Rapid purification of a high-affinity plasminogen activator from human blood plasma by specific adsorption on fibrin/Celite

(affinity chromatography/fibrinolysis/protease/mechanism of action)

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ABSTRACT A preparation of fibrin precipitated over a solid Celite (diatomaceous earth) matrix that selectively binds 50–70% of the plasminogen activator present in human blood plasma is described. Affinity chromatography of plasma on fibrin/Celite followed by gel filtration led to a 29,000-fold purification of the plasminogen activator. The activator, referred to as the high-affinity plasminogen activator, is characterized by its ability to be strongly adsorbed by fibrin. Smaller amounts of other plasminogen activators and essentially all plasminogen were not bound to fibrin. The high-affinity plasminogen activator is a single-chain unstable protease with a molecular weight of 65,000–70,000. The high-affinity plasminogen activator has a low specific activity (500 CTA units/mg) compared to tissue or urine plasminogen activators (100,000–200,000 CTA units/mg) (CTA, Committee on Thrombolytic Agents).

Plasminogen activators are proteases, found in nearly all animal tissues and many body fluids (1). The blood plasminogen activators appear to play a key role in the degradation of fibrin clots by converting the proenzyme plasminogen, to its active form, plasmin, by limited proteolysis. The mechanism of plasminogen activation, which leads to selective proteolytic degradation of fibrin by plasmin, is a comparatively nonspecific protease, poorly understood. This is largely due to difficulties encountered in purifying the plasminogen activator of blood. Much of our current knowledge of the molecular biology of fibrinolysis is based on studies using plasminogen activators of extravascular origin, principally urokinase, because it is the only purified human plasminogen activator available. It has been recognized that plasminogen activators found in some tissues (2) or blood (3) bind to fibrin clots. We have used this observation to purify the blood protease. The gel-like consistency of fibrin made it impractical for this purpose due to its limited surface area and poor flow characteristics. By contrast, a preparation of fibrin deposited on a solid adsorptive matrix was found to have properties suitable for affinity chromatography. This preparation was used to purify the major plasminogen activator found in the blood plasma of healthy individuals.

Plasma Preparation. Blood was obtained from healthy volunteers who had rested or exercised vigorously on a treadmill for 15 min. The blood was collected into glass tubes containing 3.8% sodium citrate (0.1 vol). Plasma was prepared by centrifugation at 5000 × g for 10 min at 5°C. Plasma was recentrifuged for 10 min to make it absolutely clear before subjecting it to chromatographic separation.

Enzyme Assays. Plasminogen activator activity of plasma was determined from the euglobulin precipitate (4) spotted on plasminogen-rich fibrin plates as described below.

Plasminogen activator activity in the fractions eluted from fibrin/Celite and after gel filtration was determined by spotting the fractions directly on plasminogen-rich fibrin plates. Plasminogen-rich fibrin plates were prepared from commercial human fibrinogen as described by Brakman (5). One percent human fibrinogen solution (1.2 ml) was mixed at room temperature with 10 ml of sodium barbitol buffer, pH 7.8, containing 1.7 mM CaCl₂ and 0.7 mM MgCl₂. Clotting was induced by adding 0.5 ml of thrombin (10 units/ml). Samples were spotted on the fibrin plates in triplicate along with a urokinase reference standard and a buffer control. Activity was expressed as the area of the lysed zone after incubation (17 hr, 37°C). Committee on Thrombolytic Agents (CTA) units were estimated from a standard semilogarithmic plot of CTA units versus lysis area obtained with reference standard urokinase at concentrations giving lysis areas ranging between 50 and 300 mm². The buffer used for the dilution of the standard urokinase was the same as the one in which the plasminogen activator to be assayed was dissolved.

Plasminogen-free fibrin plates were prepared by heating the above plates (1 hr, 80°C). Absence of plasminogen was established by functional assay (urokinase, 25 CTA units incubated for 17 hr, 37°C).

Plasminogen activity was determined after 2-hr incubation of a sample (0.2 ml) with urokinase (25 CTA units in 50 μl) and then spotting the sample on a plasminogen-free fibrin plate (17 hr, 37°C).

Plasmin was determined by spotting the test sample directly on a plasminogen-free fibrin plate (17 hr, 37°C).

