Purification of Plasminogen Activators from Human Seminal Plasma

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Two plasminogen activators (1 and 2) were isolated from human seminal plasma by high-speed centrifugation, Sephadex-gel filtration and ion-exchange chromatography. The activators were shown to be homogeneous by polyacrylamide-disc-gel electrophoresis at pH 8.3 and 4.5, and by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The molecular weights of activators 1 and 2 were estimated as 69000 and 74000. Their amino acid compositions are very similar, both being high in aspartic acid, glutamic acid, serine, glycine and leucine, and low in methionine, tryptophan, tyrosine, isoleucine and histidine. Activators 1 and 2 each possess 16 cysteine residues. Both activators have isoelectric points of approx. 7.0, are stable over a wide pH range at temperatures up to 60°C, but lose activity at higher temperatures, particularly under very basic or acidic conditions. They are not inhibited by EDTA, Mg^{2+} and Ca^{2+} at 10 mM concentrations, but their activity decreases on addition of 10 mM-cysteine or Fe^{2+} and 6-aminohexanoate or sera from pregnant women. The precipitin band formed between urokinase and its antiserum is continuous with the precipitin bands formed between the seminal plasminogen activators and the urokinase antiserum. Antisera to urokinase inhibit both the activity of urokinase and the seminal plasminogen activators.

The presence of fibrinolytic activity in human seminal plasma has been known for many years (Huggins & Neal, 1942; Harvey, 1949; Oettle, 1950; von Kaulla & Shettles, 1953; Ying *et al.*, 1956), and this topic has been reviewed by Zaneveld *et al.* (1975). This activity is mostly due to two proteins: (1) plasminogen activator and (2) a proteolytic enzyme named seminin or chymotrypsin-like enzyme (Lundquist *et al.*, 1955; Syner & Moghissi, 1972; Fritz *et al.*, 1972). The seminal activator has been subjected to some very preliminary studies, but has never been purified to any degree. It is derived primarily from the Cowper's gland and urethra (Kester, 1971), but can also be found in fairly high concentrations in the prostate gland and seminal vesicles, mostly associated with the vascular endothelium (Kester, 1969, 1971). The activator is virtually absent from the vas deferens, epididymis or testis. Results from our laboratory obtained by the split-ejaculation technique confirm these observations (Tauber *et al.*, 1975).

Two plasminogen activators were suggested to be present in human seminal plasma, one being absorbed and one being excluded by Sephadex G-200 (Hisazumi, 1970; Suominen & Niemi, 1970). Crude

seminal plasma was used for these studies, however, and it appears likely that the very-high-molecular-weight activator represents a complex between the activator and a presently unknown high-molecular-weight protein. Plasminogen-activator activity of crude seminal preparations is inhibited by 6-aminohexanoate and aminomethylcyclohexanecarboxylate, and is stable from pH 4 to 8 (Hisazumi, 1970; Nijs *et al.*, 1971). Increasing the acidity of the seminal plasma results in a rapid destruction of the activator. This is most probably caused by the activation of seminal pepsinogen at that pH (Nijs *et al.*, 1971; Lundquist & Seedorf, 1952), which in turn causes the digestion of the activator (Rasmussen & Albrechtsen, 1960). Removal of the pepsinogen from the seminal plasma, or the use of prostatic preparations that are normally devoid of pepsinogen (Hisazumi, 1970), results in activator stability at low pH, similar to urokinase (Rasmussen & Albrechtsen, 1960). Evidence is also available for the presence of a pro-activator with activity similar to that of streptokinase (von Kaulla & Shettles, 1953; Rasmussen & Albrechtsen, 1960; Hisazumi, 1970; Nijs *et al.*, 1971) and of an inhibitor of the plasminogen activator (Nijs *et al.*, 1971) in human seminal plasma and/or in human prostatic tissue.

To determine the physiological role of a protein it is highly advantageous to work with pure preparations. Since we were interested in evaluating the

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Abbreviation used: SDS, sodium dodecyl sulphate.

function of the seminal plasminogen activator in the coagulation–liquefaction process of human semen, we purified this activator and determined some of its characteristics. The results are reported in the present paper. A preliminary communication of some of the data has been published (Zaneveld *et al.*, 1974).

