[Chem. Pharm. Bull. 29(9)2624—2630(1981)]

Purification and Properties of a Tissue Plasminogen Activator from Hog Kidney

Shinji Soeda and Atsuo Nagamatsu*

Faculty of Pharmaceutical Sciences, Fukuoka University, Nakamura, Nishi-ku, Fukuoka, 814-01, Japan

(Received February 7, 1981)

A procedure was developed for the purification of a tissue plasminogen activator from hog kidney. It involves six consecutive steps: (1) extraction of the tissue plasminogen activator from acetone-dried hog kidney with 0.3 M potassium acetate buffer, pH 4.2; (2) ammonium sulfate precipitation; (3) hydrophobic chromatography on *n*-butyl-Sepharose; (4) affinity chromatography on concanavalin A-Sepharose; (5) gel filtration on hydrophilic vinylmonomer (Toyopearl HW-55); (6) affinity chromatography on fibrin-Sepharose. The purified tissue plasminogen activator had an activity of 13000 IU per mg of protein as assessed by plasminogen activation and had no direct fibrinolytic activity. The apparent molecular weight of the purified tissue plasminogen activator was 60000 as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Carbohydrate analyses of the purified plasminogen activator preparation showed it to contain 54.0 μg of sialic acid and 175.0 μg of hexose per mg of protein. As assessed by plasmin-catalyzed hydrolysis of D-Val-Leu-Lys-pNA, the functional activity of the purified tissue plasmigogen activator was markedly stimulated by addition of fibrin, whereas the interaction with fibrin had a slightly stimulating effect on the activity of human urokinase. Treatment with concanavalin A or glycosidases resulted in 75% or 70% loss of activity of tissue plasminogen activator, respectively, but had no effect on human urokinase.

Keywords——tissue plasminogen activator; hydrophobic chromatography; affinity chromatography; fibrin-bound activator; glycosidase treatment

Much attention has recently been focused on the possible role of the fibrinolytic system in a number of pathological phenomena, including malignant cell transformation.[2] Fibrinolysis is mediated by a group of enzymes called plasminogen activators which convert serum plasminogen to the active proteolytic enzyme plasmin. Plasminogen activators have been found in a number of tissues including kidney,[3] and many attempts have been made to purify the plasminogen activator, known as tissue activator. Human urokinase, the plasminogen activator in urine and the only activator available in a highly purified form, is produced by the kidney cells.[4] Normal human embryonic kidney (HEK) cell cultures are a particularly rich source of plasminogen activator, and comparisons of immunological, physicochemical and enzymic properties indicate that the enzyme isolated from HEK cell-conditioned culture media is similar to human urokinase.[5,6] However, there are striking differences in the effect of 6-aminohexanoic acid or α_1-antitrypsin on fibrinolysis caused by human urokinase or porcine tissue activator,[7,8] and in the affinity for fibrin of the various tissue activators and human urokinase.[9–11] Although the renal plasminogen activator has been isolated from rabbit kidney and some of its properties have been compared with those of human urokinase,[12] there is still much controversy regarding its molecular weight, homogeneity, structure and enzymic reactivity.

The purpose of the present paper is to describe a purification procedure, including affinity chromatography on fibrin-Sepharose, for tissue plasminogen activator from hog kidney as well as some properties of the purified enzyme in comparison with those of human urokinase, with emphasis on the marked differences in the activating effect of fibrin and in the importance of carbohydrate residues of two activators.

Materials and Methods

The following reagents were obtained commercially: *n*-butylamine, potassium thiocyanate, galactose, mannose, concanavalin A, bovine serum albumin and casein (Wako Pure Chem. Co., Osaka, Japan); α-methyl-D-mannoside (Tokyo Kasei Co., Tokyo, Japan); L-lysine HCl and D-Val–Leu–Lys–pNA (Daiichi Chem. Co., Tokyo, Japan); N-acetylneuraminic acid (Sigma Chem. Co., St.Louis, Mo., U.S.A.); glycosidase mixed type (Seikagaku Kogyo Co., Tokyo, Japan); human plasminogen (25 CU/vial) and human plasminogen-rich fibrinogen (AB KABI, Stockholm, Sweden); bovine thrombin (500 NIH units/vial) and human urokinase (6000 IU/vial) (Mochida Pharm. Co., Tokyo, Japan); Sepharose 4B and concanavalin A-Sepharose (Pharmacia Fine Chem. Co., Stockholm, Sweden); Toyopearl HW-55 (Toyo Soda Co., Tokyo, Japan). *n*-Butyl-Sepharose was prepared by coupling *n*-butylamine with Sepharose 4B using CNBr as described by Shaltiel,[13] fibrin-Sepharose was prepared according to the method of Aasted[14] and lysine-Sepharose was prepared as described by Wiman and Wallén.[15] Hog kidneys were obtained as fresh as possible from the slaughterhouse and stored at −70°C.

