Purification and Characterization of Human Vascular Plasminogen Activator Derived from Blood Vessel Perfusates*

Bernd R. Binder,‡ Jocelyn Spragg,$ and K. Frank Austen
From the Physiological Institute of the Medical Faculty, University of Vienna, Vienna A-1090, Austria; and the Departments of Medicine, Harvard Medical School, and the Robert B. Brigham Division of the Affiliated Hospitals Center, Inc., Boston, Massachusetts 02115

A vascular plasminogen activator obtained by perfusion of the vessels of the lower extremities of human cadavers has been purified to apparent homogeneity by sequential precipitation in ammonium sulfate, reverse ammonium sulfate gradient solubilization, hydrophobic chromatography on octyl-Sepharose, and gel filtration on Sephadex G-75 and G-150. The isolation procedures were carried out in the presence of 0.2 to 1.0 M NaCl and 0.1 M arginine, as these conditions improved the maintenance of functional activity at each isolation step. The purified vascular plasminogen activator had an activity of 10,000 to 40,000 CTA units/mg of protein as assessed by plasminogen activation and had no direct fibrinolytic activity. The isoelectric point of the purified vascular plasminogen activator was in the range of 7.8 to 8.8 with a peak at 8.2. The apparent molecular weight of the purified vascular plasminogen activator was in the range of 70,000 to 75,000 by gel filtration and by sucrose density ultracentrifugation and was in the same range when the [3H]-diisopropyl phosphorofluoridate-labeled protein was analyzed by sucrose density gradient centrifugation. On reduction and alkylation followed by sodium dodecyl sulfate-gel electrophoresis, the [3H]diisopropyl phosphorofluoridate-labeled vascular plasminogen activator exhibited the same molecular weight of 67,000 as the unreduced labeled protein. The functional activity of the purified vascular plasminogen activator was unaffected by concentrations of the IgG fractions of antisera specific for urokinase or plasma prekallikrein which completely inhibited the fibrinolytic activity of urokinase and kallikrein, respectively. Thus the human vascular plasminogen activator is a distinct cationic single polypeptide chain serine esterase.

The fibrinolytic activity of human plasma uncovered by the streptococcal substance (1) streptokinase (2) was subsequently shown to depend upon activation of a precursor designated plasminogen (3). Shortly thereafter it was demonstrated that extracts of human tissues had fibrinolytic activity which also acted by converting plasminogen to plasmin (4). These tissue extracts contained a plasminogen-activating activity termed tissue activator which was stable to both acid treatment and heating at 60°C for 30 min (5). The demonstration that postmortem blood contained an increased amount of plasminogen-activating activity and that much of this activity was acid- and heat-stable was interpreted as indicating its derivation from the vascular bed (5). When frozen sections of human tissues such as heart, skeletal muscle, and aorta were overlayered with plasminogen-rich fibrin, it was found that the endothelial cells were the predominant source of plasminogen-activating activity (7).

Perfusion of the vessels of the lower extremities of human cadavers yielded an activity termed vascular plasminogen activator (6, 9), which after partial purification gave an approximate molecular weight of 64,000 by gel filtration in high salt buffers and was shown to convert plasminogen to plasmin (10, 11). Vascular plasminogen activator, purified 200-fold by serial precipitation, anion exchange chromatography, and gel filtration, had an activity of 2,300 CTA units/mg of protein, the stability of which was dependent upon the presence of high salt concentrations (10). By use of both high salt- and arginine-containing buffers, the vascular plasminogen activator from the perfused vessels of the lower extremities of human cadavers has now been purified to apparent homogeneity. Purified vascular plasminogen activator is composed of a single polypeptide chain with a molecular weight of 67,000 and exhibits a plasminogen-activating activity of 10,000 to 40,000 CTA units/mg of protein.

