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PURIFICATION AND CHARACTERIZATION OF RAT PLASMA ANTITHROMBIN III

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Summary

Antithrombin III was purified from rat plasma in 70% yield by salting out with $(NH_4)_2SO_4$, affinity chromatography on heparin-Sepharose 4B, and ion-exchange chromatography on DE-52. The preparation was homogeneous as judged by polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate, by analytical ultracentrifugation, and by immunochemical analysis. It was composed of a single polypeptide chain whose molecular weight was estimated to be about 64 000 both by sodium dodecyl sulfate polyacrylamide gel electrophoresis and by sedimentation equilibrium analysis. Antithrombin III was a glycoprotein containing 3.6% glucosamine, 0.2% fucose, 2.5% mannose, 1.6% galactose and 3.9% sialic acid. Isoelectric focusing in polyacrylamide gel revealed four bands in the range of pH 4.7—4.9, indicating the microheterogeneity.

Rat antithrombin III inhibited bovine α-thrombin by forming an equimolar complex. Kinetics of this reaction were studied by gel electrophoresis in sodium dodecyl sulfate. When the inhibitor was allowed to react with an excess amount of thrombin, the initial equimolar complex with a molecular weight of 110 000 was cleaved gradually to a product with a molecular weight of 97 000, which was further cleaved to a second product with a molecular weight of 85 000.

Introduction

Human plasma has been shown to contain a number of serine protease inhibitors, which are most commonly found in α-globulin fraction [1]. Antithrombin III is unique among these inhibitors in having its action enhanced by hepa-

rin, and it inhibits a variety of plasma proteases, such as thrombin, factor IXa, factor Xa, factor XIa or factor XIIa [2]. It is suggested that antithrombin III may play an important role in the regulation of hemostatic processes. For example, familial antithrombin III deficiency was shown to be associated with increased risk of thromboembolic phenomena [3]. Robinson et al. [4] also showed that the elevated levels of this inhibitor resulted in bleeding tendency. Further studies, however, have been hampered for the lack of suitable experimental animal systems.

Recently, antithrombin III has been purified and characterized not only from human origin [5], but also from several species of mammal, such as ox [6], horse [6], dog [7] and rabbit [8]. These inhibitors are shown to resemble one another in their chemical and physicochemical properties, suggesting that these proteins may play essentially the same role in the pathophysiology of mammals. Since these large animals are not suitable for experimental studies, we attempted to develop a model system using the rat. The present paper describes the initial approach to this problem. Antithrombin III was purified from rat plasma and its chemical and physicochemical properties were determined.

Materials and Methods

Materials. Bovine fibrinogen (fraction I) and trasylol were obtained from Sigma Chem. Co. Bovine α-thrombin was prepared from topical thrombin (Parke-Davis Co.) by chromatography on two successive columns [9,10]. Heparin-Sepharose 4B was prepared by the method of Danishefsky et al. [11].

Purification of rat plasma antithrombin III. Rat blood was collected in plastic tubes containing trasylol (400 Kallikrein inhibitor units/ml), 1% EDTA, and 0.9% NaCl in a final ratio of 1 : 10, and the plasma was separated by centrifugation. Antithrombin III was absorbed onto heparin-Sepharose 4B from the plasma supernatant after precipitation at 40% saturation with $(NH_4)_2SO_4$, and then eluted with 50 mM Tris-HCl (pH 7.5) containing 1.5 M NaCl, subsequent to prior elution with the same buffer containing 0.4 M NaCl. The eluate was further chromatographed on DEAE-cellulose column using a linear gradient of 0—0.2 M NaCl in 50 mM Tris-HCl (pH 7.5). Antithrombin III was eluted as a single peak at approx. 0.1 M NaCl.

Assay of biologic activities. Fibrinogen-clotting activity was determined by the method of Lundblad et al. [12]. The standard curve was obtained from determining the clotting time of various concentrations of α-thrombin. One arbitrary unit was defined as that amount of thrombin which clotted 0.3 ml of the fibrinogen solution in 15 s at 37° C. Antithrombin activity was measured in a two stage assay similar to that of Rosenberg and Damus [5]. One inhibitory unit (I.U.) was defined as that amount of antithrombin III which inhibited one unit of α-thrombin.

