

# Degradation Products of Fibrinogen by Elastase-Like Neutral Protease from Human Granulocytes

## CHARACTERIZATION AND EFFECTS ON BLOOD COAGULATION IN VITRO

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**ABSTRACT** We investigated the effect of elastase-like neutral protease isolated from human granulocytes on human fibrinogen. Dependent on enzyme concentration and time of incubation, the elastase-like protease induced a progressive degradation of fibrinogen. Analysis of the remaining polypeptide chains showed a high susceptibility of the A $\alpha$ - and low susceptibility of the  $\gamma$ -chain of fibrinogen towards the proteolytic action of the enzyme. The split products were characterized by polyacrylamide gel electrophoresis and two-dimensional immunoelectrophoresis. They showed antigenic determinants of fibrinogen and of plasmin-induced proteolysis products D and E. The cleavage fragments isolated by gel chromatography had distinct molecular weights. Coagulability of fibrinogen by thrombin was inhibited according to the concentration of the protease and the time of incubation. Split products of fibrinogen with higher molecular weight prolonged the coagulation time of native fibrinogen, whereas low molecular weight fragments were ineffective.

## INTRODUCTION

The ability of leukocyte enzymes to degrade fibrin at neutral or slightly alkaline pH was first demonstrated by Rulot (1) and Opie (2). Further studies indicated that both fibrinogen and fibrin are susceptible to the action of leukocyte enzymes (3–5) and that the intrinsic enzymatic system of the leukocytes is distinct from plasmin (6–10). Apparently, intracellular plasminogen does not account for fibrinolysis, as only very low plasminogen levels have been detected in polymorphonuclear leukocytes (8, 11, 12).

Recently, Ohlsson and Olsson (9), Ohlsson (13), and

Schmidt et al. (14–17) showed that human polymorphonuclear granulocytes contain two neutral proteases capable of degrading fibrinogen. A chymotrypsin-like protease, which predominantly hydrolyses peptide bonds adjacent to the carboxyl group of phenylalanine, exhibited only a weak reactivity with fibrinogen. An elastase-like protease (ELP),<sup>1</sup> which preferentially acts on peptide bonds with valine and alanine as the carboxyl group donor, showed strong activity with fibrinogen comparable to that of trypsin (16, 17).

More detailed studies on fibrinolysis by leukocyte proteases have been performed only with crude leukocyte extracts (10, 12). The fibrinogen degradation products formed by the leukocyte extracts were found to be completely different from those generated by plasmin.

Our studies with purified human granulocyte elastase, however, show a similarity between the plasmin-produced D and E fragments and the proteolysis products of degradation by ELP. Until now, the process of fibrinogen degradation by leukocytes has been mainly considered to involve the release of thromboplastic substances from leukocytes, triggering the formation of thrombin. This would result in consumption of clotting factors and subsequent activation of circulating plasminogen, followed by the appearance of D and E fragments (18–20). The detection of D and E split products in the bloodstream is therefore regarded as a major indicator for the clinical syndrome of intravascular coagulation (18, 19). The results given below suggest that the appearance of D and E fragments is not necessarily a specific marker for plasmin fibrinolysis.

Other mechanisms, e.g. direct fibrinogenolysis by

*Received for publication 17 August 1977 and in revised form 22 November 1977.*

<sup>1</sup>Abbreviations used in this paper: ELP, elastase-like protease; Fbg, fibrinogen; SDS, sodium dodecyl sulfate.

ELP, may be equally responsible for the production of D and E fragments. This study investigates in detail the ability of purified human ELP to degrade human fibrinogen. The resulting split products were characterized with respect to their antigenic determinants, which were compared with those of native fibrinogen and plasmin split products. The ability of fibrinogen fragments to influence blood coagulation in vitro was also investigated.

## METHODS

Purified human plasminogen-free fibrinogen ( $\Phi$ TXF 40), plasmin fragment D standard ( $\Phi$ TFV 04), and fragment E standard ( $\Phi$ TFW 04), anti-human fibrinogen serum ( $\Phi$ RCH 04), anti-fragment D serum ( $\Phi$ TNU 04), and anti-fragment E serum ( $\Phi$ TNV 04), streptokinase ( $\Phi$ BSK 04), plasminogen ( $\Phi$ RKA 04), thrombin ( $\Phi$ RHT 18), and 90–95% pure  $\alpha_1$ -antitrypsin (free of other human plasma anti-proteases, 171175) were obtained from Behring-Werke AG, Marburg/Lahn, West Germany.

