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Purification and Characterization of Dog Urinary Kallikrein¹⁾

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Two forms of *N*- α -tosyl-L-arginine methyl ester (TAME) hydrolyzing activities were found in dog urine and one of them was confirmed to be dog urinary kallikrein (DUK, E.C. 3.4.21.8).

Mainly by chromatographic techniques, the kallikrein was purified about 1390-fold from the dialyzed dog urine with the overall yield of 36% of vasodilator activity.

The specific activities of the finally purified preparation of DUK were 1250 kallikrein units(KU)/A₂₈₀ of vasodilator activity and 11.3 μ mol/min/A₂₈₀ of TAME esterolytic activity, and the preparation was homogeneous on disc gel electrophoresis and ultracentrifugal analysis. The molecular weight of DUK was estimated to be approximately 2.6 to 2.9 $\times 10^4$ by vertical plate polyacrylamide gel electrophoresis, gel filtration on a Sephadex G-100 column and ultracentrifugation, and the isoelectric point of this enzyme was also determined as pI 4.2.

Various properties such as pH stability, optimum pH for esterolytic activity and heat stability were similar to those of dog renal kallikrein.

Keywords—purification of kallikrein; dog urine; vasodilator activity; esterolytic activity; estimation of molecular weight

Urinary kallikrein (EC 3.4.21.8) is considered to be produced in the kidney.³⁾ Through investigation into the correlation between urinary and renal kallikreins in the rat, Nustad also suggested that urinary kallikrein could be traced to the renal organ.⁴⁾ On the other hand, many studies on the kallikrein-kinin system in the kidney and urine have been carried out by researchers in the clinical field.⁵⁾ The administration of aldosterone caused increased excretion of the urinary kallikrein into the urine, and some renal diseases were also discovered to increase its excretion. In these investigations, rather impure renal and urinary kallikreins were used. For the study of the kallikrein-kinin system in the renal organ, the kallikrein preparation should be purified and characterized as far as possible. The authors' previous paper⁶⁾ reported the purification of dog renal kallikrein (DRK) and showed that DRK is similar to partially purified dog urinary kallikrein (DUK).

The present paper describes the purification and characterization of DUK, and compares the properties of this purified kallikrein with those of DRK and human urinary kallikrein (HUK).

Materials and Methods

Dog Urine—Dog urine, the starting material of this investigation, was kindly supplied by Y. Otaki, Department of Pharmacology, School of Medicine, Teikyo University. This was directly collected from the bladder by catheterization and the collected dog urine was stored in the frozen state until use.

Materials—The following materials were obtained commercially: *N*- α -tosyl-L-arginine methyl ester (TAME), *N*- α -benzoyl-L-arginine ethyl ester (BAEE), *N*- α -tosyl-L-lysine methyl ester (TLME) and synthetic bradykinin (Protein Research Foundation, Osaka, Japan); *N*- α -benzoyl-L-arginine methyl ester (BAME, Serva Chemical Co., West Germany); DEAE-cellulose (Green Cross Drug Mfg. Co., Osaka, Japan); Sephadex

G-75, G-100 and DEAE-Sephadex A-50 (Pharmacia AB, Sweden); soybean trypsin inhibitor and cytochrome *c* (Sigma Chemical Co., U.S.A.); myoglobin (Miles-Servac Co., England); γ -globulin (human heavy chain, Tokyo Kasei Co., Tokyo, Japan); bovine serum albumin and egg white albumin (Daiichi Chemical Co., Tokyo, Japan).

Assay of Kallikrein Activities—Vasodilator activity was determined by the method of Moriya *et al.*,⁷⁾ by measuring the increase of femoral arterial blood flow following the injection of a sample into a dog, and was expressed as kallikrein unit (KU).⁸⁾ Esterolytic activities were measured both by the colorimetric method with chromotropic acid⁹⁾ at pH 8.0, 30°C, and by the spectrophotometric method¹⁰⁾ at pH 8.0, 25°C. All esterolytic activities were expressed in terms of μ mol of substrate hydrolyzed per min. Kinin releasing activity was determined according to the method previously reported by Moriya's group.¹¹⁾ Partially purified bovine kininogen containing LMW and HMW kininogen, purified in our laboratory, was used as substrate and the activity was expressed in terms of μ g equivalent to bradykinin released per min at 30°C, pH 8.0.