MATERIALS AND METHODS

Human fibrinogen (Fraction I, B grade, Calbiochem), thrombin, bovine topical (Parke-Davis); urokinase, M, = 31,500 (Leo Pharmaceutical Products, Denmark) and urokinase, M, = 54,000 (Serono Products, Freiburg, FRG); Sephadex G-150, G-75, G-25, octyl-Sepharose, Sephacryl S-200, and sizing standards (human serum albumin, transferrin, ovalbumin, and chymotrypsinogen A) (Pharmacia); diaminoacetic acid grade I (Calbiochem), L-arginine hydrochloride, lodoacetamide, and diithiothreitol (Sigma); Indubiose A 45 agarose (Fisher); acrylamide, bisacrylamide, sodium dodecyl sulfate (Bio-Rad); [3H]diisopropyl phosphorofluoridate ([HDPF] (New England Nuclear); diisopropyl phosphorofluoridate (DFP) (Aldrich); [14C]formaldehyde (Amersham); antibodies against human transferrin and ovalbumin (Behringwerke); Ampholines, pH 3.5 to 10 (LKB); Amicon apparatus and Diaflow membrane UM-10 (Amicon); and Collodion bags 10 S7S (Schleicher and Schuell) were obtained as noted.

Anti-urokinase serum was prepared by injecting rabbits intracutaneously with 1,000 CTA units of either the M, = 31,500 or the 54,000 urokinase in complete Freund's adjuvant followed by five additional injections at 10-day intervals of 100 CTA units of urokinase in Al(OH)3. Seven days after the last injection, blood was obtained from the carotid artery. An IgG fraction was prepared from the serum by

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To whom reprint requests should be addressed in Boston.

1 The abbreviations used are: CTA, Committee on Thrombolytic Agents; SDS, sodium dodecyl sulfate, DFP, diisopropyl phosphorofluoridate.
ammonium sulfate fractionation and ion exchange chromatography (12); subsequent gel filtration on Sephacryl S-200 in 0.05 M Tris-HCl, 0.1 M NaCl buffer, pH 8.0, removed any fibrinolytic activity seen on plasminogen-rich fibrin plates. The IgG anti-urokinase and the normal rabbit IgG prepared by the same procedure were concentrated to a final protein concentration of approximately 3 mg/ml. Goat anti-mal rabbit IgG prepared by the same procedure were concentrated to plasminogen-rich fibrin plates. The IgG anti-urokinase and the normal rabbit IgG were stored at -70°C. Pooled supernatants of at least four perfusions were used as starting material for each purification of vascular plasminogen activator.

For protein determination the method of Lowry et al. (15) was used with human albumin as a standard. Concentration of vascular plasminogen activator-containing samples were performed by positive pressure filtration through a Diaflo UM-10 membrane or by filtration through collodion bags retaining proteins with M, greater than 25,000.

Functional Assay of Fibrinolytic Activities—Fibrinolytic activity was assayed on human plasminogen-rich or plasminogen-free fibrin/agarose plates. Commercial human fibrinogen was used as a source of plasminogen-rich fibrinogen and as starting material for preparation of plasminogen-free fibrinogen (16). Removal of plasminogen was established by functional and antigenic analysis. For both types of fibrin/agarose plates, a final concentration of fibrinogen of 1.5 mg/ml in Michaelis buffer, pH 8.4 containing 10⁻⁴ M CaCl2 was used; the final fibrinogen concentration was 0.5% in the agarose plates.

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Plasminogen-activating activity was also assayed in a two-step procedure which involved activation of plasminogen and assay of the plasmin formed on plasminogen-free fibrin/agarose plates. Twenty microliters of the samples to be tested were incubated with 20 μl of purified human plasminogen (470 μg/ml) in 0.1 M phosphate buffer, pH 7.4, for 1 h at 37°C. Ten microliters of each incubation mixture were applied to plasminogen-free fibrin/agarose plates and incubated for 6 h at 37°C. Standardization of the two-step procedure was accomplished by incubating serial dilutions of M, = 54,000 urokinase with plasminogen and plotting the diameter of lysis zones produced by the plasmin formed versus log CTA units/ml of urokinase. Plasminogen incubated in buffer alone showed no lytic activity. To test the direct fibrinolytic activity, samples were applied to the plasminogen-free fibrin/agarose plates without preincubation with plasminogen.

Analytical Techniques—Disc gel electrophoresis was performed in duplicate gels in 7.5% polyacrylamide at pH 4.3 or 9.3 according to the method of Maurer (19). One gel was fixed and stained with Coomassie brilliant blue (20) and the other was cut into 2-mm slices.
tions containing more than 10 CTA units/ml of vascular plasminogen activator were pooled and concentrated about 100-fold by positive pressure filtration through a UM-10 Diaflo membrane before Sephadex G-75 gel filtration.