ELP was isolated from extracts of granules of polymorphonuclear leukocytes as published previously (15). The purity of the enzyme was at least 96% (17). The protease was stored at  $-20^\circ\text{C}$  in 0.05 M Tris/HCl buffer, pH 7.5, containing 0.15 M NaCl. Enzyme and fibrinogen dilutions were performed in the same buffer.

**Fibrinogen digestion by ELP.** Fibrinogen (Fbg), 2 mg/ml, was incubated with 0.1 vol of ELP at a ratio of 0.3, 1.2, 5.0, and 20.0  $\mu\text{g}$  ELP/mg Fbg at  $37^\circ\text{C}$  and the reaction was stopped after different time periods (10 min, 1, 4, and 24 h) by 0.1 mM diisopropylfluorophosphate. For coagulation tests, ratios of 0.005–5.0  $\mu\text{g}$  enzyme/mg Fbg were applied; in these experiments proteolysis was terminated by addition of 50  $\mu\text{g}$   $\alpha_1$ -antitrypsin/ $\mu\text{g}$  ELP.

**Fbg digestion by plasmin.** Fbg, 2 mg/ml, was incubated for 1 and 24 h with 0.1 vol of plasminogen (0.06, 0.25, 1.0, and 4.0 Committee of Thrombolytic Agents (CTA) U/mg Fbg). The reaction was stopped by addition of 1,000 Kallikrein inhibitor units (KIU) Trasylol (Bayer Werke AG, West Germany).

**Two-dimensional immunoelectrophoresis** according to Clarke and Freeman (21) was performed in 1% agarose as described previously (22). Electrophoresis was carried out at 220 V (4–5 V/cm) for 135 min (first dimension) and at 190 V (3–4 V/cm) for 18 h (second dimension). The antisera for the second dimension were 0.25–0.75% anti-human Fbg, anti-fragment D, and anti-fragment E sera. Either Fbg (2 mg/ml), fragment D (0.1 mg/ml), or fragment E standard (0.05 mg/ml) served as a control. All slides were stained with Coomassie Brilliant Blue R 250 (Serva GmbH, Heidelberg, West Germany) in acetic acid:ethanol:water = 100:450:450.

**Polyacrylamide gel electrophoresis** in presence of sodium dodecyl sulfate (SDS) was performed according to Weber and Osborn (23) using 5% gels for nonreduced and 10% gels for reduced and alcyated (0.01 M dithioerythrol, 0.01 M iodoacetamide, Serva GmbH) incubation mixtures. The gels were subjected to electrophoresis at 8 mA/gel for 5 h and stained after fixation for 1 h at  $60^\circ\text{C}$  with 12.5% trichloroacetic acid (TCA) with Coomassie Brilliant Blue G 250.

**Gel chromatography.** Incubation mixtures with 5  $\mu\text{g}$  ELP/mg Fbg were concentrated on Amicon PM 10 membranes (Amicon Corp. Scientific Sys. Div., Lexington, Mass.), which retain proteins with mol wt  $> 10,000$ , dialyzed against 0.05 M Tris/HCl buffer, pH 7.5, containing 1 M NaCl, and separated on Bio-Gel A 1.5 m (Bio-Rad Laboratories, Richmond, Calif.)

equilibrated with the same buffer (Pharmacia column K 26/100, Pharmacia Inc., Piscataway, N. J.)

**DEAE chromatography of Fbg split products.** 100 mg Fbg was digested with 500  $\mu\text{g}$  ELP for 1 h. The digestion products were concentrated and separated according to their molecular weight by Amicon ultrafiltration (Amicon Corp. Scientific Sys. Div.) as described above. The sample with mol wt  $> 10,000$ , dialyzed against the starting buffer (0.1 M sodium phosphate), was applied to DEAE-cellulose (Whatman DE 32 [Whatman, Inc., Clifton, N. J.], Pharmacia column K 9/30 [Pharmacia Inc.]) and eluted by a continuous gradient of 0.0–0.5 M potassium phosphate and 0.1–0.0 M sodium phosphate, pH 8.0.

