Bulletin of Okayama Prefectural Junior College Vol.34 50~58 (1991)

Biochemical and Immunological Properties of Canine Urokinase, Highly Purified from Bladder Urine

Hiroyuki SUMI, Nobuyoshi NAKAJIMA, Koji SASAKI* and Shigeru MORIYAMA*

* Biochemistry Section, Central Research Laboratories Zeria USA, Inc, New York.

Key words : Urokinase, Plasminogen activator, Benzamidine-Sepharose, Double immunodiffusion analysis.

SUMMARY

The physicochemical and thrombolytic properties of two different molecular forms of urokinase and their precursor protein(prourokinase) have been reported [*J. Biol. Chem.* 258: 8014, 1983; *Progress in Fibrinolysis* 6; 165, 1988]. In the present study, canine urokinase was first isolated from bladder urine and highly purified by the chromatographies with aluminum magnesium silicate, benzamidine-Sepharose and Sephadex G-100. The purified material showed a molecular weight of 52, 000 daltons and had a specific activity of 61, 000IU/mg protein using fibrin substrate and 294, 800IU/mg protein using chromogenic substrate (pyro-glutamyl-glycyl-arginine-p-nitroanilide). Canine urokinase did not form immunoprecipitin line with antibody to human urokinase in the double immunodiffusion analysis and its activity was not either neutralized with both antibodies to human urokinase and human melanoma tissue plasminogen activator.

INTRODUCTION

Plasminogen activators (PAs) were isolated from various animal tissues [1-4] biological fluids [5-7] and cell culture media [8-11]. Human urokinase (UK) isolated from urine [12-18], the most well-known PA, has been used in clinical fields as a thrombolytic agent [19]. However, there are few reports in the purifications and some properties of UK from other mammals. PAs were classified into two groups according to their biochemical and immunological properties; one is "urokinase-type PA (u-PA)", with a molecular weight of under 60,000 daltons, which is neutralized by antihuman UK antibody [5-9], and

the other is "tissue-type PA (t-PA)", with a molecular weight of over 60,000 daltons, which is not neutralized by antibody to human UK but quenched by antibody to porcine heart t-PA or human melanoma t-PA [1, 3, 4, 11]. Recently,We demonstrated the existence of both u-PA and t-PA in dog plasma after oral administration of human UK [20]. But the biochemical characteristics of canine UK was unknown. Present report described the isolation and purification of canine UK from bladder urine of beagle dogs and its biochemical and immunological properties in comparison with those of human UK.

MATERIALS AND METHODS

Canine urine

Male beagle dogs (LR-E strain) of 12–15 months old were used. Canine urine was directly collected from bladder using a polyethylene catheter, and aprotinin (Trasylol; Bayer, West Germany) was immediately added to be a final concentration of 100 kallikrein inhibition unit (KIU)/ml urine. The urine sample was stored at −80℃ until use.

Purification of canine UK

Canine UK was isolated and purified from bladder urine by the serial methods, aluminum magnesium silicate (AMS; Fuji Chemicals, Japan) adsorption chromatography, benzamidine-Sepharose (Bz-Sepharose) affinity chromatography [21] and Sephadex G-100 gel filtration. The molecular weight of canine UK was estimated with molecular weight standards; bovine serum albumin (Mr=67,000), ovalbumin (Mr=43,000) and α-chymotrypsinogen A (Mr=25,000).

Enzyme assays

Amidolytic activity was measured by the method of Barlow and Marder [22] using pyro-glutamyl-glycyl-arginine-p-nitroanilide (S−2444; Kabi, Sweden). Fibrinolytic activity was determined according to the method of Walton [23] and a unit of canine UK was defined with human high molecular weight UK as a standard. The plasminogen activator activity of canine UK was confirmed by the activation of bovine plasminogen (Daiichi Chemicals, Japan) with plasminogen-free fibrin agar plate (Daiichi Chemicals). Protein was determined by absorbance measurement at 280 nm or by the protein-dye binding method [24] with bovine serum albumin as a standard.