Estimation of protein. Protein concentrations were determined spectrophotometrically at 280 nm. The absorption coefficient, $A^{1\%}_{1cm}$ at 280 nm, for bovine α-thrombin was assumed to be 17.9 [13] and that for rat antithrombin III was estimated as 5.9 (see Results).

Electrophoresis. Polyacrylamide gel electrophoresis in the presence and

absence of sodium dodecyl sulfate was carried out as described by Weber and Osborn [14] and by Davis [15], respectively. Analytical isoelectric focusing was carried out in cylindrical polyacrylamide gels containing 7.5% acrylamide and 2% LKB 3/10 carrier anpholyte as described by Righetti and Drysdale [16].

Chemical, ultracentrifugal, and immunochemical analyses. Experimental details for the determinations of amino acid and carbohydrate compositions, analytical ultracentrifugation, and immunochemical analyses were described previously [17].

Results

Purification of rat antithrombin III

Table I summarizes the results of a purification procedure. Starting from the $(NH_4)_2SO_4$-fractionated plasma, a purification of about 1600-fold was achieved with a recovery of about 70%. The purified preparation of antithrombin III showed a single band after polyacrylamide gel electrophoresis under non-denaturing conditions (Fig. 1A). Sodium dodecyl sulfate polyacrylamide gel electrophoresis before and after reduction also showed a single band (Fig. 1B). Virtually no change in electrophoretic mobility was observed after reduction, suggesting that rat antithrombin III was composed of a single polypeptide chain. When subjected to ultracentrifugal analysis with schlieren optics, this preparation sedimented as a single symmetrical peak, of which sedimentation coefficient, $s^0_{20,w}$, was estimated to be 4.2 S. Upon double immunodiffusion, the preparation gave a single precipitin line with an antiserum developed in the rabbit. These data indicate the homogeneity of this material, which was used for subsequent studies.

Molecular weight of rat antithrombin III

Sedimentation equilibrium analyses showed the linear relationship between logarithms of concentration and squares of radial distance. Molecular weight was estimated to be 64 400, assuming a partial specific volume of 0.722 ml/g, which was calculated from amino acid and carbohydrate composition. Essentially the same value, i.e. 64 000, was obtained when molecular weight was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Absorption coefficient

The ultraviolet absorption spectrum of rat antithrombin III in 50 mM Tris-

TABLE I

PURIFICATION OF ANTITHROMBIN III FROM RAT PLASMA

Step	Total protein (mg)	Total activity (I.U.)	Specific activity (I.U./mg protein)	Purification (-fold)
1. Plasma $(NH_4)_2SO_4$ supernatant	16 766.2	3040	0.18	1
2. Heparin-Sepharose 4B	12.9	2512	195	1082
3. DE-52	7.8	2250	288	1599