**Coagulability of Fbg after incubation with ELP.** Fbg (2 mg/ml) was incubated with 0.1 vol of increasing concentrations of ELP (0.035–0.35  $\mu\text{g}$ /mg Fbg) at  $37^\circ\text{C}$  for various periods of time, ranging from 5 min to 24 h. At the indicated intervals 0.1-ml aliquots of the reaction mixture were removed and incubated with 0.2 ml thrombin (0.3 NIH/ml) and the coagulation time was measured. 0.1 ml Fbg (2 mg/ml) plus 0.2 ml thrombin (0.3 NIH/ml) served as a control.

**Anticoagulant activity of ELP-generated Fbg split products was tested in the following manner:** Fbg was incubated with 0.1 vol ELP as described above. After 5 and 10 min of incubation at  $37^\circ\text{C}$ , 50  $\mu\text{g}$   $\alpha_1$ -antitrypsin/mg ELP was added. 0.1 ml of Fbg (2 mg/ml), 0.1 ml of this incubation mixture, and 0.1 ml of thrombin (1.2 NIH U/ml) were mixed and the clotting time was measured. Controls were either 0.1 ml Fbg, 0.1 ml buffer, and 0.1 ml thrombin, or 0.2 ml Fbg and 0.1 ml thrombin.

## RESULTS

During the action of purified ELP on human Fbg, a number of high and low molecular weight split products were produced depending on enzyme concentration and time of incubation.

Fig. 1 shows a SDS-polyacrylamide gel electrophoresis of unreduced Fbg which was incubated with

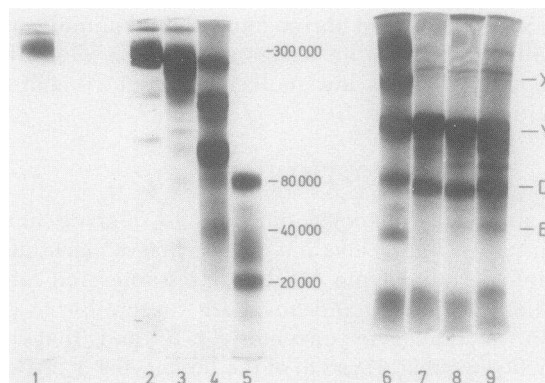


FIGURE 1 Separation of Fbg split products after a 1-h incubation with ELP by SDS-polyacrylamide gel electrophoresis on 5% gels under nonreducing conditions. Gel 1 shows the undegraded Fbg. Gel 2 demonstrates Fbg incubated with 0.3, gel 3 with 1.2, gel 4 with 5.0, and gel 5 with 20.0  $\mu\text{g}$  ELP/mg Fbg for 1 h. Gels 6–9 show an Fbg degradation for 1 h with 0.06, 0.25, 1.0, and 4.0 CTA U plasminogen/mg Fbg, activated by streptokinase.

different concentrations of ELP (gels 2–5) and plasmin (gels 6–9) for 1 h (ELP left part, plasmin right part of the figure). During fibrinolysis with different plasmin concentrations, almost identical fragments resulted, whereas increasing amounts of ELP induced a progressive fibrinolysis to a fragment somewhat larger (mol wt = 300,000) than fragment X (mol wt = 270,000). This was followed by rapid cleavage into a number of smaller fragments at higher enzyme concentrations. At an ELP concentration of 5  $\mu\text{g}/\text{mg}$  Fbg, one line corresponding in molecular weight to fragment Y (mol wt = 140,000) was observed. An additional protein band with a mol wt  $\approx$  40,000, similar to fragment E, was visible in this gel. This fragment also appeared at the highest ELP concentration used (20  $\mu\text{g}/\text{ml}$  Fbg). The largest proteolysis product arising at this enzyme concentration corresponded in molecular weight to the plasmin-induced fragment D. SDS-polyacrylamide gel electrophoresis of a 24-h incubation (not shown) demonstrated an increase of proteolysis with increasing ELP concentrations, whereas the pattern of plasmin fibrinogenolysis remained constant at different plasmin concentrations.