Experimental

Materials and equipment

Fresh human semen was obtained from healthy donors by masturbation after 3–4 days of abstinence. Immediately after liquefaction, which usually required 10–20 min, the semen was centrifuged at 1500 rev./min for 15 min at room temperature (20°C). The supernatant seminal plasma was separated from the precipitated spermatozoa by decanting, and formed the starting material. It was kept at –20°C until further fractionation. Buffers were prepared as described by Gomori (1955) and included 0.1 M-sodium acetate buffer, pH 5.6, containing 0.05 M-NaCl; 0.1 M-Tris/HCl buffer; 0.1 M-sodium citrate buffer, pH 6.0; and 0.5 M-sodium barbiturate buffer, pH 7.8, containing 0.1 M-NaCl. Chemicals and biochemicals used were SDS (Aldrich Chemical Co., Milwaukee, WI, U.S.A.); 2-mercaptoethanol (Eastman-Kodak, Rochester, NY, U.S.A.); fibrinogen (bovine; 90% clottable, plasminogen-rich, Schwarz-Mann, Orangeburg, NY, U.S.A., lot. no. Y1641); agarose (Behringwerke AG, Marburg/Lahn, W. Germany); agar (Difco Lab., Detroit, MI, U.S.A.); human albumin (purified, Behringwerke), bovine albumin and ovalbumin (Schwarz-Mann).

Enzymes were obtained from Leo-Pharmaceutical (Copenhagen, Denmark) [human urokinase, 2210–2300 Ploug units/vial; a Ploug unit being defined as the lysis time of a fibrin clot by a standard urokinase preparation (Ploug & Kjeldgaard, 1957)] and Sigma Chemical Co., St. Louis, MO, U.S.A. (urease type VII and catalase). Sephadex G-75, G-200 and CM-50 were bought from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A., and Freund's adjuvant was from Hyland Lab, Costa Mesa, CA, U.S.A.

The following equipment and test systems were used for the experiments: LKB microimmuno-diffusion equipment (LKB Instruments, Gelman Instruments); microzone electrophoresis system (Beckman Instruments); disc electrophoresis system (Canalco); molecular-weight-estimation kit (Schwarz-Mann); Grafar gel-punch assembly with Wilson plates (Grafar Corp.); dialysis bags (Fisher Scientific Co.); Kodak projector slide plates, contrast, 8.3 cm × 10.2 cm (3.25 in × 4 in); and Immunoplates (Hyland Division of Travenol Lab., lot no. 0771 F010A1).

Methods

Activator and enzyme assays. Plasminogen activator activity was evaluated by a slight modification of the fibrinogen plate method of Astrup & Mullertz (1952) and Schumacher & Schill (1972). These plates consist of a mixture of bovine fibrinogen and plasminogen, where the activation of plasminogen to plasmin causes the digestion of the fibrinogen. Heating destroys plasminogen (Lassen, 1952; Schumacher & Schill, 1972). Differentiation can therefore be made between plasminogen activator activity and proteolytic activity when both heated and unheated fibrinogen plates are used. The major change from previous methodology was the addition of 12.5 mM-CaCl₂ to the fibrinogen plate. This enhanced the sensitivity of the system and increased the visibility of the digestion rings.

To prepare the unheated fibrin plates, 1 g of agarose and 138 mg of CaCl₂ were added to 60 ml of 0.1 M-sodium barbiturate buffer, pH 7.0, containing 0.1 M-NaCl, and heated in a boiling-water bath for 20 min. Bovine fibrinogen (300 mg) was dissolved in 40 ml of the same buffer by gently stirring for 40 min at room temperature. The agarose solution was cooled to 50°C, mixed with the fibrinogen solution for 30 s and immediately poured on the LKB frames. This resulted in an agarose layer that was 1.2 mm thick. Several 2 µl holes were punched in the agarose with the gel punch. The samples were applied with a 20 µl Hamilton syringe.

The activator was expressed in CTA (Committee on Thrombolytic Agents) units (Johnson *et al.*, 1968). Stock urokinase solutions with an activity of 1578 CTA-units/ml were prepared in 0.85% NaCl, containing 0.5% human albumin and 0.1% EDTA, and stored at –20°C. Dilutions were prepared in 0.15 M-NaCl. Fresh solutions were made each time an assay was performed. Diffusion took place at room temperature for 16 h. The activity of the activator was determined from the diameter of the digestion zone by plotting against standard urokinase dilutions using a semilogarithmic graph. Lowest concentration of assayable plasminogen activator was 1.5–2.0 CTA units/ml.