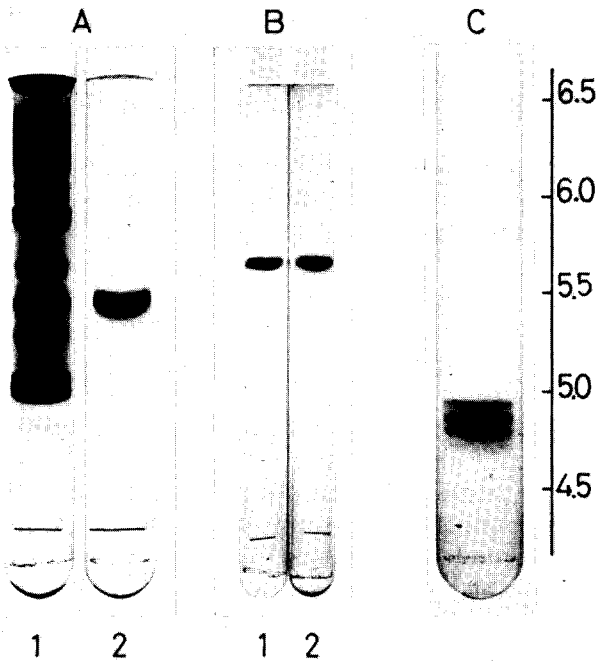

Fig. 1. Gel electrophoresis of rat antithrombin III. (A) Polyacrylamide gel electrophoresis of rat plasma and the purified antithrombin III. A standard polyacrylamide disc gel electrophoresis (7.5% gel and Tris/glycine buffer, pH 8.3) was used. Electrophoresis was performed at 3 mA per gel for 1 h. Gel 1, diluted plasma (100 μg of protein); gel 2, purified antithrombin III (10 μg of protein). Protein bands were stained with Coomassie brilliant blue. The wire inserted at the bottom of the gel shows the position of tracking dye. (B) Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the purified antithrombin III. 10 μg of antithrombin III (15 μl) were mixed with 15 μl of 20 mM sodium phosphate buffer (pH 7.2) containing 2% sodium dodecyl sulfate and 50% glycerol and heated at 100°C for 5 min in the presence and absence of 2.5% 2-mercaptoethanol. The sample was then applied on a 5.0% gel column, and electrophoresis was performed at 7 mA per gel for 4 h. Gel 1, non-reduced antithrombin III; gel 2, reduced antithrombin III. Protein bands were stained with Coomassie brilliant blue. The wire inserted at the bottom of the gel shows the position of tracking dye. (C) Gel isoelectric focusing of rat antithrombin III. Antithrombin III (20 μg of protein) was subjected to isoelectric focusing. After 12 h at 450 V (4°C) one gel of each pair was stained with bromophenol blue, and the other was cut into 1 mm sections. Each slice was soaked overnight in 1 ml of water, and the pH of each solution was measured. Arabic numerals at right side indicate pH gradient along the gel.

HCl buffer (pH 7.5) containing 0.15 M NaCl showed a maximum at 279—280 nm. The ratio of the absorbance of antithrombin III at 280 nm to its absorbance at 260 nm was 1.37. An absorption coefficient ($A^{1\%}_{1cm}$, at 280 nm) was found to be 5.92.

Isoelectric point of rat antithrombin III

The microheterogeneity of rat antithrombin III was found by isoelectric focusing in polyacrylamide gel (Fig. 1C). Antithrombin III formed four bands in the region of pH 4.7—4.9.

Amino acid and carbohydrate composition

The chemical compositions of rat antithrombin III are shown in Table II, together with comparative values for human, bovine and horse antithrombins

TABLE II

AMINO ACID AND CARBOHYDRATE COMPOSITION OF RAT ANTITHROMBIN III IN COMPARISON WITH VALUES REPORTED FOR OTHER SPECIES

Except where noted, the values for amino acids for the rat are the averages of 24, 48, and 72-h hydrolyses. Data in parentheses indicate g/100 g of glycoprotein. Data for human, bovine and horse are from Kurachi et al. [6].

Components	mol/mol glycoprotein			
	Rat	Human	Ox	Horse
Amino acid				
Lys	28.7 (6.52)	36.7	32.0	29.9
His	3.6 (0.86)	5.3	6.4	7.7
Arg	17.5 (4.73)	23.5	23.2	19.6
Asp	44.8 (9.25)	47.7	45.5	40.5
Thr *	19.7 (3.64)	25.5	28.1	23.4
Ser *	28.8 (4.70)	32.9	33.9	27.1
Glu	47.9 (10.9)	54.1	43.9	48.1
Pro	15.8 (2.82)	21.9	18.2	20.5
Gly	16.0 (1.86)	19.1	18.1	16.3
Ala	21.2 (2.93)	32.5	29.0	25.8
Cys 1/2 **	4.3 (1.14)	6.1	6.3	6.1
Val	30.1 (5.48)	27.5	29.2	19.9
Met	7.0 (1.61)	10.9	8.9	9.3
Ile	19.2 (3.91)	21.1	24.5	18.1
Leu	37.4 (7.61)	39.5	42.4	29.6
Tyr	8.5 (2.39)	7.3	9.6	8.9
Phe	34.9 (8.94)	33.7	34.0	25.8
Try ***	6.6 (1.90)	6.3	5.8	7.9
Carbohydrate				
Man	9.0 (2.52)			
Fuc	0.6 (0.15)			
Gal	5.8 (1.62)			
GlcN	12.9 (3.59)			
NeuN	8.1 (3.90)			

* Extrapolated to zero hydrolysis time.
** Determined as cysteic acid in the performic acid-oxidized sample.
*** Determined spectrophotometrically.