Plasminogen activator activity of purified preparations was also determined by fluorometric assay with the plasmin substrate 7-(D-valyl-L-leucyl-L-lysaminomido)-4-(trifluoromethyl)coumarin (6).

Preparation of Fibrin/Celite. Hyflo Super-Cel or Celite (20 g) was freed of its very fine particles by suspending in water (4 liters) for 2 hr and discarding the supernatant after most of the Celite had sedimented. The procedure was performed three times. The Celite was filtered and washed repeatedly on a

Abbreviations: CTA, Committee on Thrombolytic Agents; iPr₂P-F, diisopropyl fluorophosphate; εAlx, ε-aminoheptane acid (ε-amino caprylic acid).

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Buchner funnel with 0.05 M sodium phosphate buffer, pH 7.4, containing 0.1 M NaCl and 1 mM EDTA (buffer A). The washed Celite was suspended in buffer A (50 ml) and mixed with 2% fibrinogen solution (50 ml). The suspension was stirred magnetically at 30 ± 1°C while 200 units of bovine thrombin in 2 ml of 0.15% NaCl was added dropwise. After the addition of thrombin was completed (1 min), the suspension was stirred for 15 min at 30°C; then it was immediately filtered under suction and washed with buffer A (four times, 100 ml each). The washed solid was suspended in buffer A and packed in a column (1.5 × 50 cm) with the help of buffer A. The volume of the fibrin/Celite packed under gravity was 72 ± 6 ml. The column was washed with buffer A (800 ml) at room temperature for 24 hr, then with 500 ml of 0.01 M sodium phosphate buffer, pH 7.4, containing 1 mM EDTA, 0.3 M NaCl, 0.2 M arginine, and 5 mM e-aminohexanoic acid (eAhx, e-aminocaproic acid) (buffer C) at 4°C, and finally it was equilibrated with 500 ml of 0.01 M sodium phosphate buffer, pH 7.4, containing 1 mM EDTA, 0.3 M NaCl, and 5 mM eAhx (buffer B) at 4°C.

The amount of fibrin deposited on Celite was determined by treating 1 ml (settled volume) of the washed fibrin/Celite with 5 ml of 1 M NaOH at 90°C for 15 min and measuring the absorbance at 280 nm. The amount precipitated was found to be 7 mg of fibrin per ml of settled volume of fibrin/Celite.

Plasminogen activity in the washed fibrin/Celite was determined by incubating a suspension (0.5 ml) of fibrin/Celite with 50 CTA units of urokinase for 2 hr at 37°C and assaying an aliquot on a plasminogen-free fibrin plate. Presence of thrombin in the washed fibrin/Celite was determined by incubating the suspension with 1% fibrinogen solution.

Precipitation of Fibrin in the Presence of Other Solid Matrices. Fibrinogen was also converted to fibrin in the presence of CaCO₃, Sephadex G-25 fine, Sepharose 4B, or activated charcoal. In each case, the ratio of fibrinogen to solid matrix was 0.05:1.0 and the conversion to fibrin was accomplished as above.

Protein Determination. Protein concentrations were estimated by the method of Lowry (7) as modified by Peterson (8). Solutions containing low protein concentrations were concentrated after dialysis against 1% aqueous NaDodSO₄.

Concentration and Dialysis of Enzyme Solutions. Solutions were concentrated by using an Amicon 8 MC ultrafiltration apparatus fitted with Amicon YM 10 membrane. Dialysis was performed in the same equipment in the deadiiffusion mode.

Gel Electrophoresis. Polyacrylamide/NaDodSO₄ gel electrophoresis was performed as described by Weber and Osborn (9) in 7.5% gels. One milliliter of the purified plasminogen activator was heated at 100°C for 3 min in the presence of 2% NaDodSO₄, dialyzed for 48 hr against several changes of 0.05% NaDodSO₄ in 0.5 mM sodium phosphate buffer, pH 7.0, and lyophilized. To minimize proteolytic cleavage, samples were treated with 25 mM benzamidine hydrochloride/25 mM eAhx/0.5 mM peptostin/0.5 mM o-phenanthroline/5 mM EDTA/10 mM N-ethylmaleimide before denaturation with NaDodSO₄. Electrophoresis was performed, with and without reduction, at 5 mA per gel for 4 hr. Gels were stained with 0.25% Coomassie blue for 2 hr and destained by diffusion for 24 hr with methanol/acetic acid/water in the ratio 1:1:5.175 (vol/vol) with a small amount of mixed-bed resin (AG 501-X8D, Bio-Rad) as described by Weber and Osborn (9).