Proteolytic enzyme tests were performed by using heated fibrinogen plates and the gelatin-covered Kodak plates (Schumacher, 1971). The former were prepared in the same way as the unheated plates except that the fibrinogen agar mixture was heated for 60 min at 85°C before being applied to the plate. The photoplates allowed the qualitative evaluation of proteinases that hydrolyse gelatin (in our case seminin). For this purpose, 30 µl drops were added to the gelatin plates, incubated for 16 h at 37°C in a moist chamber and rinsed under a stream of water. Digestion of the gelatin was shown by a clearing of the areas below the drop.

Inhibition and stability studies. For these experiments, the plasminogen activator activity was always adjusted to approx. 18 CTA units/ml. The effect of 6-aminohexanoate was evaluated by mixing 5 μ l of activator in 0.1M-sodium citrate buffer, pH 6.0, with 5 μ l of different 6-aminohexanoate concentrations (0.5 mM to 2.5 M) in the same buffer. After incubation for 2 h at room temperature, activity was tested on unheated fibrin plates. As control, activator was incubated with 5 μ l of 0.1M-sodium citrate buffer under identical conditions. Similar inhibitor studies were done with diluted and undiluted sera from pregnant women in their third trimester. The effect of FeCl₂, MnCl₂, CaCl₂, cysteine and EDTA on seminal plasminogen activator activity was determined by incubating each with the activator solutions for 30 min at various temperatures. The final concentration of each compound was 10 mM. Inhibitor tests with antisera or control sera were performed by mixing 10 μ l of various dilutions with 20 μ l of seminal plasminogen activator and incubating for 1 h at room temperature before assay on unheated fibrin plates.

The stability of the seminal activator under acid and alkaline conditions and at various temperatures was evaluated by dissolving the purified freeze-dried material at a concentration of 10 CTA units/ml in 100 μ l of 0.4M-Tris/acetate buffer with pH adjusted from 2 to 13 by the addition of HCl or NaOH, and incubating in a water bath at 27, 50, 60 and 70°C for 15 or 30 min. The solutions were cooled at 4°C for 10 min and tested for activity at room temperature.

Electrophoretic analyses. Microzone electrophoresis was performed on cellulose acetate membranes at pH 8.5 for 20 min at 3.5 mA. The membrane was cut in half, and one part placed on top of an unheated fibrin plate for 18 h at room temperature to assess the location of the plasminogen activator. The other half was stained for protein by fixation in 3% (w/v) sulphosalicylic acid and 3% (w/v) trichloroacetic acid for 10 min, followed by staining with 0.0025% nigrosin in 2% (v/v) acetic acid. Comparative studies were made with human serum, diluted 1:10 in 0.9% NaCl.

Analytical polyacrylamide-disc-gel electrophoresis was performed at pH 8.3 and 4.5 in 4 mm $\times$ 50 mm, 7.5% cross-linked gels as described by Brewer & Ashworth (1969). The upper gel was omitted. The basic buffer consisted of 28.4 mM-glycine and 5 mM-Tris, and 0.001% Bromophenol Blue was used as tracking dye. Disc gel electrophoresis at acid pH was performed with 48 mM-potassium acetate buffer, and 0.001% Methyl Green as tracking dye. The test solution was mixed with 10 μ l of 50% (w/v) sucrose, layered on top of the gel, and 1.5 mA per gel was applied. To show the protein bands, the gels were stained for 1 h with 1% Amido Black in

7% acetic acid. To determine the location of plasminogen activator, unstained gels were sliced transversely into 2 mm sections and layered on top of unheated fibrin plates.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with 7% cross-linked gels and a buffer containing 0.1% SDS was carried out in 60 mm $\times$ 4 mm glass tubes as described by Weber & Osborn (1969), except that the samples were not dialysed but were heated at 100°C for 2 min in 50 mM-Tris/HCl, pH 7.0, containing 2% (w/v) SDS and 4% 2-mercaptoethanol. The running buffer (pH 8.5) consisted of 6 g of Tris, 28.8 g of glycine and 10 ml of 20% (w/v) SDS made up to 2 litres. Bromophenol Blue dye (0.001%) was used as marker. A current of 2.5 mA per gel was applied. The gels were fixed and stained in methanol/acetic acid/water solution (5:1:5 by vol.) containing 0.25% Amido Black and destained for 20 h in the same solution without Amido Black.

