SUBSTRATES OF HAGEMAN FACTOR

I. Isolation and Characterization of Human Factor XI (PTA) and Inhibition of the Activated Enzyme by α_1-Antitrypsin

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The contribution of a coagulation factor to the enzymatic cascade known as the intrinsic coagulation pathway is dependent upon its rate of activation and the rate of destruction of the activated enzyme. Considerable progress has recently been made in our understanding of the mechanism of activation and inhibition of coagulation Factor XI or partial thromboplastin antecedent (PTA).[1] Activation of the precursor form (Pre-PTA) is dependent upon contact activation of Hageman factor (1) as well as upon a feedback mechanism in which a second Hageman factor substrate, prekallikrein, is activated to kallikrein (2) which in turn further activates Hageman factor (3). In the absence of this intrinsic control (Fletcher factor or prekallikrein deficiency) (4), the rate of activation of Hageman factor and, in turn, pre-PTA is diminished (5). Once activated, PTA is known to be inhibited by the inhibitor of the activated first component of complement (CĪ INH) (6) as well as by antithrombin III (7).

In this manuscript we describe a method for the purification of pre-PTA which separates it from other identifiable proteins and demonstrate that it is directly converted to activated PTA by incubation with trace quantities of activated Hageman factor or Hageman factor prealbumin fragments. When we sought the plasma inhibitors of activated PTA, a new α_1-globulin PTA inhibitor of mol wt 65,000 was isolated and identified as α_1-antitrypsin.

Materials and Methods

Bradykinin triacetate (Sandoz Pharmaceuticals, Ltd., Basel, Switzerland) was used as the standard for native bradykinin. Hexadimethrine bromide (Aldrich Chemical Co., Inc., Milwaukee, Wis.), enzodiffusion fibrin plates and streptokinase (Hyland Division, Travenol Laboratories, Inc., Costa Mesa, Calif.), hemostatic phosphatide (cephalin) and benzoyl-d,l-arginine-p-nitroanilide (Nutritional Biochemical Corp., Cleveland, Ohio), α_1-antitrypsin immunodiffusion plates (Meloy Laboratories, Inc., Springfield, Va.), concanavalin A Sepharose (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), and antisera to IgG, β_2-glycoprotein I, α_1-antitrypsin, α_2-macroglobulin, CĪ INH, α_2-anti-

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[1] Abbreviations used in this paper: CĪ INH, inhibitor of the activated first component of complement; CM, carboxymethyl; PTA, partial thromboplastin antecedent; PTC, plasma thromboplastin component; PTT, partial thromboplastin time; QAE, quaternary aminoethyl; SDS, sodium dodecyl sulfate; SP, sulphopropyl.

thrombin (antithrombin III), α_1-antichymotrypsin and inter-α-trypsin inhibitor (Behring Diagnostics, Inc., Woodbury, N. Y.) soybean, lima bean, and ovomucoid trypsin inhibitors (Sigma Chemical Co., St. Louis, Mo.) were obtained as indicated.

Hageman factor-deficient plasma, PTA-deficient plasma, and plasma thromboplastin component (PTC)-deficient plasmas were obtained from Sera-Tec Biologicals, New Brunswick, N. J. α_1-anti-trypsin-deficient plasma was a gift from Dr. Charles Reed (University of Wisconsin Medical School, Madison, Wis.). Trasylol was provided by Dr. Marion Webster, NIH, Bethesda, Md.

Preparation and Assay of Plasma Proteins. Fresh plasma was utilized as the source of Hageman factor fragments, pre-PTA, prekallikrein, the Hageman factor dependent plasminogen proactivator, α_2-macroglobulin, α_1-antitrypsin, C$\overline{1}$ INH, α_1-chymotrypsin inhibitor and inter-α-trypsin inhibitor. For the preparation of plasma, blood was collected in plastic acid-citrate dextrose packs which were made 360 μg/ml in hexadimethrine bromide (36 mg/ml solution in 0.15 M saline) to inhibit activation of Hageman factor. The blood was centrifuged at 900 g for 20 min at 4°C and the plasma separated with plastic pipettes. Plastic columns were utilized throughout all chromatographic procedures to minimize activation of Hageman factor. However, glass tubes were used to collect the fractions unless unactivated Hageman factor was sought (5). Samples were concentrated by ultrafiltration through a UM-10 membrane (Amicon Corp., Lexington, Mass.).

Alkaline disc gel electrophoresis and isoelectric focusing in polyacrylamide gels were performed as previously described (2, 8). 7½% acrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) were prepared and run according to the method of Weber and Osborn (9) in glass tubes measuring 5 mm × 125 mm. An anti-IgG immunoadsorbant consisting of rabbit antibody to human IgG coupled to 6B Sepharose was prepared as previously described (10). The column was run in 0.003 M PO$_4$ buffer containing 0.35 M sodium chloride and all samples were dialyzed against this buffer before application to the column. Use of this higher ionic strength buffer was found to minimize nonspecific adsorption to the column and did not interfere with the binding of the IgG. Protein quantitation on a weight basis was determined by micro-Kjeldahl analysis.