In Fig. 2 an electrophoretic separation of the same samples of ELP- and plasmin-degraded Fbg is shown under reducing conditions. The first gel represents the typical pattern of the three Fbg subunits. Both enzymes rapidly split the A $\alpha$ -chain of Fbg. With increasing concentrations of plasmin the B $\beta$ -chain was completely degraded, whereas the  $\gamma$ -chain was not attacked. In contrast, the elastase gradually digested all three chains of Fbg. On incubation with the lowest ELP concentration, the B $\beta$ - and the  $\gamma$ -chains were apparently little effected. A fragment with a molecular

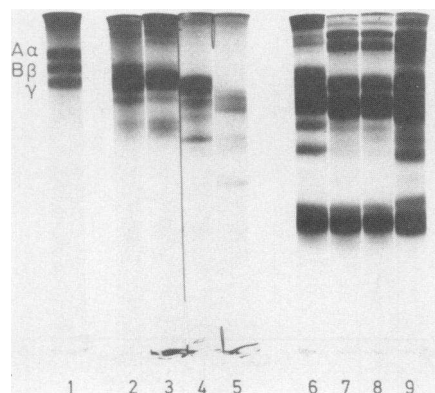


FIGURE 2 Separation of Fbg degradation products by SDS-polyacrylamide gel electrophoresis under reducing conditions. Gel 1 shows the pattern of reduced undegraded Fbg, gels 2–5 the pattern after a 1-h incubation with 0.3 (gel 2), 1.2 (gel 3), 5.0 (gel 4), and 20.0 (gel 5)  $\mu\text{g}$  ELP/mg Fbg. Gels 6–9 show Fbg after a 1-h degradation with 0.06, 0.25, 1.0, and 4.0 CTA U/mg Fbg.

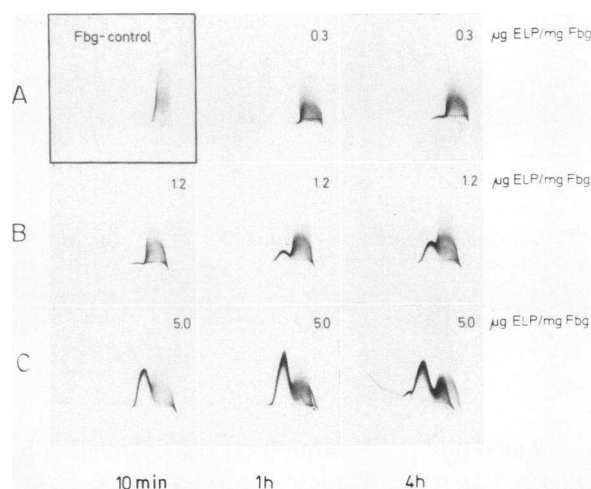


FIGURE 3 Two-dimensional immunoelectrophoresis of ELP-degraded Fbg with anti-Fbg serum in the second dimension. The amounts of ELP incubated with fibrinogen were 0.3  $\mu\text{g}/\text{mg}$  Fbg for row A, 1.2  $\mu\text{g}/\text{mg}$  Fbg for row B and 5.0  $\mu\text{g}/\text{mg}$  Fbg for row C. The times of incubation noticed below the plates were 10 min, 1 h, and 4 h. Undegraded Fbg as a control is shown on the inset.

weight between the B $\beta$ - and the  $\gamma$ -chains, however, appeared. This fragment, together with the B $\beta$ - and the  $\gamma$ -chains, disappeared at the highest ELP concentrations.

To characterize the degradation products generated by ELP immunologically, two-dimensional immunoelectrophoresis was performed. Fig. 3 demonstrates a series of two-dimensional immunoelectrophoreses with anti-Fbg serum in the second dimension. Fbg was incubated with 0.3, 1.2, and 5.0  $\mu\text{g}$  ELP/mg Fbg for 10 min, and 1 and 4 h. One to six precipitation arcs were formed depending on the incubation time and the ELP concentration. Each represents a fragment with antigenic determinants of Fbg. A similar but not identical pattern was observed with 20  $\mu\text{g}$  ELP/mg Fbg (not shown), indicating progressive proteolysis. To exclude the loss of very fast migrating split products, the length of the first dimension was doubled. Additional precipitation lines were not detected (not shown).

