Purification of Rat Fibrinogen and its Constituent Chains

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Rat fibrinogen was purified from rat plasma by using lysine–Sephacrose chromatography, repeated precipitation with 25%–satd. $(\text{NH}_4)_2\text{SO}_4$ and gel chromatography on Sepharose 6B. To minimize proteolytic activity, rats were injected intravenously with Trasylol before bleeding and the collected blood was treated with Trasylol and di-isopropyl phosphorofluoridate. A 95%–clottable preparation was obtained in 70–75% yield; it proved to be free of factor XIII and plasminogen. It showed a single band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and on disc electrophoresis in 8M-urea. Alanine was the only detectable N-terminal amino acid. After reduction and modification of the thiol groups, the material could be separated into three distinct chains ($\text{A}\alpha$, $\text{B}\beta$ and γ) by pore-limit polyacrylamide slab-gel electrophoresis in the presence of sodium dodecyl sulphate. The amino acid compositions of the whole fibrinogen and of the separated modified chains were determined. The molecular weights were 61 000, 58 000 and 51 000 for $\text{A}\alpha$ -, $\text{B}\beta$ - and γ -chains respectively. Our results for the chains are in contrast with previous reports on rat fibrinogen [Bouma & Fuller (1975) *J. Biol. Chem.* **250**, 4678–4683; Stemberger & Jilek (1976) *Thromb. Res.* **9**, 657–660], in which no separation between $\text{A}\alpha$ - and $\text{B}\beta$ -chains was achieved on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis for 3 h. Evidence is presented that this is probably due to $\text{A}\alpha$ -chain degradation as a result of incomplete inhibition of proteolytic enzymes during the purification. Complete inhibition of proteolytic activities is essential in all steps of the present purification procedure.

For the development of immunohistochemical procedures for the study of disseminated intravascular coagulation with the rat as a model, monospecific antisera against rat fibrinogen are required. It was realized that commercially available antisera did not fulfil all criteria for specificity (H. Craane, J. J. Emeis, F. Haverkate, W. Nieuwenhuizen & J. Lindeman, unpublished work). Therefore it was decided to prepare rat fibrinogen as an antigen for the production of a specific antiserum in our own laboratory. The present paper describes the purification and partial characterization of rat fibrinogen and its constituent chains. Major differences were found between our preparation and those reported by others (Bouma & Fuller, 1975; Stemberger & Jilek, 1976). In particular, the occurrence of 'normal' $\text{A}\alpha$ -chains in our preparation when the preparation is protected against proteolytic degradation on all steps of the procedure must be noted. This is the first report describing the complete separation and purification of all three chain types from rat fibrinogen.

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Abbreviation used: SDS, sodium dodecyl sulphate.

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Experimental

Materials

Di-isopropyl phosphorofluoridate was obtained from Serva, Heidelberg, Germany, (2-bromoethyl)-trimethylammonium bromide was from Aldrich, Beerse, Belgium, and lysine–Sephacrose was from Pharmacia Fine Chemicals, Uppsala, Sweden. Trasylol was purchased from Bayer, Leverkusen, Germany, rat immunoglobulin G (lot no. 27) was from Miles Laboratories Inc. (Marquette 10-Z11; Amsterdam, The Netherlands), human fibrinogen was from KABI AB, Stockholm 30, Sweden, and plasminogen-free bovine fibrinogen (Poviet) was from Organon–Teknika, Oss, The Netherlands. Rabbit anti-(rat plasminogen) was a gift from Dr. D. W. Traas (D. W. Traas, Gaubius Institute, Leiden; unpublished work).