Preparation of Fibrin Clot——Human plasminogen-free fibrinogen, prepared from commercial fibrinogen by affinity chromatography on lysine-Sepharose, was dissolved in 0.1 M sodium phosphate buffer, pH 7.4, at concentration of 1% clottable protein. Then thrombin (5 NIH units/ml) was added to the fibrinogen solution and the mixture was shaken until clotting was complete. The clot of fibrin thus obtained was homogenized in an Ultra Turrax and centrifuged at $3000 \times g$ for 20 min. The precipitate was washed with 0.2 M sodium phosphate buffer, pH 7.4, and stored in the same buffer until use.

Enzyme Assays——Fibrinolytic activity was assayed on human plasminogen-rich fibrin plates prepared according to the method of Alkjaersig *et al.*[16] Standardization of the plasminogen-rich fibrin plates was performed with serial dilutions of urokinase containing 1.0 to 4.0 IU in 20 μl. The zones of lysis were between 14 and 22 mm in diameter after 18 h of incubation at 37°C and the standard curve was plotted as the log concentration of urokinase *versus* the diameter of the lysis zone. Tissue plasminogen activator samples were diluted in 0.1 M borate buffer, pH 8.0, containing 0.1% Triton X-100, and applied in the same way as the standards, and serial dilutions of standard urokinase were included on each plate.

Plasminogen-activating activity was also assayed in a two-step procedure which involved activation of plasminogen and assay of the amidolytic activity of the plasmin formed using D-Val–Leu–Lys–pNA as a substrate. Five hundred microliters of tissue plasminogen activator solution (50 IU/ml) and urokinase solution (50 IU/ml) to be tested were incubated with 600 μl of human plasminogen (5 CU/ml) in 0.1 M sodium phosphate buffer, pH 7.4, containing 25% (w/v) glycerol at 37°C with or without fibrin clots. At appropriate times 200-μl aliquots were removed and used for the determination of amidolytic activity of plasmin (ΔA_{405}/min) as described by Teger-Nilson *et al.*[17]

To test the direct fibrinolytic activity, samples were applied to fibrin plates heated to 80°C for 30 min to inactivate plasminogen.

Protein Quantitation——Protein was quantitated by ultraviolet absorption measurement at 280 nm in a 1 cm quartz cuvette or by the Folin method according to Lowry *et al.*,[18] using bovine serum albumin as the reference.

Polyacrylamide Gel Electrophoresis——Polyacrylamide gel electrophoresis in the presence of SDS was carried out on 7.5% gels according to Weber and Osborn.[19] The standard proteins used as markers for molecular weight determinations were plasminogen (92000), bovine serum albumin (68000), α-subunit of RNA polymerase (39000) and soybean trypsin inhibitor (21500). Calibration curves were determined for unreduced proteins.

Binding Test to Fibrin-Sepharose——Two milliliters of each activator solution (500 IU/ml) was applied to a fibrin-Sepharose column (1.0×5.0 cm) equilibrated with 0.05 M sodium phosphate buffer, pH 7.5, containing 20 mM L-lysine, followed with the same buffer containing 1.6 M KSCN. Fractions of 3 ml were collected at a flow rate of 30 ml per h.

Carbohydrate Analysis——Hexoses were determined using the phenol/H_2SO_4 method[20] on prehydrolyzed samples (2 M HCl, 110°C for 3 h, in a vacuum). A mixture of 50% galactose and 50% mannose was used as a standard. Sialic acid was analyzed by the method of Warren[21] using N-acetylneuraminic acid as a standard.