Molecular Weight Determination. Molecular weight of the purified plasminogen activator was determined according to the method of Andrews (10) by gel filtration on Sephadex G-100 superfine. Bovine serum albumin, ovalbumin, and chymotrypsinogen were used as markers. Molecular weight was also determined by NaDodSO₄ gel electrophoresis with bovine serum albumin, ovalbumin, chymotrypsinogen, and ribonuclease as markers, run on a separate gel.

Inhibition of High-Affinity Plasminogen Activator by iPrP-F. Plasminogen activator, 2 ml, (ca. 6 μg in 0.05 M sodium acetate buffer, pH 4.6, containing 0.5 M NaCl, 5 mM EDTA, and 0.1 M arginine) was neutralized by adding 1 M NaOH to a final pH of 7.5. Then 0.2 ml of [1.3-14C]iPrP-F [0.04 μCi (1 Ci = 3.7 × 10¹² becquerels), 0.4 μmol] in polypropylene glycol was added to give a final concentration of 0.2 μM. The solution was incubated at 4°C for 24 hr. Aliquots (30 μl) were removed and assayed for activity by the fibrin-plate method. The remaining solution was treated for 3 min at 100°C with 5% NaDodSO₄, exhaustively dialyzed against 0.1% NaDodSO₄, and analyzed, with or without reduction, by NaDodSO₄ gel electrophoresis. The protein bands, stained with Coomassie blue, were cut out in 2-mm-thick slices, treated for 6 hr at room temperature with 40 μl of water and 0.3 ml of Protosol, and suspended in 5 ml of Aquasol overnight, and radioactivity was measured.

The control experiment was run with polypropylene glycol not containing iPrP-F.

Purification of Plasminogen Activator. All steps were carried out at 2–4°C. Only plastic or silicone-treated glassware was used. In some experiments, benzamidine-HCl was added to all the buffers in addition to the constituents described below.

Step 1. Affinity chromatography of plasma fibrin/Celite. Freshly prepared plasma (150–200 ml) obtained from rested or exercised volunteers was mixed with heparin (10 units/ml of plasma) and an aqueous solution of 100 mM eAhx containing 20 mM EDTA (50 μl/ml of plasma). The solution was applied at the rate of 50–80 ml/hr to fibrin/Celite (packed loosely in a silicone-treated glass column, 1.5 × 50 cm, to a volume of 50 ml) previously equilibrated with buffer B. When volumes of plasma over 200 ml were used, a batch method was substituted for the column adsorption. After adsorption, fibrin/Celite was packed in a column. After the column had been washed with 12–15 column volumes of buffer B, plasminogen activator was eluted with the equilibration buffer containing 0.2 M arginine. Active fractions (79–89 in Fig. 1) were pooled and concentrated to 0.5 ml under nitrogen pressure [30 pounds/inch² (207 kPa)].

Step 2. Gel filtration of the plasminogen activator eluted from the fibrin/Celite column. The concentrated plasminogen activator solution was filtered at 2.5 ml/hr through a column of Sephadex G-100 superfine (1.5 × 90 cm, 125 ml) previously equilibrated with 0.05 M sodium acetate buffer, pH 4.6, containing 0.5 NaCl, 5 mM EDTA, and 0.1 M arginine. The most active fractions (fractions 27–31, Fig. 2) were combined and stored at −20°C in 1-ml portions.

RESULTS

Precipitation of Fibrin in the Presence of Various Solid Matrices. Treatment of fibrinogen with thrombin in the presence of Celite resulted in flocculation of the particles but not in gel formation.

The clumps of fibrin/Celite settled easily and showed good flow properties when packed into columns. Washing of the fibrin/Celite with the arginine-containing buffer (buffer C) removed some uncharacterized protein. There was no detectable plasminogen or thrombin present in fibrin/Celite.