Purification of fibrinogen

Plasma. At 65 h before exsanguination, male Wistar rats (Centraal Proefdierenbedrijf TNO, Zeist, The Netherlands) weighing 200–300 g were injected intramuscularly with 1 ml of commercial turpentine to induce a hyperfibrinogenaemic state (Weimer & Humelbaugh, 1967). The animals were injected intravenously with 500 k-i.u. of Trasylol (1 k-i.u. of Trasylol

equals 2.15 nmol or 0.14 μg) in 0.5 ml of 0.9% (w/v) NaCl immediately before blood collection. Blood was withdrawn from the abdominal aorta into a pre-cooled plastic syringe containing 0.5 ml of a solution of Trasylol (200 k-i.u./ml), 1% (w/v) EDTA and 0.9% NaCl. After the blood had been transferred to a plastic tube containing another 0.5 ml of this solution, 0.1 ml of 0.1 M-di-isopropyl phosphorofluoridate in dry propan-2-ol was added. The contents of the tube were immediately mixed and cooled to 0°C. Plasma was obtained by centrifugation at 1200 g for 20 min at 4°C (Sorvall, HS-4 rotor). Platelets were removed by centrifugation for another 20 min at 23 500 g (Sorvall, SS-34 rotor). Trasylol solution (10 000 k-i.u./ml) was added to give a final concentration of 100 k-i.u./ml. The plasma was then diluted with 2 vol. of Trasylol/phosphate buffer (0.15 M- KH_2PO_4 , 3 mM-EDTA and 10 k-i.u. of Trasylol/ml, adjusted to pH 7.5 with 5 M-KOH). Di-isopropyl phosphorofluoridate was added to a final concentration of 1 mM.

The diluted plasma was processed immediately. All subsequent purification steps were carried out at 4°C.

Removal of plasminogen. This was carried out by the method of Matsuda *et al.* (1972) by passing the diluted plasma through a column of lysine-Sepharose.

$(\text{NH}_4)_2\text{SO}_4$ fractionation. Fibrinogen was precipitated from the plasminogen-free diluted plasma by adding solid $(\text{NH}_4)_2\text{SO}_4$ to give 25% saturation. After 1 h, the precipitate was collected by centrifugation for 20 min at 4800 g (Sorvall, GSA rotor). The precipitate was dissolved in the original volume of Trasylol/phosphate buffer and the fibrinogen was reprecipitated with 25%-satd. $(\text{NH}_4)_2\text{SO}_4$ as described above. The precipitated material was dissolved in a minimal amount of the Trasylol/phosphate buffer and di-isopropyl phosphorofluoridate was added to a final concentration of 1 mM.

Gel filtration on Sepharose 6B. The dissolved $(\text{NH}_4)_2\text{SO}_4$ -precipitated material was applied to a column (100 cm $\times$ 4 cm) of Sepharose 6B equilibrated with Trasylol/phosphate buffer. Fractions with a clottability of at least 95% were pooled, treated with di-isopropyl phosphorofluoridate and stored in the frozen state.

Reduction, modification and separation of fibrinogen chains. A sample (45 ml; 1.3 mg/ml) of the pooled fibrinogen fractions after gel filtration was freeze-dried and dissolved in 4.3 ml of 8 M-urea, pH 8.5, containing 1 M-Tris/HCl and 10 mM-EDTA (final concentration 40 μM). The pH was adjusted to 8.5 and 2-mercaptoethanol was added to a final concentration of 120 mM. After incubation of the mixture for 3 h at 37°C in the dark under N_2 , (2-bromoethyl)-trimethylammonium bromide was added to a concentration of 1.2 M to thialaminylate the chains (Itano & Robinson, 1972). After incubation for 24 h, the mixture was dialysed against 5% (v/v) acetic acid and

freeze-dried. The thialaminylated chains were separated by a semi-preparative version of pore-limit polyacrylamide slab-gel electrophoresis (see below under 'Analytical methods'). Bands representing the individual chains, and visible because of the fluorescence introduced by partial dansylation (Gros & Labouesse, 1969), were cut out and extracted with 0.1% SDS. After dialysis against water, the chain preparations were freeze-dried.

Analytical methods

Determination of fibrinogen. Fibrinogen was determined gravimetrically as fibrin by the method of Astrup *et al.* (1965).