Purification of Human Pre-PTA. 2,500 ml of human plasma was dialyzed for 12 h against three changes of 0.003 M PO$_4$ buffer pH 8.3 and applied to a 15 cm × 100 cm column of quaternary aminoethyl (QAE) Sephadex equilibrated with the same buffer. The effluent containing three Hageman factor substrates, pre-PTA, prekallikrein, and plasminogen proactivator (10) was concentrated to 400 ml, the pH brought to 6.0 with 1 N HCl, and the mixture fractionated in a 9 cm × 72 cm column of sulphopropyl (SP) Sephadex equilibrated with 0.003 M PO$_4$ buffer pH 6.0. The column was washed with five liters starting buffer and a gradient of 12 liters starting buffer, and 12 liters 0.003 M PO$_4$ buffer pH 6.0 containing 0.35 M NaCl was applied. Pre-PTA eluted at a conductivity of 21mS at the far end of the salt gradient and, as previously reported, was well separated from both prekallikrein and plasminogen proactivator (10). The pre-PTA peak was then concentrated to 5.0 ml and fractionated on a 5 × 100 cm column of Sephadex G-150 equilibrated with 0.003 M PO$_4$ buffer 0.15 M NaCl run at 15 ml/h. Pre-PTA eluted along the ascending limb of the IgG peak at a mol wt of 175,000 whereas contaminating prekallikrein and plasminogen proactivator eluted later along the descending limb of the IgG peak (10). When the pre-PTA peak was pooled and concentrated to 25 ml, neither prekallikrein nor plasminogen proactivator were detected. When this fraction was further concentrated 10-fold, prekallikrein and plasminogen proactivator were still not detectable. This pre-PTA pool was then passed over the anti-IgG immunoadsorbant and the effluent concentrated to 10 ml. The final preparation corrected the PTT of PTA-deficient plasma from a control time of 12 min to 2.5 min in the presence of kaolin and gave a PTT of 14 min when tested in the absence of kaolin. No correction of the PTT of PTC deficient plasma was observed. When the purified material was assessed by alkaline disc gel electrophoresis and SDS gel electrophoresis, a single band was observed in each gel (Fig. 1). The molecular weight of pre-PTA when determined on SDS gels (Fig. 2), revealed a mol wt of 163,000 based upon the standards indicated. When the preparation was subjected to isoelectric focusing in 4% polyacrylamide gels from pH 7–10, a single band was again obtained (Fig. 3). Elution and assay of an unstained gel run simultaneously indicated that this band represented pre-PTA and possessed an isoelectric point between pH 8.8 to 9.4, the peak activity being at pH 9.0 to 9.1.

PTA Assays. Pre-PTA was assayed by determining its ability to shorten the partial thromboplastin time (PTT) of PTA-deficient plasma. 50 μl PTA source was incubated for 2 min at 37°C with 50 μl "kaolin-cephalin" reagent (20 mg kaolin plus 20 μl cephalin [6 mg/ml] in 2 ml phosphate-buffered saline) and 50 μl PTA-deficient plasma in 10 × 75 mm plastic tubes. 50 μl 0.05 M CaCl$_2$ was added and
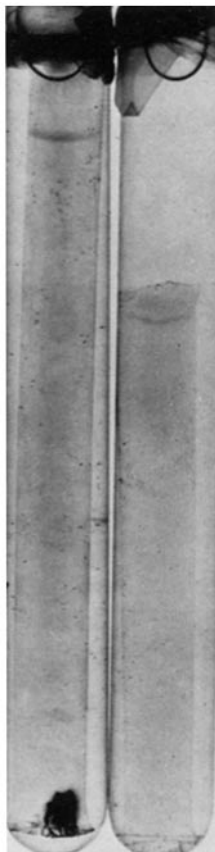
Fig. 1. SDS gel electrophoresis (left) and alkaline disc gel electrophoresis at pH 9.3 (right) of purified pre-PTA.

the clotting time determined at room temperature. The tubes were tilted every 30 sec and the end point defined as the time interval required for the clot to adhere to the plastic tube. Activated PTA was assayed in the same fashion; however, kaolin was then omitted from the cephalin reagent. For studies in which inhibition of activated PTA was examined, the inhibitor source was incubated with an equal volume of activated PTA for 30 min at 37°C and the residual clotting activity of the PTA was determined. The PTA content expressed as a percentage of normal plasma was determined by comparing the PTT of serial dilutions of the preparation with the PTT of serial dilutions of a pool of 10 normal plasmas whose PTA content was set at 100%.

Activated Hageman Factor. Activated Hageman factor was prepared by sequential chromatography of plasma on QAE Sephadex, Carboxymethyl (CM) cellulose, Sephadex G-150, and diethylaminoethyl (DEAE) cellulose as previously described (5). The preparation was active upon all three Hageman factor substrates, pre-PTA, prekallikrein, and plasminogen proactivator. When utilized at a concentration of 15 μg/ml, it corrected the coagulation defect of Hageman factor-deficient plasma from a control time of 30 min to 1 min 40 sec in the presence or absence of kaolin indicating that the preparation was completely activated. Its coagulation and kinin-generating activity were associated with a band observed in the cathodal region of an alkaline disc gel and revealed no evidence of fragmentation. It had no effect upon PTA-deficient plasma.

Hageman Factor Fragments. The Hageman factor prealbumin fragments were purified by chromatography of plasma on QAE Sephadex followed by rechromatography on QAE Sephadex, SP Sephadex, Sephadex G-100, and elution from alkaline disc gels after electrophoresis as previously
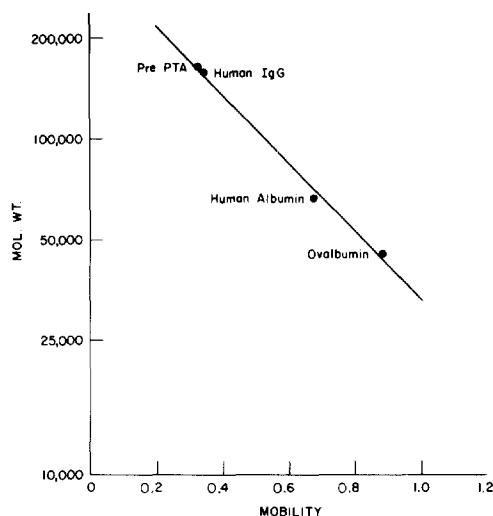
FIG. 2. Molecular weight estimation of pre-PTA as assessed by SDS gel electrophoresis. Standards utilized were human IgG (160,000), human albumin (68,000), and ovalbumin (45,000).

described (2). After extensive dialysis the fragments were concentrated by ultrafiltration and were routinely utilized at 25 μg/ml. When assayed functionally, 5 μl of Hageman factor fragment generated 100 ng bradykinin after incubation with 0.2 ml fresh plasma for 2 min at 37°C.