III [6]. Rat antithrombin III was found to have a total carbohydrate content of 11.8%, consisting of mannose, fucose, galactose, glucosamine and sialic acid. While traces of xylose and glucose were detected, galactosamine was not detected. The amino acid compositions of rat antithrombin III is very similar to that of antithrombin III isolated from human, bovine and horse.

Interaction of bovine α-thrombin with rat antithrombin III

The reaction of rat antithrombin III with bovine α-thrombin was followed by assaying the remaining thrombin activity. Time courses for the inhibition of α-thrombin by various amounts of antithrombin III are shown in Fig. 2A. At initial time period, the amounts of inactivated thrombins increased linearly with time and then levelled off. The maximum inhibition was also linear with the concentration of antithrombin III up to 0.5 μM (Fig. 2B). Extrapolation of the linear portion of the curve to 100% inhibition gives a value of 0.88 μM (Fig. 2B), which is close to the α-thrombin concentration, i.e. 0.97 μM, employed in

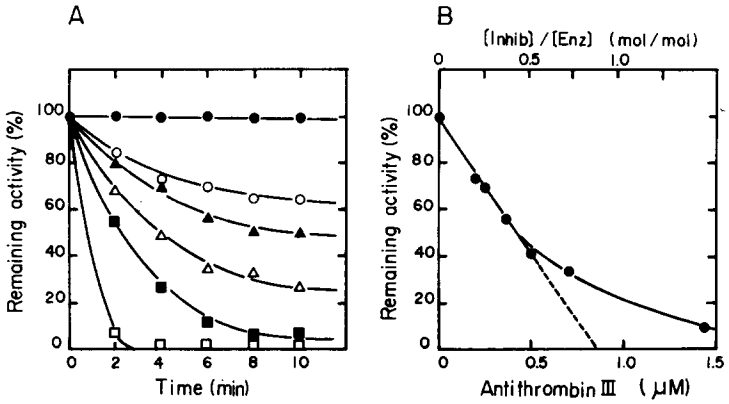

Fig. 2. Inhibition of α-thrombin by antithrombin III. (A) Time course of inhibition reaction. The reaction mixture in a total volume of 0.2 ml contained 0.97 μM α-thrombin and none (●), 0.25 μM (○), 0.36 μM (▲), 0.71 μM (△), 1.44 μM (■) and 3.60 μM (□) antithrombin III. At various time periods of incubation, aliquots (20 μl) were removed and assayed for thrombin-clotting activity as described in the text. (B) Inhibition of α-thrombin-clotting activity as a function of antithrombin III concentration. Activities remaining after 6 min incubation are plotted against the concentration of antithrombin III. The linear portion of the curve is extrapolated to obtain 100% inhibition value (- - - - -).