Clottability assay. Clottability was determined by measuring the A_{280} of the fibrinogen solution and filtered supernatant remaining after clotting with thrombin. The value after clotting was corrected for the thrombin without fibrinogen (Birken *et al.*, 1975).

Detection of plasminogen in rat fibrinogen. The removal of plasminogen by use of lysine-Sepharose was checked by the Ouchterlony (1968) double-diffusion test.

Factor XIII detection. The detection of traces of Factor XIII activity was carried out essentially as described by Finlayson & Morton (1972).

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis in SDS was performed as described by Weber & Osborn (1969) with and without prior reduction with 2-mercaptoethanol for 16 h at 37°C. Disc electrophoresis was carried out as described by Ornstein & Davis (1964) in the presence of 8 M-urea in 5% polyacrylamide gels at pH 8.3. Slab gels with an acrylamide concentration gradient ranging from 15 to 25% containing 0.1% (w/v) SDS were prepared by means of a Gradipore gradient maker (Gradient Pty., P.O. Box 342, Lane Cove, N.S.W. 2066, Australia). After application of the samples, the slabs were run for 24 h at 15°C in a Pharmacia gel electrophoresis apparatus GE4 with a current of 50 mA/plate. Samples were pretreated with dansyl chloride (Gros & Labouesse, 1969) to allow detection by fluorescence in u.v. light.

Amino acid analysis. Amino acid analyses were performed as described by Spackman *et al.* (1958) with a Technicon NC1 instrument, equipped with a column (length, 65 cm) of C2 resin (Technicon, Rotterdam, The Netherlands). Samples were hydrolysed at 110°C for 24, 48 and 72 h in evacuated sealed tubes containing 5.7 M-HCl. Corrections for destructive losses of threonine and serine were made by extrapolation to zero time.

N-Terminal amino acids. These were determined as described by Gray (1967) by Dr. H. M. Verhey (Department of Biochemistry, State University, Utrecht, The Netherlands).

Antisera. After collection of pre-immunization serum, adult New Zealand rabbits were injected intramuscularly (in the thighs and shoulders) with 1 ml of fibrinogen solution [1 mg of fibrinogen/ml of 0.9% (w/v) NaCl] emulsified with 1 ml of Freund's complete adjuvant. Booster injections with 0.5 ml of the antigen solution, emulsified in 0.5 ml of Freund's complete adjuvant, were given at intervals of 4 weeks. Antiserum was collected 2 weeks after the second booster injection.

Antiserum was tested in a double-diffusion test (Ouchterlony, 1968) and by immunoelectrophoresis (Grabar, 1964).

Results

Fibrinogen

Rat fibrinogen was obtained in an overall yield of 70–75% and had a clottability of at least 95%. Even

after prolonged incubation, no γ dimers or $A\alpha$ polymers could be detected. Hence rat fibrinogen was judged to be free of factor XIII (Fig. 1). It was also free of plasminogen (Fig. 2), as can be deduced from the absence of immunoreactivity with rabbit anti-(rat plasminogen). The preparation showed a single band on SDS/polyacrylamide-gel electrophoresis (Fig. 3) and on disc electrophoresis in 8M-urea (Fig. 4) without prior reduction with 2-mercaptoethanol. After reduction and thialaminylation, three distinct bands were seen (Fig. 3) after electrophoresis for 3 h.

Fig. 1. Detection of factor XIII activity in the rat fibrinogen preparations

SDS/polyacrylamide-gel electrophoresis was carried out in 10% acrylamide gels after reduction with 2-mercaptoethanol as described in the text. (1) Rat fibrinogen after gel filtration on Sepharose 6B; (2) rat fibrinogen after $(\text{NH}_4)_2\text{SO}_4$ precipitation.

Fig. 2. Detection of plasminogen-like immunoreactivity in the rat fibrinogen preparations by double immunodiffusion. Central well contained rabbit anti-(rat plasminogen). Outer wells contained diluted plasma, before lysine-Sepharose affinity chromatography (1 and 3) and after lysine-Sepharose 4B chromatography (2).

