ISOLATION OF ANTITHROMBIN III FROM HUMAN PLASMA: ITS SEPARATION FROM α_1-ANTITRYPSIN[1]

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Summary

Plasma was adsorbed with $Al(OH)_3$ in a ratio of 8 : 2. The gel was washed free of entrapped plasma and antithrombin III and α_1-antitrypsin eluted by repeated washing with 0.36 M ammonium phosphate, pH 8.1. The crude inhibitor preparation was subjected to chromatography on QAE-Sephadex A-50 at pH 8.0, followed by gel filtration on Sephadex G-200. In these two preparative steps the two inhibitors eluted together. However, they were separated by rechromatography on QAE-Sephadex at pH 7.4, following which they were recovered in highly purified form, α_1-antitrypsin by passage through concanavalin-A-Sepharose and antithrombin III through heparin-Sepharose.

Introduction

Antithrombin activity in human serum was first observed by Morawitz in 1905 (1). Brinkhouse et al. in 1939 (2) first noted that heparin was effective as an anticoagulant only in the presence of a plasma factor. Subsequently, an intimate relationship was described between the antithrombin activity of plasma and heparin (3, 4), since heparin was found to enhance up to 100-fold the thrombin neutralizing activity of antithrombin. Concentrated but impure preparations of antithrombin III had been available for some time (5, 6), but pure inhibitor preparations have not been prepared until recently (7, 8, 9, 10). By sucrose density gradient ultracentrifugation antithrombin III has a sedimentation coefficient of 3.8 (unpublished observations). Its isoelectric point is 5.11. Its molecular weight was estimated to be 65,000 (8) or 63,200 (9). It contains 13.4% carbohydrate (8).

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Antitryptic activity of serum was first reported by Ferni and Pernosi in 1894 (11). About 90% of the antitryptic activity is localized in the α_1-globulin zone and the bulk of this is due to α_1-antitrypsin. In recent years several investigators reported the isolation of highly purified preparations of α_1-antitrypsin (8, 12, 13, 14, 15, 16, 17). This inhibitor has an S-value of approx. 3.8, a pI of 4.9, a diffusion coefficient of 5.2, a partial specific volume of 0.646 and an extinction coefficient of 5.3. The molecular weight was first estimated to be 45,000 but more recent data indicate 53,000-54,000. It contains 12.2% carbohydrate.

In our first studies we succeeded in separating antithrombin III and α_1-antitrypsin by chromatography on QAE-Sephadex, Sephadex G-200 and SP-Sephadex from kininogens (18), but the two inhibitors could only be separated from each other by preparative electrophoresis in Pevicon (19). Following the publication of Rosenberg and Damus (9) we separated the two inhibitors by isoelectric focusing (20, 21). To our knowledge recent investigators who took advantage of the adsorption of antithrombin III onto $Al(OH)_3$ did not take into account the adsorption of α_1-antitrypsin to the gel. The latter inhibitor desorbs together with antithrombin III and is present in the eluates. Since the yield by isoelectric focusing is relatively poor and only small quantities can be prepared, attempts were made to isolate and separate antithrombin III and α_1-antitrypsin by a series of chromatographic steps. Chromatography on QAE-Sephadex at pH 7.4 separates the two inhibitors and each can then in turn be obtained in highly purified form following passages through concanavalin-A-Sepharose and heparin-Sepharose respectively.

Materials and Methods

Preparation of plasma. Initially the inhibitors were isolated from defibrinated plasma, according to Rosenberg and Damus (9), as previously described (20, 21).

In subsequent studies when the inhibitors were isolated from large quantities of plasma obtained for the isolation of factor XII, prekallikrein and plasminogen, the defibrination and adsorption with Ba^{++} carbonate steps were omitted. The plasma obtained from the Canadian Red Cross was centrifuged at 3050g for 30 minutes and the precipitates discarded. Varying amounts of plasma were used ranging between 500 and 4000 ml. The plasma was adsorbed with $Al(OH)_3$ (Amphogel, unflavoured, Wyeth Laboratories, Windsor, Ont.) in a ratio of 2 parts gel to 8 parts plasma (v/v). The Amphogel was stirred gently for 15 minutes at 24° and then separated by centrifugation (2000g for 15 minutes). The gel precipitates were washed with cold 0.15 M NaCl, recentrifuged and the protein eluted with 0.36 M ammonium phosphate,

pH 8.1. The elution was twice repeated, the eluates concentrated to one-tenth of the initial plasma volume and dialysed against the starting buffer of the first chromatographic step.