Immunological studies

Rabbit antibodies to human low molecular weigh UK (Mr=33,000) were prepared by the method of Sasaki et al [20]. Neutralization assay of canine UK by both anti-human UK IgG and anti-human melanoma t-PA immunoglobulin (Biopool, Sweden) was carried out on plasminogen-rich fibrin agarose plate. Double immunodiffusion analysis was per-

formed by the method of Ouchterlony [25].

RESULTS

Purification of canine UK

Two liters of the frozen canine urine thawed at room temperature was centrifuged to remove the precipitates at 1,600 g for 10 min at 4°C and diluted with an equal volume of distilled water. The pH of diluted urine was adjusted to 7.0 with 4 mol/l HCl and applied to a column (4.5 × 13 cm) of AMS equilibrated with 10 mmol/l phosphate buffer, pH 7.0, containing 5 KIU/ml aprotinin and the column was washed with 4 mol/l urea in the same buffer. At the first purification step, AMS to be the specific adsorbent for basic proteins was employed for the concentration of canine UK. Canine UK adsorbed on the AMS column was eluted with 0.1 mol/l phosphate buffer, pH 7.4, containing 8 mol/l urea, 2 mol/l NaCl and 5 KIU/ml aprotinin (Fig. 1). After Bz-Sepharose chromatography, canine UK was separated into two molecular forms by gel filtration on Sephadex G-100 column. The chromatographic profile showed a main active peak with Mr=52,000 and a minor one with Mr=31,000 (Fig. 2). The degradation of high molecular type canine UK was prevented by the addition of aprotinin to all solvents during purification except for the final gel filtration. Canine UK with Mr=52,000 had a specific fibrinolytic activity of 61,000 IU/mg protein and a specific amidolytic activity of 294,800 IU/mg protein. The amidolytic activity of purified canine UK corresponding to 140 IU was never inhibited by 100 KIU of aprotinin.

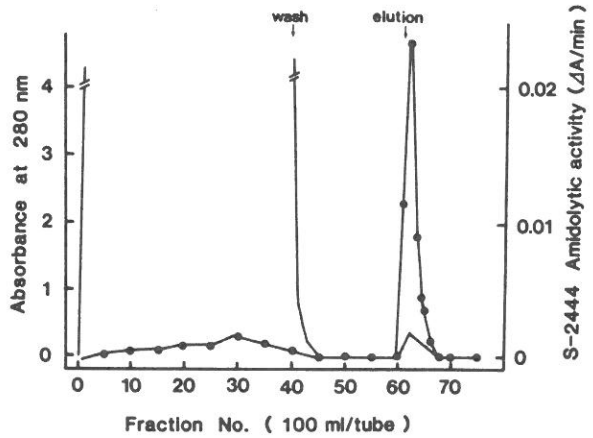

Fig. 1 AMS adsorption chromatography of canine bladder urine. Two litters urine was applied to a column (4.5 × 13 cm) and the adsorbed activator was eluted with 0.1mol/l phosphate buffer, pH 7.4, containing 8 mol/l urea, 2 mol/l NaCl NaCl and 5 KIU/ml aprotinin. The eluted fractions (#61–70) were pooled and dialyzed. ———, absorbance at 280 nm; ●———●, S-2444 amidolytic activity.