For the molecular-weight determinations, urease (mol.wt. 80000), bovine serum albumin (mol.wt. 67000), catalase (mol.wt. 60000) and ovalbumin (mol.wt. 45000) were used as references. The molecular weight of the plasminogen activator was estimated by extrapolation after plotting the migration coefficients of the reference proteins on semi-logarithmic paper.

Amino acid analyses and protein determinations. The amino acid composition of the seminal plasminogen activators was determined as described by Spackman *et al.* (1958) by using a Durrum model D 500 amino acid analyser. Acid hydrolyses were performed for 24, 48 or 72 h. The amino acid residues per molecule were calculated by assuming 42 alanine residues per molecule of activator 1 and 47 alanine residues per molecule of activator 2, based on the molecular weights of these plasminogen activators. Cysteine was determined after performic acid oxidation (Hirs, 1956). Tryptophan was measured by differential spectrophotometry (Edelhoch, 1967).

Protein concentrations were estimated at A_{280} , assuming that 1 mg of protein/ml gives an absorbance of 1.0 in a 1 cm light-path.

Immunological studies. Antisera against human urokinase were prepared in guinea pigs after first heat inactivating (70°C, 16 h) 1.5 ml of a commercial preparation containing 6500 CTA units of urokinase/ml, 0.1 mg of EDTA/ml and 5 mg of human albumin/ml. The preparation was subsequently mixed with 0.5 ml of Freund's adjuvant and injected subcutaneously into the axillary and inguinal regions. Then 4 weeks after this injection, six booster injections each consisting of 0.35 ml of inactivated urokinase preparation without adjuvant were administered intraperitoneally at 2-day intervals. Each animal received therefore a total of 22100 CTA units of inactivated urokinase. At 10

days after the last booster injection, the guinea pigs were bled by cardiac puncture and the sera prepared. Control serum was obtained by cardiac puncture before the immunization procedure. To retain a good antibody titre the same animals were re-boostered once every 6 weeks subcutaneously with 1.5 ml of the inactivated urokinase preparation mixed with 0.5 ml of Freund's adjuvant.

Double-immunodiffusion Ouchterlony plate tests were performed using either Hyland or Grafar Wilson plates (Crowle, 1961) and 10 ml of a 1% agar solution in 0.05 M-sodium barbiturate buffer, pH 7.8, containing 0.1 M-NaCl was poured on a plate, resulting in a 1.8 mm-thick layer. Holes were punched so that the central hole (6.8 mm diameter) was 10 mm away from the six peripheral holes (2.8 mm diameter), which were 11 mm apart.

Results

Isolation of seminal plasminogen activator

Step I: ultracentrifugation. Whole human seminal plasma has a plasminogen activator activity of 0.79 CTA units/mg of protein that is stable for at least 3 months at -20°C . After thawing, the seminal plasma was centrifuged at $131\,000g$ for 5 h in a SW27 swinging-bucket rotor to remove all insolubles. The supernatant was decanted and either immediately frozen with solid CO_2 and acetone and stored in the freezer at -20°C , or directly used in step II.

Step II: Sephadex G-75 gel filtration. This step as well as the next was performed at 4°C with a flow rate controlled at 8 drops/min by a peristaltic pump. The eluate was collected in plastic tubes to minimize surface absorption of the activators. High-speed-centrifuged seminal plasma (7 ml) was applied to an $80\text{ cm} \times 5\text{ cm}$ column with a void volume of 940 ml and eluted with 0.1 M-sodium-acetate buffer,

pH 5.6, containing 0.05 M-NaCl. Plasminogen activator activity appeared in the void volume, although always toward the end, indicating limited retardation by the Sephadex gel (Fig. 1). The subsequent protein peak contained all the semin. This enzyme has mol.wt. 33000 (Syner & Moghissi, 1972). The plasminogen activator fractions with highest activity and not contaminated with semin were pooled and either dialysed at 4°C against 6×5 litres of water over a 48 h period, then freeze-dried and stored, or with an Amicon filtration apparatus desalted and concentrated to 5 ml by using a UM-10 membrane with a cut-off point at a mol.wt. of 10000. The concentrated solution was subsequently dialysed at 4°C against 6×1 litre of 0.1 M-sodium citrate buffer, pH 6.0, over a 24 h period and immediately used in step III. Such treatments had no effect on the plasminogen activator activity.