Protein Concentration—The protein concentration was estimated by measuring the absorbancy at 280 nm in a 1 cm width cuvette.

Electrophoresis—Disc gel electrophoresis was performed by the modified method of Davis¹²⁾ using 7% polyacrylamide gel columns at 0.04 M Tris-glycinate buffer pH 8.6. Vertical plate polyacrylamide gel electrophoresis was carried out based on the modified method of Raymond and Weintraub¹³⁾ in 7.5% gel with 0.08 M Tris-EDTA borate buffer (pH 9.2) containing 0.1% (w/v) sodium dodecyl sulfate (SDS). After electrophoresis, the gel was stained for protein with 0.2% (w/v) Coomassie Brilliant Blue in 20% (v/v) methanol containing 5% (v/v) acetic acid.

Isoelectric Focusing—Isoelectric focusing with the Ampholine system was done using the apparatus of LKB Produkter AB as developed by Vesterberg and Svensson.¹⁴⁾ The pH range of carrier ampholyte was 3.5 to 5.0 and electrophoresis was performed for 40 h at 500 V constant voltage with the cooling system set at 4°C.

Ultracentrifugal Analysis—Sedimentation equilibrium analysis for the determination of molecular weight was carried out with a Spinco ultracentrifuge model E.

Results

Purification of Dog Urinary Kallikrein

All steps were carried out at 0 to 4°C.

Step 1. DEAE-cellulose Adsorption and Elution—Dog urine (4100 ml) was dialyzed against tap water overnight. The dialyzed dog urine (4900 ml) was added to an equal volume of tap water and adjusted to pH 7.0 with 1 N NaOH. DEAE-cellulose (50 g) was added to the diluted dialyzed dog urine and packed in a column (3 cm \times 50 cm) after 2 h of adsorption. The initial eluate with 0.05 M Tris-HCl buffer at pH 7.0 had neither vasodilator nor TAME esterolytic activity. The second eluate (240 ml) with 0.05 M Tris-HCl buffer containing 0.15 M NaCl had TAME esterolytic activity, but not vasodilator activity. This fraction contained a new arginine ester hydrolyzing enzyme(s) of dog urine. The kallikrein containing solution (300 ml) was eluted with 0.05 M Tris-HCl buffer containing 0.5 M NaCl; this fraction possessed most of the vasodilator activity and about 30% of the TAME hydrolyzing activity (Fig. 1).

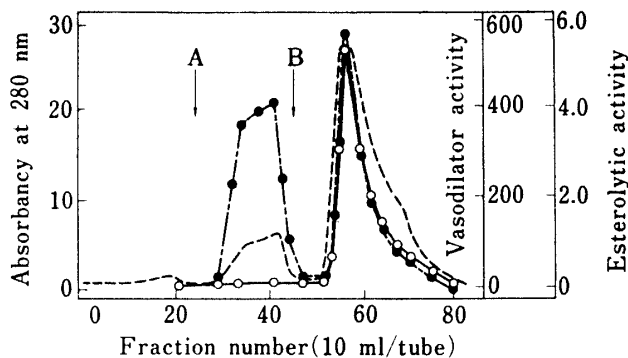


Fig. 1. Separation of Kallikrein and Arginine Esterase in Dialyzed Dog Urine by DEAE-cellulose Adsorption

For details of the procedure, see the text (Step 1). A, 0.05 M Tris-HCl buffer (pH 7.0) containing 0.15 M NaCl; B, 0.05 M Tris-HCl buffer (pH 7.0) containing 0.5 M NaCl; ●, esterolytic activity; ○, vasodilator activity; ···, absorbancy at 280 nm.

Step 2. DEAE-cellulose Chromatography—The kallikrein fraction (Total A_{280} = 3200) obtained from Step 1 was dialyzed against deionized water overnight and concentrated to

approximately 30 ml by DEAE-cellulose adsorption. The concentrated crude kallikrein solution was further dialyzed against 0.05 M Tris-HCl buffer (pH 7.0) containing 0.05 M NaCl for 6 h. The dialyzed crude kallikrein solution (36 ml) was applied to a DEAE-cellulose column (2.5 cm × 90 cm) pre-equilibrated with the same buffer. The column was eluted with a gradient elution system using the same buffer containing 0.05 to 0.5 M NaCl. The elution profile is shown in Fig. 2A. Most of the TAME hydrolyzing activity was eluted at fraction numbers 56 to 68 (6 ml/tube) and these fractions were pooled (78 ml). The total absorbancy at 280 nm of this preparation was 330.