As described above, in proteolysis of Fbg by ELP, some fragments were obtained which were similar in molecular weight to those generated by plasmin. Two-dimensional immunoelectrophoresis using anti-D and anti-E serum showed that fragments sharing antigenic determinants of the plasmin-induced D and E fragments appeared at certain stages of ELP degradation. Two-dimensional immunoelectrophoresis of an ELP Fbg incubation mixture (5  $\mu\text{g}$  ELP/mg Fbg, 1 h) with anti-Fbg, anti-D, and anti-E serum is shown in Fig. 4. Fig. 4A shows the reaction of the degraded Fbg with anti-Fbg serum. As already shown in Fig. 3,

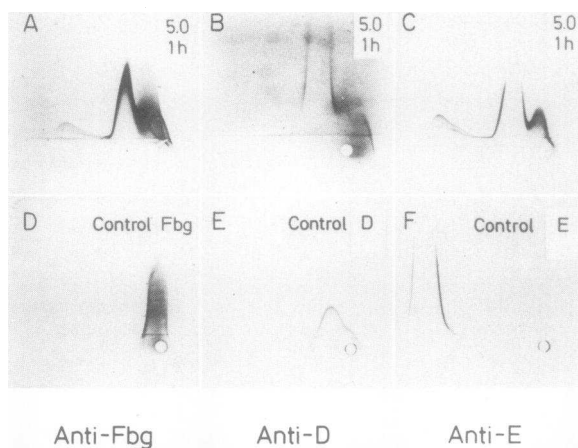


FIGURE 4 Two-dimensional immunoelectrophoresis of Fbg after a 1-h incubation with 5  $\mu$ g ELP/mg Fbg. In the second dimension anti-fibrinogen, anti-D, and anti-E serum were used (left to the right). The lower plates show the appropriate controls fibrinogen (D), standard D (E), and standard E (F).

at least six peaks were formed under these conditions of digestion. Three arcs appeared in the position of native Fbg. With anti-D serum, only two precipitation arcs were visible, the first in the same position as Fbg and the second in the same position as the D fragment (Fig. 4B). With anti-E serum a pattern similar to that observed with anti-Fbg serum resulted (Fig. 4C). The two arcs at the position of native Fbg were, however, missing. The fast migrating peak, which had the same position as the control E below, only became visible with anti-

Fbg and anti-E serum. Since there is no cross-reaction between anti-D and anti-E serum, it can be concluded that this precipitation arc represents an E-like fragment. In addition, the possible existence of a D-like fragment is postulated owing to the similarity between the positions of the control D below and the intermediate peak of the reaction mixture. This D-like fragment is similar, but not identical with the D standard as shown by its cross-reaction with anti-E serum (Fig. 4C). To separate the D- and E-like fragments, the ELP Fbg incubation mixture (5  $\mu$ g ELP/mg Fbg, 1 h) was applied to a gel chromatography column (Bio-Gel A 1.5 m). Single fractions were obtained that reacted either with anti-D or anti-E serum, as shown by countercurrent electrophoresis (Fig. 5). D- and E-like fragments were also isolated by DEAE chromatography using a decreasing sodium and an increasing potassium phosphate gradient. The elution pattern showed three separated protein peaks at a sodium:potassium ratio of first, 200 meq Na:20 meq K (I), second, 60 meq Na:600 meq K (II), and third, 35 meq Na:700 meq K (III). The pool of fraction I reacted with anti-Fbg and anti-D serum. The pool of fraction II reacted weakly with anti-Fbg and with anti-E serum, while the pool of fraction III exhibited only a weak reaction with anti-Fbg serum, but a strong reaction with anti-E serum.

It was of interest to know how the enzyme affects the thrombin coagulation of Fbg. The experiments showed that the clotting of Fbg was inhibited depending on the incubation time and the enzyme concentration.