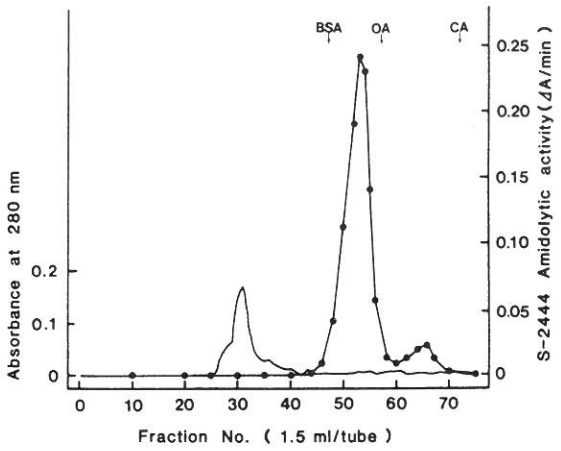

Fig. 2 Gel filtration of canine UK from Bz-Sepharose chromatography on Sephadex G-100. Lyophilized sample fron Bz-Sepharose was dissolved in 0.5 ml of 0.1 mol/l phosphate buffer, pH 7.4, containing 1 mol/l NaCl and applied to a column (1.4 × 95 cm) eqvilibrated with the same buffer. The eluted fraction (#47-54) were pooled, dialyzed and lyophilized. ———, absorbance at 280 nm; ●———●, S-2444 amidolytic activity: BSA, bovine serum albumin (Mr=67,000); OA, ovalbumin (Mr=43,000); CA, α-chymotrypsinogen A (Mr=25,000).

Table I. Purification of canine UK from bladder urine.

	Protein OD 280 nm	Activity IU	Yield %	Specific activity IU/OD 280 nm	Purification factor
Urine	(138, 770)	18, 660	100		
AMS	200	15, 290	84	80	1
Bz-Sepharose	3. 75	13, 750	74	3, 850	48
Sephadex G-100	0. 07	2, 540	14	36, 290	454

The activity was measured by fibrin agarose plate method (see text for details).

In the absence of aprotinin during the purification steps, the proportion of low molecular type activator (Mr=31,000) increased. The result of the purification steps is summarized in Table I. Canine UK with Mr=52,000 was purified 454-fold over the eluted fraction from AMS column. In the presence of plasminogen, the purified canine UK as well as human UK completely converted plasminogen to plasmin, but did not lyse fibrin in the absence of plasminogen (Fig. 3).

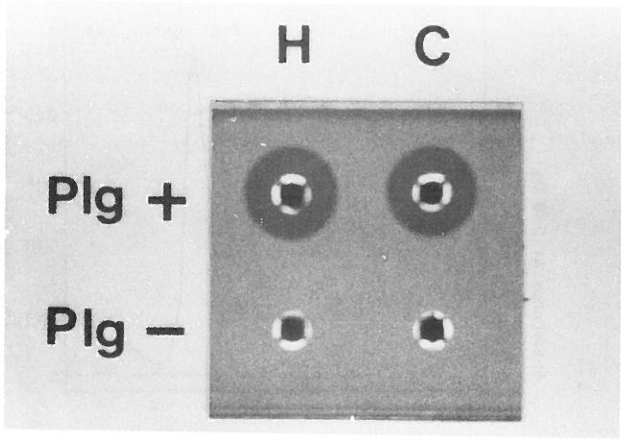

Fig. 3 Plasminogen activator activity of canine UK. Ten μl of activator (1,500 IU/ml) dissolved in 0.1 mol/l phosphate butter and the same volume of bovine plasminogen (5 casein units/ml) in 50 m mol/1 Tris buffer (pH 9.0) containing 20 mmol/l lysine, 0.1 mol/l NaCl, 25 % glycerol were mixed and incubated for 30 min at 37℃ (Plg +). Ten μl of the reaction mixture was placed in a hole of plasminogen-free agar plate and incubated for 4 hr at 37℃. Fibrinolytic activity of activator alone was performed by the same manner in the absence of plasminogen (Plg -). H; human UK, C; canine UK.