Plasminogen. Plasminogen was prepared by affinity chromatography of 100 ml of plasma utilizing a lysine-Sephadex column and epsilon aminocaproic acid elution as described by Deutsch and Mertz (11). The plasminogen was concentrated, dialyzed overnight at 4°C against 0.003 M PO₄ buffer pH 8.0 containing 0.15 M NaCl, and further fractionated by Sephadex G-100 gel filtration (10). The plasminogen peak was pooled, concentrated, and adjusted to 200 μg/ml for routine use. The preparation contained 28 azocaseinolytic U/ml (12).

α_1-*Antitrypsin.* α_1-antitrypsin was assayed functionally by its ability to inhibit the esterase activity of trypsin upon benzoyl-*d,l*-arginine-*p*-nitroanilide (13) and was quantitated on a weight basis by radial immunodiffusion against monospecific antibody.

Kallikrein and Prekallikrein. Kallikrein was assayed by incubation of 0.2 ml enzyme source with 0.2 ml heat-inactivated plasma for 2 min at 37°C and the bradykinin generated determined by bioassay (2). Prekallikrein was assayed by incubation of 0.1 ml prekallikrein source with 0.1 ml Hageman factor fragments (25 μg/ml) for 5 min at 37°C. 0.2 ml heat-inactivated plasma was then added, the mixture incubated for 2 min at 37°C and the bradykinin generated determined.

Plasminogen Activator and Plasminogen Proactivator. Plasminogen activator was assayed by incubating 20 μl of plasminogen activator source with 20 μl plasminogen (200 μg/ml) for 1 h at 37°C and the plasmin generated determined by fibrin plate (10). Plasminogen proactivator was assayed by incubation of 10 μl plasminogen proactivator with 10 μl Hageman factor fragments (25 μg/ml) for 10 min at 37°C. 20 μl plasminogen (200 μl) was added, the mixture incubated for 1 h at 37°C and the plasmin generated determined.

Results

Activation of Pre-PTA and Inhibition of the Activated Enzyme. 1 ml of the preparation of pre-PTA (7.5 μg/ml) was incubated with 10 μl of Hageman factor fragments (0.25 μg) or 10 μl buffer for 48 h at 4°C. Activation of the pre-PTA was observed only with the preparation containing the Hageman factor fragments (Table I) which corrected the deficient plasma from a 4% PTA level (PTT 11 min) to an 82% level as indicated by the PTT of 2.5 min in the presence or

absence of kaolin. Incubation of the Hageman factor fragments with buffer alone for 48 h at 4°C did not alter their ability to activate prekallikrein or plasminogen proactivator and they had no significant affect upon the partial thromboplastin time of PTA-deficient plasma. Before incubation, the mixture of pre-PTA and Hageman factor fragments did not shorten the PTT of PTA-deficient plasma beyond that of the PTA preparation alone. In this fashion the PTA preparation could be activated utilizing only a trace quantity of activated Hageman factor. Alkaline disc gel electrophoresis and SDS gel electrophoresis of the activated PTA revealed a single band indistinguishable from that shown for pre-PTA in Fig. 3. The same result was obtained when equal volumes of either intact activated Hageman factor or more concentrated preparations of Hageman factor fragments were incubated with pre-PTA for 5 min at 37°C. Pre-PTA was not activated upon incubation with either kallikrein or plasminogen activator and no autoactivation was observed when preparations containing a mixture of pre-PTA and activated PTA were incubated for 4 h at 37°C.

The ability of DFP and TLCK to inhibit activated PTA was tested in the following fashion. ½ ml of activated PTA was incubated for 90 min at 37°C with buffer or with each inhibitor at concentrations ranging from 5×10^{-5} M to 5×10^{-2} M. The mixture was dialyzed against two changes of 4 liters 0.003 M PO_4 buffer 0.15 M NaCl for 24 h and assayed for residual PTA activity. Incubation of PTA with buffer alone revealed a PTT of 3 min indicating that the PTA preparation was not inactivated nonspecifically during the incubation and dialysis steps. Incubation of activated PTA with increasing concentrations of
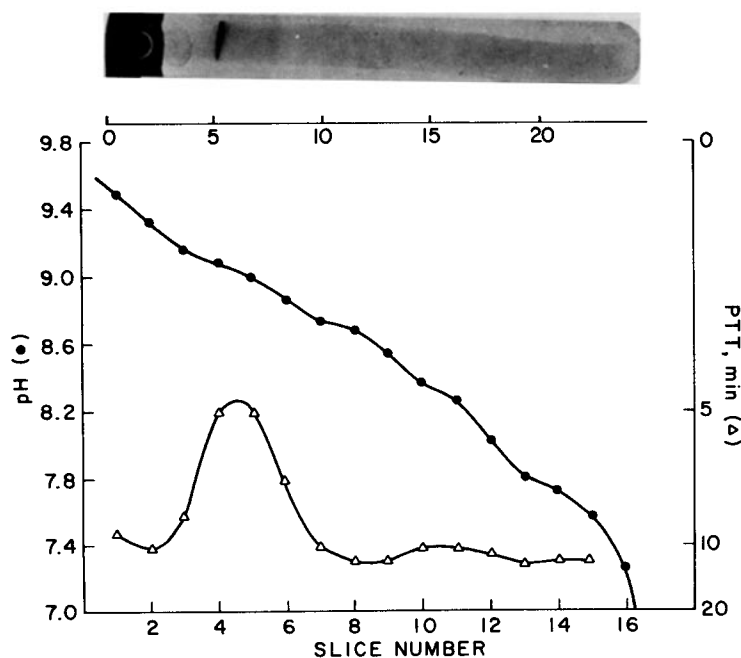

FIG. 3. Isoelectric focusing of pre-PTA in polyacrylamide gels run with pH 7–10 ampholytes. Beneath is shown a duplicate gel which was sliced, eluted, and assayed for pH and pre-PTA activity.