Fbg became uncoagulable after a 10-min incubation

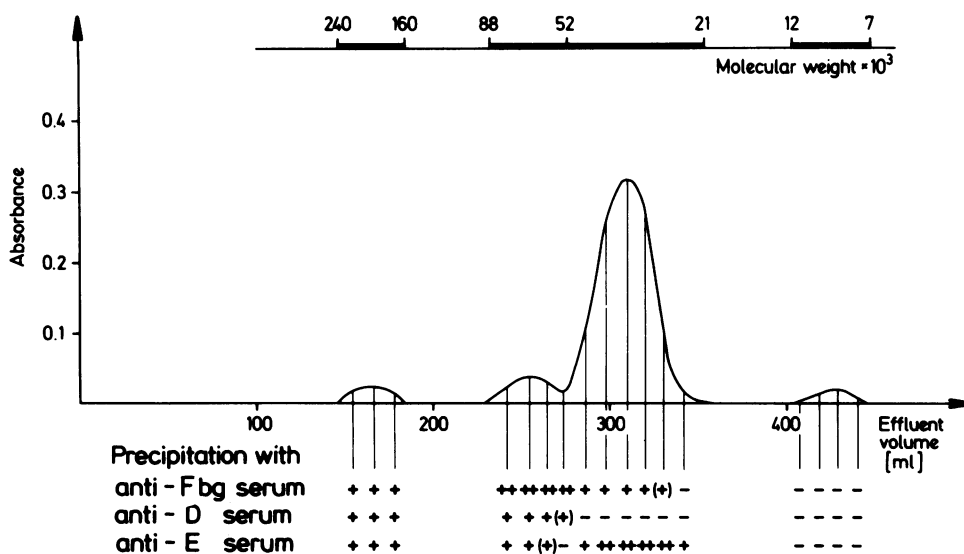


FIGURE 5 Gel chromatography on Bio-Gel A 1.5 m of 100 mg Fbg incubated with 500  $\mu$ g ELP for 1 h. The precipitation of single fractions with anti-Fbg, anti-D, and anti-E serum was tested in countercurrent electrophoresis. The intensity of the precipitation lines is expressed by ++, +, (+), or -.

with 0.3  $\mu\text{g}$  ELP/mg Fbg, whereas no change in the appearance of the native Fbg was observed in SDS-polyacrylamide gel electrophoresis and in two-dimensional immunoelectrophoresis under similar conditions. The coagulation time of Fbg therefore, is a very sensitive method for determining Fbg degradation by ELP.

Finally, the anticoagulant activity of the Fbg fragments produced after a 5- and 10-min incubation with different concentrations of ELP was tested (Fig. 6). At a certain stage of degradation (1.5 and 1.75  $\mu\text{g}$  ELP/mg Fbg), split products were formed that completely inhibited the coagulation of Fbg. These split products could not be coagulated independently. Addition of higher thrombin concentrations to the uncoagulable samples resulted in clotting again. As demonstrated by the abrupt decrease of the curves, further degradation led to fragments which no longer interfered with Fbg coagulation.

## DISCUSSION

Apart from the classical system of fibrin digestion by plasmin, Fbg- and fibrin-degrading enzymes have been described in several cells and tissues, including polymorphonuclear leukocytes (1-9, 14-17). Although plasminogen and its proactivator may be present within polymorphonuclear granulocytes in small amounts (8, 11, 12), a number of recent results demonstrate a plasmin-independent fibrinolytic system due to leukocyte proteases (10, 12-17).

Polymorphonuclear granulocytes frequently accumulate intra- and extravascularly at fibrin deposits

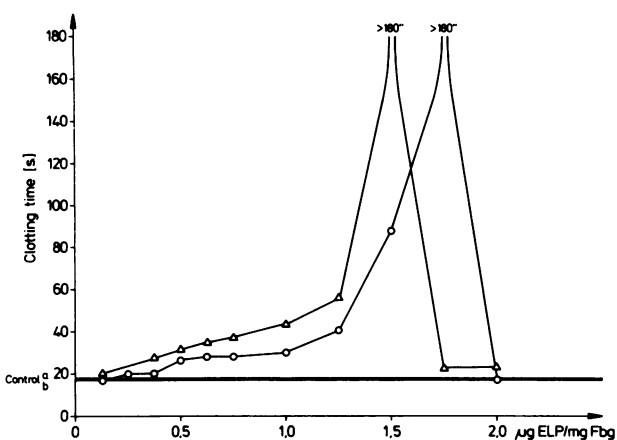


FIGURE 6 Anticoagulant activity of Fbg split products. The split products were generated by different enzyme concentrations (abscissa) and incubation times of 5 min (○) and of 10 min (△). The curves show the influence of Fbg split products generated at certain times of incubation and enzyme concentrations on the Fbg coagulation. Control a: 0.2 ml Fbg and 0.1 ml thrombin; 18.2 s. control b: 0.1 ml Fbg, 0.1 ml buffer, and 0.1 ml thrombin; 17.6 s.

