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Purification and Some Properties of Hog Renal Kallikrein

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Following the activation of hog kidney cortex homogenate with acetone, kallikrein was purified about 317-fold by diethylaminoethyl-cellulose adsorption, acetone precipitation and chromatography on Sephadex G-75, diethylaminoethyl-Sephadex A-50 and Sephadex G-100, and affinity chromatography on Trasylol-Sepharose 4B.

The final purified preparation of hog renal kallikrein had a kinin-forming activity of 35.1 μg bradykinin eq/min/mg, and appeared to be homogeneous in polyacrylamide gel electrophoresis. This enzyme was a glycoprotein with a molecular weight of 50000 as determined by gel filtration on a column of Sephadex G-100.

The hog renal kallikrein had an optimum pH of 9.5 and was stable at pH 8.0. This enzyme was hardly inhibited by soybean trypsin inhibitor, but was moderately sensitive to ovomucoid and more sensitive to Kunitz inhibitor. These properties were compared with those of hog pancreatic kallikrein.

Keywords——hog renal kallikrein; affinity chromatography; kinin-forming; kallikrein inhibition; molecular properties of kallikrein

Although it has not been proven conclusively that urinary kallikrein is formed in and secreted by the kidney, it has been suggested that renin (EC 3.4.99.19) and kallikrein (EC 3.4.21.8) are located in the same kidney subcellular fraction.[1] Within the submaxillary gland, kallikrein and renin activity seem to be closely associated at a subcellular level.[2] Margolius *et al.*[3] demonstrated that measurement of urinary kallikrein revealed lower levels in patients with essential hypertension than in a control population, normal levels in renal-artery stenosis, and raised levels in phaeochromocytoma and primary aldosteronism. Their findings in hypertensive disease afford additional reasons for considering possible relationships between the kallikrein-kinin and renin-angiotensin systems, even though the pathologic and physiologic functions of renal kallikrein are not yet clear. In view of the probable pathologic and physiologic functions of renal kallikrein, it seemed worthwhile to develop a simple means of purifying the enzyme to facilitate its further study. The purposes of the present work were to purify kallikrein from hog kidney cortex and to compare the properties of hog renal kallikrein (HRK) with those of hog pancreatic kallikrein (HPK).

Materials and Methods

Materials——The following reagents were obtained commercially: bovine serum albumin, ovalbumin, bovine chymotrypsinogen and horse cytochrome c (Boehringer Mannheim Yamanouchi Co., Tokyo); Sephadex G-100, Sephadex G-75, DEAE-Sephadex A-50, DEAE-cellulose and Sepharose 4B (Pharmacia Fine Chemicals Co., Sweden); soybean trypsin inhibitor and ovomucoid (Sigma Chemical Co., U.S.A.); Trasylol (Bayer AG, F.R.G.); benzoyl-L-arginine ethyl ester (BAEE) HCl and synthetic bradykinin (Protein Research Foundation, Osaka); D-Pro–Phe–Arg–pNA and D-Val–Leu–Arg–pNA (Daiichi Chemical Co., Tokyo): Trasylol-Sepharose 4B was prepared by coupling Trasylol with Sepharose 4B using CNBr as described by Oza *et al.*[4] High molecular weight (HMW) and low molecular weight (LMW) kininogens were prepared from bovine plasma by the method of Komiya *et al.*[5] HPK used was purified from a commercial product (Bayer AG, F.R.G.) by affinity chromatography on a Trasylol-Sepharose 4B column. Hog kidneys were obtained as fresh as possible from the slaughterhouse and stored at −80°C.

Methods——The esterolytic activity of kallikrein was determined by the BAEE esterase method using a high-speed liquid chromatography procedure devised by us.[6] The amidolytic activity of kallikrein was measured spectrophotometrically at 25°C using D-Pro–Phe–Arg–pNA and D-Val–Leu–Arg–pNA as substrates

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according to Claeson *et al.*[7] The kinin-forming activity was estimated by the rat uterus assay as described by Moriwaki *et al.*[8] Synthetic bradykinin (BK) served as a reference for kinin-forming activity determination. Three different substrates were used: heat-treated bovine plasma, bovine HMW and LMW kininogens. Inhibition experiments with proteinase inhibitors were performed at 30°C for 30 min by mixing various quantities of inhibitor with a known quantity of enzyme in 50 mM Tris-HCl buffer (pH 8.0) containing 50 mM NaCl. Kallikrein activity was measured by high-speed liquid chromatography with 10 mM BAEE as the substrate. The molecular weight was determined by gel filtration on a Sephadex G-100 column according to Moriwaki *et al.*[9] Gel electrophoresis was performed in 7% polyacrylamide gels using 50 mM Tris-glycine buffer at pH 8.3, and electrophoresis was carried out at 2 mA per gel for 1 h. Proteins were stained for sugar moieties with the Schiff reagent. Noncarbohydrate proteins were detected with Coomassie brilliant blue R-250. Protein concentration was determined spectrophotometrically by measuring the absorbance at 280 nm with a cell of 1 cm light path, assuming tentatively that the value of $E_{280}^{1\%}=10.0$.