DFP revealed a dose-dependent inactivation of PTA as shown in Fig. 4. In contrast a concentration of 10^{-3} M TLCK did not inhibit activated PTA.

The effect of various trypsin inhibitors upon the coagulant activity of activated PTA was next examined. Incubation of activated PTA with lima bean trypsin inhibitor and ovomucoid trypsin inhibitor at concentrations up to 1 mg/ml did not yield inhibition. However, soybean trypsin inhibitor and trasylol could not be evaluated because their addition to the reagent mixture without any incubation with the PTA preparation inhibited coagulation. We therefore incubated ½ ml activated PTA with either buffer, ½ ml of soybean trypsin inhibitor (1 mg/ml), or ½ ml trasylol (10,000 U/ml) for 30 min at 37°C. The mixtures were dialyzed against 0.003 M PO₄ buffer pH 8.0 and each was passed over a 1.9 cm x 15 cm column of QAE Sephadex. 30 ml of effluent were collected and concentrated to 1.0 ml. This effluent was free of any inhibitors as assessed by the ability of each effluent to inhibit the kinin-generating activity of kallikrein or the esterase activity of trypsin. The effluents were then assayed for activated PTA. PTA that had been incubated with buffer alone had a clotting time of 4 min while the soybean

TABLE I

Activation of Pre-PTA by Hageman Factor Fragments

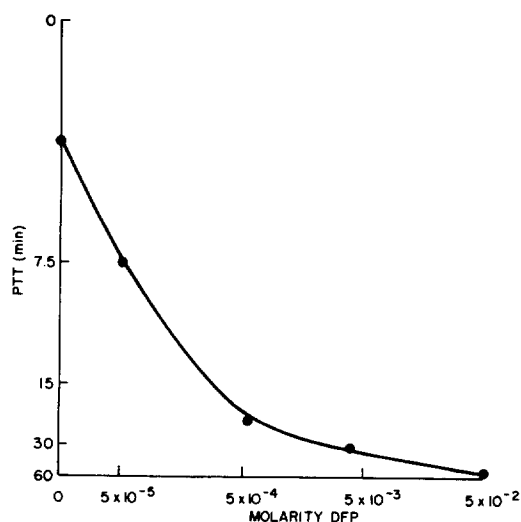| Sample	Time of incubation	Reagent added to PTA-deficient plasma	PTT
	h		*min*
Pre-PTA + buffer	48	Cephalin	11
Pre-PTA + HFf	None	Cephalin	11
Pre-PTA + HFf	48	Cephalin	2.5
Pre-PTA + buffer	48	Cephalin + kaolin	2.5
Buffer + HFf	48	Cephalin	10.5

FIG. 4. Inhibition of activated PTA after incubation with varying concentrations of DFP.

trypsin inhibitor and trasylol effluents clotted in 19 min and 12 min respectively indicating that both these inhibitors are effective upon activated PTA.

Identification of α_1-Antitrypsin as a Plasma Inhibitor of Activated PTA. In order to isolate the plasma inhibitors of activated PTA, the QAE Sephadex column utilized to isolate pre-PTA was washed with a series of buffers of increasing ionic strength, the fractions concentrated to 500 cc, and assayed for various inhibitors by electroimmunodiffusion as indicated in Table II. PTA inhibition was not observed in the 0.12 M NaCl fraction which contained α_2-macroglobulin; however, inhibition was observed in the 0.2 M NaCl fraction containing α_1-antitrypsin, α_1-antichymotrypsin, antithrombin III, and $\overline{CI}$ INH, and to a lesser degree in the 0.3 M NaCl fraction which contained inter-α-trypsin inhibitor as well as these latter inhibitors in lesser concentration.

The 0.2 M NaCl fraction was therefore dialyzed against 0.003 M PO_4 buffer pH 8.1 and applied to a 9 cm x 72 cm column of QAE Sephadex equilibrated with the same buffer. The column was first washed with 4 liters of starting buffer and then

TABLE II

*Isolation of Plasma Inhibitors by Batch Elution of QAE Sephadex**

	0.003 M PO_4	0.003 M PO_4 0.06 M NaCl	0.003 M PO_4 0.12 M NaCl	0.003 M PO_4 0.20 M NaCl	0.003 M PO_4 0.30 M NaCl
α_2-Macroglobulin	−	−	+	−	−
α_1-Antitrypsin	−	−	−	+	±
α_1-Chymotrypsin inhibitor	−	−	−	+	±
Anti-thrombin III	−	−	−	+	±
$\overline{CI}$ inhibitor	−	−	−	+	±
Inter α-trypsin inhibitor	−	−	−	−	+

* The location of each inhibitor is reported as either positive (+) or negative (−) as assessed by electroimmunodiffusion against monospecific antisera to each of the proteins sought. A fraction containing an inhibitor in lesser concentration (by inspection) than the major fraction is designated (±).