Sephadex G-75 was equilibrated in a 0.075 M potassium acetate buffer containing 0.3 M NaCl, 0.1 M arginine, and 0.01 M EDTA, pH 4.2, in a column (2.5 x 90 cm). The concentrated vascular plasminogen activator from the octyl-Sepharose step was applied to the column and filtered in the equilibrating buffer at a flow rate of 7 ml/h with 5-ml fractions collected. A protein peak appeared at the void volume of the column, and a single peak of vascular plasminogen activator was recovered in the descending limb of the protein peak, corresponding to a molecular weight of 65,000 to 75,000. The marker proteins transferrin, ovalbumin, and chymotrypsinogen A were filtered separately. Active fractions of the vascular plasminogen activator were pooled and concentrated 10-fold by positive pressure filtration through a Diaflo UM-10 membrane and filtered on Sephadex G-150.

Sephadex G-150 was equilibrated in a 0.075 M potassium acetate buffer containing 0.3 M NaCl, 0.1 M arginine, and 0.01 M EDTA, pH 4.2, in a column (2.5 x 90 cm). The pooled concentrate from the Sephadex G-75 column was applied to the Sephadex G-150 column and filtered in the equilibrating buffer at a flow rate of 7 ml/h with 5-ml fractions collected. A protein peak was present in the void volume of the column and showed some degree of tailing (Fig. 3). A single peak of vascular plasminogen activator was seen in the region corresponding to a molecular weight of approximately 70,000 to 75,000 as determined from the location of sizing proteins run separately. Active fractions containing no apparent optical density at 280 nm were pooled, divided into aliquots, and frozen at 70°C. This material contained 10,000 to 40,000 CTA units/mg of protein (Table I) as assayed in plasminogen-rich fibrin/agarose plates and had no activity on plasminogen-free fibrin/agarose plates. For the analytical procedures the purified vascular plasminogen activator was concentrated 20-fold before use.

Characteristics of the Vascular Plasminogen Activator—Purified vascular plasminogen activator was subjected to alkaline and acid polyacrylamide disc gel electrophoresis in replicate gels. When a 5-µg portion of vascular plasminogen activator was subjected to alkaline disc gel electrophoresis at pH 9.3, no distinct protein band was observed and functional activity was recovered at the origin and tailing into the upper quarter of the gel. The same amount of material was subjected to acid disc gel electrophoresis at pH 4.3, and plasminogen activator activity was eluted from two slices in the middle of the gel corresponding to the position of a faintly staining band in the same region of a replicate gel. The isoelectric point of the vascular plasminogen activator was determined by applying 10 µg to a sucrose gradient containing Ampholines from pH 3.5 to 10. All of the applied material was recovered in the pH range of 7.8 to 8.8 with a peak of functional activity at 8.2.

The ability of DFP to inhibit the functional activity of the vascular plasminogen activator was assessed with material

![Fig. 1. Reverse ammonium sulfate gradient solubilization of vascular plasminogen activator at pH 7.0. The linear ammonium sulfate gradient, from 2 to 0 M, was started at the point indicated by the arrow.](http://www.jbc.org/)

![Fig. 2. Hydrophobic chromatography of the vascular plasminogen activator recovered from reverse ammonium sulfate gradient solubilization (Fig. 1). A linear gradient from 0% to 50% ethylene glycol was begun at the point indicated by the arrow.](http://www.jbc.org/)

![Fig. 3. Filtration of the vascular plasminogen activator recovered from the Sephadex G-75 step on Sephadex G-150 at pH 4.2. Sizing standards filtered separately were located at the positions indicated. HSA, human serum albumin.](http://www.jbc.org/)