The specific activity of the plasminogen activator in the pooled fractions was typically 9.25 CTA units/mg of protein. A 3–4-fold increase in total plasminogen activator activity was consistently found after the Sephadex gel filtration.

Step III: Sephadex CM-50 ion-exchange chromatography. Either 30 mg of the freeze-dried material from step II dissolved in 0.1 M-sodium citrate buffer, pH 6.0, or 5 ml of the concentrated solution was applied to a column ($35\text{ cm} \times 4\text{ cm}$) of Sephadex CM-50 packed, equilibrated in 0.1 M-sodium citrate buffer, pH 6.0, and subsequently eluted with 330 ml of a continuous NaCl gradient from 0 to 2.25 M in the same citrate buffer, but at pH 6.3. Two peaks of activator activity were found (Fig. 2), appearing at 0.75 M-NaCl (1) and the other at 0.85 M-NaCl (2). Activators 1 and 2 possessed typical specific activities of 293 and 226 CTA units/mg respectively. Each peak of activator activity corresponded exactly to the protein peak and was perfectly symmetrical. A summary of the purification of the plasminogen activators is given in Table 1.

Fig. 1. Sephadex G-75 gel filtration of high-speed-centrifuged human seminal plasma

The column ($80\text{ cm} \times 5\text{ cm}$) was eluted with 0.1 M-sodium acetate buffer, pH 5.6, containing 0.5 M-NaCl. Each fraction contained 7 ml. ○, Protein (A_{280}); ●, plasminogen activator (CTA units/fraction). The most active fractions, uncontaminated with semin, were pooled.

Fig. 2. *Sephadex CM-50 ion-exchange chromatography of a human seminal plasminogen activator preparation purified on Sephadex G-75*

The column (35 cm × 4 cm) was equilibrated and first eluted with 0.1 M-sodium citrate buffer, pH 6.0, and subsequently with a continuous NaCl gradient (—). Each fraction contained 3 ml. ●, Protein (A_{280}); ○, plasminogen activator (1 and 2) (CTA units/fraction). For details of peaks see the text.

Table 1. *Purification of plasminogen activator from human seminal plasma*

	Total protein (mg)	Total activity (CTA units)	Specific activity (CTA units/mg)	Purification (fold)
Pooled seminal plasma	408.0	323.0	0.79	1
Centrifugation at 130000g for 4h	323.0	239.0	1.00	1.2
Sephadex G-75 gel filtration	88.47	820.0	9.26	12
Sephadex CM-50 ion-exchange chromatography				
activator 1	0.415	121.50	293	371
activator 2	0.240	54.20	226	286

The two fractions containing plasminogen-activator activity were pooled separately, dialysed against 6 × 5 litres of water over a 36 h period and freeze-dried. Both plasminogen activators lose 40% of their activity if kept in solution at 4°C for 4 weeks, although they are not affected by relatively short periods of dialysis and freeze-drying.

Criteria for homogeneity and molecular-weight estimations

After micro-zone electrophoresis at pH 8.5 of the concentrated step-II material, the band with plasminogen activator activity was located in the cationic area between the β_2 - and γ -globulins of human serum, indicating an isoelectric point of approx. 7.0. The same preparation showed one major and one minor slow-moving band and three fast-moving bands in the pH 8.3 polyacrylamide-

disc-gel-electrophoresis system. The plasminogen-activator activity was associated with the slow-moving fractions. Disc electrophoresis of the step II-material at pH 4.5 produced one large band which migrated slowly and three small faster-moving bands.

After step III, activators 1 and 2 each produced only one band in the pH 8.3 and pH 4.5 disc-gel-electrophoretic systems, of slightly different mobilities. Both migrated slowly in the pH-8.3 and rapidly in the pH-4.5 system. The location of these bands corresponded to the plasminogen activator bands of step II.