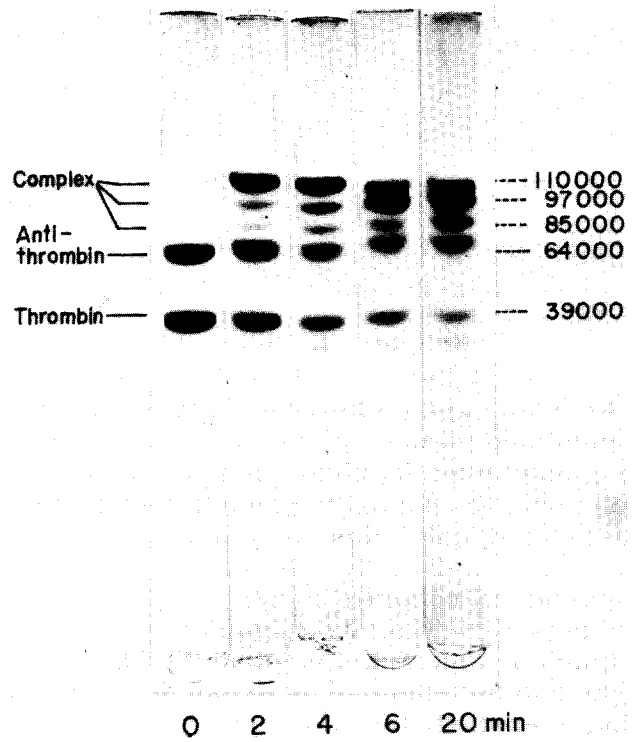

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoretic analysis of interaction of α-thrombin and antithrombin III. A mixture of α-thrombin and antithrombin III (molar ratio 1 : 1) was incubated at 37°C under the same conditions as in Fig. 2. Aliquots (15 μl) were removed at given times and subjected to electrophoresis under non-reduced condition as described in the legend to Fig. 1B. An estimate of molecular weight for each band is indicated on the right.

these experiments. These data suggest that antithrombin III inhibits α-thrombin by the formation of a 1 : 1 complex. When these experiments were carried out in the presence of heparin (10 μg/0.2 ml of reaction mixture), the reaction was completed within 1 min. Heparin had no effect, however, on the stoichiometry of this complex formation (data not shown).

Kinetics of this complex formation was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Figs. 3 and 4). When equimolar amounts of antithrombin III and α-thrombin were mixed, a new slow-moving band with a molecular weight of approx. 110 000 was distinctly observed at short incubation time. This suggests the formation of a stable complex, which was composed of 1 mol of α-thrombin and 1 mol of antithrombin III since the molecular weight of the complex is close to the summation of those of two proteins. On longer incubation, however, this complex decomposed to two products with molecular weight of approx. 97 000 and 85 000, respectively (Fig. 3). When antithrombin III was added in excess, both an initial and a partially

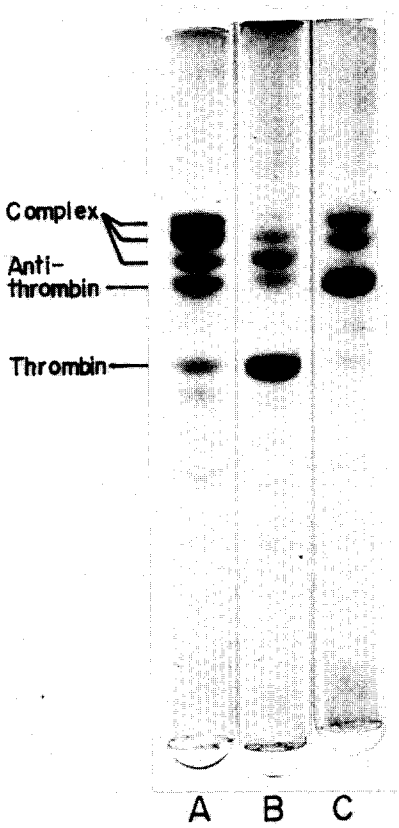

Fig. 4. Sodium dodecyl sulfate polyacrylamide gel electrophoretic analysis of interaction of α-thrombin and antithrombin III at various molar ratios. α-Thrombin and antithrombin III solutions were mixed, incubated at 37°C for 20 min, and subjected to electrophoresis under non-reduced condition as described in the legend to Fig. 1B. The molar ratios of α-thrombin and antithrombin III were 1 : 1 in gel A; 3 : 1 in gel B, and 1 : 3 in gel C.

degraded complexes were found (Fig. 4). On the other hand, when α-thrombin was present in excess, both the initial and partially degraded complexes disappeared but a complex with molecular weight of approx. 85 000 was formed (Fig. 4). These results suggest that the degradation of the complex was due to the limited proteolysis by free enzyme in the reaction mixture.

Discussion

The present results indicate that sufficient amounts of highly purified antithrombin III can be prepared from rat plasma with heparin-Sepharose affinity chromatography followed by ion-exchange chromatography on DE-52. Recently, van Ruijven-Vermeer and Nieuwenhuizen [18] demonstrated that minor proteolytic degradation which sometimes occurs during the purification of plasma proteins was completely prevented by adding trasylol and EDTA. Accordingly, in the present studies, we prepared plasma using trasylol and EDTA as anticoagulant. Judging from the results of sodium dodecyl sulfate (Fig. IA and B) and immunochemical analysis, it seems unlikely that rat antithrombin III obtained in the present study was subjected to proteolytic modification.