washed with 4 liters of 0.003 M PO_4 buffer pH 8 containing 0.12 M NaCl. A 10 × 10 liter linear salt gradient was then applied utilizing the buffer containing 0.12 M NaCl as the starting buffer and 0.003 M PO_4 buffer pH 8 containing 0.30 M NaCl as the limit buffer. As shown in Fig. 5, antithrombin III was eluted at a conductivity of 19mS followed by α_1-antitrypsin and α_1-antichymotrypsin at 22mS and somewhat later by $\overline{CI}$ INH at 26 mS. PTA inhibition was observed through the entire region from tubes 350–550. However, the peak activity appeared to coincide with the mixture of α_1-antitrypsin and α_1-antichymotrypsin. Tubes 400–450 were therefore concentrated to 10.0 ml and fractionated on Sephadex G-100 as shown in Fig. 6 A. The optical density observed at 280 mμ was due primarily to albumin. α_1-antichymotrypsin eluted along the ascending limb of the albumin peak at an approximate mol wt of 72,000 and α_1-antitrypsin eluted along the descending limb of the albumin peak and had an estimated mol wt of 65,000. Gel filtration of $\overline{CI}$ INH reveals a mol wt of approximately 175,000; it is thus considerably larger and elutes at the void volume of G-100. When the
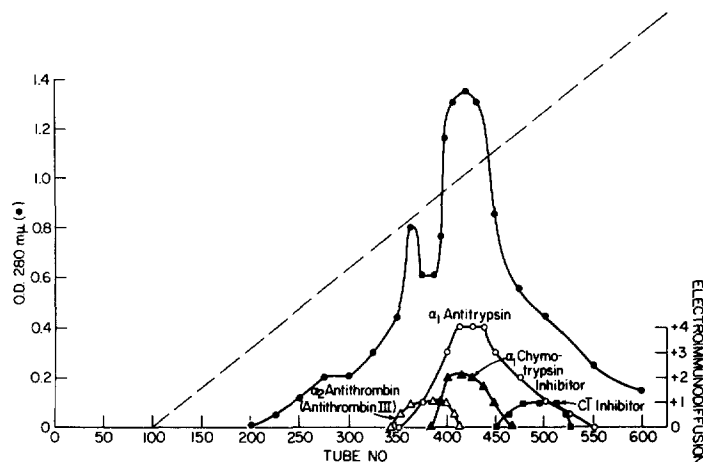
FIG. 5. QAE Sephadex chromatography of the 0.2 M NaCl eluate indicated in Table II demonstrating the elution profile of the four α-globulin inhibitors. The inhibitors were located by electroimmunodiffusion against monospecific antisera and the elution profile estimated by inspection utilizing a zero to +4 scale. The dashed line indicates the conductivity of the salt gradient between zero and 38mS.
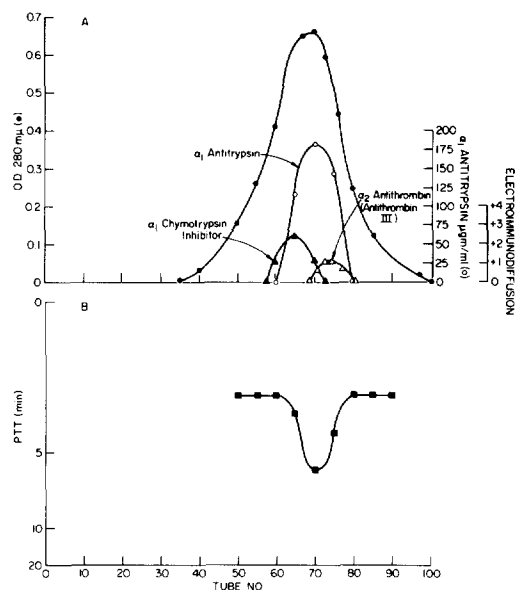

FIG. 6. (A) Sephadex G-100 gel filtration of the concentrate of tubes 400–450 obtained from QAE Sephadex (Fig. 5). (B) Beneath is shown the assay for inhibition of activated PTA.

column was assayed for PTA inhibition, the peak of inhibition observed coincided with the elution profile of α_1-antitrypsin (Fig. 6). When tubes 60–80 were concentrated to 5 ml, complete inhibition of activated PTA was obtained (PTT > 1 h). However, the concentrate contained detectable antithrombin III which was not evident when the column was assayed directly.

The concentrate was then passed over a 5 cm x 12 cm column of Con A Sepharose in order to bind α_1-antitrypsin via its carbohydrate moiety (14). The effluent contained albumin and α_1-chymotrypsin inhibitor and was concentrated back to 5 ml in order to assay it for PTA inhibitory activity. This effluent did not inhibit activated PTA, thus α_1-chymotrypsin inhibitor did not contribute to the inhibitory activity of the G-100 fraction. The column was next eluted with 150 cc 0.1 M methyl-α-D glucopyranoside, the eluate concentrated to 5 ml, dialyzed against 0.003 M PO_4 buffer 0.15 M NaCl, and assayed for α_1-antitrypsin, antithrombin III, and PTA inhibitory activity. This fraction possessed all of the PTA inhibitory activity and contained 2.5 mg/ml α_1-antitrypsin. By electroim-munodiffusion antithrombin III was also detectable; however, its presence at this stage of purification was variable. In order to remove any residual antithrombin III and other trace contaminants, this fraction was recycled on a 3.8 cm x 42 cm column of QAE Sephadex column run in the same fashion as described above utilizing a gradient of 2 liters in each bucket. Only the peak tubes of α_1-antitrypsin as assayed by quantitative immunodiffusion were then pooled and concentrated to 2 mg/ml. Polyacrylamide gel electrophoreses of the α_1-antitryp-sin preparation after the Sephadex G-100 step, Con A Sepharose step, and final QAE Sephadex step are shown in Fig. 7. The final preparation contained a single band which was shown to represent α_1-antitrypsin by elution and assay of an unstained disc gel run simultaneously. The preparation was then serially diluted and 25 μl of each dilution incubated with 25 μl of purified activated PTA (10 μg/ml) for 20 min at 37°C and assayed for residual activated PTA. A dose-response inhibition was obtained as shown in Fig. 8; inhibition was first detectable at α_1-antitrypsin concentrations of 25–30 μg/ml. At the upper limit of normal plasma concentration (4,400 μg/ml) complete inhibition of PTA was obtained since the preparation did not clot in over 1 h. A time course of inhibition was performed utilizing 1,200 μg/ml α_1-antitrypsin incubated with 20 μg/ml activated PTA as shown in Fig. 9. Essentially no inhibition of activated PTA was obtained if it was mixed with α_1-antitrypsin but not incubated; as the incubation time at 37°C was increased, inhibition was observed such that the PTT at 30 min was 10 min compared to an initial PTT of 2 min 20 sec.