Glycosidase Treatment——A 200-μg portion of each sample was treated with mixed glycosidases (10 μg) containing at least 12 glycosidic enzymes for 4 h at 37°C in a total volume of 200 μl of 0.02 M acetate buffer, pH 5.5, containing 0.1 M NaCl. At appropriate times 20-μl aliquots were removed and assayed on human plasminogen-rich fibrin plates.

Purification of Tissue Plasminogen Activator——All steps were performed at 4°C.

Step 1. Extraction: The frozen hog kidneys were partially thawed and minced in a meat grinder. The minced tissue was suspended in 4 l acetone/kg of tissue, homogenized in a Waring blender and filtered. The filter cake was resuspended in acetone and the homogenizing process was repeated three times. The delipidated powder obtained from 3 kg of tissue (about 400 g) was suspended in 9 l of 0.3 M potassium acetate buffer, pH 4.2, and the suspension was gently stirred overnight. After centrifugation at $15000 \times g$ for 20 min,

the precipitate was reextracted with 4.5 l of the same buffer for 5 h. After centrifugation, both supernatants were combined.

Step 2. Ammonium Sulfate Precipitation: Powdered ammonium sulfate was slowly added to the extract to 65% saturation. The solution was stirred overnight. The precipitate was collected by centrifugation at $15000 \times g$ for 20 min. The precipitate was dissolved in 1.7 l of 0.01 M sodium phosphate buffer containing 3 M NaCl and the pH was adjusted at 7.5 with 1 M NaOH. After stirring for 3 h, the pH was readjusted and the solution was centrifuged at $15000 \times g$ for 20 min. The sediment was discarded.

Step 3. *n*-Butyl-Sepharose Chromatography: One-third volume of the supernatant from Step 2 (about 600 ml) was applied to a column of *n*-butyl-Sepharose (2.5 × 20 cm), equilibrated with 0.01 M sodium phosphate buffer, pH 7.5, containing 3 M NaCl. After washing with the equilibrating buffer, elution was performed with a linear gradient from 3 to 0 M NaCl in 0.01 M sodium phosphate buffer, pH 7.5 (total volume 800 ml). Fractions of 3 ml were collected at a flow rate of 60 ml per h. This chromatographic step was repeated three times.

Step 4. Concanavalin A-Sepharose Chromatography: The active fractions from Step 3 were combined (380 ml) and applied to a concanavalin A-Sepharose column (1.5 × 12 cm), equilibrated with 0.01 M sodium phosphate buffer, pH 7.5, containing 1 M NaCl. After washing with the same buffer, elution was performed with a linear gradient (total volume 400 ml) from 0 to 0.6 M α-methyl-D-mannoside in 0.01 M sodium phosphate buffer, pH 7.5, containing 1 M NaCl. Fractions of 5 ml were collected at a flow rate of 20 ml per h.

Step 5. Gel Filtration on Hydrophilic Vinylmonomer (Toyopearl HW-55): The active fractions from Step 4 were combined and concentrated to 15 ml by ultrafiltration with a Diaflow PM-10 membrane. The concentrated solution was gel filtered through a Toyopearl HW-55 column (2.5 × 90 cm) in 0.01 M sodium phosphate buffer, pH 7.5, containing 1.6 M KSCN. Fractions of 4 ml were collected at a flow rate of 30 ml per h.

Step 6. Fibrin-Sepharose Chromatography: To the combined active fractions from Step 5, 35 ml of fibrin-Sepharose gel was added and this solution was equilibrated by dialysis against 0.05 M Tris-HCl buffer, pH 8.0. The gel was packed into a column (1.5 × 35 cm) and washed with the equilibrating buffer until the absorbance of the eluate at 280 nm returned to the base line, then adsorbed protein was eluted with the same buffer containing 1.6 M KSCN. Fractions of 4 ml were collected at a flow rate of 40 ml per h. Plasminogen activator-containing fractions were combined, divided into aliquots, and frozen at −70°C.

Results

Purification of Tissue Plasminogen Activator

The results of the purification procedure are summarized in Table I.