Step 3. Sephadex G-100 Gel Filtration—The pooled DUK preparation obtained from step 2 was concentrated on DEAE-cellulose and dialyzed against 0.05 M Tris-HCl buffer containing 0.1 M NaCl. The dialyzed DUK solution (22 ml) was applied to a Sephadex G-100 column (2.5 cm × 90 cm). The column was eluted with same buffer and fractions (6 ml/tube) were collected. The elution profile is shown in Fig. 2B. The kallikrein was eluted in fractions 54 to 64, and these were pooled. The total absorbancy at 280 nm of this preparation was 47.

Step 4. DEAE-Sephadex A-50 Chromatography—The pooled active fraction of DUK from step 3 (66 ml) was applied to a DEAE-Sephadex A-50 column (1.5 × 90) pre-equilibrated with 0.05 M Tris-HCl buffer pH 7.0 containing 0.1 M NaCl. The column was eluted with a gradient elution system using the same buffer containing 0.1 to 0.6 M NaCl. The elution profile of this step is shown in Fig. 2C. TAME esterolytic activity was eluted in fractions 50 to 58 (3.5 ml/tube) and these fractions were pooled (30 ml). The total absorbancy at 280 nm of the pooled fraction was 18 and specific activities of esterolysis of TAME and vasodilator were 8.9 $\mu\text{mol}/\text{min}/A_{280}$ and 820 KU/A_{280} , respectively.

Step 5. Sephadex G-75 Gel Filtration—The active preparation of DUK from step 4 was concentrated and dialyzed against 0.05 M Tris-HCl buffer at pH 7.0 containing 0.05 M NaCl, then