Fig. 3. SDS/polyacrylamide-gel electrophoresis of rat fibrinogen and its chains

Gels were run for 3 h at a current of 5 mA/tube as described in the text. (1) Non-reduced rat fibrinogen after Sepharose 6B chromatography in 5% acrylamide gel; (2) reduced rat fibrinogen in 10% acrylamide gel; (3) $A\alpha$ -chain of rat fibrinogen in 10% acrylamide gel; (4) $B\beta$ -chain of rat fibrinogen in 10% acrylamide gel; (5) γ -chain of rat fibrinogen in 10% acrylamide gel.

Fig. 4. Disc electrophoresis of rat fibrinogen. Disc electrophoresis was carried out in 8M-urea, pH 8.5, as described by Ornstein & Davis (1964), in 5% acrylamide gel.

Table 1. *Amino acid composition of rat fibrinogen and its constituent chains*

Tryptophan was not determined. For the chains, cysteine was determined as L-(2-[(2-amino-2-carboxyethyl)thio]-ethyl)trimethylammonium hydroxide, which emerged from the column just before ammonia.

Amino acid	Amino acid content (residues/molecule)				
	Whole fibrinogen	A α -chain	B β -chain	γ -chain	2(A α +B β + γ)
Asparagine plus aspartate	385	58	68	48	348
Threonine	218	40	38	31	218
Serine	260	39	43	33	230
Glutamine plus glutamate	321	66	59	54	358
Proline	148	23	23	15	122
Glycine	283	57	51	47	310
Alanine	123	24	26	25	150
Half-cystine	52	3	14	9	52
Valine	118	30	26	14	140
Methionine	69	13	13	12	76
Isoleucine	122	26	19	18	126
Leucine	163	35	29	30	188
Tyrosine	100	13	13	21	94
Phenylalanine	80	16	12	18	92
Lysine	214	32	33	30	190
Histidine	55	10	8	11	58
Arginine	151	28	25	18	142
No. of residues	2862	513	500	434	2894
Mol. wt.	340125	60600	58740	51840	342340

$A_{1\text{cm}}^{1\%}$ of rat fibrinogen in 0.01M-sodium acetate, pH 5.0, was 15.4 at 280 nm, according to the protein content found by amino acid analysis. Table 1 shows the amino acid composition of rat fibrinogen. Alanine was the only detectable *N*-terminal amino acid. The pI of rat fibrinogen was 5.4, as determined by isoelectric focusing in thin-layer polyacrylamide gels (Manual LKB-Producter A.B., S-16125 Bromma 1, Sweden).

Fibrinogen chains

In preliminary experiments, we found that the separation of A α - and B β -chains was not possible by ion-exchange chromatography. Therefore the separation by pore-limit polyacrylamide slab-gel electrophoresis in the presence of SDS was chosen. Fig. 3 shows that completely pure A α -, B β - and γ -chains were obtained. Their molecular weights were estimated by SDS/polyacrylamide-gel electrophoresis by using proteins of known molecular weights as reference. Rat fibrinogen A α -, B β - and γ -chains appeared to have molecular weights of 61000, 58000 and 51000 respectively. Summation of the values found for the constituent chains of rat fibrinogen thus yields a molecular weight of 340000 for whole fibrinogen. This is close to the molecular weights found for fibrinogens from other mammalian species (Doolittle, 1973). The amino acid compositions of A α -, B β - and γ -chains are listed in Table 1. In agreement with the results of Bouma & Fuller (1975), no *N*-terminal amino acid was detected in the γ -chain.

Fig. 5. Immunological cross-reactivities of monospecific rabbit anti-(rat fibrinogen) (central well) with other fibrinogens

Wells (1), (3) and (5) contained the rat fibrinogen preparation that was used as the antigen; well (2), rat plasma; well (4), bovine plasma; well (6), human plasma.