In order to confirm that α_1-antitrypsin is definitely responsible for the observed PTA inhibition, 50 cc of either normal plasma (α_1-anti-trypsin 3,200 μg/ml) or α_1-antitrypsin-deficient plasma (α_1-antitrypsin $<$ 2 μg/ml) were fractionated through the G-100 step and assayed for PTA inhibition. As shown in Fig. 10, the normal plasma contained α_1 antitrypsin as well as α_1-antichymotrypsin and antithrombin III (10 A) and when assayed possessed a peak of PTA inhibitory activity (10 B). However, the α_1-antitrypsin deficient plasma possessed both α_1-antichymotrypsin and antithrombin III but no α_1-antitrypsin (10 C) and did not exhibit a peak of PTA inhibitory activity (10 D). When tubes 50–80 from both columns were concentrated and assayed for PTA inhibition, the concentrate from the normal plasma gave complete inhibition (PTT $>$ 1 h). However, the concentrate from the α_1-antitrypsin-deficient plasma prolonged the PTT from 2.5 min to 8 min. The PTA inhibitor present in this fraction of the α_1-antitrypsin-deficient plasma was isolated by elution from Con A Sepharose
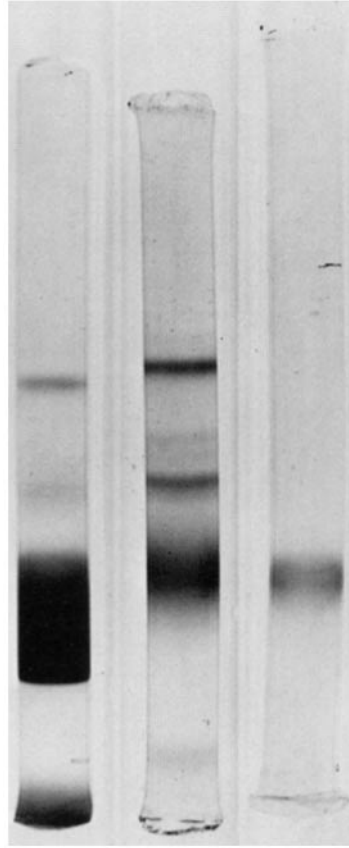
FIG. 7. Disc gel electrophoresis of α_1-antitrypsin after each of the final three steps of purification. From left to right the gels shown were obtained after chromatography on Sephadex G-100, Con A Sepharose, and rechromatography on QAE Sephadex.
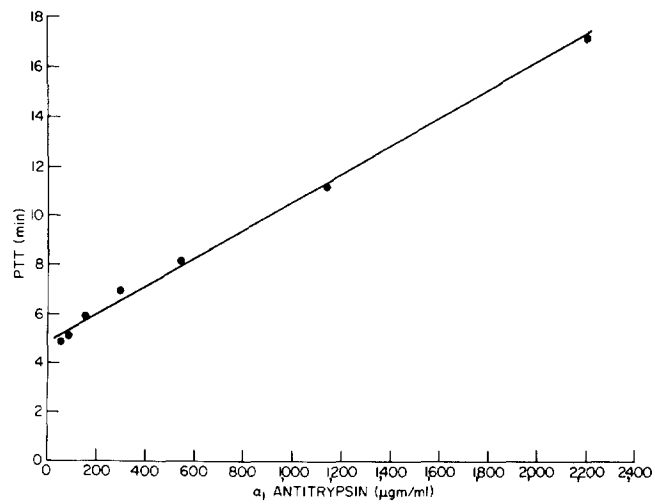

FIG. 8. Dose-response inhibition of 10 μg activated PTA by α_1-antitrypsin after incubation for 20 min at 37°C.
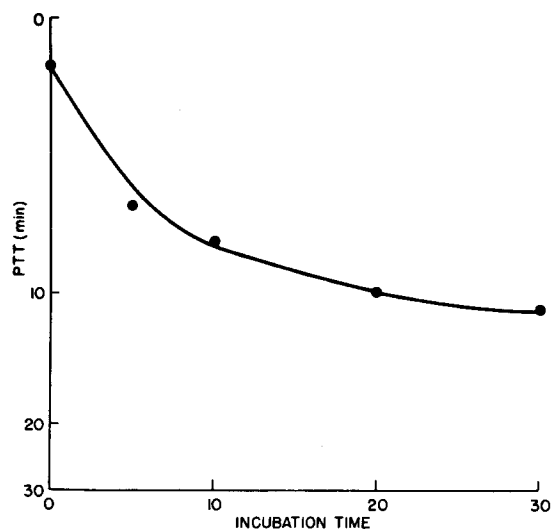
FIG. 9. A time-course of inhibition of 20 μg activated PTA by α_1-antitrypsin at a concentration of 1,200 μg/ml.

and rechromatography on QAE Sephadex. This inhibitor was identified as antithrombin III by its elution position on QAE Sephadex and alkaline disc gel electrophoresis and its enhanced activity after incubation with heparin (7).