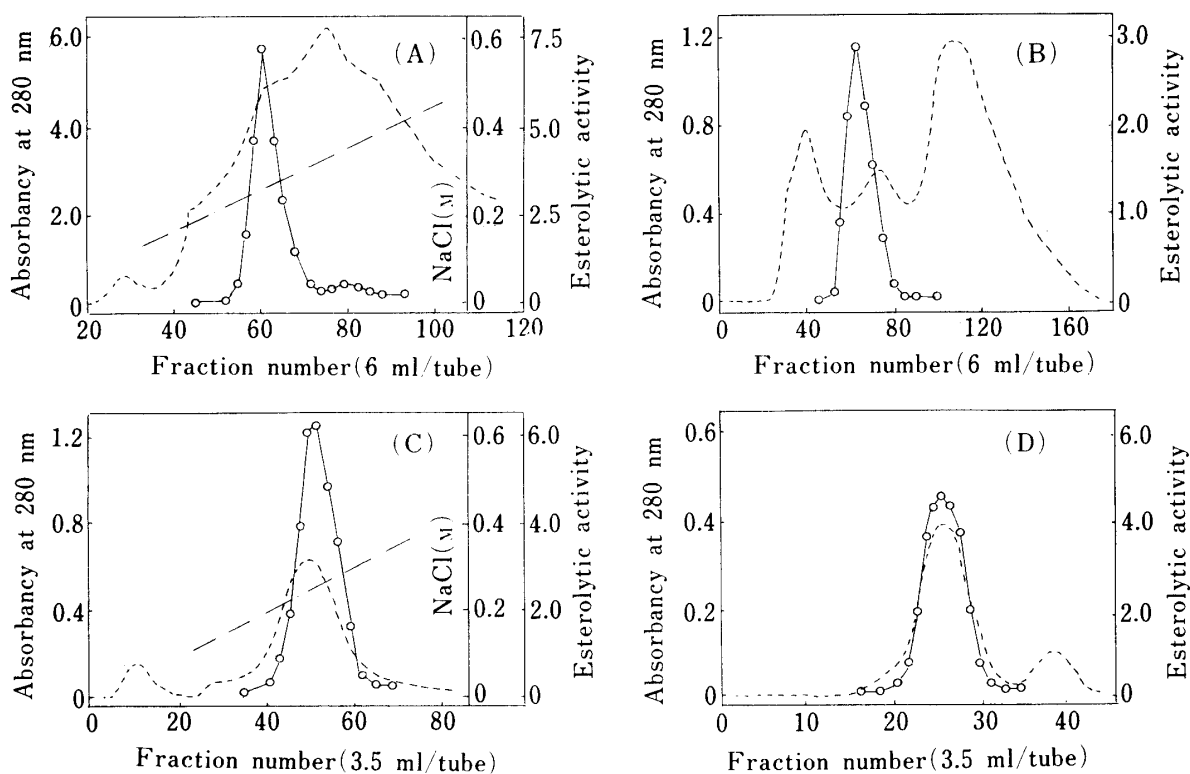


Fig. 2. Purification of Dog Urinary Kallikrein by (A) DEAE-cellulose Chromatography, (B) Sephadex G-100 Gel Filtration, (C) DEAE-Sephadex A-50 Chromatography, and (D) Sephadex G-75 Gel Filtration

For details of the procedure, see the text (steps 2 to 5). \circ —, esterolytic activity; ---, absorbancy at 280 nm; —, concentration of NaCl.

10 ml of dialyzed DUK solution was applied to a Sephadex G-75 column (1.5 cm × 80 cm) pre-equilibrated with the same buffer. The elution profile of this step is shown in Fig. 2D, and most of the TAME hydrolyzing activity was eluted in fractions 24 to 30 (3.5 ml/tube). The total absorbancy at 280 nm of this preparation was 8.0 and specific kallikrein activities (assayed in terms of esterolysis of TAME and vasodilator) were 11.4 $\mu\text{mol}/\text{min}/A_{280}$ and 1250 KU/A_{380} , respectively.

The results of purification of DUK are shown in Table I, and Table II summarizes the specific activities of purified DUK assayed by various chemical and biological procedures. The specific activity, measured by vasodilator assay, of purified DUK was 1390 times more than that of the initial dialyzed dog urine, and the final preparation represented 36% recovery of kallikrein activity (vasodilator assay).

TABLE I. Summary of the Purification of Dog Urinary Kallikrein

Step	Procedure	Protein Total protein (A_{280})	Vasodilator activity			Esterolytic activity		
			Total activity (KU)	Specific activity (KU/A_{280})	P.F. ^{a)}	Total activity ($\mu\text{mol}/\text{min}$)	Specific activity ($\mu\text{mol}/\text{min}$ $/A_{280}$)	P.F. ^{a)}
1	Dialyzed dog urine (4900 ml)	31200	28300	0.9	1	1370	0.044	1
	DEAE-cellulose adsorption							
	0.15 M NaCl eluate	600	900	1.5	1.6	750	1.25	28.4
2	0.5 M NaCl eluate	3200	25000	7.9	8.8	400	0.13	2.8
	DEAE-cellulose chromatography	331	22100	66.7	74	255	0.77	17.5
3	Sephadex G-100 gel filtration	47	19000	410	456	184	3.96	90
4	DEAE-Sephadex A-50 chromatography	18	13800	821	913	149	8.88	202
5	Sephadex G-75 gel filtration	8	10100	1250	1390	92	11.3	256

a) Purification factor.

TABLE II. Biological and Esterolytic Activities of Purified Dog Urinary Kallikrein

Biological activities			Esterolytic activity
Vasodilator (KU/A_{280})	Kinin releasing ($\mu\text{gBK}^{\text{a)}}$ / min/A_{280})	Ratio ($\text{ngBK}^{\text{a)}}$ / min/KU)	TAME hydrolyzed ($\mu\text{mol}/\text{min}/A_{280}$)
1250	11.5	92	11.3

a) BK: bradykinin.

Biological activities were measured by the vasodilator and kinin releasing assay methods.^{7,10)} Esterolytic activity was determined by colorimetry with chromotropic acid.⁹⁾

Homogeneity

The finally purified preparation of DUK showed a single band in disc electrophoresis on a polyacrylamide gel column (Fig. 3A), and result of the sedimentation equilibrium analysis (Fig. 3B) gave a linear functional relationship. These results suggested that the DUK was homogeneous.

