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TERMINAL PLASMIN DEGRADATION PRODUCTS D AND E OF DUCK FIBRINOGEN AND THEIR EFFECT ON POLYMERIZATION OF FIBRIN*

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Duck fibrinogen (Mr 320 000) treated with streptokinase-activated human plasminogen in the presence of calcium ions was hydrolysed to terminal core fragments D and E. They were isolated from the digest by: (1) ion-exchange chromatography on DEAE-cellulose, (2) gel filtration on Sephadex G-100, and (3) affinity chromatography with the use of fibrin monomers coupled to CNBr-activated Sepharose. When the native D fragment, D1, was additionally digested by plasmin in the presence of EDTA, more degraded forms D2 and D3 appeared. Molecular weight of D1, D2, D3, and E estimated on SDS-polyacrylamide gel electrophoresis is 100 000, 89 000, 60 000 and 50 000, respectively. It was found that after reduction with 2-mercaptoethanol the fragments D1 and D3 consisted each of three polypeptide chains: α, β, γ; the γ-chain of D3 remnant was more degraded (Mr 24 000) as compared with the γ-chain of D1 remnant (Mr 42 000). Polymerization of both duck and pig fibrin monomers was inhibited by fragments D1 but not by D3.

Bird fibrinogen, like mammalian fibrinogen (Blombäck & Blombäck, 1972: Doolittle, 1975) is a soluble plasma glycoprotein built of three pairs of non-identical polypeptide chains Aα, Bβ, and γ2 which form two half-molecule units held together by disulphide bonds. Molecular weight of bird fibrinogen has been found to be about 320 000 (Krajewski et al., 1980). Different functional domains important for protofibril formation were found to exist in its molecule (Cierniewski et al., 1980). Preliminary experiments showed

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that bird fibrinogen, in comparison with the mammalian one, is more sensitive to plasmin proteolysis, but the molecule seems to be organized into the same two terminal D, and one central E, domains (Krajewski et al., 1980).

Digestion of human fibrinogen in the presence of calcium ions resulted in the terminal degradation products: D and E of M, 90 000 and 50 000, respectively (Nussenzweig et al., 1961: Marder & Budzyński, 1974). When proteolysis was carried out in the presence of EDTA or EGTA, three kinds of fragment D appeared, of M, 94 000 (D₁), 88 000 (D₂) and 83 000 (D₃), (Furlan et al., 1975; Olexa & Budzyński, 1981). It has been shown that human fragment D₁ but not D₃ inhibited fibrin monomer polymerization (Beliser et al., 1975: Williams et al., 1981).

The aim of our study was to isolate both D and E fragments from duck fibrinogen digest and to compare their effect on polymerization of fibrin monomers in homo- and heterologous systems.

MATERIALS AND METHODS

Preparation of fibrinogen. Duck and pig fibrinogens were isolated from fresh pooled plasma by the cold ethanol precipitation technique (Doolittle et al., 1967). The coagulability of fibrinogen preparations used in thrombin tests was 96 - 98%.

Plasmin digestion of fibrinogen. Plasminogen prepared from fresh human plasma by the method of Deutsch & Mertz (1970) and activated with streptokinase was utilized for the cleavage of duck fibrinogen. In typical experiments, 500 mg of fibrinogen was dissolved in 25 ml of 0.15 m-Tris/HCl buffer, pH 7.4, containing 5 mm-CaCl₂ and 0.02% sodium azide. The ratio of plasminogen to substrate in the reaction mixture was 1:200 (w/w). The incubation was carried out at 37°C for 24 h. Proteolysis was stopped with trasylool (10 000 KIU).

Fractionation and purification of terminal degradation products. Fragments D and E were isolated from the fibrinogen digest by three methods: (1) ion-exchange chromatography on DEAE-52 cellulose column (Nussenzweig et al., 1961): (2) gel filtration on Sephadex G-100 (Gardlund et al., 1972): (3) affinity chromatography with the use of fibrin monomers coupled to CNBr-activated Sepharose 4B (Heene et al., 1979).

D₃ Remnant obtained from fragment D₁ by further plasmin hydrolysis in the presence of 5 mm-EDTA was purified on Sephadex G-100.