Both activators 1 and 2 produced only 1 band after SDS/polyacrylamide-gel electrophoresis (Fig. 3). From ten different experiments which produced almost identical results, the molecular weight of activator 1 was estimated as 69000 and that of activator 2 as 74000. This corroborated the results obtained by Sephadex-gel filtration (step II), since

Fig. 3. SDS/polyacrylamide-gel electrophoresis of activator 1 (A1) and activator 2 (A2) after final purification. See the text for further details.

activators 1 and 2 were always slightly retarded by Sephadex G-75.

Amino acid composition

The amino acid compositions of activator 1 and activator 2 are very similar (Table 2). Both are high in aspartic acid and glutamic acid, serine, glycine and leucine, whereas relatively small amounts of methionine, tryptophan, tyrosine, isoleucine and histidine are present. Both activators possess 16 cysteine residues. From the amino acid analyses, a molecular weight of 69 331 can be calculated for activator 1 and 73 803 for activator 2.

Stability

At pH 6.0 in 0.1M-sodium citrate buffer, both activators were stable if heated at 60°C for 30 min but lost 20–40% of their activity at 70°C for 30 min. Fig. 4 shows the activity of the activators at various pH values and temperatures after incubation times of 15 min. Under these conditions both activators were stable at 37, 50, 60 and 70°C over a wide pH range. The activators were labile only at extremely

acidic and basic pH and at temperatures of 60°C and above. Curves obtained at 4°C and at room temperature looked similar to the ones at 37°C.

Effect of ions, cysteine and EDTA

EDTA, Mg^{2+} , Mn^{2+} and Ca^{2+} at 10 mM had little effect on plasminogen activator activity over a fairly wide temperature range (Fig. 4). At that same concentration and at 60°C, cysteine completely destroyed the activities of the activators whereas Fe^{2+} decreased activator 1 activity to 40% and inactivated activator 2 completely. After incubation at 70°C, 10 mM- Fe^{2+} inhibited both activators totally. As control, the influence of 10 mM- Fe^{2+} and 10 mM-cysteine on plasmin (2.5 CTA units/ml) was evaluated, but no effect on the enzyme was noted.

Inhibition studies

Undiluted sera from pregnant women inhibited the activity of both activators completely. Only a 25% inhibition was obtained if the sera were diluted 5-fold, whereas 10-fold dilutions did not inhibit either of the seminal activators at all.

6-Aminohexanoate completely inhibited both activators down to 10 mM concentrations, below which the inhibitory effect rapidly diminished, so that 1 mM solutions produced only a 10% decrease in activator activity. Inhibition was monophasic, i.e., did not show a brief decrease in the middle of the inhibition curve, and was the same for both inhibitors.

Immunological studies

Human urokinase antisera inhibited the activity of the seminal plasminogen activators completely at 1:1 and 1:2 serial dilutions. Higher dilutions produced gradually smaller inhibitory effects, respectively 97, 61, 33 and 11% for 1:4, 1:8, 1:16 and 1:32 dilutions, until a 1:64 dilution, when the activators were no longer inhibited. Similar results were obtained with urokinase, although the antisera prevented its activity to a greater extent. At these same dilutions, control sera had no effect on the activity of either the seminal plasminogen activators or urokinase.

The urokinase/albumin mixture that was used for injection into guinea pigs produced several bands with its antiserum, the major one being continuous with albumin (Plate 1). A second precipitin band was continuous with the single precipitin band formed between the antiserum and pure urokinase and was also continuous with the single precipitin bands formed with the seminal plasminogen activators. No spurring was observed (Plate 1). The precipitate seen around the central well is due to the diffusion and precipitation of basic proteins in the agar.

EXPLANATION OF PLATE I

Immunodiffusion on an Ouchterlony plate.

Wells: 1, guinea-pig urokinase antiserum; 2, 0.2% human albumin; 3, human urokinase (1150 Ploug units) containing albumin; 4, activator 1; 5, activator 2; 6, purified human urokinase (4600 Ploug units; not containing albumin); 7, partially purified seminal plasminogen activator preparation (step II).