Chromatography. All glassware was siliconized and plastic test tubes and pipettes were used. Depending on the protein concentration, columns of varying sizes were packed with QAE-Sephadex A-50, equilibrated with 0.1 M Tris-HCl, pH 8.0. The dialysed $Al(OH)_3$ eluates were chromatographed with either a gradient or with steps. In case of a gradient the starting buffer and the same buffer containing 0.2 M NaCl as the limiting buffer were used. With stepwise elution (with large volumes) 0.075 M and 0.12 M NaCl respectively were added to the equilibrating buffer.

Elution of antithrombin III and α_1-antitrypsin in all chromatographic procedures was monitored with (a) immunodiffusion, using monospecific antibody against the inhibitors (Behringwerke, Hoechst Pharmaceuticals, Toronto, Ont.) and (b) inhibition of thrombin and trypsin respectively. Chromatographic fractions were added to thrombin (Park Davis, 1000 units/ml; Detroit, Mich.) or trypsin (Worthington; TPCK 200 U/mg; Freehold, N. J.) and the inhibition of the arginine esterase activity of these enzymes determined. In the assay 4 NIH units of thrombin hydrolysed approximately 260 mμ moles of BAEe and 3.3 μg of trypsin hydrolysed the same amount of synthetic ester. The results are expressed as % inhibition.

For gel filtration fractions containing the inhibitors were pooled and concentrated by positive pressure ultrafiltration (PM-10 membrane, Amicon Corp., Lexington, Mass.). In the case of small volumes 25 ml was applied to a 5 x 100 cm column (Pharmacia, Toronto, Ont.) and recycled through a second column of the same size. The columns were packed with Sephadex G-200 fine, equilibrated with 0.5 M NaCl in 0.1 M Tris-HCl, pH 8.0. Larger volumes (150-200 ml) were applied to a 10 x 100 column and recycled. The elution of these fractions was monitored as described above.

Pooled fractions were again concentrated, dialysed against 0.05 M Na phosphate buffer, pH 7.4, containing 0.02 M NaCl. Columns were packed with QAE-Sephadex, equilibrated with the phosphate buffer and the samples were applied. Elution was carried out with a gradient, the limiting buffer being phosphate containing 0.12 M NaCl.

The α_1-antitrypsin-containing fractions were subsequently passed through a QAE-Sephadex column equilibrated with 0.1 M Tris-HCl, pH 9.0, the limiting buffer of the gradient being 0.2 M NaCl.

The α_1-antitrypsin obtained with QAE-Sephadex (pH 9.0) was passed through columns of concanavalin-A, linked to Sepharose 4B, according to Liener et al. (15), whereas those containing antithrombin III were chromatographed on a heparin-Sepharose 4B column by the procedure of Damus and Wallace (10), slightly modified. The 0.3 M NaCl step was omitted, i.e. the gradient followed the equilibrating buffer. The heparin linked to Sepharose 4B was obtained from Wilson Chemical Corp., Chicago, Ill. and further purified by the method of Iverius (22). Some columns were prepared by the method of Miller-Anderson et al. (23).

Additional procedures. Protein elution was monitored spectrophotometrically at 280 nm and the concentration of pooled partially or highly purified fractions determined by the method of Lowry et al. (24). The method of Mancini et al. (25) was used to ascertain the concentration of the inhibitors (M-Partigen plates, Behringwerke). Polyacrylamide disc gel electrophoresis was carried out at alkaline pH by the method described by Davies (26). Sodium dodecyl sulfate disc gel electrophoresis was done according to Weber and Osborn (27). Immunodiffusion and immunoelectrophoresis was done by standard procedures (28).