Isoelectric Point

The result of isoelectric focusing using the Ampholine system as shown in Fig. 4 gave only

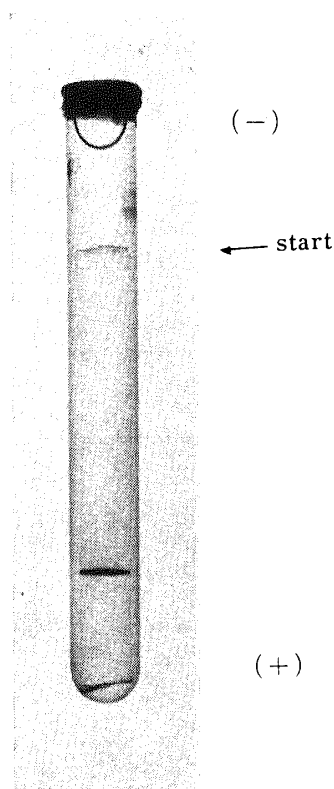


Fig. 3A. Disc Gel Electrophoresis of Purified Dog Urinary Kallikrein

Tris-glycinate buffer (pH 8.6) was used and the electrophoresis was carried out with a constant current of 2.5 mA/disc column at 4°C.

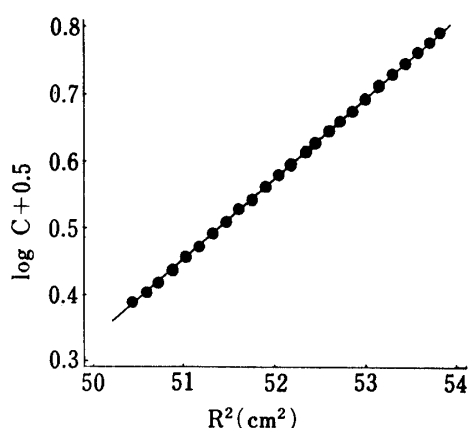


Fig. 3B. Sedimentation Equilibrium Centrifugation of Dog Urinary Kallikrein

The initial concentration of protein was 2 $A_{280}/100$ ml of 0.1 M phosphate buffer (pH 7.0). The plots were read from an interference of the equilibrium distribution at 13410 rpm and 10°C for 190 min.

one active peak (measured by vasodilator assay using the dog), having isoelectric point with pI 4.2.

Correlation Between Esterolytic and Vasodilator Activities

The relationship between the TAME hydrolyzing and the vasodilator activities of DUK from Steps 2 to 5 is shown in Fig. 5. The coefficient of correlation between the two assay methods was 0.846 with no significant difference ($p < 0.05$), and one $\mu\text{mol}/\text{min}$ of TAME hydrolyzing activity at pH 8.0, 30°C corresponded to 101 ± 12 KU (mean \pm S.E.).

Estimation of Molecular Weight

The approximate molecular weight of DUK was estimated to be 2.7 to 2.9×10^4 by gel filtration on a Sephadex G-100 column using the method of Andrews¹⁵⁾ and by SDS vertical plate polyacrylamide gel electrophoresis, as shown in Fig. 6. Furthermore, the molecular weight estimated by sedimentation equilibrium analysis was found to be 2.6×10^4 (Fig. 3B).

Optimum pH and pH Stability

Optimum pH for TAME esterolytic activity was determined with 0.08 M modified Britton-Robinson's widerange buffer (pH range of 5.0 to 11.0), and the maximum relative activity was observed at pH 8.8. The pH dependency of stability of DUK was investigated by measuring its esterolytic action on TAME using the same buffer, and DUK was most stable at pH 8.5. These results (shown in Fig. 7) were similar to those renal and human urinary kallikreins.^{6,16)}

Heat Stability

The effect of the heat treatment of DUK, measured in terms of TAME hydrolyzing activity at pH 8.0, is summarized

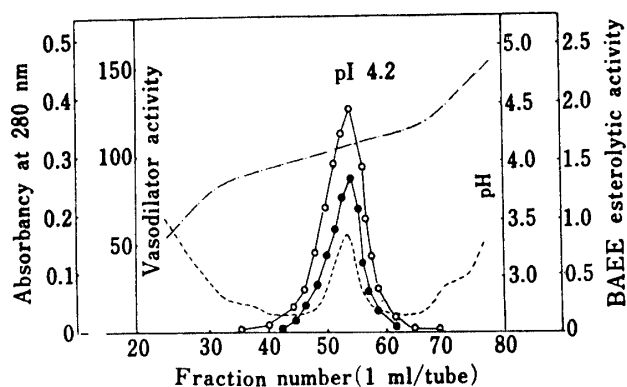


Fig. 4. Isoelectric Focusing of Purified Dog Urinary Kallikrein

- - - - , pH value; -O-, vasodilator activity; ...; absorbancy at 280 nm; O, BAAE esterolytic activity.