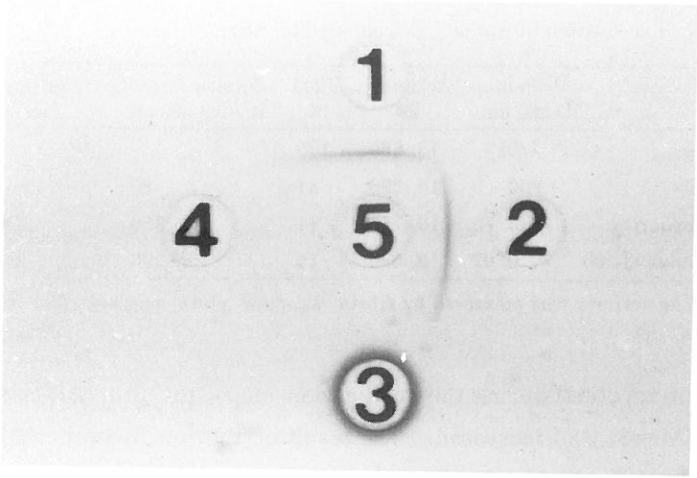

Fig. 4 Ouchterlony analysis of canine UK. Each well contained 10 μl of the sample dissolved in 50 m mol/l veronal buffer, pH 8.6 and immunodiffusion was performed for 48 hr at room temperature. Well 1, human high molecular weght UK (10,000 IU/ml); well 2, human low molecular weight UK (10,000 IU/ml); well 3, 50-fold concentrated canine bladder urine; well 4, canine UK (10,000 IU/ml); well 5, antihuman low molecular weight UK IgG (0.8 mg/ml).

Immunological studies

The immunological properties of canine UK were determined by antibody neutralization assay and double immunodiffusion analysis. The activity of canine UK (5 IU/ml) was not

quenched by at least 500 μg/ml of the anti-human UK IgG and the anti-human melanoma t-PA immunoglobulin (Table Ⅱ). Whereas 5 IU/ml of human UK was completely neutralized by 5 μg/ml of the anti-human UK IgG but not inhibited by at least 500 μg/ml of the anti-human melanoma t-PA immunoglobulin. By the Ouchterlony technique, 100 IU of canine UK and canine bladder urine (50-fold concentrated) corresponding to 50 IU of the activator showed no immuno-precipitin line with the anti-human UK IgG (Fig. 4).

Table Ⅱ Neutralization analysis of canine UK and human UK by both anti-human UK and anti-human melanoma t-PA antibodies

Plasminogen activator	Antibody	Residual activity (%)				
		Antibody concentration (μg/ml)				
		0.05	0.5	5	50	500
Canine UK	Anti-UK	102	106	102	98	102
	Anti-t-PA	101	97	102	102	102
Human UK	Anti-UK	94	60	0	0	0
	Anti-t-PA	99	103	104	101	98

Ten μl of activator (10 IU/ml) dissolved in 0.1 mol/l phosphate buffer, pH 7.4, was added to an equal volume of the antibody in concentration from 0.1 to 1,000 μg/ml in the same buffer. After incubation for 30 min at 4℃, 10 μl of the reaction mixture was placed on a plasminogen-rich fibrin agarose plate and incubated for 16 hr at 37℃ with serial UK standard solutions (0.625-10 IU/ml).

DISCUSSION

Canine UK was first isolated and purified from bladder urine by the serial methods, AMS adsorption, Bz-Sepharose affinity chromatografy and Sephadex G-100 gel filtration. AMS adsorption was effective for rapid separation and concentration of the crude canine UK fraction, because the proteins with low isoelectric point such as canine urinary kallikrein [26] and esterase [27] could not adsorbed on the column.

According to Sephadex G-100 gel filtration, the molecular weights of two forms of canine UK were determined to be 52,000 and 31,000, respectively. These values were very similar to those of human UKs [6]. Canine UK with Mr=52,000 was also comparable to rat UK (Mr=56,000) [7], canine vascular tissue PA (Mr=48,000) [2], and canine u-PA (Mr=50,000) [13]. Increase of low molecular type of canine UK with Mr=31,000 in the abcence of aprotinin suggested that canine UK with Mr=31,000 was caused by the degradation of high molecular type with proteolytic enzyme(s) as well as human UK was done.

The specific amidolytic activity of canine UK which was not inhibited by an excess amount of aprotinin was about 5-fold higher than the specific fibrinolytic activity. It is defined that amidolytic activity is equivalent to fibrinolytic activity in human UK [6]. The specific fibrinolytic and amidolytic activities of canine UK were also about 0.5-fold and

about 2.7-fold in comparison with those of human UK, respectively. These results suggest that canine UK differs from human UK in ezymatic properties.