Some physicochemical properties of rat antithrombin III are summarized in Table III, together with comparative values for the human, bovine, equine, canine, and rabbit antithrombins III. They are glycoproteins containing 9—16% carbohydrates, and resemble one another not only in physicochemical properties but in chemical compositions (Table II). The molar ratio of each monosaccharide of rat antithrombin III is very similar to that of most plasma glycoproteins [19], suggesting that the rat inhibitor has a complex type of oligosaccharide unit. Danishefsky et al. [20] reported that human antithrombin III was complexed tightly with β-glucosylceramide which was extractable with lipid

TABLE III

SOME PHYSICOCHEMICAL PROPERTIES OF RAT ANTITHROMBIN III IN COMPARISON WITH VALUES REPORTED FOR OTHER SPECIES

The data for human antithrombin III have been compiled from Kurachi et al. [6], Danishefsky et al. [20] and Nordenman et al. [21]; those for bovine antithrombin III from Kurachi et al. [6] and Nordenman et al. [21]; those for equine antithrombin III from Kurachi et al. [6]; those for canine antithrombin III from Damus and Wallace [7], and those for rabbit antithrombin III from Yin et al. [8].

Property	Rat	Human	Ox	Horse	Dog	Rabbit
Molecular weight	64 000	58 000	57 000	53 000	77 000	67 000
$s^0_{20,w}$ (S)	4.2	4.2	4.1			
Partial specific volume (ml/g)	0.72	0.72	0.72	0.71		
$A^{1\%}_{1cm}$ at 280 nm	5.9	5.7	6.0	7.7	8.6	
A_{280}/A_{260}	1.37					
Ratio:polar/apolar amino acid *	1.37	1.34	1.29	1.49		
p*I*	4.7—4.9	4.9—5.3	4.5—5.0			
Carbohydrate content (%)	11.8	9.0—10.4	9.9—11.5	15.9		

* Ratios of polar/apolar amino acids were calculated as described by Hatch [23].

solvents. Since traces of glucose were also detected in rat antithrombin III, the existence of such glycolipid must be explored.

Rat antithrombin III migrated on isoelectric focusing in polyacrylamide gel as four narrow bands, having isoelectric points of pH 4.7—4.9 (Fig. 1C). The similar observations have been made for human antithrombin III [21] which showed 2—4 bands and for bovine antithrombin III [21] which showed 4—6 bands. These bands of human antithrombin III were converted to a single homogeneous component by treatment with neuraminidase [22]. Although the origin of the microheterogeneity of rat antithrombin III is not clear at present, it may be due to the presence of variable amounts of sialic acid.

In the present experiments, the formation of a stable complex between bovine α-thrombin and rat antithrombin III was shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The stability of the complex in the presence of 1% sodium dodecyl sulfate (pH 7.2) at 100°C for 5 min suggests that it may involve a covalent bond between α-thrombin and antithrombin III. Rosenberg and Damus [5] reported a similar undissociable equimolar complex of human antithrombin III and thrombin. The present results show that the molecular weight of the complex is 110 000 (Fig. 3), which is very close to the summation of molecular weights of both proteins. Results obtained with experiments in which the clotting activity of α-thrombin was examined at increasing concentrations of antithrombin III also suggested a formation of 1 : 1 complex between enzyme and inhibitor (Fig. 2). Figs. 3 and 4 indicate that the complex with a molecular weight of 110 000 was degraded to a product of molecular weight 97 000, which was further degraded to a second product of molecular weight of 85 000 by the action of free enzyme. Similar proteolytic degradation of the enzyme-inhibitor complex was also found in the reaction of human antithrombin III and thrombin [5].

Thus, the present results on the isolation and characterization of rat antithrombin III revealed that the rat can be used as an experimental model for studying the biological roles of antithrombin III in a number of pathological or physiological processes.

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