### Table I

Purification of the vascular plasminogen activator

<table>
<thead>
<tr>
<th>Material</th>
<th>Pooled volume</th>
<th>Protein content</th>
<th>Activity</th>
<th>Specific activity</th>
<th>Accumulated yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting material</td>
<td>2000–3000</td>
<td>0.3–2.0</td>
<td>1–14</td>
<td>0.9–10</td>
<td>100</td>
</tr>
<tr>
<td>Resolubilization after ammonium sulfate</td>
<td>600–800</td>
<td>0.3–1.2</td>
<td>25–68</td>
<td>68–157</td>
<td>53–86</td>
</tr>
<tr>
<td>Octyl-Sepharose chromatography</td>
<td>600–800</td>
<td>0.04–0.08</td>
<td>20–56</td>
<td>250–1250</td>
<td>32–48</td>
</tr>
<tr>
<td>Gel chromatography on Sephadex G 75</td>
<td>50–75</td>
<td>0.004–0.04</td>
<td>15–40</td>
<td>4,000–10,000</td>
<td>5–9</td>
</tr>
<tr>
<td>Gel chromatography on Sephadex G-150</td>
<td>50–75</td>
<td>0.002–0.003</td>
<td>15–50</td>
<td>10,000–40,000</td>
<td>3–5</td>
</tr>
</tbody>
</table>
Human Vascular Plasminogen Activator

recovered at the reverse ammonium sulfate step. A sample of the material was dialyzed at 4°C against 2 liters of 0.1 M potassium phosphate buffer, pH 7.5, for 4 h, and 200 μl containing 25 CTA units/ml were made 5 × 10⁻³ M in DFP. The mixture was incubated in a collodion bag for 5 h at 25°C, and dialyzed for 4 h at 4°C against 1 liter of 0.075 M potassium acetate buffer containing 0.3 M NaCl, 0.1 M arginine, and 0.01 M EDTA at pH 4.2. A replicate sample without DFP was similarly incubated and dialyzed. The functional activity of each sample was determined in plasminogen-rich fibrin plates. The crude vascular plasminogen activator incubated for 5 h without DFP yielded 14 CTA units/ml, whereas the DFP-treated sample contained 2 CTA units/ml, representing 86% inhibition of function by DFP. Additional replicates diluted with DFP-containing buffer that had been dialyzed or with buffer alone indicated the complete removal of DFP at the time of assay. [³H]DFP was then employed to label the active site of purified vascular plasminogen activator.

[³H]DFP was incorporated by incubating 15 μg of purified vascular plasminogen activator in 10⁻⁴ M [³H]DFP at 4°C for 12 h in a final volume of 0.1 ml of neutralized 0.075 M potassium acetate buffer containing 0.3 M NaCl, 0.1 M arginine, and 0.01 M EDTA. After sedimentation for 12 h at 175,000 × g at 4°C in either 0.1 M phosphate buffer, pH 7.2, or 0.075 M potassium acetate buffer containing 0.3 M NaCl, 0.1 M arginine, and 0.01 M EDTA at pH 4.2, the samples were analyzed for radioactivity, plasminogen-activating activity, and molecular weight by sucrose density gradient centrifugation and by SDS-gel electrophoresis with and without reduction. Molecular weights calculated from sucrose density gradient centrifugation for both unlabeled and labeled vascular plasminogen activator were the same and were approximately 72,000. The recovery of the labeled and the unlabeled vascular plasminogen activator was 89% and 97%, based upon radiolabel and functional assay, respectively.

The alkylated, unreduced [³H]DFP-treated vascular plasminogen activator presented as a single peak with a molecular weight of 67,000 on SDS-gel electrophoresis (Fig. 4). After reduction and alkylation, the radioactivity was again present as a single peak with the same molecular weight, indicating that vascular plasminogen activator was composed of a single polypeptide chain. The recovery of radioactivity for both samples was approximately 95%. Under the same experimental conditions, a commercial preparation of chymotrypsin A showed one band upon alkylation followed by SDS-gel electrophoresis and two additional bands upon reduction and alkylation followed by SDS-gel electrophoresis.

A separate 30-μg sample of [³H]DFP-treated vascular plasminogen activator was incubated with 10 μl of [¹⁴C]formaldehyde in a final volume of 0.3 ml as described (23). After separation of the bound and free radioactivity by filtration on Sephadex G-25, the protein was subjected to SDS-gel electrophoresis with and without reduction and alkylation. The [³H] radioactivity was present in the same single region of the gel in both reduced and unreduced samples, and the [¹⁴C] label was also found in a single region of both gels completely coincident with the [³H] label. Ninety-six and ninety-three per cent of the applied [³H] and [¹⁴C], respectively, were recovered in the single radioactive peak in the unreduced gel, and 94% and 90% were recovered in the same region of the reduced gel. Unlabeled vascular plasminogen activator, admixed with the unreduced doubly labeled enzyme prior to electrophoresis, was identified functionally in plasminogen-rich fibrin plates, and had the same mobility as the [³H] and [¹⁴C] radioactivity; 85% of the applied functional activity was recovered in the unreduced sample, while the reduced enzyme was functionally inactive.