Analytical procedure. Fibrinogen, fibrinogen digest, fragments D₁, D₃ and E were analysed by polyacrylamide gel (5%) electrophoresis in 0.1% SDS as described by Weber & Osborn (1969). After reduction with 2-mercapto-ethanol, the electrophoresis was carried out in 12.5% gel according to
Fig. 1. SDS-polyacrylamide gel (5%) electrophoresis of duck fibrinogen (Fg, panel a) and its terminal plasmin degradation products (panel b). Duck fibrinogen was digested with human plasmin (plasmin to fibrinogen ratio 1:200) for 24 h. Three kinds of fragments D1, D2 and D3 (degradation products) and a single fragment E were separated in the gel.

T. Krajewski et al. (facing p. 146).
Fig. 2. Affinity chromatography of the duck fibrinogen digest on duck fibrin monomers (homologous system) or pig fibrin monomers (heterologous system) coupled to CNBr-Sepharose 4B, according to Heene et al. (1979). Non-adsorbed material (mainly fragments E) were eluted with 0.05 M-Tris/H₃PO₄ buffer, pH 7.8 (buffer A). Specifically adsorbed fragments D were eluted in both systems with 0.05 M-Tris/H₃PO₄ buffer, pH 5.3, containing 1 M-NaCl (buffer B). Strongly adsorbed material (early plasmin degradation products and fibrinogen) were eluted with buffer B containing in addition 3 M-urea (B₃) or 6 M-urea (B₆). The SDS-polyacrylamide (5%) gels correspond to the digest and to each eluted peak.
Swank & Munkres (1971). Gels were stained for protein with Coomassie Brilliant Blue and for carbohydrate with the periodic acid-Schiff reagent (Kapitany & Zebrowski, 1973). Molecular weight of the analysed peptides was estimated by SDS-polyacrylamide gel electrophoresis.

Protein concentration of fibrinogen and of the plasmin terminal degradation products was determined by the microbiuret method (Itzhaki & Gill, 1964) and spectrophotometrically at 280 nm using the extinction coefficients of 15.5, 20.8 and 10.2 for duck fibrinogen and D and E fragments, respectively.

Inhibition of fibrin monomer polymerization by fragments D and E. Fibrin monomers were prepared according to Belitsser et al. (1968). Polymerization of fibrin monomers in the presence of fragments D₁, D₃ and E was determined spectrophotometrically at 600 nm as described by Latašlo et al. (1962).

Reagents. DEAE-52 cellulose was obtained from Whatman Biochemicals Ltd (England): Sephadex G-100, CNBr-Sepharose 4B, Lysine-Sepharose 4B from Pharmacia Fine Chemicals (Uppsala, Sweden): acrylamide, \( N,N' \)-bisacrylamide, \( N,N,N',N' \)-tetramethylenediamine (Temed), Tris, ammonium persulphate. Coomassie Brilliant Blue R and Fuchsin basic, all from Serva (Heidelberg, F.R.G.): sodium dodecyl sulphate (SDS) from B.D.H. Chemicals Ltd (England): calcium chloride from Sigma Chem. Co. (St. Louis, Mo., U.S.A.): trasylol (traskolan) from Polfa (Jelenia Góra, Poland): other reagents were of analytical grade, supplied by POCh (Gliwice, Poland).

RESULTS

The hydrolysis of duck fibrinogen by activated plasminogen resulted in formation of the D:E complex as the major high-molecular-weight product. It dissociated into fragments D and E in the presence of 1% SDS (Fig. 1). Although plasmin digestion was performed in the presence of calcium ions, a small amount of fragment D₁ was further hydrolysed to the intermediate forms D₂ and D₃ of lower molecular weight (Fig. 1).

Fragments D and E isolated from the terminal products of duck fibrinogen proteolysis by means of DEAE-52 cellulose chromatography and Sephadex G-100 filtration, appeared to be homogeneous in SDS-polyacrylamide gel electrophoresis. During purification of fragment D₁ by affinity chromatography it was observed that this fragment was specifically adsorbed irrespective whether duck or pig monomers were coupled to CNBr-Sepharose (Fig. 2). When fragment D₁ was repeatedly hydrolysed by plasmin in the presence of EDTA, a more degraded form, D₃ of 

\( M_r \), 80 000 was isolated from the digest.

The densitometric scanning of the gels after electrophoresis of the reduced samples of fragments D₁, D₃ and E is shown in Fig. 3. It was
found that fragment D₁ consisted of polypeptides with \( M, 44\,000, 42\,000 \) and 15 000, corresponding to the \( \beta, \gamma \) and \( \alpha \)-chain remnants, respectively. Fragment D₃ had the same submolecular pattern as D₁ except that \( \gamma \)-chain was more degraded (\( M, 24\,000 \)). The reduced fragment E migrated in the gel as a triple band with \( M, 9000, 8000 \) and 6500, corresponding to the \( \alpha, \beta \) and \( \gamma \)-chain fragments, respectively. Submolecular composition and some physico-chemical properties of all the above-studied plasmin degradation products are shown in Table 1.