The activity of canine UK with Mr=52,000 was not neutralized by both the antibody to human UK and the antibody to human melanoma t-PA. Canine UK did not form immunoprecipitin line with the anti-human UK antibody. Kucinski et al. reported that PAs in urine of nonprimetes, such as dog, pig, rabbit and guinea pig, were not inhibited by anti-human UK anti-serum [28]. Rat UK, however, cross-reacted with antibody to human UK [7]. In this experiment, canine UK was immunologically distinguished from human UK and melanoma t-PA. These findings indicate that the immunological reactivity of mammalian UK relates with a common phylogenetic derivation as well as the other proteins.

This study provides the information about biochemical and immunological differences between canine UK and human UK. It is important that the biochemical and immunological properties of UK from different animal urine are characterized to understand the physiological roles of PAs in tissues or organs.

ACKNOWLEGEMENTS

The authors wish to thank Prof. R. Nakamura, Nagoya University and Prof. H. Mihara, Miyazaki Medical College for useful comments on this report.

REFERENCES

1 Wallen, P. ; Bergsdorf, N. ; Ranby, M. : Purification aod identification of two structural variants of porcine tissue plasminogen activator by affinity adsorption of fibrin. *Biochim. Biophys. Acta* **719** ; 318–328 (1981).

2 Highsmith, R. F. : Isolation and properties of plasminogen activator derived from canine vascular tissue. *J. Biol. Chem.* **256** : 6788–6795 (1981).

3 Kok, P. : Separation of plasminogen activators from human uterine tissue and comparison with activators from human urine and porcine tisse. *Thromb. Heamostas.* (Stuttg.) **41**: 718–733 (1979).

4 Kirchheimer, J. ; Knller, A. ; Binder, B.R. : Isolation and characterization of plasminogen activators from hyperplastic and malignant prostate tissue. *Biochim. Biophis. Acta* **797**: 256–255 (1984).

5 Wun, T.C. ; Schleuning, W. D. ; Reich, E. : Isolation and characterization of urokinase from human plasma. *J. Biol. Cem.* **257** : 3276–3283 (1981).

6 Shibatani, T. ; Kakimoto, T. ; Chibata, I. : Purification of high molecular weight urokinase from human urine and camparative study of two active forms of urokinase. *Thromb. Heamostas.* (Stuttg.) **49** : 91–95 (1983).

7 Huber, K. ; Kirchheimer, J. ; Binder, B. R. : Isolation of rat urokinase characterization and enzyme kinetic analysis, comparison with human urokinase ; in Davidson, Bachmman, Bouvier, Kruithof, Progress in fibrinolysis chap. 4, pp. 168–170 (Churchill Livingstone, Edionburgh 1983).

8 Vdtterlein, D. ; Bell, T. E. ; Young, P. L. ; Roblin, R. : Immunological quantitation and immunoad-

sorption of urokinase-like activities secreted by human cell. *J. Biol. Chem.* **255**: 3665–3672 (1980).

9 Bykowska, K. ; Levin, E. G. ; Rijken, D. C. ; Loskutoff, D. J. ; Collen, D. : Characterization of a plasminogen activator secreted by cultured bovine aortic endothelial cells. *Biochim, Biophis. Acta* **703**: 113–115 (1980).

10 Goldfarb, R. H. ; Quigley, J. P. : Purffication of plasminogen activator from rous sarcoma virus transformed chick embryo fibrobrasts traced with the tumor promoter phorbol 12-myristate 13-acetate. *Biochemistry* **19** : 5463–5471 (1980).

11 Rijken, D. C. ; Collen, D. : Purification and characterization of the plasminogen activator secreted by human melanoma cells in culture. *J. Biol. Chem.* **256**: 7035–7041 (1981).