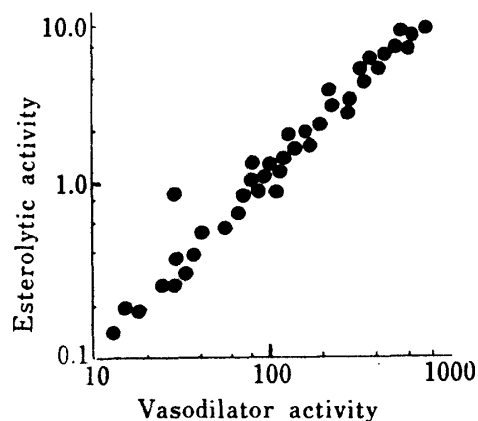


Fig. 5. Correlation Between Esterolytic and Vasodilator Activities of Dog Urinary Kallikrein

The ordinate shows TAME hydrolyzing activity as determined by the colorimetric method with chromotropic acid at pH 8.0, 30°C, and the abscissa gives vasodilator activity, measured by the arterial blood flow method in the dog.

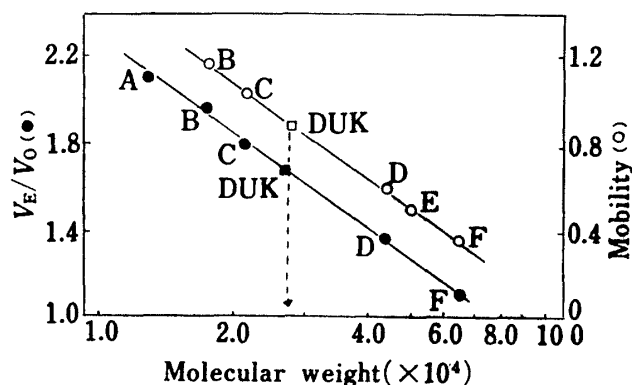


Fig. 6. Estimation of the Approximate Molecular Weight of Dog Urinary Kallikrein by Gel Filtration and SDS Vertical Plate Polyacrylamide Gel Electrophoresis

Authentic cytochrome *c*(A), myoglobin(B), soybean trypsin inhibitor(C), egg white albumin(D), γ -globulin (human heavy chain, E) and bovine serum albumin (F) were used as markers.

Gel filtration (●) was performed with a Sephadex G-100 column (1.5 x 80 cm), equilibrated with 0.05 M Tris-HCl buffer (pH 7.0) containing 0.1 M NaCl, and blue dextran was used for determination of the void volume. SDS electroresis (○) was carried out at a constant 200 V at 4°C.

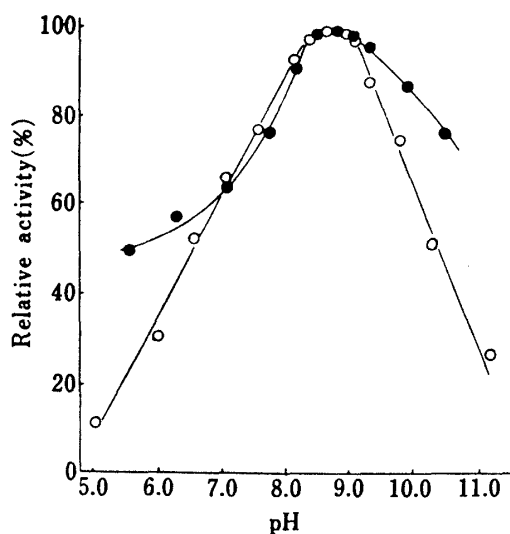


Fig. 7. pH Dependencies of Activity and Stability of Dog Urinary Kallikrein

The pH dependencies of the activity and stability were determined by the esterolytic method with chromotropic acid using TAME as the substrate at various pH's in 0.08 M, modified Britton-Robinson's wide-range buffer. To determine the pH dependency of stability, the enzyme was preincubated for 18 h in the same buffer. The activity was expressed as a percentage of that of the optimum pH.

○, pH activity; ●, pH stability.