Table I. Purification of Tissue Plasminogen Activator from Hog Kidney

Step	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg)	Yield (%)	Purification factor
1. Acetate extraction	12100	42400	3.5	100	1.0
2. $(NH_4)_2SO_4$ precipitation	9200	36800	4.0	87	1.1
3. *n*-Butyl-Sepharose	530	29200	55	69	17
4. Concanavalin A-Sepharose	67	16100	240	38	69
5. Toyopearl HW-55	5.4	15100	2800	37	800
6. Fibrin-Sepharose	0.8	10400	13000	25	3700

To increase the hydrophobic interaction, *n*-butyl-Sepharose chromatography was carried out in the presence of 3 M NaCl, so that the fractionation was started in 3 M NaCl. Desorption of the activator was achieved by gradually lowering the ionic strength. The active fractions gave a 79% recovery of activator activity with a 17-fold increase in purity. The tissue plasminogen activator was found to be bound to the immobilized lectin concanavalin A. The elution was performed with a gradient from 0 to 0.6 M α-methyl-D-mannoside. Assuming that the gradient was linear, it was calculated that the tissue plasminogen activator eluted between 0.25 and 0.45 M α-methyl-D-mannoside. The specific activity was increased about 4 times. The gel filtration separated the activator from the greater part of the high molecular weight materials (including concanavalin A). The specific activity was increased by a factor 12.

The tissue plasminogen activator preparation obtained after gel filtration lost its activity when KSCN was removed by dialysis. The loss in activity might have been due to the aggregation of activator protein at low ionic strength. To prevent such aggregation, fibrin-Sepharose gel was added to the combined active fractions from Step 5 and this suspension was equilibrated by dialysis against 0.05 M Tris–HCl buffer, and then the gel was packed into a column. As shown in Fig. 1, the activator activities were fully adsorbed on fibrin-Sepharose and were eluted with Tris–HCl buffer containing 1.6 M KSCN in a single peak, which contained 69% of the activity and had a purity of 13000 IU/mg protein. This final preparation, tested on plasminogen-free fibrin plate at a concentration of 1000 IU/ml, lacked any proteolytic activity.

Molecular Properties

The homogeneity of purified tissue plasminogen activator was examined by unreduced SDS-polyacrylamide gel electrophoresis at pH 7.0, and one broad single protein band appeared (the insert in Fig. 1) corresponding to a molecular weight of 60000. Furthermore, an estimate of the molecular weight was made by Toyopearl HW-55 gel filtration as described by Inoue *et al.*[22] The molecular weight was calculated to be 60000.

The analytical data for sugar are presented in Table II, compared with those of urokinase. The purified tissue plasminogen activator contained 175 μg of hexose and 54 μg of sialic acid per mg of protein.

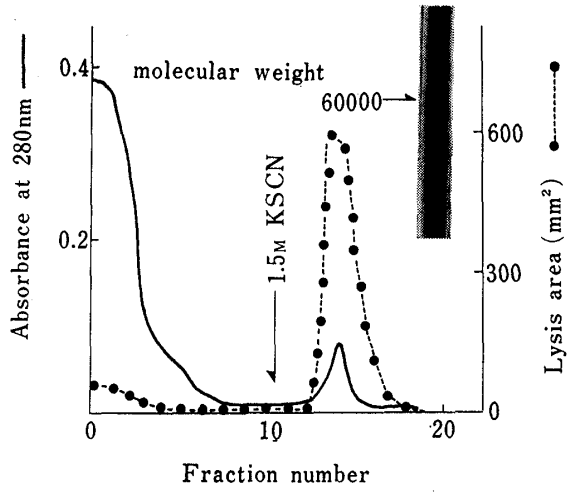

Fig. 1. Fibrin-Sepharose Chromatography of Partly Purified Tissue Plasminogen Activator (Step 5 Material)

Conditions were as described in "Materials and Methods." The activities of the fractions were determined by the human plasminogen-rich fibrin plate method.