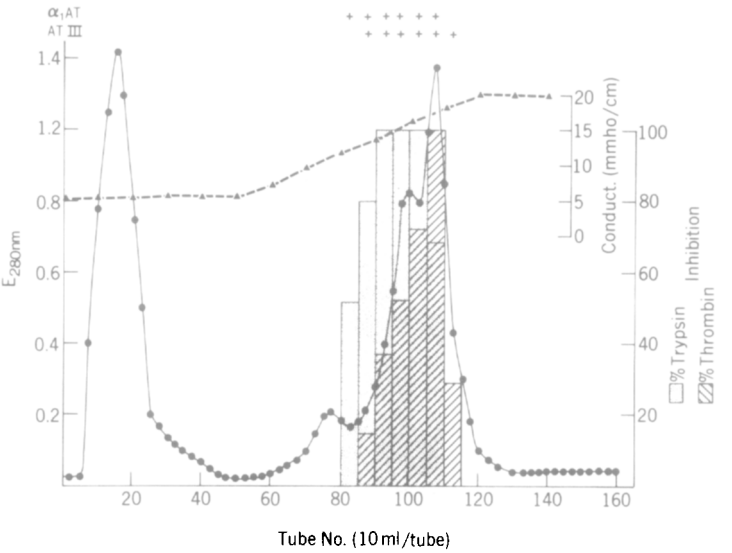

Figure 1. Chromatography of 50 ml of $Al(OH)_3$ eluates of human plasma (total O.D. units 2900) on a 2.5 x 20 cm column of QAE-Sephadex A-50, equilibrated with 0.1 M Tris-HCl (pH 8.0). Elution was carried out with a linear gradient, the limiting buffer containing 0.2 M NaCl. The elution was monitored by immunodiffusion, as shown on the top of the illustration and by inhibition of trypsin and thrombin respectively, represented by the bars (see Materials and Methods).

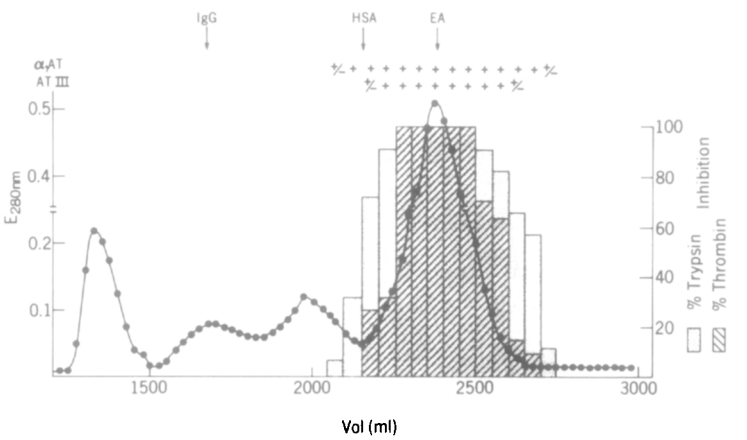

Figure 2. Gel filtration on Sephadex G-200 of inhibitor-containing fraction obtained by QAE-Sephadex. Two interconnected columns (5 x 92 and 5 x 89 cm) were packed with the gel equilibrated with 0.1 M Tris-HCl, containing 0.5 M NaCl. The volume of the applied sample was 25 ml and the total O.D. units 210. The elution volume of the markers immunoglobulin G (IgG), human serum albumin (HSA) and egg-albumin (EA) are shown by arrows.

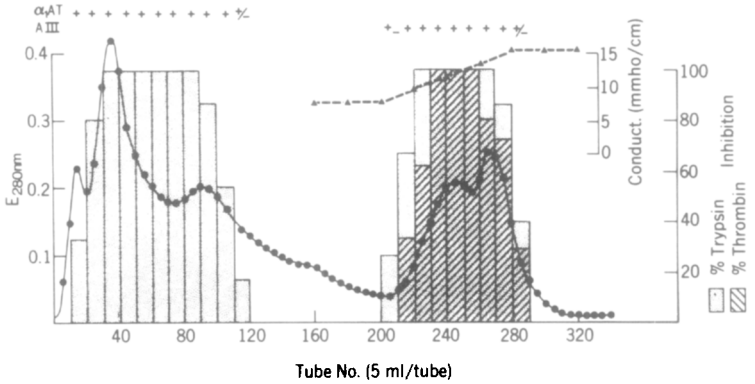

Figure 3. Rechromatography of sample recovered by Sephadex G-200 (total O.D. 90 units) on a 2.5 x 5 cm column of QAE-Sephadex A-50, equilibrated with 0.05 Na phosphate buffer containing 0.02 M NaCl (pH 7.4). Alpha$_1$-antitrypsin eluted in the excluded peak and antithrombin III with a gradient, the limiting buffer containing 0.12 M NaCl.

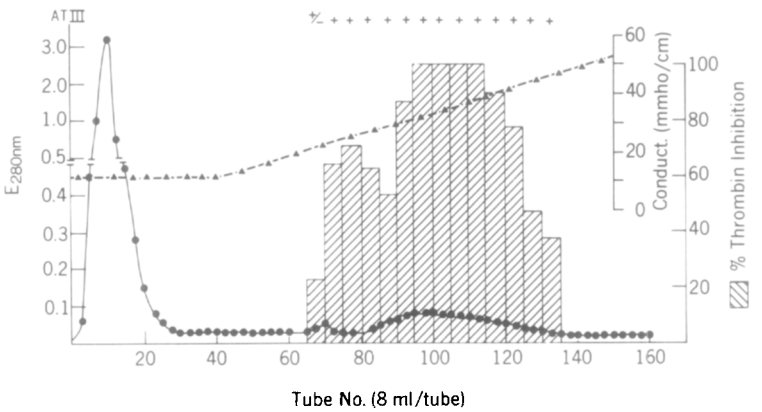

Figure 4. Chromatography of partially purified antithrombin III (total O.D. 160 units) on a 1.5 x 8 cm column of heparin-Sepharose 4B. The column was equilibrated with 0.1 M Tris-HCl, containing 0.145 M NaCl (pH 7.4), followed by a gradient with the limiting buffer containing 0.8 M NaCl.