Results and Discussion

Purification of HRK

The following purification steps were carried out at 4 °C unless otherwise stated. Kallikrein activities were measured against BAEE and heat-treated bovine plasma during the purification procedures.

Step 1. Extraction——From the autolyzed (15—20 °C, 24 h) and homogenized kidney cortex (2000 g), HRK was extracted with diluted acetone (30% v/v) as described by Webster and Prado for HPK.[10]

Step 2. DEAE-cellulose Batchwise Adsorption——Kallikrein activities in the extract from step 1 were adsorbed on DEAE-cellulose at pH 6.7, and eluted with 1600 ml of 0.5 M sodium phosphate buffer (pH 6.3). The eluate was dialyzed overnight against running tap water.

Step 3. Acetone Precipitation——The dialyzed solution from step 2 was redialyzed overnight against 0.065 M sodium acetate containing 0.035 M NaCl, and the pH was adjusted to 5.4 with 5 M acetic acid. The solution was cooled in an ice bath and an equal volume of acetone was added while stirring. After standing overnight, the precipitate, which should contain about 5.7% of the enzyme activity and 70% of the protein, was removed by centrifugation at $3500\times g$ for 10 min, and discarded. The concentration of acetone in the supernatant was raised to around 60% (v/v). The following day the precipitate, which should contain 3.1% of the enzyme activity and 9.6% of the protein, was centrifuged off at $7800\times g$ for 10 min, and dissolved in 200 ml of distilled water. The pH of the solution was adjusted to 7.0.

Step 4. Sephadex G-75 Gel Filtration——The solution from step 3 was concentrated to 20 ml by ultrafiltration with an Amicon PM-10 membrane. The concentrated solution was filter-

TABLE I. Summary of the Purification of HRK

Steps	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Purity	Kinin-forming activity	
					μg BK eq/min/mg	Purity
Extraction	61068.8	1764	0.029	1		
DEAE-cellulose	7228.4	575	0.080	3		
Acetone precipitation						
0—50%	5053.1	32.9	0.007			
50—60%	692.3	17.9	0.026	1	0.07	1
Sephadex G-75	60.7	12.3	0.20	7	0.78	11
DEAE-Sephadex A-50	3.85	11.3	2.9	100	10.7	153
Sephadex G-100	2.05	10.7	5.2	179	19.5	279
Trasylol-Sepharose	0.52	4.8	9.2	317	35.1	501

The esterolytic activity was expressed in terms of esterolytic units (EU) equal to μmol of BAEE hydrolyzed per min at 30°C in 50 mM Tris-HCl buffer, pH 8.0.

The rat uterus assay was calibrated with synthetic BK, and the amount of BK-equivalent in the test solution was determined as described by Moriwaki *et al.*[8]

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ed through a Sephadex G-75 column (2.5×91 cm). The active fractions were pooled (104 ml).

Step 5. DEAE-Sephadex A-50 Chromatography——The pooled fraction of HRK was concentrated to approximately 5 ml as described in step 4, and applied to a DEAE-Sephadex A-50 column (1.5×31 cm) equilibrated with 0.2 M ammonium formate. The kallikrein activity was eluted with a linear gradient of 0.2 to 0.8 M ammonium formate and of pH 6.7 to 6.85, and the active fractions were pooled (77 ml).

Step 6. Sephadex G-100 Gel Filtration——The active fraction was concentrated to 2.5 ml as described in step 4 and applied to a Sephadex G-100 column (1.5×85 cm) equilibrated with 0.05M Tris–HCl buffer (pH 7.4) containing 0.05 M NaCl. The active fractions were combined (84 ml), concentrated to 2 ml as described in step 4 and dialyzed overnight against 0.1 M bicarbonate buffer (pH 7.8) containing 0.5 M NaCl.

Step 7. Affinity Chromatography——The dialyzed HRK solution was further purified by affinity chromatography with Trasylol-Sepharose 4B according to the method of Oza *et al.*[4] as modified by Levinsky *et al.*[11]

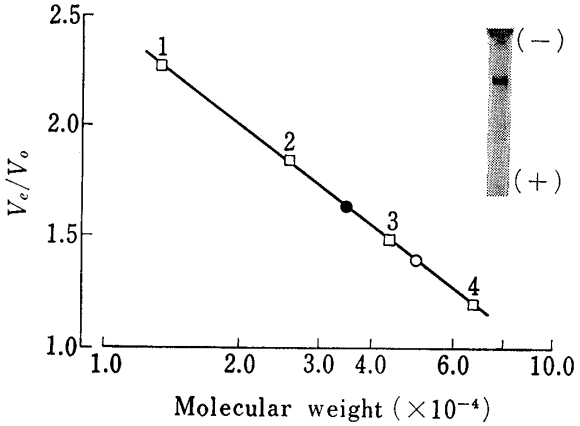

Fig. 1. Gel Electrophoresis and Estimation of the Molecular Weight of HRK

One mg each of cytochrome c, chymotrypsinogen, ovalbumin, bovine serum albumin and blue dextran and 0.5 mg each of HRK and HPK was dissolved in 1 ml of Tris-HCl buffer (0.1 M, pH 7.7), and applied to a Sephadex G-100 column (1.5×90 cm). The flow rate was maintained at 11.0 ml per h. Eluates of each sample were checked for absorbance at 280 nm for proteins and at 620 nm for blue dextran. 1, cytochrome c; 2, chymotrypsinogen; 3, ovalbumin; 4, bovine serum albumin: ○, HRK; ●, HPK.