![Image](https://example.com/image.png)

**Fig. 3.** Densitometric scanning of gels (12.5%) after electrophoresis of reduced fragments D₁, D₃ and E. The numbers above the particular peaks correspond to molecular weight (\( \times 10^{-3} \)) of the constituent polypeptides. The sequence of polypeptide chains of fragments D₁ and D₃ is \( \beta, \gamma, \alpha \), and remnant E, \( \alpha, \beta, \gamma \).

The influence of fragments D₁, D₃ and E on fibrin polymerization is presented in Fig. 4. Fragment D₁ inhibited fibrin monomer polymerization in both homo- and heterologous systems, but E remnant did not, and even accelerated the process in the homologous system. The same experiments carried out with fragment D₃ indicated that this degraded form of fragment D₁ was completely devoid of antipolymerizing activity.
Fig. 4. Effect of duck fibrinogen fragments D₁, D₃ and E on polymerization of the duck (upper part) and pig (lower part) fibrin monomers. Molecular ratio of fibrin monomers to fragment D₁, D₃ or E was 1:1.
Table 1

Submolecular composition and some physico-chemical properties of duck fibrinogen and its subunits as well as plasmin degradation products

<table>
<thead>
<tr>
<th>Fibrinogen and its fragments</th>
<th>Chain remnants</th>
<th>Number of subunits per mol</th>
<th>Molecular weight</th>
<th>Carbohydrate content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen ( (M_r 320 000) )</td>
<td>( \alpha )</td>
<td>2</td>
<td>59 000*</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>( \beta )</td>
<td>2</td>
<td>53 000*</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>( \gamma )</td>
<td>2</td>
<td>48 000*</td>
<td>+</td>
</tr>
<tr>
<td>Fragment D₁ ( (M_r 100 000) )</td>
<td>( \alpha )</td>
<td>1</td>
<td>15 000</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>( \beta )</td>
<td>1</td>
<td>44 000</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>( \gamma )</td>
<td>1</td>
<td>42 000</td>
<td>–</td>
</tr>
<tr>
<td>Fragment D₂ ( (M_r 89 000) )</td>
<td>( \alpha )</td>
<td>1</td>
<td>15 000</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>( \beta )</td>
<td>1</td>
<td>44 000</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>( \gamma )</td>
<td>1</td>
<td>33 000</td>
<td>–</td>
</tr>
<tr>
<td>Fragment D₃ ( (M_r 80 000) )</td>
<td>( \alpha )</td>
<td>1</td>
<td>15 000</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>( \beta )</td>
<td>1</td>
<td>44 000</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>( \gamma )</td>
<td>1</td>
<td>24 000</td>
<td>–</td>
</tr>
<tr>
<td>Fragment E ( (M_r 44 000) )</td>
<td>( \alpha )</td>
<td>2</td>
<td>9 000</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>( \beta )</td>
<td>2</td>
<td>8 000</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>( \gamma )</td>
<td>2</td>
<td>6 500</td>
<td>+</td>
</tr>
</tbody>
</table>

According to Krajewski et al. (1980).

DISCUSSION

Removal by thrombin of negatively charged fibrinopeptides A and B from the NH₂-terminal region of the fibrinogen molecule generates fibrin monomers which spontaneously aggregate to form fibrin clot (Blombäck & Laurent, 1958; Doolittle, 1975). Recently it has been found that cleavage of only fibrinopeptide A is sufficient to produce all of the structures seen on polymerization by thrombin (Fowler et al., 1981). The general mechanism of fibrin formation has been suggested to be based on the interaction of binding sites “a” and “b” located in the COOH-terminal region (fragment D) of a fibrinogen molecule, with polymerizing sites “A” and “B” situated in the NH₂-terminal part (fragment E) of the neighbouring molecule (Olexa & Budzyński, 1980: Blombäck et al., 1981).