(4, 24, 25). This concentration of cells may be facilitated by chemotactic agents including chemotactic fibrinopeptides (25). Once localized, active release of proteolytic enzymes from the granulocytes may be induced by various mechanisms. Fibrinolysis proceeds at neutral pH, where most cathepsins are inactive (3, 16, 26, 27). In this respect, the neutral granulocyte protease ELP may be of particular importance: ELP is actively secreted during frustrated phagocytosis on nonphagocytatable surfaces, in the presence of antigen-antibody complexes, complement components, and in the presence of endotoxin, whereas the chymotrypsin-like protease remains within the cells (28, 29).

In two recent studies, Bilezikian and Nossel (10) and Plow and Edgington (12) investigated the effect of leukocyte extract on Fbg (10, 12). The leukocyte extract caused a rapid cleavage of all three primary constituent chains of fibrinogen including the  $\gamma$ -chain. The ability of the extract to attack the  $\gamma$ -chain of Fbg primarily and rapidly was used to distinguish the enzymes from plasmin, trypsin, thrombin, and a number of bacterial proteases. Since a fragment of high molecular weight remained, even after prolonged incubation, it was concluded that leukocyte proteases exhibit a restricted specificity for a limited number of specific peptide bonds (21). These results obtained with crude extract do not conform with the findings with pure enzyme used in this study. Under comparable conditions (as calculated from the inhibition of Fbg coagulation time after 1 h) the  $\text{A}\alpha$ -chain of Fbg was rapidly destroyed by ELP. In contrast, the  $\text{B}\beta$ - and the  $\gamma$ -chain appeared to be little affected, whereas incubation with crude extracts resulted in degradation of these subunits. Only at higher ELP concentrations was the  $\text{B}\beta$ - and then the  $\gamma$ -chain fully degraded. The band observed at mol wt = 300,000 in SDS-polyacrylamide gel electrophoresis under unreduced conditions, was similar to that reported by Bilezikian and Nossel (10) and Plow and Edgington (12). The different specificity of the leukocyte extract may be due to the presence of other intracellular proteases. Possibly only the ELP is of major importance in extracellular proteolysis of Fbg, since it is the only protease to be actively secreted. As demonstrated in SDS-polyacrylamide gel electrophoresis (Fig. 1), during certain stages of proteolysis by ELP, fibrinogen split products were formed similar in molecular weight to those generated by plasmin. In addition, two-dimensional immunoelectrophoresis using anti-D and anti-E antisera demonstrated that fragments showing antigenic determinants of fragments D and E had appeared. Furthermore, separation of split products which only reacted with anti-D or anti-E serum, respectively, was achieved by anion-exchange and gel chromatography.

Although these results may suggest a similarity in

the action of ELP and plasmin on Fbg, a striking difference between ELP and plasmin becomes evident on comparison of the patterns of fibrinogenolysis in SDS-polyacrylamide gel electrophoresis after long incubation periods. Plasmin showed an identical cleavage pattern at different concentrations, indicating a limited proteolysis. In contrast, ELP (dependent on its concentration) caused a varying pattern of split products, even after 24 h of incubation.

These results show that ELP, like other proteolytic enzymes with low specificity (17), induces progressive proteolysis. The coagulation experiments suggest a possible role of ELP in blood coagulation. Depending on incubation time and concentration, ELP degraded Fbg until it became uncoagulable. In addition, the ELP-produced Fbg split products inhibited the thrombin-induced generation of fibrin. This inhibition is ascribed to high molecular weight fragments, since only fragments obtained by low enzyme concentrations and short incubation times were effective. The anticoagulant effect of these fragments was overcome by increasing the thrombin concentration. This suggests a competitive inhibition of thrombin by these cleavage fragments.

Direct proteolysis of Fbg and of other coagulation factors by ELP indicate the existence of an alternative origin of coagulation disorders independent of both thrombin and plasmin action (14, 22, 30). This is supported by studies showing a correlation between circulating ELP and coagulation defects in patients with acute leukemia and septicemia (22). In addition, intravenous injection of ELP in green monkeys induced a marked drop in several coagulation factors including Fbg, and produced an increase in Fbg degradation products and severe bleeding complications (30).

The appearance of Fbg fragments sharing antigenic determinants with the plasmin-derived proteolysis products in these patients and in the animal experiment may therefore be due to a direct proteolysis of ELP rather than plasmin fibrinolysis. A number of clinical syndromes may therefore represent ELP-induced clotting factor deficiencies, which would require special treatment.

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