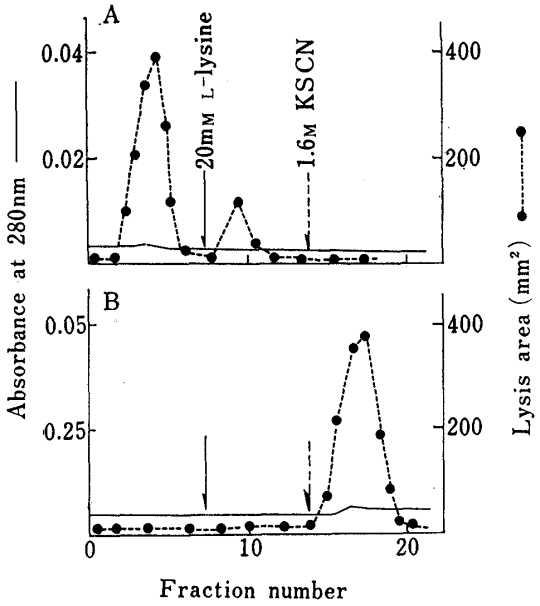

Fig. 2. Binding of Human Urokinase and Purified Tissue Plasminogen Activator to Fibrin-Sepharose

Test conditions were as described in "Materials and Methods," and the activities of the fractions were determined as described in Fig. 1.
A: urokinase, B: tissue plasminogen activator.

TABLE II. Carbohydrate Analyses of the Tissue Plasminogen Activator and Urokinase

Protein	Carbohydrate content (μg/mg)	
	Sialic acid	Hexoses
Tissue activator	54.0	175.0
Urokinase	15.5	24.5

Difference in the Binding to Fibrin-Sepharose of Purified Tissue Plasminogen Activator and Urokinase

The tissue plasminogen activator was bound much more strongly than urokinase to fibrin-Sepharose at protein concentrations producing the same degree of fibrinolytic activity. As shown in Fig. 2A, the greater part of urokinase activities appeared in the effluent immediately after the void volume, and a small amount of the activities adsorbed on fibrin-Sepharose was eluted with the phosphate buffer containing 20 mM L-lysine. The tissue plasminogen activator activities were adsorbed on the column completely and could be eluted with the phosphate buffer containing 1.6 M KSCN, but not with the L-lysine-containing buffer. The results are shown in Fig. 2B.

Effect of Fibrin on the Activation of Plasminogen

Plasminogen-activating activities of the tissue plasminogen activator and urokinase were measured by a two-step procedure using D-Val-Leu-Lys-pNA as a substrate. It has been shown that the interaction with fibrin has a marked stimulating effect on activation with the tissue plasminogen activator but does not in the case of urokinase. The effect of fibrin is clearly demonstrated in Fig. 3A. Further, the effects of fibrinogen and some other proteins on plasminogen-activating activity of the tissue plasminogen activator were examined. As shown in Fig. 3B, fibrinogen had a slightly stimulating effect, while the other proteins had no apparent effect.

Inhibitory Effect of Concanavalin A

When the tissue plasminogen activator (250 IU) was treated with large amounts (300—400 μg) of concanavalin A at 37°C for 10 min, 75% loss of activity was observed. The activity

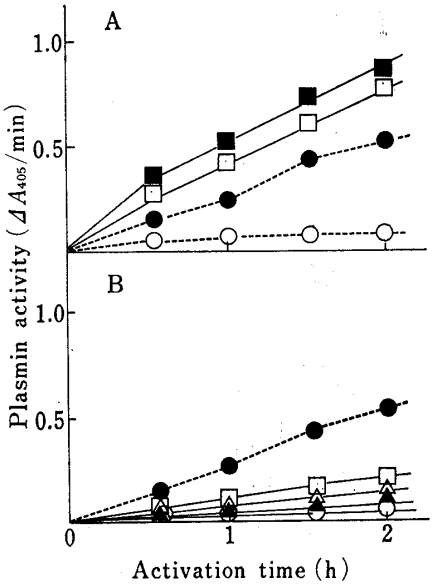

Fig. 3. Effects of Fibrin and Some Other Proteins on the Activation of Plasminogen

Test conditions were as described in "Materials and Methods." Fibrin in the test system were used at a concentration of 5% clottable protein, and other proteins in the test system were used at a concentration of 5%. A: ○, tissue plasminogen activator alone; ●, tissue plasminogen activator with fibrin clot; □, urokinase alone; ■, urokinase with fibrin clot. B: □, tissue plasminogen activator with fibrinogen; △, with albumin; ▲, with casein; ○, with heated fibrin clot; ●, with fibrin clot.

