HIGH-PERFORMANCE CHROMATOGRAPHIC METHOD FOR THE PURIFICATION OF TISSUE-TYPE PLASMINOGEN ACTIVATOR

OSAMU MATSUO*, YUMIKO TANBARA, KIYOTAKA OKADA, HIDEHARU FUKAO, HIROSHI BANDO and TETSUHIRO SAKAI

Department of Physiology, Kinki University School of Medicine, Sayama 589 (Japan)

(First received June 16th, 1986; revised manuscript received July 15th, 1986)

SUMMARY

High-performance affinity chromatography was performed on five ligand-bound columns in an attempt to purify tissue-type plasminogen activator (t-PA), which is a glycoprotein with a high affinity for fibrin and also has two Kringle structures and finger-domain in its molecule. The five columns were concanavalin A-5PW, *p*-aminobenzamidine-5PW, imidinodiacetic acid-5PW, boric acid-5PW and lysine-5PW. All five were able to rapidly separate t-PA from contaminating proteins, with high resolution and recovery.

INTRODUCTION

Recently there has been great progress in the study of blood fibrinolysis[1-3]. A new plasminogen activator (tissue-type plasminogen activator, t-PA), which differs from urinary plasminogen activator (urokinase, UK), has been found to induce plasminogen activation on the surface of fibrin. Formerly, t-PA was purified from tissue homogenates of the heart, ovary or uterus by precipitation with ammonium sulphate, acid precipitation or gel filtration[4-6]. However, the development of affinity chromatography has made it easy and convenient to purify t-PA[7]. Further, the introduction of tissue culture medium as a starting material has promoted the use of high-performance liquid chromatography (HPLC) for rapid separation because of the low protein contamination.

In the present study, affinity chromatography with HPLC was employed for the purification of t-PA from melanoma (Bowes) tissue culture medium.

MATERIALS AND METHODS

The following high-performance affinity chromatography columns were prepared with TSK gel G5000PW, which is a hydrophilic resin-based material of large pore size for high-performance gel filtration, with a particle diameter of 10 μg: (1) concanavalin A-5PW; (2) *p*-aminobenzamidine-5PW; (3) iminodiacetic acid-5PW; (4) boric acid-5PW; (5) lysine-5PW. All affinity chromatographic measurements were

carried out with a computer-controlled multipump (CCPM) at a flow-rate of 1.0 ml/min and with a variable wavelength UV detector (Toyo Soda Kogyo). Proteins were usually detected at 280 nm, unless stated otherwise. The ligands were concanavalin A (Seikagaku Kogyo, Tokyo), *p*-aminobenzamidine dihydrochloride (Sigma, St. Louis, MO, U.S.A.), iminodiacetic acid (Tokto Kasei, Tokyo), *m*-aminophenylboronic acid hemisulphate (Aldrich, Milwaukee, WI, U.S.A.) and L-lysine hydrochloride (Nakarai Kagaku, Kyoto).

The equilibration buffer and elution buffer were (1) 50 mM Tris–HCl (pH 7.5) containing 0.01% Tween 80; 0.4 M methyl α-D-mannopyranoside and 0.6 M potassium thiocyanate dissolved in the equilibration buffer for concanavalin A-5PW; (2) 50 mM Tris–HCl (pH 7.5) containing 0.01% Tween 80; 1 M potassium thiocyanate dissolved in the equilibration buffer for *p*-aminozamidine-5PW; (3) 50 mM Tris–HCl (pH 8.0) containing 0.5 M sodium chloride and 0.01% Tween 80; 0.1 M glycine dissolved in the equilibration buffer for iminodiacetic acid-5PW; (4) 50 mM Tris–HCl (pH 8.5) containing 50 mM magnesium chloride and 0.01% Tween 80; 0.4 M methyl α-D-mannopyranoside and 0.6 M potassium thiocyanate dissolved in the equilibration buffer for boric acid-5PW; (5) 50 mM Tris–HCl (pH 7.5 containing 0.01% Tween 80; 0.2 M arginine and 0.8 M potassium thiocyanate dissolved in the equilibration buffer for lysine-5PW.

The t-PA activity was measured by the fibrin-film method using plasminogen-rich fibrinogen (bovine, Organon Teknika) and thrombin (bovine, Mochida Pharmaceuticals)[8].