When fibrinogen was treated with thrombin in the presence of CaCO₃, Sephadex, or Sepharose, the resulting fibrin formed a characteristic gel. Only activated charcoal behaved in a manner similar to Celite.

Purification of Plasminogen Activator. Affinity chromatography on fibrin/Celite. Passage of blood plasma, obtained from rested or exercised subjects, through a column of fibrin/Celite resulted in the adsorption of 50–70% of the plasminogen acti-
pressor activity. The remaining plasminogen activator activity emerged in the breakthrough peak and the washings. Extensive washing of the column removed only small amounts of plasminogen activator activity. Increasing the concentration of NaCl in the elution buffer to 0.5 M also failed to elute the bound plasminogen activator. Addition of arginine to the elution buffer, however, led to the elution of the bound plasminogen activator. The elution profile of the fibrin/Celite column is presented in Fig. 1.

Some fibrinogen/fibrin-reactive material identified by antibody-coated latex particles (Thrombo-Wellco Test) also emerged from the column on application of the arginine-containing buffer. Control experiments with a fibrin/Celite column through which no plasma had been passed showed that washing with the arginine-containing buffer also eluted some fibrinogen/fibrin-reactive material from the column.

Almost all the plasminogen eluted unretarded from the column. The earlier fractions eluted with arginine contained a trace of plasminogen and, therefore, were not pooled with the high-affinity plasminogen activator peak.

Chromatography of blood plasma on a column of Celite, under conditions similar to those used with fibrin/Celite, did not result in adsorption of plasminogen activator.

**Gel filtration of high-affinity plasminogen activator peak**

The protein contaminants, consisting mainly of fibrin and fibrinogen derivatives, which were eluted with arginine from the fibrin/Celite column were easily separated from the activator by gel filtration on a column of Sephadex G-100 superfine. The major protein peak appeared in the void volume of the column, followed by a single peak of plasminogen activator (Fig. 2).

In runs employing batch adsorption, an unidentified protein peak closely preceding the plasminogen activator peak was observed. This contaminant was rarely encountered when the column adsorption method was used, probably because it is discarded with the earlier fractions eluted with arginine.

When less than 200 ml of plasma obtained from resting volunteers was used, approximately 29,000-fold purification over the starting plasma (euglobulin precipitate) was achieved with an 18% yield in two steps. The specific activity of the final product was 500 CTA units/mg. A summary of a typical purification is presented in Table 1.

Processing larger amounts of plasma by the batch adsorption method reduced the yield significantly. This was due to larger elution volumes, which required larger membranes for concentration, and most of the activator was irreversibly lost on the membranes.

When plasma from strenuously exercised individuals was used as the starting material, the initial activity was 5–10 times higher than in the resting plasma. However, on gel filtration, 80–95% of the activity was lost, compared to a 40–50% loss when plasma from resting volunteers was used.

**Rechromatography of the plasminogen activator activity in the breakthrough peak of fibrin/Celite**. When the plasminogen activator in the breakthrough peak of the fibrin/Celite column (Fig. 1) was rechromatographed on another column of fibrin/Celite, the activity reemerged in the breakthrough peak. An appreciable amount of activity, however, was lost during the rechromatography step.

**Enzymatic Activity of the Purified High-Affinity Plasminogen Activator**. Incubation of plasminogen-rich fibrin plate with purified plasminogen activator demonstrated lysis presumably.
due to plasminogen generation. Further direct evidence of plasminogen elaboration was obtained by using the fluorescent substrate 7- (p-valyl-L-leucyl-L-lysaminido)-4-(trifluoromethyl) coumarin, which showed plasminogen activation by the purified high-affinity activator.

Gel Electrophoresis of Purified High-Affinity Plasminogen Activator. NaDodSO₄ gel electrophoresis of the plasminogen activator peak eluted from fibrin/Celite column showed two major bands and one minor band and some material that did not enter the gel (Fig. 3). Electrophoresis of the plasminogen activator peak obtained by gel filtration demonstrated either a single band corresponding to 40,000 daltons or two bands corresponding to 70,000 and 40,000 daltons. When inhibitors of proteolytic enzymes were added prior to denaturation with NaDodSO₄, a single band at 40,000 daltons was never observed; instead there was a predominant band corresponding to 70,000 daltons and a minor band at 40,000 daltons.