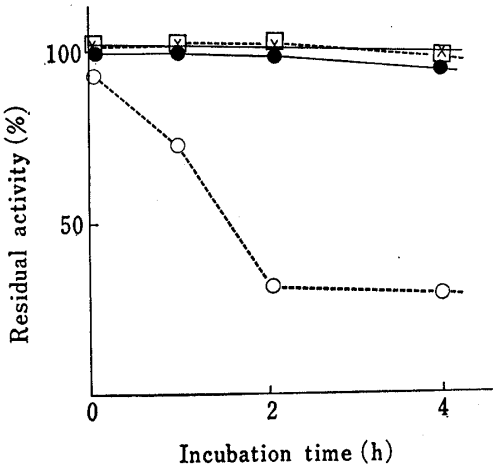

Fig. 4. Inhibitory Effect of Glycosidase Treatment on the Activity of Tissue Plasminogen Activator

Test conditions were as described in "Materials and Methods." ●, tissue plasminogen activator; ○, tissue plasminogen activator treated with glycosidases; ×, urokinase; □, urokinase treated with glycosidases.

Protein	Released hexoses
Tissue activator	75%
Urokinase	50%

was completely restored on the addition of α-methyl-D-mannoside at a concentration of 0.2 M. No inhibitory effect of this material on urokinase was observed.

Glycosidase Treatment of Tissue Plasminogen Activator

As shown in Fig. 4, the tissue plasminogen activator, when treated with glycosidases in 0.02 M acetate buffer, pH 5.5, at 37°C for 4 h, released 75% of the hexose content of the molecule and a 70% loss of activity was observed. However, urokinase was not affected by the treatment with glycosidases.

Discussion

The purification procedure described in the present paper yielded a highly purified tissue plasminogen activator, representing a 3700-fold increase in specific activity from that of the acetate extract of acetone-dried hog kidneys (Table I). The purification procedures were carried out in the presence of 1 M NaCl or 1.6 M KSCN as the presence of one of these reagents proved to be essential for the maintenance of functional activity at each step.

The apparent molecular weight of the tissue plasminogen activator from hog kidneys was calculated to be 60000 both by unreduced SDS-polyacrylamide gel electrophoresis and by gel filtration on Toyopearl HW-55. This molecular weight is comparable to those of tissue plasminogen activators from hog heart (59000),[23] and from hog ovaries (60000),[24] but differs from the molecular weight of an activator from hog parotid glands (25000).[25]

Tissue plasminogen activator is usually compared with human urokinase because no tissue plasminogen activator standard or no urokinase standard of other species is available. Studies on the comparison of human urokinase with hog renal tissue plasminogen activator show that it is possible by simple assay methods to demonstrate marked differences between human urokinase and hog renal tissue plasminogen activator. The two activators exhibit marked differences in the mechanism by which they induce hydrolysis of D-Val-Leu-Lys-pNA. Addition of fibrin markedly accelerates the rate of plasminogen activation by the tissue plasminogen activator, while it only slightly or moderately influences the rate of activation by urokinase (Fig. 3A). This is most likely related to the fact that hog renal tissue plasminogen activator has a much higher affinity for fibrin-Sepharose than urokinase (Fig. 2). Camiolo *et al.*[26] assumed that activation of plasminogen by the tissue plasminogen activator from hog ovaries mainly depends on the fibrin-bound activator, because the formation of fibrin markedly accelerated the rate of plasminogen activation by the tissue plasminogen activator. Our findings also support this hypothesis.

The strong affinity of the tissue plasminogen activator for concanavalin A-Sepharose and the results of carbohydrate analyses indicate that tissue plasminogen activator in hog kidneys is a glycoprotein. The interaction of free concanavalin A with the tissue plasminogen activator resulted in loss in the activator activity, and treatment of the tissue plasminogen activator with glycosidases also induced inactivation of the activator (Fig. 4), while concananvalin A and glycosidases had no inhibitory effect on the activity of urokinase. Thus, carbohydrate residues of the tissue plasminogen activator might play an important role in the plasminogen-activating activity of the tissue plasminogen activator, whereas carbohydrate residues of urokinase do not play such a role.

Acknowledgement The authors wish to thank Professor Yuichi Yamamura, President of Osaka University, for his encouragement.

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