The purity of proteins was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Weber and Osborn[9]. The proteins in the gels were visualized by silver staining[10].

The molecular weight and plasminogen activator activity were measured simultaneously using electrophoretic enzymography[11]. A fibrin film was produced in polyacrylamide gel, and the position of the plasminogen activator appearing as a clear zone in the fibrin film yielded the molecular weight.

Tissue culture of melanoma (Bowes) was performed as described elsewhere[12], and the monolayered cells were cultured with essential Eagle medium for harvesting. In the conventional method, t-PA was purified from tissue culture medium according to the methods of Rijken and Collen[13]. Human urokinase was purified according to Holmberg *et al.*[14], and had molecular weights of 53 000 and 33 000.

The activity of t-PA was expressed in international units using standard t-PA (Lot 83/517)[15].

RESULTS AND DISCUSSION

Elution profile of t-PA by high-performance affinity chromatography

Since t-PA is a glycoprotein, high-performance chromatography on concanavalin A-5PW (75 mm × 7.5 mm I.D.) was carried out as follows. After equilibrating the column with 50 mM Tris–HCl (pH 7.5) containing 0.01% Tween 80, 1 ml of melanoma (Bowes) tissue culture medium was injected. First the unbound protein was eluted (Fig. 1), and then bound protein was eluted with a linear gradient (0–0.4 M methyl α-D-mannopyranoside and 0–0.6 M potassium thiocyanate). The t-PA was eluted as a single peak at 0.4 M methyl α-D-mannopyranoside and 0.6 M potassium
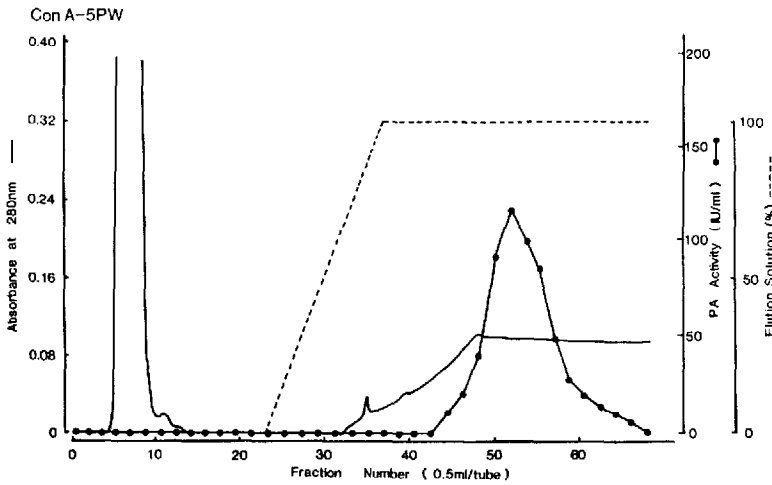
Fig. 1. High-performance affinity chromatography on concanavalin A-5PW. A 1-ml volume of tissue culture medium was applied to concanavalin A-5PW (75 mm × 7.5 mm) and eluted with a linear gradient of methyl α-D-mannopyranoside (0–0.4 M) and potassium thiocyanate (0–0.6 M).

thiocyanate. All of the t-PA activity was eluted and none was observed in the unbound portion. Thus, the separation of t-PA from other contaminating proteins by high-performance affinity chromatography on concanavalin A-5PW was rapid, with high resolution and recovery.

High-performance affinity chromatography on p-aminobenzamidine-5PW (75 mm × 7.5 mm I.D.) was carried out as follows. After equilibrating the column with 50 mM Tris–HCl (pH 7.5) containing 0.01% Tween 80, 3 ml of melanoma (Bowes) tissue culture medium were injected. First the unbound protein was eluted, and then
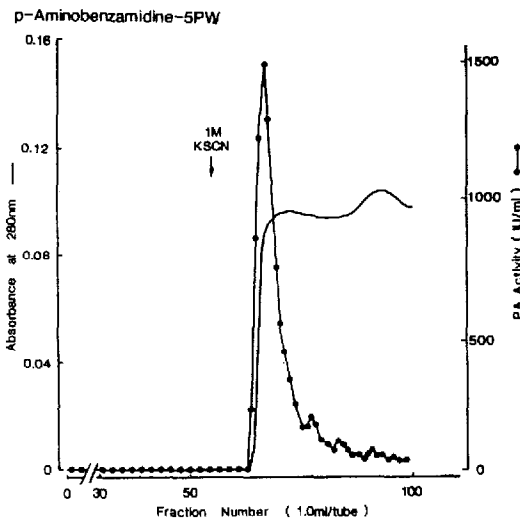