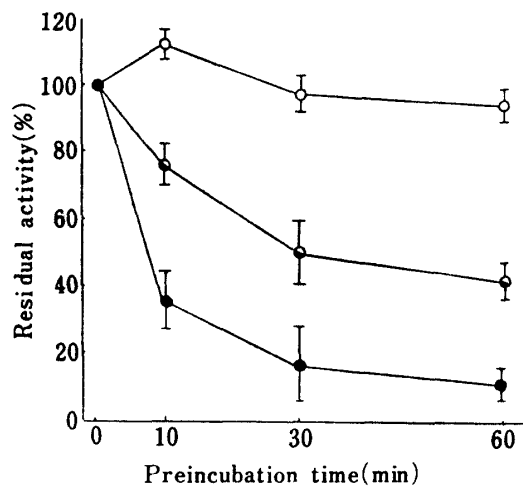


Fig. 8. Effect of Heat Treatment on the Esterolytic Activity of Dog Urinary Kallikrein

Purified DUK at pH 8.0 was heated at the indicated temperatures for 10, 30 and 60 min. The residual TAME esterolytic activity was measured by the colorimetric method with chromotropic acid. The activity was expressed as a percentage of the control (untreated).

○, 50°C; ◐, 75°C; ●, 100°C.

in Fig. 8. DUK was very stable and 95% of the activity remained even after treatment at 50°C for 60 min and the results were similar to those for dog renal and human urinary kallikreins.^{6,16)}

Kinetic Properties

The kinetic parameters for the purified DUK using *N*-substituted arginine and lysine

esters were calculated by means of Lineweaver-Burk plots. The results are summarized in Table III and are in good agreement with other previous results for various glandular kallikreins.¹⁷⁾ K_1 values of *N*- α -tosyl-L-lysine chloromethyl ketone (TLCK) and diisopropyl fluorophosphate (DEP) against DUK with TAME as the substrate at pH 7.0 were calculated to be 6.2 and 10.0×10^{-3} M, respectively, and were similar to those against human urinary kallikrein.^{16,18)}

TABLE III. Kinetic Parameters for the Hydrolysis of Synthetic *N*-substituted Arginine and Lysine Esters by Purified Dog Urinary Kallikrein

Substrate	K_m (mM)	V_{max} ($\mu\text{mol}/\text{min}/A_{280}$)	Kcat (s^{-1})	Kcat/ K_m (s/mM)
TAME	0.35	21.6	4.2	11.9
BAEE	0.55	23.7	4.6	8.3
BAME	0.58	28.2	5.4	9.4
TLME	1.14	6.9	0.12	0.1

Esterolytic activities were measured both by the spectrophotometric method¹⁹⁾ using TAME, BAEE and BAME as substrates at pH 8.0, 25°C, and by the colorimetric method with chromotropic acid⁹⁾ using TLME as the substrate at pH 8.0, 30°C. Enzyme concentration was 4.2 μM .

Discussion

High kallikrein activity (6 to 10 KU of vasodilator activity per ml of dialyzed urine) was found in the dog urine, in conflict with other accounts.¹⁹⁾ The kallikrein content in the dialyzed dog urine was about 30 times more than that of human urine, according to the calculation shown in reference 20. The ratio of kallikrein content in the dog urine to that in dog kidney cortex (KU per dialyzed one-day urine/KU per g wet weight of kidney cortex) was estimated to be 30 to 140, and this value is 15 to 40 times more than that of kallikrein contained in human urine and kidney cortex (unpublished data). This result might suggest that the turnover of kallikrein in the dog kidney is very rapid in comparison with that in the human kidney.

DUK was finally purified as a homogeneous preparation (Fig. 3) having only one component of pI 4.2 (Fig. 4). This is in contrast to some renal and urinary kallikreins which show micro-heterogeneity (multiple active components) such as the kallikreins from the renal organ of the dog,⁶⁾ and rat⁴⁾ and human urines.^{16,21)}