Monospecific antisera to urokinase and plasma prekallikrein were used to study the effects of these materials on the function of vascular plasminogen activator. Fifty CTA units of urokinase and 7 CTA units of vascular plasminogen activator were incubated with various dilutions of the IgG fraction of normal rabbit serum and of antiserum to urokinase for 30 min at room temperature in 0.05 M Tris-HCl, 0.1 M NaCl, pH 8.0. Ten microliters of each reaction mixture were assayed on plasminogen-rich fibrin plates. The nonspecific IgG fraction had no effect on the plasminogen-activating activity of either urokinase or vascular plasminogen activator (Fig. 5). The IgG fraction of the immune serum from rabbits immunized with M₈ 31,500 urokinase completely inhibited the urokinase activity at a 1:4 dilution and gave 75% inhibition when diluted 1:16. In contrast, the immune IgG had no effect on the activity of vascular plasminogen activator. When the IgG fraction was obtained from rabbits immunized with M₈ 54,000 urokinase, there was 100% inhibition of urokinase activity at all dilutions tested, namely 1:1 to 1:32, whereas no dilution inhibited the function of vascular plasminogen activator.

Incubation of serial 2-fold dilutions of an IgG fraction of immune serum to plasma prekallikrein with Hageman factor-activated partially purified prekallikrein for 60 min at 37°C inhibited kallikrein activity in a dose-response fashion (Fig. 6). The fibrinolytic activity of kallikrein was measured by activation of plasminogen as quantitated in plasminogen-free fibrin plates. No dilutions of the anti-plasma prekallikrein IgG fraction inhibited the capacity of 10 CTA units of vascular plasminogen activator.

**Fig. 5.** Analysis of the capacity of serial dilutions of an IgG fraction of anti-urokinase serum to inhibit the fibrinolytic activity of urokinase (C) and vascular plasminogen activator (A) in a plasminogen-rich fibrin plate. An IgG fraction of normal rabbit serum was also incubated with either urokinase (O) or vascular plasminogen activator (A).
plasminogen activator to convert added plasminogen to plasmin in the two-step assay.

**DISCUSSION**

Perfusion of the vessels of lower extremities of human cadavers yields a plasminogen-activating activity termed vascular plasminogen activator (8, 10), which has now been purified to apparent homogeneity. The isolation procedures were carried out in the presence of 0.3 to 1.0 M NaCl and 0.1 M arginine as these conditions proved essential to the maintenance of activity at each step. After precipitation in 2 M (NH₄)₂SO₄, the vascular plasminogen activator was recovered by reverse ammonium sulfate gradient solubilization in the fractions containing approximately 0.5 M (NH₄)₂SO₄ (Fig. 1). Hydrophobic chromatography on octyl-Sepharose also permitted the use of a high salt and arginine-containing buffer system and yielded vascular plasminogen activator as a single peak during gradient elution with ethylene glycol (Fig. 2). Gel filtration on Sephadex G-75 revealed a single peak of activity in the inclusion volume, but overlapping with the descending limb of the predominant protein peak. A final Sephadex G-150 gel filtration step separated a single peak of activity at an approximate molecular weight of 70,000 to 75,000 (Fig. 3) from the contaminating proteins which appeared in the void volume. The overall recovery of vascular plasminogen activator from six different preparations ranged from 3 to 5% relative to the activity in the starting perfusate (Table I). The apparent loss of vascular plasminogen activator during reverse ammonium sulfate gradient solubilization and during octyl-Sepharose chromatography, approximately one-third at each step, was largely due to the way in which the fractions were pooled. The subsequent losses appeared to be due to the lability of the partially purified enzyme, particularly during the concentration steps, and may be the result of separating a stabilizing factor from the enzyme during gel filtration. The final product showed an activity of 10,000 to 40,000 CTA units/mg of protein.