Fig. 2. Elution profile on p-aminobenzamidine-5PW. A 3-ml volume of tissue culture medium was applied to p-aminobenzamidine-5PW (75 mm × 7.5 mm), washed with 1 M sodium chloride buffer solution and eluted with 1 M potassium thiocyanate buffer solution.

the column was washed with 1 *M* sodium chloride dissolved in the equilibration buffer. Finally, 1 *M* potassium thiocyanate dissolved in the equilibration buffer was used to elute the bound protein, as a single peak (Fig. 2). All of the t-PA activity was eluted and none was observed in the unbound portion or in the portion eluted with 1 *M* sodium chloride solution. Thus, the separation of t-PA from other contaminating proteins by high-performance affinity chromatography on *p*-aminobenzamidine-5PW was rapid, with high resolution and recovery.

Using two different high-performance metal chelate affinity chromatography columns, an iminodiacetic acid column and a boric acid column, purification of t-PA was carried out as follows. The iminodiacetic acid column (75 mm × 8.0 mm I.D.) was equilibrated with 50 m*M* Tris–HCl (pH 8.0) containing 0.5 *M* sodium chloride and 0.01% Tween 80, and 20 ml of zinc chloride (5 mg/ml) were applied to the column. This amount of zinc chloride was sufficient to saturate the column with zinc ion. After the column had been re-equilibrated with the initial buffer, 30 ml of melanoma (Bowes) tissue culture medium were applied. The unbound protein passed through first, and the bound protein was then eluted with a linear gradient of glycine (0–0.1 *M*). As shown in Fig. 3, the bound protein was eluted at a glycine concentration of 0.067 *M*. The t-PA was eluted as two peaks: the first peak appeared at a glycine concentration of 0.083 *M*, just after the protein peak; the second and major peak appeared at a glycine concentration of 0.1 *M*. All of the t-PA activity was eluted and none was observed in the unbound portion. Thus, the separation of t-PA from other contaminating proteins was effectively achieved by high-performance iminodiacetic acid affinity chromatography. When boric acid-5PW was used, the column (75 mm × 7.5 mm I.D.) was equilibrated with 50 m*M* Tris–HCl (pH 8.5) containing 50 m*M* magnesium chloride and 0.01% Tween 80, and 1 ml of melanoma (Bowes) tissue culture medium was applied. The unbound protein passed through first, and
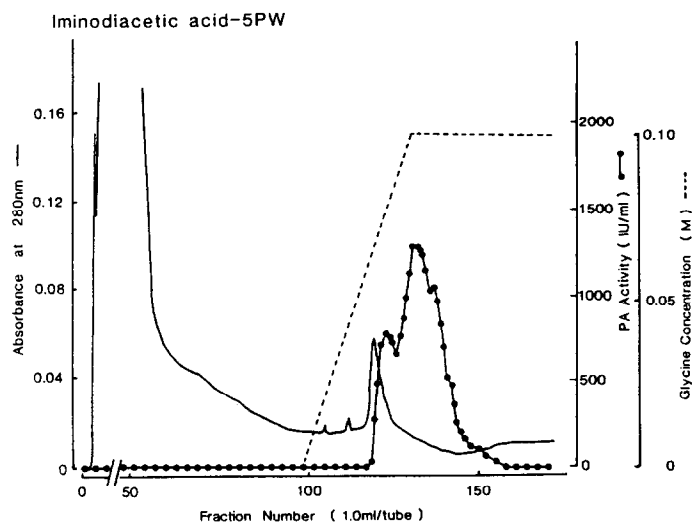

Fig. 3. High-performance affinity chromatography on iminodiacetic acid-5PW. A 30-ml volume of tissue culture medium was applied to iminodiacetic acid-5PW (75 mm × 8.0 mm), which had previously been equilibrated with buffer containing zinc chloride, and eluted with a linear gradient of glycine (0–0.1 *M*).

the bound protein was then eluted with a linear gradient of 0–0.4 M methyl α-D-mannopyranoside and 0–0.6 M potassium thiocyanate. As shown in Fig. 4, the t-PA was eluted as a single peak and no activity was observed in the unbound portion. Thus, high-performance boric acid affinity chromatography is also useful for the separation of t-PA from other contaminating proteins, with high resolution and recovery.