Results

The Al(OH)$_3$-eluates were extensively dialysed against 0.1 M Tris-HCl, pH 8.0, until the conductivity of the sample reached that of the Tris-buffer. QAE-Sephadex A-50 columns were equilibrated with the above Tris-buffer and the samples applied. After eluting the excluded peak, a linear gradient between the starting buffer and 0.2 M NaCl was applied to the

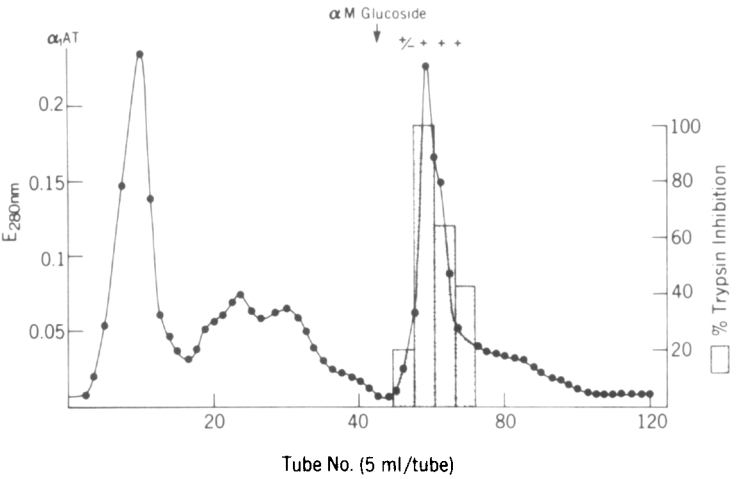

Figure 5. A 1.5 x 5 cm column was packed with concanavalin-A-Sepharose 4B equilibrated with 0.05 M Na phosphate buffer, containing 0.1 M NaCl, pH 7.4. When the protein had reached baseline 0.1 M α-methyl glucoside in phosphate buffer and applied with the α_1-antitrypsin eluted.

column (Fig. 1). More recently, with larger volumes, the protein was eluted in steps (0.075, 0.12, 0.2 M NaCl), the antithrombin III and α_1-antitrypsin eluting with 0.12 M NaCl. After concentrating the fractions containing the two inhibitors by positive pressure ultrafiltration, aliquots were applied to a column of Sephadex G-200 and recycled through a second similar column (Fig. 2). Some of this material was passed directly through a heparin-Sepharose column. However, in order to separate α_1-antitrypsin the sample containing the two inhibitors had to be rechromatographed on QAE-Sephadex. After several pilot runs 0.05 M Na phosphate, 7.4 was found to separate the two inhibitors, as shown in Figure 3. The remaining protein contaminants were separated from the antithrombin III on a column of heparin-Sepharose (Fig. 4). The α_1-antitrypsin (see Fig. 3) was passed through a third column of QAE-Sephadex equilibrated with 0.1 M Tris-HCl (pH 9.0) containing 0.05 M NaCl. All protein adsorbed to the column and was eluted with a linear gradient to 0.2 M NaCl. Two peaks eluted, the α_1-antitrypsin eluting in the first, smaller peak. This material was passed through a column of concanavalin-A-Sepharose. As shown in Figure 5 the inhibitor eluted in a sharp peak after application of α-methyl glucoside.