Bird fibrinogen appears to be similar in many respects to that of mammalian origin (Krajewski et al., 1980). The present experiments have shown that two kinds of terminal plasmin degradation products of duck fibrinogen (fragments D and E) occur in the digest predominantly in a non-covalent complex D:E. The fibrinogen exhaustively hydrolysed with plasmin in the presence of calcium ions yielded, in addition to E remnant, mainly one type of fragment D \( (D₁, M_r 100 000) \). When fragment D₁ was additionally digested by plasmin in the presence of EDTA, fragment
D₃ (Mₑ 80 000) has been found to be the main product of the reaction. Both D₁ and D₃ remnants are composed of α, β and γ-chains, but the length and molecular weight of the γ-chains of D₁ (42 000) and D₃ (24 000) are different. Thus it is clear that the γ-chain of fragment D₃ is devoid of a piece of peptide from the COOH-terminal end. Similar differences in the molecular weight and biological properties between fragments D of human fibrinogen have also been observed by many authors (Furlan et al., 1975; Van Ruijven-Vermeer et al., 1979; Olexa & Budzyński, 1981).

It has also been established that, in a human fibrinogen molecule, there are three calcium binding sites, two of which are localized in the COOH-terminal region of the γ-chains in fragments D (Marguerie, 1977; Lawrie & Kemp, 1979; Nieuwenhuizen et al., 1979) and that binding of calcium ions to the D domains of the fibrinogen molecule protects its γ-chains against further digestion by plasmin (Haverkate & Timan, 1977; Nieuwenhuizen et al., 1981).

Our studies on the effect of the duck fragments D₁, D₃ and E on fibrin monomer polymerization have shown that D₁ but not D₃ strongly inhibits this process both in homo- and heterologous systems. This is in agreement with earlier studies on anticoagulant activities of the products of human fibrinogen degradation by plasmin, carried out by Larrieu et al. (1972). They have found that fragment D acts primarily by inhibiting fibrin monomer polymerization, while fragment E competes with the receptor site(s) on fibrinogen for the active center of enzyme and thus prolongs clotting time. It has also been demonstrated (Williams et al., 1981; Knoll et al., 1984) that fragments D do not affect the release of fibrinopeptides but they directly block the bimolecular reaction of activated fibrin monomers. According to the data presented by Furlan et al. (1983), inhibition of fibrin monomer aggregation by fragments D depends on the concentration of calcium ions in the system, and the effect is strongly enhanced on decreasing Ca²⁺ concentration from 1 to 0.1 mM.

The fact that fragment D contains a polymerizing site was known earlier (Kudryk et al., 1973; Matthias et al., 1973) but Haverkate et al. (1979) and Lawrie & Kemp (1979) proved that the anticoagulant property of D₁ remnant (D₁rem) was due to its γ-chain carboxy-terminal 13 000 molecular weight piece, which protects against plasmin attack when fragment D₁ is prepared in the presence of calcium ions. Olexa & Budzyński (1981) isolated from the γ-chain of fragment D₁ a peptide built of 37 amino acid residues, encompassing the positions 273-410, which competed for the binding site in E₁ domain of neighbouring fibrin monomer, and inhibited protofibril formation. This peptide was missing in an inactive fragment D₃. Nieuwenhuizen et al. (1982) have shown that this anticoagulant activity of fragment D₁ was due to its COOH-terminal γ-stretch of Mₑ 9000 followed by a piece of Mₑ 4000 possessing a calcium binding site.
The present results are in agreement with the previous findings (Cier-
niewski et al., 1980) and support the idea that there exists in the duck
fragment D₁, but not in D₃, a polymerizing site responsible for the
interaction with duck or pig fibrin monomers. Behaviour of E remnant in
the experiments seems to confirm our knowledge on the general mechanism
of polymerization of fibrin monomers. It is concluded that there are equivalent
polymerizing sites for the COOH-terminal binding domains of duck fragment
D₁ located in the NH₂-terminal region of both duck and pig fibrin
monomers, and that in the duck fibrinogen molecule a polymerizing site(s)
is placed in the Mᵣ 18 000 piece of the carboxy-terminal γ-chain of
fragment D₁. Such a site is absent in the D₃ remnant.

REFERENCES


Enhanced anticoagulant activity of fragment D formed during plasmin hydrolysis of


Blombäck, B. & Laurent, T. C. (1958) Studies on the action of thrombin on bovine fibrinogen


III. Molecular model of the plasmin-resistant disulfide knot in monomeric fragment D.

Furlan, M., Rupp, Ch. & Beck, E. A. (1983) Inhibition of fibrin polymerization by fragment D
is affected by calcium, Gly-Pro-Arg and Gly-His-Arg. Biochim. Biophys. Acta, 749,
25 - 32.

products of human fibrinogen. I. Isolation and characterization of fragments E and D


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