Discussion

Recently, considerable progress has been made in the isolation of the substrates of activated Hageman factor both as activated enzymes and in precursor form. The first chromatographic separation of activated PTA from kallikrein (15) and pre-PTA from prekallikrein (8) was achieved by Kaplan and Austen utilizing sulfoethyl Sephadex. Harpel also prepared activated PTA free of kallikrein by incubating the mixture with α_2-macroglobulin and then separating the activated PTA from the kallikrein-α_2-macroglobulin complex (16). Three step procedures for the isolation of pre-PTA were then reported (10, 17) utilizing QAE Sephadex, Sephadex G-150, and SP Sephadex or utilizing Pevikon block electrophoresis rather than a gel filtration step (18). In the studies presented herein, we have found a minimum of four steps essential for the purification of pre-PTA. The molecule is isolated largely in precursor form if precautions are taken in the handling of plasma in order to minimize activation of Hageman factor. Once pre-PTA was obtained in the QAE Sephadex effluent, it was stable and could be readily processed through the other purification steps. A minimum of three columns were required in order to completely separate it from the other Hageman factor substrates prekallikrein and plasminogen proactivator (10) since the SP Sephadex concentrate was invariably contaminated with both these enzymes; pre-PTA is the first substrate eluted from Sephadex G-150 and it is thereby then obtained free of these trace contaminants. After these three steps approximately 80% of the protein content is still due to IgG which is completely removed utilizing the immunoadsorbant. Approximately 20% of the plasma pre-PTA is
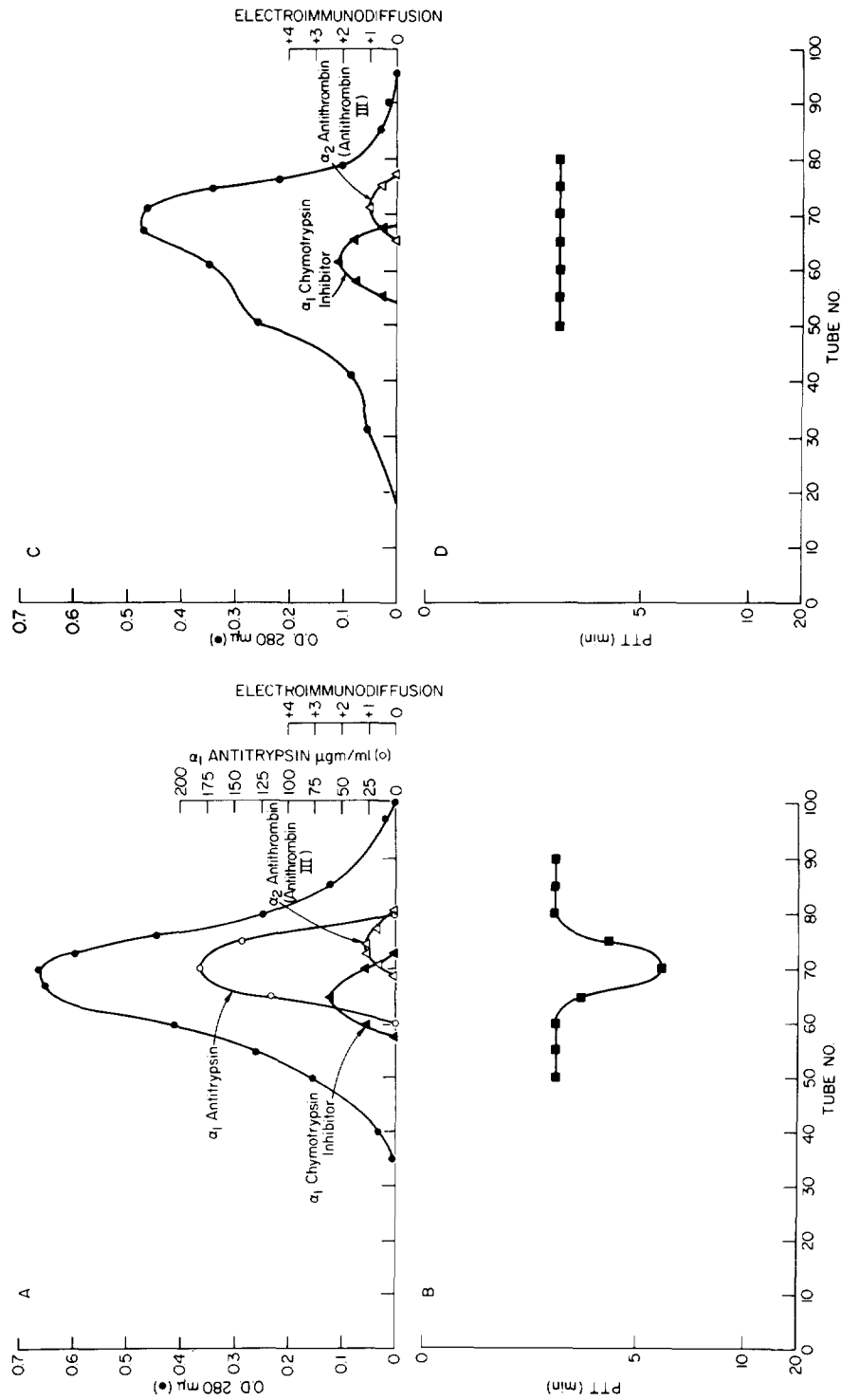
Fig. 10.   Sephadex G-100 chromatography of the QAE Sephadex fractions obtained from normal plasma (A) and α_1-antitrypsin deficient plasma (C). Beneath are shown the assay of each column for PTA inhibition.

recovered after the three chromatographic steps and further losses during the immunoadsorbant step yields a final recovery of 10–15%.