When the separation of t-PA on lysine-5PW was performed, the column (75 mm × 7.5 mm I.D.) was equilibrated with 50 mM Tris–HCl (pH 7.5) containing 0.01% Tween 80, and 3 ml of melanoma (Bowes) tissue culture medium were applied. The unbound protein passed through first, and the column was then washed with 0.3 M sodium chloride dissolved in the equilibration buffer. The bound protein was eluted with 0.2 M arginine and 0.8 M potassium thiocyanate. The t-PA was eluted as a single, sharp peak. All of the activity was observed in the eluted portion and none was observed in the unbound portion. Thus, high-performance lysine-5PW affinity chromatography was effective for the separation of t-PA from other proteins in a rapid and convenient manner.

Purification of t-PA

In each of the above experiments, a single-stage affinity chromatography with HPLC was employed to purify t-PA from tissue culture medium. In order to obtain t-PA as a single band in SDS-PAGE, several variations of affinity chromatography were investigated. Among them, the combination of iminodiacetic acid-5PW, concanavalin A-5PW and p-aminobenzamidine-5PW was found to be excellent. A 30-ml volume of tissue culture medium (872.7 I.U.) was first applied to iminodiacetic acid-5PW, and the eluted t-PA was then applied to concanavalin A-5PW. On washing the column, the bound t-PA was eluted with 0.4 M methyl α-D-mannopyranoside.
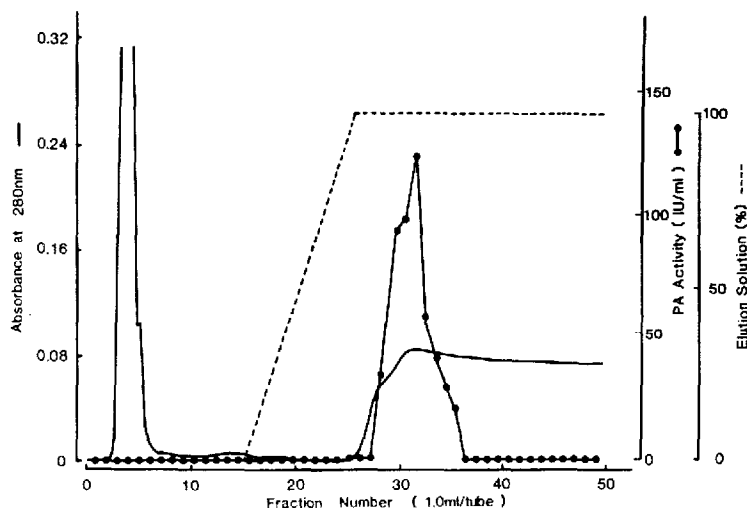
Boric acid-5PW

Fig. 4. Elution profile on boric acid-5PW. A 1-ml volume of tissue culture medium was applied to boric acid-5PW (75 mm × 7.5 mm) and eluted with a linear gradient of methyl α-D-mannopyranoside (0–0.4 M) and potassium thiocyanate (0–0.6 M).