Table 2. Amino acid composition of seminal plasminogen activators 1 and 2

Data are expressed as amino acid residues/molecule, assuming 42 alanine residues/molecule of activator 1 and 47 alanines residues/molecule of activator 2 (see the text). Threonine and serine values are based on linear extrapolations to zero time. Half-cystine was determined by performic acid oxidation, and tryptophan by differential spectrophotometry.

Amino acid	Activator	...	Time of hydrolysis						Value taken		Nearest integer	
			24h		48h		72h					
			1	2	1	2	2	1	2	2	1	2
Lysine			33.5	34.0	32.8	33.5	32.8	33.4	33.0	33.6	33	34
Histidine			14.1	12.4	13.5	12.3	13.9	12.3	13.8	12.3	14	12
Arginine			25.9	28.1	26.1	26.9	25.7	27.8	25.9	27.6	26	28
Aspartic acid			52.9	57.0	51.5	55.7	53.2	56.6	52.5	56.4	53	56
Threonine			26.3	26.8	23.4	23.9	18.7	19.8	31.2	31.9	31	32
Serine			38.8	43.8	31.8	35.1	19.7	21.1	50.1	58.0	50	58
Glutamic acid			68.4	72.1	65.9	70.4	66.1	72.0	66.8	71.5	67	72
Proline			26.2	26.9	23.8	24.8	26.8	26.9	25.6	26.2	26	26
Glycine			52.5	58.0	50.4	55.7	51.6	58.9	51.5	57.5	52	58
Alanine			42.0	47.0	42.0	47.0	42.0	47.0	42.0	47.0	42	47
Cystine (half)									16.0	16.0	16	16
Valine			35.4	37.2	34.7	36.4	34.3	37.7	34.8	37.1	35	37
Methionine			6.5	6.2	6.3	6.1	6.1	5.9	6.3	6.1	6	6
Isoleucine			16.2	16.5	16.0	16.1	15.9	15.9	16.0	16.2	16	16
Leucine			40.5	42.2	39.8	42.2	39.9	41.8	40.1	42.1	40	42
Tryptosine			15.7	14.4	13.5	13.6	12.0	12.3	13.7	13.4	14	13
Phenylalanine			21.4	22.1	20.3	21.4	19.7	21.1	20.5	21.5	21	22
Tryptophan									5.0	9.0	5	9
No. of residues:											547	548
Mol.wt.:											69 331	73 803

Fig 4. Effect of temperature and pH on the activity of the human seminal plasminogen activators (1 and 2) with incubation periods of 15 min

The plasminogen activator activity at pH7 and 37°C represents the 100% value. See the text for further details. ●, 37°C; ▲, 50°C; ■, 60°C; ○, 70°C; A1, activator 1; A2, activator 2.

Discussion

Although the presence of plasminogen activator in seminal plasma has been known for many years, this is the first report of its isolation. Two activators

were obtained in pure form by a relatively simple three-step procedure. Although the third step (Sephadex CM-50 ion-exchange chromatography) resulted in a significant loss of total activator activity compared with the previous step, the high

Fig. 5. Effect of ions, cysteine and EDTA (10 mM final concn.) on the activity of the human seminal plasminogen activators (1 and 2) at various temperatures with incubation periods of 30 min

See the text for further details. ○, EDTA; □, cysteine; △, MgCl₂; ●, FeCl₂; ■, CaCl₂; ▲, MnCl₂; △, untreated. A1, activator 1; A2, activator 2.

degree of purity obtained, judging from the electrophoretic analyses, justifies its use. Initially there was some concern that the final plasminogen activator proteins were minor activator fractions, because of this rather large loss of activity during the final step. However, consistently the following results were obtained: (1) after the initial step (high-speed centrifugation to remove all the insoluble material), the total activity dropped by approx. 25%; (2) after the subsequent Sephadex G-75 gel filtration step, the overall activity increased about 4-fold; (3) after the final Sephadex CM-50 ion-exchange chromatography, the activity dropped again approx. 4-5-fold, but was only 25% less than that present after step 1. The overall loss in activity, compared with the starting material, is no greater than that usually found during purification. Further, no other plasminogen activator activity is present in any other fractions eluted after gel filtration or ion-exchange chromatography. The final activator proteins are therefore the only plasminogen activators in human seminal plasma.