An apparent molecular weight of 70,000 to 75,000 was obtained by both gel filtration (Fig. 3) and sucrose density gradient centrifugation. Further, when the active site was labeled with [³H]DFP and the protein subjected to alkylation and SDS-gel electrophoresis, the molecular weight was 67,000. The inactivation of the vascular plasminogen activator by DFP was studied only with crude material because the dialysis step required to remove DFP before assay resulted in almost total loss of activity of untreated purified vascular plasminogen activator. That the [³H]DFP labeled the purified activator was determined from the fact that the labeled protein had the same molecular weight on sucrose density gradient centrifugation as the unlabeled and functionally intact vascular plasminogen activator. When the purified labeled vascular plasminogen activator was subjected to reduction and alkylation followed by SDS-gel electrophoresis, it exhibited the same molecular weight as the unlabeled labeled protein (Fig. 4). Thus, the vascular plasminogen activator is a single poly peptide chain serine esterase.

The purified vascular plasminogen activator exhibited an isoelectric point in the range of pH 7.8 to 8.8 with a peak at 8.2 for four different preparations. Although the analytic alkaline disc gel electrophoresis revealed no contaminant and the acid disc gel showed correspondence between the faintly stained protein band and the plasminogen-activating function of the eluate of the same region of a duplicate gel, the quantity of protein applied and available was too limited to exclude completely minor contaminante on the basis of protein staining. However, when purified vascular plasminogen activator, which had been labeled at the active site with [³H]DFP (Fig. 4), was also labeled with [¹⁴C]formaldehyde, a single region of ¹⁴C was recovered after SDS-gel electrophoresis and that region corresponded exactly to the area in which ³H radioactivity was identified, and, under nonreducing conditions, to the area of functional activity. Thus, the purified vascular plasminogen activator does not have detectable protein contaminants.

The vascular plasminogen activator, like other human activators of plasminogen such as urokinase and plasma kallikrein (24, 25), is a highly cationic protein. Kallikrein can be distinguished from the vascular plasminogen activator by its molecular weight of 108,000 on gel filtration (25), of 88,000 on SDS-gel electrophoresis (26), and by its heavy and light chain composition as revealed after reduction (26). The activity of vascular plasminogen activator was not inhibited by concentrations of antibodies to human plasma prekallikrein which suppressed the plasminogen-activating function of kallikrein derived by Hageman factor activation of prekallikrein (Fig. 6).

Additional Hageman factor-dependent plasminogen activator activities have been recognized (27) and attributed to a partially degraded prekallikrein (26) and to plasma thromboplastin antecedent (28), neither of which would give a single chain on reduction and alkylation. Urokinase has been identified in two forms by SDS-gel electrophoresis, an Mₖ = 47,000 protein comprised of two chains (28) and an Mₖ = 31,500 single chain polypeptide which may represent the heavy chain of the larger molecule (28). Urokinase has been further distinguished from the partially purified vascular plasminogen activator in studies demonstrating that despite similar Kᵦ values for plasminogen activation, their specificity for synthetic amino acid ester substrates differed (10). Furthermore, both the studies of Aoki (10) and those of our own (Fig. 5) reveal that antibodies to urokinase in concentrations which totally suppress urokinase function have no effect on the vascular plasminogen activator. Thus, the vascular plasminogen activator may be distinguished from kallikrein and urokinase by its chain structure and apparent lack of antigenic identity. Synthesis of an activator of plasminogen has recently been demonstrated for endothelial cells derived from rabbit vena cava and maintained in continuous culture; however, the activator has not yet been characterized by any physicochemical criteria (29).

Tissue activators from two different sources have been purified to homogeneity, namely from pig heart and from cultured human pancreatic carcinoma cells. The pig heart plasminogen activator (30) purified to homogeneity by different procedures exhibited a molecular weight of 32,000 (31) and 63,000 (32), respectively, and was cationic based upon its mobility in acid disc gels (31). The plasminogen activator secreted by cultured human pancreatic carcinoma cells ex-
hibited a molecular weight of 55,000 and a pI of 8.6 (33). Additional plasminogen-activating activities have been recognized in human neutrophils activated with concanavalin A (34), human tumor cell lines of rhabdomyosarcoma (35) and melanoma (35, 36), activated mouse macrophages (37), and hog ovaries (38). In each case, the activator was identified functionally and characterized in terms of size by gel filtration or SDS-polyacrylamide gel electrophoresis with and without ['H]DFP label. For the most part, the activity fell within a molecular weight range of 48,000 to 70,000. Thus, all the tissue activators including the vascular plasminogen activator fall within a comparable size range and, where data are available, are cationic.

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