Molecular Weight of the Plasminogen Activators. The major protein band obtained in the presence of proteolytic inhibitors corresponded to a molecular weight of 70,000. Only in the absence of protease inhibitors did a second band of molecular weight 40,000 become predominant or exclusive.

Analytical gel filtration of the purified plasminogen activator along with bovine serum albumin, ovalbumin, and chymotrypsinogen as markers showed a single peak of activity that coincided with the serum albumin peak, indicating a molecular weight of the high affinity plasminogen activator of 65,000–70,000. This corresponded to the band seen on NaDodSO₄ gel electrophoresis in the presence of proteolytic enzyme inhibitors. The plasminogen activator that did not bind to the fibrin/Celite column showed two peaks of activity on gel filtration, corresponding to molecular weights of 145,000 (30% of the unadsorbed activator) and 70,000 (70% of the unadsorbed activator).

Stability of the High-Affinity Plasminogen Activator. The plasminogen activator eluted from the fibrin/Celite column with arginine buffer was stable for several days at 4°C and could be stored at −20°C for at least 1 week without apparent loss in the activity.

Plasminogen activator after gel filtration lost activity rapidly when stored at −20°C in 0.05 M sodium acetate buffer, pH 4.6, containing 0.5 M NaCl, 5 mM EDTA, and 0.1 M arginine.

Reaction of Purified High-Affinity Plasminogen Activator with IP₃₇-P-F. When plasminogen activator was incubated with 0.2 mM IP₃₇-P-F at 4°C for 24 hr, 50% of the enzymatic activity was lost. The enzyme control incubated under identical conditions lost more than 90% activity. This suggests that IP₃₇-P-F has a partial stabilizing effect under these conditions and that the high-affinity plasminogen activator is relatively resistant to IP₃₇-P-F inhibition compared to other serine proteases. Plasminogen activator treated with [³⁵S]IP₃₇-P-F showed no incorporation of radioactivity.

DISCUSSION

The low concentration of plasminogen activator normally present in blood and the difficulty in separating it from inhibitors and other proteins have hindered previous attempts to isolate this protease from blood. Radcliffe and Heinze (11) achieved a 5000-fold purification over starting plasma but their activator was "far from pure" with "many bands" present on gel electrophoresis. More recently, Binder et al. (12) have purified to homogeneity, with a 3–5% yield, a plasminogen activator from cadaver limb perfusates believed to be identical with the vascular plasminogen activator found in blood. Although the molecular weight of 70,000–75,000 reported by these authors is comparable to that of our high-affinity plasminogen activator, the low specificity of the plasminogen activator isolated from plasma compared to the cadaver limb plasminogen activator indicates that the two enzymes may not be identical.

The two-step purification procedure used in the present study overcomes most of the problems previously encountered and provides a comparatively high yield of plasminogen activator. For affinity chromatography, a suitable matrix (Celite) that adsorbs fibrinogen and permits fibrin formation while preventing gelation is required. The fibrin/Celite provides a suitable medium for specific adsorption of the plasminogen activator from blood plasma. Fibrin is the essential component, because Celite alone did not adsorb plasminogen activator.

Affinity chromatography on fibrin/Celite adsorbs plasminogen activator selectively. With the exception of some soluble fibrin that leaches out from the column and possibly some fibrin or fibrinogen from the plasma, few other proteins were present in the arginine eluates. Gel electrophoresis of the plasminogen activator peak eluted from fibrin/Celite revealed only two major bands and one minor band. Plasminogen, which has been shown to bind to fibrin (13), was eluted in the breakthrough peak and only a minute trace of it was detected in the first few fractions eluted with arginine. The presence of thrombin, which is also known to bind to fibrin (14), may have been obscured by the heparin that was added to the plasma.

The tight binding of the high-affinity blood activator to fibrin is a property not shared by all plasminogen activators in blood, as indicated by the presence of activator activity in the breakthrough peak. Some of this activity corresponded to a much higher molecular weight (145,000) than the high-affinity plasminogen activator. Plasminogen activators from tissue or urine may be similarly characterized and distinguished according to their affinity for fibrin by using the fibrin/Celite column.