Antisera

In immunoelectrophoresis, undiluted antiserum against rat fibrinogen showed a strong immuno-

reaction with rat fibrinogen and a weak reaction with another protein present in rat plasma and in rat serum. With antiserum diluted 10-fold, this second precipitation line was not observed. Absorption of the antiserum with rat immunoglobulins G resulted in the complete disappearance of this precipitation line. This indicates that the fibrinogen preparation used as antigen and pure by all other criteria must have contained trace amounts of immunoglobulins G, although we were unable to demonstrate the presence of immunoglobulins G in this preparation immunologically. Human and bovine fibrinogen showed lines of partial identity with rat fibrinogen when tested against rabbit anti-(rat fibrinogen) (Fig. 5).

Discussion

Rat fibrinogen preparations have been described by Bouma & Fuller (1975) and Stemberger & Jilek (1976). These preparations have properties that differ from those reported for other mammalian species, particularly their low clottability (Stemberger & Jilek, 1976) and the nearly identical molecular weights found for the A α - and B β -chains (Bouma & Fuller, 1975; Stemberger & Jilek, 1976). In contrast with those reports, our method for the purification of rat fibrinogen consistently results in a high recovery of a highly clottable, stable material that can be separated after reduction into three different chains by analytical SDS/polyacrylamide-gel electrophoresis in as short a time as 3 h. The difference in molecular weight between A α and B β chains as found in the present investigation was only 3000, which is less than in other mammalian species. It is known (Collen *et al.*, 1974) that A α -chains in fibrinogen are highly susceptible to proteolytic attack. Minor proteolytic degradations further diminish the small difference in molecular weight between A α - and B β -chains, thus making the discrimination between A α - and B β -chains on analytical SDS/polyacrylamide-gel electrophoresis in a 3 h run impossible. Collen *et al.* (1974) further suggested that ϵ -aminohexanoic acid may not completely prevent degradation of fibrinogen *in vitro*, as was assumed by Bouma & Fuller (1975). Evidence supporting this suggestion was found when we purified rat fibrinogen in the presence of 1 mM- ϵ -aminohexanoic acid only (Bouma & Fuller, 1975). Under those conditions, we obtained rat fibrinogen with a shortened A α chain (Fig. 6), but with alanine as the only N-terminal amino acid.

Furthermore, Stemberger & Jilek (1976) found that the solubility of their preparation was high, giving rise to low yields when conventional precipitation methods, such as (NH₄)₂SO₄ precipitation, were applied. They obtained much higher yields by using other methods developed for the precipitation of human fibrinogen with high solubility (Jilch & Hörmann, 1974). The high solubility of some

Fig. 6. SDS/polyacrylamide-gel electrophoresis of rat fibrinogen prepared as described by Bouma & Fuller (1975) (1) Non-reduced rat fibrinogen after Sepharose 6B chromatography purified in the presence of ϵ -aminohexanoic acid (5% polyacrylamide gel); (2) the same preparation after reduction (10% polyacrylamide gel).

fractions of human fibrinogen has been attributed to the presence of partially degraded A α -chains (Pizzo *et al.*, 1972; Jilch & Hörmann, 1974; Jilch *et al.*, 1976). The rat fibrinogen preparation of Stemberger & Jilek (1976) was only clottable to the extent of 69%, which is comparable with human fibrinogen containing partially degraded A α -chains. Our preparations exhibited no precipitating and clottability properties differing from those found for native fibrinogens from other species. The findings mentioned above strongly suggest that, in the rat, the C-terminal part of the A α -chains is rapidly degraded by proteolytic enzymes. Whether this is due to the molecular structure of rat fibrinogen itself or to a proteolytic apparatus that is more readily activated in the rat than in other species is not known at present.

However, this study shows that under the conditions of the present purification procedure, it is essential to use inhibitors more effective than ϵ -aminohexanoic acid to obtain fibrinogen that is suitable for metabolic studies.

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