Based on the results of gel filtration in a Sephadex G-100 column and SDS vertical plate polyacrylamide gel electrophoresis (Fig. 6), the molecular weight of this kallikrein was concluded to be approximately 2.7 to 2.9×10^4 , while ultracentrifugal analysis (Fig. 3B) gave a value of about 2.6×10^4 . These results were in good agreement with those for multiple components of human urinary kallikrein separated by isoelectric focusing,^{16a)} but are slightly different from the result for dog renal kallikrein (molecular weight 3.8×10^4)⁶⁾ However, the data for the multiple components of dog renal kallikrein, separated by isoelectric focusing (molecular weights of 2.4×10^4 each, unpublished data), were in good agreement with those for the present kallikrein. The molecular weights of the kallikrein contained in human urine^{16,21,22)} and those of kallikrein from the horse,²³⁾ the hog,²⁴⁾ the rabbit,²⁵⁾ the rat⁴⁾ and the renal organs of the human²⁶⁾ and rat³⁾ was reported to be 2.7 to 4.7×10^4 , 3.1×10^4 , 3.6×10^4 , 3.2 to 3.6×10^4 , 4.7 to 4.9×10^4 and 3.8×10^4 , respectively. These results suggest that the molecular weight of the renal kallikrein may be somewhat greater than that of urinary kallikrein, and that the urinary kallikrein is a partial degradation product of the renal kallikrein. DUK was found to have a smaller molecular weight than DRK (3.8×10^4 before separation into multiple components), in accord with the above view.

The correlation between the esterolytic activities such as TAME and BAEE hydrolyzing actions and vasodilator activity of the glandular kallikreins was described by Webster *et al.*²⁷⁾ Their correlation was used by Moriya as a basis for the TAME esterolytic assay method, in place of the vasodilator assay method for kallikrein. It is well known that the ratio of vasodilator to TAME or BAEE esterolytic activities of the most highly purified glandular kallikreins is held to be constant. In the present investigation, hydrolysis of one μmol of TAME per min at 30°C, pH 8.0, corresponded to 101 ± 12 (mean \pm S.E.) KU of vasodilator activity (Fig. 5). This result is in contrast to the ratios of kallikreins in the kidney of the dog⁶⁾ and in human urine^{16a)} which were calculated to be 58 and 24 KU per μmol hydrolyzed TAME at 30°C, pH 8.0, respectively.

Some properties of the esterolytic actions of the present kallikrein, including substrate specificity,²⁹⁾ are similar to those of the kallikreins in the renal organ of the dog⁶⁾ and in human urine.^{16a)} Furthermore, the inhibitory actions of proteinase inhibitors on the esterolytic activities of kallikreins in human urine¹⁶⁾ and in the renal organ and in the urine (partially purified) of the dog⁶⁾ are also similar. These results indicate that the enzymatic properties of the renal and urinary kallikreins of the dog are almost identical. Some differences of properties between urinary kallikreins of the human,¹⁶⁾ the rat⁴⁾ and the dog (present results), such as specific vasodilator and esterolytic activities and micro-heterogeneity, could be ascribed to species difference. These results suggest that the urinary kallikrein originates in the kidney. On the other hand, Fiedlar and Gerhard recently reported²⁸⁾ that the urinary kallikrein represent enzyme synthesized in the pancreas and submaxillary glands filtered *via* the blood circulation and kidney. Moreover, they did not rule out the synthesis of urinary kallikrein in the kidney, and considered that the kallikrein in the urine might be derived more from the pancreas and submaxillary glands than from the kidney. Further experimental work is necessary.

It is interesting that a large amount of a new arginine ester hydrolyzing enzyme(s) was found in the dog urine, and the percentage of this enzyme recovered by DEAE-cellulose adsorption in step 1 was about 55% (Fig. 1). This enzyme's esterolytic action on *N*-substituted arginine and lysine esters covered a wide range. The enzyme had an acidic pI value and had neither plasmin activity nor plasminogen activator activity (unpublished data). This new enzyme(s) was not contained in human urine²⁰⁾ but it was detected in rat urine,^{4a)} and similar enzyme was also discovered in the dog pancreas.³⁰⁾ A detailed report on the new arginine ester hydrolyzing enzyme(s) in dog urine will be published elsewhere.

References and Notes

- 1) Abbreviations: KU, kallikrein unit; TAME, *N*- α -tosyl-L-arginine methyl ester; BAEE, *N*- α -benzoyl-L-arginine ethyl ester; BAME, *N*- α -benzoyl-L-arginine methyl ester; TLME, *N*- α -tosyl-L-lysine methyl ester, SDS, sodium dodecyl sulfate.
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