Plasma plasminogen activator obtained during all purification runs eluted as a single peak corresponding to the elution volume of bovine serum albumin when filtered on Sephadex G-100 superfine. However, polyacrylamide/NaDodSO₄ gel electrophoresis of the preparations revealed two protein bands corresponding to molecular weights of 70,000 and 40,000. This
suggested the possibility of proteolytic cleavage during sample preparation, as encountered with certain other proteases (ref. 9, pp. 190–191). When various protease inhibitors were added, an increase in the 70,000 molecular weight band was seen. Therefore, we believe that the native enzyme has a molecular weight of 70,000 corresponding to the single peak of activity found on gel filtration, and that the lower molecular weight form noted on NaDodSO$_4$ gels is a degradation product, mainly produced during sample preparation.

When the fibrin/Celite method was applied to larger (>200 ml) plasma volumes requiring batch treatment, the yield was substantially reduced. Two problems were encountered that appeared responsible. First, after batch adsorption, we invariably found a protein contaminant that closely preceded the plasminogen activator peak on gel filtration and that could not be completely removed without sacrificing large amounts of the plasminogen activator peak. Second, concentration of plasminogen activator from larger volumes required larger membranes and a major loss occurred during concentration due to irreversible binding of the activator to the membrane surface.

The high-affinity plasminogen activator had a specific activity of approximately 500 CTA units/mg, which contrasts with the much higher specific activities of the high molecular weight human urokinase (93,500 CTA units/mg) (15), human plasminogen activator derived from cadaver limb perfusates (10,000–40,000 CTA units/mg) (12), and pig heart plasminogen activator (120,000–160,000 CTA units/mg) (16).

The unusually low specific activity of the high-affinity plasminogen activator suggests that it may be a precursor that is transformed to a more active form by some factors or proteases. Similarly, Radcliffe (17) has just reported that the low specific activity of a plasminogen activator isolated from pig heart is enhanced 60-fold by a cofactor present in blood and tissues. Furthermore, another protease of fibrinolysis, kalikrein (18), and all of the coagulation enzymes circulate in blood as inactive (19) or weakly active (20) zymogens. Finally, when the principal fibrinolytic inhibitor of blood, $\alpha_2$-antiplasmin, is congenitally absent, little or no plasmin activity is demonstrable (21), a finding that also suggests that the plasminogen activator found in blood under physiologic conditions is a proactivator or weakly active activator.

The concept of plasminogen activator binding as a mechanism of thrombolysis was proposed by Fearnley (3) and was supported by observations by Chesterman et al. (22), who used plasminogen-deficient fibrin clots, to which adsorption of urokinase and streptokinase was demonstrated. The possibility that plasminogen activator binding is mediated by trace amounts of plasminogen on the fibrin surface was excluded because purified plasminogen activator also binds to fibrin/Celite. It therefore may be postulated that the specificity of natural fibrinolysis, which restricts plasmin action to fibrin and protects fibrinogen from degradation (23–25) even in the absence of $\alpha_2$-antiplasmin (21), is mediated primarily by the unique affinity of the blood plasminogen activator for fibrin. The binding of activator to the clot activates adjacent plasminogen present in the ambient plasma or plasminogen adsorbed to fibrin. By this mechanism, the high fibrin affinity of the activator concentrates plasmin elaboration and confines proteolysis to fibrin polymer.

In conclusion, the high-affinity plasminogen activator represents 50–70% of the detectable activator found in the euglobulin fraction of plasma. It binds firmly to fibrin. This is a property sufficiently unique among plasma proteins so that the property can be used to purify the activator by simple adsorption on fibrin/Celite. The activator is highly unstable and has an apparent molecular weight of about 70,000 and an activity of approximately 500 CTA units/mg. A smaller amount of two low-affinity plasminogen activators is also present in blood; these have molecular weights of about 145,000 and 70,000. The relationship between the high- and low-affinity activators is unknown. It is postulated that fibrinolysis is mediated primarily by the high-affinity form, the properties of which help to localize plasmin action to the fibrin surface.

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