12 Sumi, H. Toki, N. Sasaki, K. and Robbins, K. C. : Oral asministration of Urokinase. *Thromb. Res.* **20** : 711–714, (1980).

13 Sumi, H., Maruyama, M. Matsuo, O., Mihara, H. and Toki, N. : Higher fibrin-binding and thrombolytic properties of single poly peptide chain-high molecular weght urokinase. *Thromb. Heamostasis* **47**: 297, (1982).

14 Sumi, H. and Robbins, K. C. : A functionally active heavy chain derived from human high molecular weight urokinase. *J. Biol. Chem.* **258**: 8014–8019, (1983).

15 Sumi, H., Maruyama, M., Yoneta, T., Toki, N. and Mihara, H. : Activation of plasma fibrinolysis after interarectal administration of high molecular weight urokinase and its derivatives. *Acta Heamatol.* **70** : 289–295, (1983).

16 Sumi, H., Toki, N., Sasaki, K. and Mihara, H. : Comparative properties of single and double polypeptide chains of high molecular weight urokinase ; in Davidson, Bachmann, Bouvier, Kruithof, Progress in Fibrinolysis. Vol. 6, pp. 165–167 (Churchill Livingstone, New York 1983).

17 Sumi, H., Seiki, M., Morimoto, N., Tsushima, H., Maruyama, M. and Mihara, M. : Plasma fibrinolysis after intraduodenal administration of urokinase in rats. *Enzyme* **33** : 121–127, (1985).

18 Toki, N., Sumi, H., Sasaki, K., Boreisha, L. and Robbins, K. C. : Transport of urokinase across the intestinal tract of normal human subjects with stimulation of synthesis and/or release of uroki nase-type proteins. *J. Clin. Invest.* **75**: 1212–1220, (1985)

19 Sherry, S. : Therapeutic fibrinolysis: past, present and future; in Paoletti, Sherry, *Thrombosis and fibrinolysis,* pp. 1–9 (Academic Press, New York, 1977).

20 Sasaki, K. ; Moriyama, S. ; Tanaka, Y.; Sumi, H. ; Toki, N. ; Robbins, K. C. : The transport of ^{125}I-labeled human high molecular weight urokinase across the intestinal tract in a dog model with stimulation of synthesis and/or release of plasminogen activators. *Blood* **66**: 69–75 (1985).

21 Holmberg, L. : Bladh, B. : Astedt, B. : Purification of urokinase by affinity chromatography. *Biochim. Biophys. Acta* **445**: 215–222 (1976).

22 Barlow, G. H. ; Marder, V. J. : Plasma urokinase levels measured by chromogenic assay after infusions of tissue culture or urinary source urokinase. *Tromb. Res.* **18**: 431–437 (1980).

23 Walton, P. L. : An improved fibrin plate method for the assay of plasminogen activator. *Clin. Chim. Acta* **13**: 680–684 (1966).

24 Bradford, M. M. : A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248–254 (1976).

25 Ouchterlony, O. : Diffusion in gel methods for immunologinal analysis. *Prog. Allergy.* **5**: 1–78 (1958).

26 Matsuda, Y. ; Miyasaki, K. ; Fujimoto, Y. ; Hojima, Y. ; Moriya, H. : Purification and chracterization of dog urinary kallikrein. *Chem. Pharm. Bull.* (Tokyo) **30**: 2512–2520 (1983).

27 Matsuda, Y. ; Fujimoto, Y. ; Akihara, S. ; Moriya, H. : Studies on acidic arginine esterase extraced in urine. I. Purification and chracterization of dog urinary arginine esterase. *Chem. Pharm. Bull.* (Tokyo) **32**: 2371–2379 (1983).

28 Kucinski, C. S. ; Fletiher, A. P. ; Serry, S. : Effect of urokinase antisernm on plasminogen activators : determination of immunologic dissimilarity between plasma plasminogen activator and urokinase. *J. Clin. Invest.* **47**: 1238–1253 (1968).

Received June, 27 1990
Accepted October, 11 1990