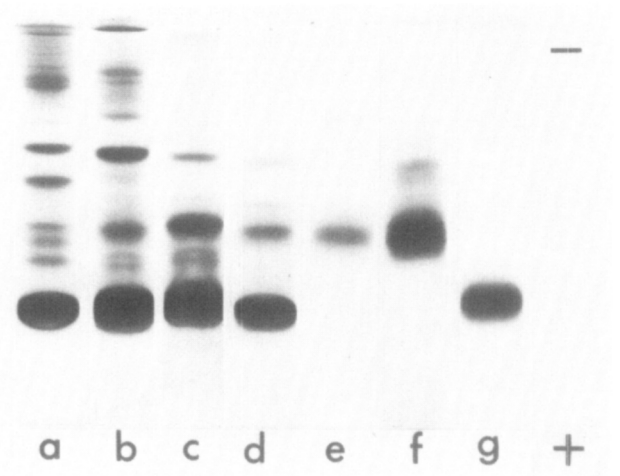

Figure 6. Alkaline disc gel electrophoretograms of the purification steps shown in Figures 1-5. Gels a, b, c and d represent $Al(OH)_3$ eluates, QAE-Sephadex at pH 8.0, Sephadex G-200 and the partially purified antithrombin preparation obtained with QAE-Sephadex at pH 7.4. The protein in these 4 gels was adjusted to approximately the same O.D. and 100 µl applied to the gels. Gel e represents the antithrombin obtained by heparin-Sepharose, of which 15 µg was applied in 50 µl, whereas gel f represents another similar preparation (QAE-Sephadex at pH 7.4 omitted) of which about 60 µg was applied in 100 µl. Gel f is the α_1-antitrypsin obtained by Con-A-Sepharose (48 µg in 50 µl).

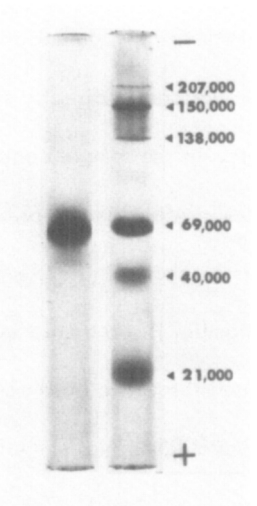

Figure 7. SDS-disc gel electrophoresis of antithrombin III (40 µg) and of markers (from the bottom of the gel): soybean trypsin inhibitor, aldolase, human serum albumin (monomer), albumin (dimer), human IgG and albumin (trimer).

TABLE 1

Purification of Antithrombin III

Sample	Total Protein (μg)	Total Activity (Units)	Sec. Activity (Units/Protein)	% Recovery	Purification Factor
$Al(OH)_3$-eluates	700,000	8,100	0.011	100.00	1.00
QAE-Sephadex (pH 8.0)	109,400	8,800	0.080	100.00	7.27
Sephadex G-200	49,000	7,050	0.143	80.0	13.00
QAE-Sephadex (pH 7.4)	15,000	4,320	0.288	61.2	26.18
Heparin-Sepharose	1,200	2,600	2.16	60.1	196.30

1 unit = absorbancy change of 0.01 optical density units per minute at 253 nm.

The electrophoretograms of the various purification steps are shown in Figure 6 and the antithrombin with marker proteins on SDS-gels in Figure 7. In various runs the molecular weight of antithrombin III ranged between 65,000-69,000.

Table 1 shows the isolation of antithrombin III from $Al(OH)_3$-eluates of 1 liter of human plasma.

Based on radial immunodiffusion of the $Al(OH)_3$-eluates the ratio of antithrombin III to α_1-antitrypsin was 1 : 1. The final recovery of α_1-antitrypsin averaged 50-60%.

Discussion

In recent years antithrombin III has been obtained in highly purified form (7,8,9,10,23). The same is true of α_1-antitrypsin (8,13,14,15,16,17). Yet there is some controvesy about their inhibitory activity. While it is generally accepted that both inhibitors inhibit trypsin, there are some conflicting reports on the ability of α_1-antitrypsin to inhibit thrombin (reviewed in Ref. 29). Antithrombin III is an excellent inhibitor of plasmin (30), whereas α_1-

antitrypsin is a poor inhibitor of this protease (21). Furthermore, antithrombin III, particularly in the presence of heparin, readily inhibits plasma kallikrein (20). On the other hand, α_1-antitrypsin, even after prolonged exposure, as suggested by Fritz et al. (31), does not inhibit kallikrein (21).

It is thus conceivable that the apparent inhibition by α_1-antitrypsin of enzymes such as thrombin, plasmin and plasma kallikrein, as reported by some investigators, was due to contamination of the preparations with antithrombin III. As described in the accompanying paper, antithrombin inhibits activated factor XII, but α_1-antitrypsin has no inhibitory effect (32). Heimburger et al. (8) reported that preparative electrophoresis was an essential step in the isolation of antithrombin III. An observation which we could confirm (19). Rosenberg and Damus (9) had to use isoelectric focusing to obtain a homogeneous preparation of antithrombin III, although they did not state whether they separated α_1-antitrypsin at that or at an earlier step. In our earlier studies (21) isoelectric focusing was found to be an essential step in the separation of α_1-antitrypsin from antithrombin III. As shown in this paper separation of the two inhibitors occurs readily by anion exchange chromatography under specific conditions. Once separated, each inhibitor can be obtained in homogeneous form by affinity chromatography.

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