TABLE I

PURIFICATION OF TISSUE-TYPE PLASMINOGEN ACTIVATOR BY HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY

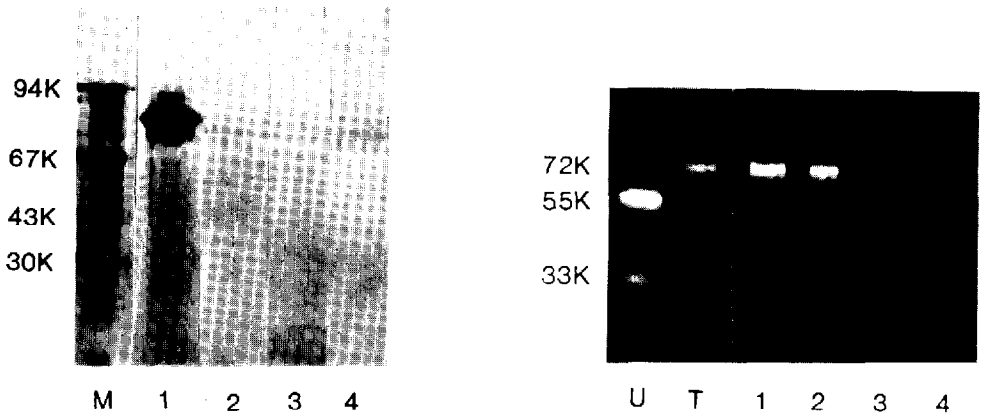| Purification step	Vol. (ml)	Absorbance at 280 nm		PA activity		Specific activity	Purification factor	Recovery (%)
		Per ml	Total	(I.U./ml)	(I.U.) Total			
Medium	30	1.300	39.0	872.7	26179	671.3	1	100
Iminodiacetic acid-5PW	12	0.023	0.276	1280.3	15364	55665	82.6	58.7
Concanavalin A-5PW	8.5	0.017	0.145	1060.2	9011	62149	92.6	34.4
p-Aminobenzamidine-5PW	17.7	0.002	0.035	407.7	7208	205928	306.8	27.5

Fig. 5. SDS-PAGE with silver staining of t-PA eluted by high-performance affinity chromatography. 1 = Starting material; 2 = protein eluted from zinc chelate-5PW; 3 = protein eluted from concanavalin A-5PW; 4 = protein eluted from p-aminobenzamidine-5PW; M = marker protein.

Fig. 6. Electrophoretic enzymography of the protein eluted by high-performance affinity chromatography. 1–4 as in Fig. 3; U = urokinase; T = t-PA purified from melanoma (Bowes) tissue culture medium by a conventional method[13].

The portion with t-PA activity was applied to p-aminobenzamidine-5PW and eluted with 1 M potassium thiocyanate buffer solution. The purification factor was about 300, and the recovery rate was 27.5% (Table I). The purity of the final product was confirmed by SDS-PAGE, which upon silver staining revealed a single band with a molecular weight of 72 000 (Fig. 5). Electrophoretic enzymography demonstrated a single active band with a molecular weight of 72 000 (Fig. 6). The above combination of affinity chromatographies is thus useful for the rapid and convenient purification of t-PA.

REFERENCES

1 O. Matsuo, D. C. Rijken and D. Collen, in P. M. Mannucci and A. D'Angelo (Editors), Urokinase: Basic and Clinical Aspects, Academic Press, London, 1982, pp. 55–61.
2 F. Bachmann and E. K. O. Kruithof, Semin. Throm. Hemostasis, 10 (1984) 6–17.
3 O. Saksela, Biochim. Biophys. Acta, 823 (1985) 35–65.
4 E. R. Cole and F. Bachmann, J. Biol. Chem., 252 (1977) 3729–3737.
5 P. Kok, Thromb. Haemostasis, 41 (1979) 718–733.
6 J. G. Corasanti, C. Celik, S. M. Camiolo, A. Mittelman, J. L. Evers, A. Barbasch, G. H. Hobika and G. Markus, J. Natl. Cancer Inst., 65 (1980) 345–351.
7 D. C. Rijken and D. Collen, J. Biol. Chem., 256 (1981) 7035–7041.
8 O. Matsuo, H. Mihara and Y. Rokushima, Acta Haematol. Jpn., 39 (1976) 298–305.
9 K. Weber and M. Osborn, J. Biol. Chem., 244 (1969) 4406–4412.
10 P. Tunón and K. E. Johansson, J. Biochem. Biophys. Methods, 9 (1984) 171–179.
11 O. Matsuo, T. Sakai, H. Bando, K. Okada, S. Nakajima, O. Takagi and S. Izaki, Haemostasis, 16 (1986) 43–50.
12 O. Matsuo, T. Sakai, C. Matsuo, Y. Nishida, K. Akazawa and H. Mihara, Acta Physiol. Latinoam., 33 (1983) 305–313.
13 D. C. Rijken and D. Collen, J. Biol. Chem., 256 (1981) 7035–7041.
14 L. Holmberg, B. Bladh and B. Åstedt, Biochim. Biophys. Acta, 445 (1976) 215–222.
15 P. G. Gaffney and A. D. Curtis, Thromb. Haemostasis, 53 (1985) 134–136.