After this work was mostly completed, a brief communication appeared by Astedt *et al.* (1976), who reported the partial purification of the plasminogen activator from human seminal plasma. Using Sephadex-gel filtration, they found an approximate mol.wt. of 67000, which is essentially identical with that of activators 1 and 2. Similar to our findings, they report that this is the only mol-

ecular-weight area in which activator activity is present. The authors did not report the occurrence of two activators at this approximate molecular weight, probably because of the impurity of their preparations and the limitations of molecular-weight estimations by Sephadex gel filtration. Although different techniques were used and only a rather impure fraction was obtained, their data correspond to ours, emphasizing the validity of our observations.

The increase in total activity of the plasminogen activators and the subsequent decrease during the final purification step are intriguing, but at present difficult to explain. They would imply the addition and removal of some activating substance, such as certain ions, to the plasminogen activator fractions. However, we could not enhance the activity of the purified plasminogen activators by adding the previous Sephadex G-75 eluates to them, nor did any of the ions tested increase activator activity or EDTA inhibit it. The increase in total plasminogen activator activity after the second step could have been due to the removal of an inhibitor. Such an inhibitor has indeed been reported in prostatic tissue (Nijs *et al.*, 1971) and blood (Bennett, 1967; Lauritsen, 1968; Hedner, 1974). Trypsin inhibitors with mol.wts. of 6200 and 10100 are present in human seminal plasma (Fink *et al.*, 1971; Zaneveld *et al.*, 1975). They can be found in the final protein peaks after Sephadex G-75 gel filtration. However, these inhibitors do not inhibit plasmin, and none of the

Sephadex fractions decreased plasminogen-activator activity. Further, the removal of inhibitor during step 2 does not explain the subsequent decrease in activity during the final step.

The seminal plasminogen activators possess rather low specific activity compared with pure urokinase or tissue activator preparations. It was initially thought that this was due to our use of bovine rather than human fibrinogen and plasminogen, i.e. a species substrate preference. Nijs *et al.* (1971) showed, for instance, that the plasminogen activator from the human prostate gland only poorly activates bovine plasminogen, whereas human plasminogen is readily activated. To determine if the same was the case for the activators from human seminal plasma, comparative assays were performed by using bovine and human fibrin assay systems. The seminal plasminogen activators gave essentially the same reaction in both systems or, if anything, an even higher one in the bovine fibrin system. Human urokinase reacted the same. The test system therefore cannot explain the low specific activity of the seminal plasminogen activators. Molecular differences are probably present between the urine activator and the seminal activators to account for this (see below).

The biochemical properties of the two activators and their amino acid compositions are very similar, and since they cross-react immunologically, it is likely that their basic molecular structures are essentially identical. It is quite possible that activator 1 is derived from activator 2 through peptide-bond cleavage by one of the seminal plasma proteinases or peptidases, resulting in the release of a peptide with a mol.wt. of 4500.

Although the urokinase preparations used for the development of antibodies were by no means pure, only a single band was formed between the urokinase antiserum and the seminal activator that was continuous with one of the bands formed between the urokinase antiserum and urokinase itself. This indicates that a single component in the urokinase preparation has immunological identity with the seminal activators, but does not show that this band is urokinase itself. However, the urokinase antisera also inhibited the activity of the plasminogen activators as well as that of urokinase, suggesting that the specific, cross-reacting antibody is indeed urokinase. Similar cross-reactions have been reported between urokinase and the tissue activators from the kidney, foetal lung and foetal ureter (Kucinski *et al.*, 1968; Bernik & Kwan, 1969; Kok & Astrup, 1969, 1972; Barlow & Lazar, 1972; Thorsen, 1973).

The inhibition of urokinase by urokinase antiserum was greater than that of the seminal activators, indicating a more effective binding of the antibodies to urokinase. Similarly, although many biochemical similarities exist between urokinase and the seminal activators, distinct differences are also present. For

instance, 6-aminohexanoate has a biphasic inhibitory effect on urokinase, but gives monophasic inhibition with the seminal activators. The molecular weight of urokinase is much less than that of the seminal activators. Thus, although the urine and seminal activators may have some structural similarities, they differ in active-site and molecular properties.

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