The activation of pre-PTA has been shown by Ratnoff et al. to be dependent upon the activation of Hageman factor (1). We have utilized trace quantities of purified Hageman factor fragments to activate pre-PTA suggesting that the activation observed is an enzymatic reaction and that a stable complex between activated Hageman factor and PTA does not form. This is consistent with the data of others (10, 15, 17–19) demonstrating that the physicochemical properties of activated PTA does not differ significantly from that of the precursor molecule. A mechanism of limited proteolytic digestion of pre-PTA by activated Hageman factor is thus suggested. Wuepper has demonstrated that reduction and alkylation of activated PTA prepared by trypsin activation of pre-PTA does not yield the same subunit structure of that of pre-PTA suggesting that cleavage of one or more bonds occurred concomitantly with activation (18). The conversion of pre-PTA to activated PTA by the Hageman factor prealbumin fragments indicates that this enzyme possesses the active site for the activation of pre-PTA as well as its known activity upon prekallikrein (2) and plasminogen proactivator (10) and confirms the observation that these fragments possess coagulant activity identifiable as Hageman factor (2, 20). Recently Schiffman and Lee have reported that they are unable to activate highly purified Factor XI with a crude preparation of partially activated Hageman factor or with kaolin absorbed Hageman factor and suggested that another factor is required for Factor XI activation (21); we have not observed evidence of such a requirement. The active site of activated PTA apparently contains a critical serine which is inhibitable by DFP but does not possess a histidine which is readily susceptible to TLCK. Utilizing an esterase assay for activated PTA, inhibition has been observed with soybean and pancreatic trypsin inhibitors (18). However, these inhibitors are difficult to evaluate in a coagulation assay. By reisolating activated PTA after incubation with each inhibitor, we could assess their effect upon its coagulant activity and found that soybean trypsin inhibitor and trasylol are inhibitors of the coagulant activity of activated PTA, whereas ovomucoid and lima bean trypsin inhibitors are not. This inhibition profile of DFP, TLCK, and trypsin inhibitors is identical to that obtained with plasma kallikrein.

When we sought the plasma inhibitors of activated PTA, we found that the major peak of inhibitory activity did not correspond to C$\overline{1}$ INH, a known inhibitor of activated PTA (6). This inhibitor was identified as α_1-antitrypsin by demonstrating that throughout the subsequent purification steps, the elution profile of α_1-antitrypsin and the PTA inhibitor peak coincided. Although antithrombin III has also been identified as a PTA inhibitor (7), its activity is greatly enhanced by heparin. In the absence of heparin, we did not observe a peak of PTA inhibition coincident with the elution profile of antithrombin III although inhibition by this factor was confirmed when fractions containing this protein were concentrated. The prominent diminution of PTA inhibitory activity when α_1-antitrypsin deficient plasma was fractionated, provides confirmatory evidence that it is α_1-antitrypsin and not an unknown, closely related contaminant which is the source of the inhibition. Nossel and Niemetz have partially purified a normal plasma inhibitor of activated PTA (22) which was an α-globulin similar to but distinguishable from antithrombin III (23) and Amir et al. (24) have isolated a similar inhibitor of mol wt 65,000 which was clearly distinguishable from C$\overline{1}$ INH

(25) and contained no apparent antitryptic or antithrombin activity. Thus, there may be yet another plasma PTA inhibitor which possesses chromatographic properties similar to α_1-antitrypsin and anti-thrombin III.

We have found that α_1-antitrypsin does not inhibit either kallikrein or plasminogen activator which further distinguishes activated PTA from these other Hageman factor substrates. The weak kallikrein inhibition observed by others to be secondary to α_1-antitrypsin (26) may, in fact, be secondary to contamination by antithrombin III (27). The prominent peak of PTA inhibition by α_1-antitrypsin observed upon fractionation of human plasma suggests that α_1-antitrypsin represents one of the major inhibitors of this coagulation factor.

Summary

Unactivated partial thromboplastin antecedent (PTA) has been purified by sequential chromatography of plasma on quaternary aminoethyl Sephadex, sulphoprophyl Sephadex, Sephadex G-150, and passage over an anti-IgG immunoadsorbant. The preparation gave a single band after alkaline disc gel electrophoresis, sodium dodecyl sulfate (SDS) gel electrophoresis and isoelectric focusing in acrylamide gels and was found to have a mol wt of 175,000 by gel filtration, 163,000 by SDS gel electrophoresis, and an isoelectric point of 8.8–9.4 (peak 9.0–9.1). Pre-PTA was activated directly by activated Hageman factor or by Hageman factor prealbumin fragments. Its coagulant activity was inhibited by DFP, soybean trypsin inhibitor and trasylol but not by lima bean trypsin inhibitor or ovomucoid trypsin inhibitor indicating that activated PTA possesses the same inhibition profile utilizing these reagents as does plasma kallikrein. A major plasma inhibitor of activated PTA was found to be a 65,000 mol wt α-globulin which was isolated free of α_1-chymotrypsin inhibitor, inter α-trypsin inhibitor, α_2-macroglobulin, and the other known inhibitors of activated PTA, the activated first component of complement ($\overline{C1}$ INH), and antithrombin III. Its physicochemical properties were identical to α_1-antitrypsin, and it was absent in α_1-antitrypsin-deficient plasma thereby identifying this PTA inhibitor as α_1-antitrypsin.

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