial action of thrombin on the remaining fibrinogen or may interfere with the polymerization of activated fibrin monomers. The addition of more fibrinogen resulted in all cases in rapid formation of a clot.

The formation of trichloroacetic acid-soluble products in systems containing protease I or II mixed with fibrinogen or fibrin was also measured. Tubes of mixtures were incubated at 37°C and trichloroacetic acid was added after different times of incubation. The precipitates were removed and the ultraviolet absorptions at 280 m$\mu$ of the supernatants were determined. The data are presented in Fig. 5 for the 0—4 h period. It will be noted that about the same absorption of the TCA-soluble products was observed in the system containing fibrin as in the system containing fibrinogen, when incubated with the same amount of protease I. The differences in amounts of ultraviolet absorbing substances released from the two substrates when incubated with protease II were significant, and consistently indicated a more rapid proteolysis of fibrinogen than of fibrin.

## DISCUSSION

Assuming an identical mode of enzymatic reaction of a proteolytic enzyme with fibrinogen and fibrin, a more rapid splitting of fibrinogen could be expected because of the homogeneous state of the reaction mixture. The restricted movement of fibrin molecules following their formation must reduce their opportunity for random contact with any soluble enzyme. It has been estab-

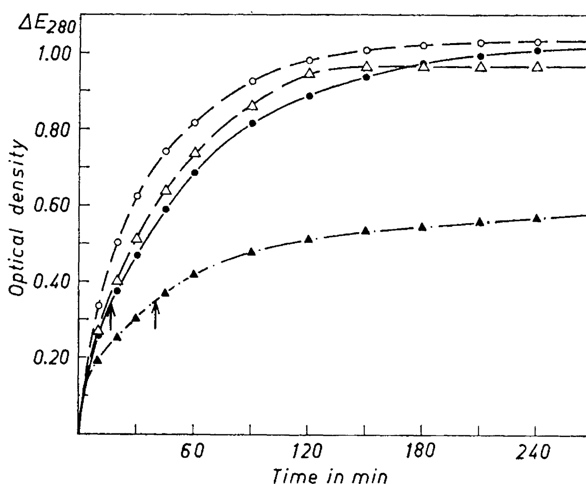


*Fig. 5.* Comparative rates of release of TCA-soluble products from fibrinogen and fibrin by incubation at 37°C with 1.5 C.U. of protease I and II per ml respectively. Initial fibrinogen concentration was 0.5 % in both series and the mixtures had a pH of 7.4. Fibrinogen digestion by protease I (○) and protease II (△). Fibrin digestion by protease I (●) and protease II (▲). The arrows indicate complete lysis.

lished that trypsin, a nonspecific protease, attacks fibrinogen with greater facility than it digests fibrin. The evidences presented above indicate also that fibrinogen is proteolyzed by protease II at a much faster rate than fibrin. Protease I, however, not only shows a stronger fibrinolytic activity than a caseinolytically equivalent amount of protease II, but also exhibits almost the same rates of fibrinogenolysis and fibrinolysis in purified systems.

If there really is no large difference in the susceptibility of fibrin and fibrinogen to the action of protease I, the lysis time of various systems containing fibrinogen and protease I would be independent of the point in time within that lysis period at which thrombin was added, providing the addition of thrombin was followed by the formation of a rigid clot. In using the opacity method, it was observed that regardless of whether the delay in the addition of thrombin was 2.5 or 10 min, the last vestiges of a visible clot disappeared at about the same time in all systems where fibrinogen and fibrin were incubated with protease I.

A comparison of the rates of release of TCA-soluble products absorbing ultraviolet light during the reaction of fibrin and of fibrinogen with protease I indicated that the rate of release from the fibrinogen system was essentially equal to that from the system containing fibrin. In contrast, the differences in the amounts released from the two substrate systems by incubation with protease II or trypsin indicated a much more rapid proteolysis of fibrinogen than of fibrin.

Purified fibrinogen and fibrin are broken down at equal rates by plasmin and there has been much dispute whether plasmin attacks fibrin specifically.

The strong adsorption of plasmin to fibrin, which has been recognized for many years, has been suggested as a probable explanation to its high fibrinolytic activity. Although it has not yet been possible to show such an adsorption of protease I to fibrin, the high activity of protease I with respect to fibrin might be explainable by the fact that this substrate fulfils the specificity requirements of protease I to a better or greater extent than do other proteins.

On the basis of the properties of protease I reported above, one is tempted to testing the therapeutic value of this substance as a thrombolytic agent, as well as for other purposes. Plasma inhibitory substances constitute, however, a severe impediment to the therapeutic use of proteolytic enzymes. As a background for the *in vivo* studies we have also studied the plasma inhibitors of both protease I and II. These results will be presented in a later publication.

## REFERENCES

1. Bergkvist, R. *Acta Chem. Scand.* **17** (1963) 1521.
2. Bergkvist, R. *Acta Chem. Scand.* **17** (1963) 1541.
3. Astrup, T. and Müllertz, S. *Arch. Biochem. Biophys.* **40** (1952) 346.
4. Pennell, R. B. *et al. Am. J. Cardiol.* **6** (1960) 449.
5. Turner, E. J. and Stewart, A. G. *Lancet* **1961** 400.

Received December 21, 1962.