The overall results of these purification steps are summarized in Table I. The purified HRK preparation showed an activity of 9.2 EU/mg protein. This value is about 317 times higher than that of the initial extract and the kinin-forming activity of the purified enzyme was determined to be 35.1 μg BK eq/min/mg when heated bovine plasma was used as a substrate.

Molecular Properties

The homogeneity of the purified HRK was examined by polyacrylamide gel electrophoresis at pH 8.3. As shown in Fig. 1, a single protein band that stained with Coomassie brilliant blue was obtained. HRK is a glycoprotein, as demonstrated by the positive periodic acid–fuchsin reaction obtained on polyacrylamide gel after electrophoresis, and the same result was obtained for HPK. A molecular weight of 50000 was obtained for HRK by gel filtration on a Sephadex G-100 column. This molecular weight is clearly different from that found for HPK (35000), which had been purified by means of affinity chromatography. The molecular weight of rat renal kallikrein was estimated to be 40000 by Porcelli *et al.*[12] They reported that the molecular weight of rat urinary kallikrein was 32000, but they suggested that the urinary kallikrein might be a partial degradation product of renal kallikrein. In our investigation, HRK was found to be a larger molecule than the other kallikreins, and this suggests that HRK obtained in the present experiment is most nearly in the native state.

Optimum pH and Heat Stability

Nustad[13] reported that the rat kidney homogenate had two pH optima at 6.5 and 8.5, using BAEE as a substrate. In our experiment, only one pH optimum, 9.5, was found for HRK, and this was higher than the optimum pH of HPK, 8.5, and not very different from the result of Kaizu and Margolius[14] for rat renal kallikrein, 9.0. If an acidic and unstable BAEE esterase was present in kidney, as reported by Nustad, it might have been removed during the purification procedures. HRK and HPK were heated at 50, 60 and 70 °C for 10 and 60 min in 0.1 M Tris–HCl buffer at pH 8.0, and the remaining BAEE esterolytic activities

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were determined. The initial activities of HRK and HPK were 1.6 EU/ml and 1.1 EU/ml, respectively. HRK was reasonably stable to heat treatment, *i.e.* about 80% of the activity was retained after heating at 60 °C for 60 min, and 60% of the activity was retained even after treatment at 70 °C for 10 min. HPK showed similar heat stability.

Kinin-forming Activity and Amidolytic Activity

Kinin-forming activity from two bovine kininogens by HRK was demonstrated using the rat uterus assay. It is well known that LMW kininogen is a good substrate of HPK. As indicated in Table II, HMW kininogen was also sensitive to HPK. HRK also reacted directly with both kininogens, but its reactivities per esterolytic activity unit were rather weaker than those of HPK.

The kinetic parameters of D-Pro-Phe-Arg-pNA and D-Val-Leu-Arg-pNA hydrolysis by HRK were determined using various substrate concentrations. From a Lineweaver-Burk plot, K_m was found to be equal to 5.5×10^{-5} M and V_{max} to 3.5×10^{-9} M/min/EU when D-Pro-Phe-Arg-pNA was used as a substrate, while K_m was found to be equal to 6.6×10^{-5} M and V_{max} to 2.8×10^{-9} M/min/EU when D-Val-Leu-Arg-pNA was used. These values of K_m and V_{max} are not very different from those given for HPK by Claeson *et al.*,[7] as indicated in Table III.

Table II. Kinin-forming Activity from Two Bovine Kininogens

Enzyme	Kinin-forming activity (μg BK eq/min/EU)	
	LMW kininogen	HMW kininogen
HRK	5.7	1.4
HPK	8.1	3.3

Table III. Kinetic Parameters for the Hydrolysis of Peptide Substrates by HRK

Substrate	K_m ($\times10^{-5}$ M)	V_{max} ($\times10^{-9}$ M/min/EU)
H-D-Pro-Phe-Arg-pNA	5.5	3.5
H-D-Val-Leu-Arg-pNA	6.6	2.8

The amidolytic activity was expressed as μmol of substrate hydrolyzed per min at 25°C in 50 mM Tris-HCl buffer, pH 9.0.

Table IV. Inhibitory Effects of Proteinase Inhibitors on Kallikreins

Inhibitor		Inhibition (%)	
		HRK	HPK
Trasylol	1 KIU	71.4	84.3
	10	78.6	100
	100	89.3	100
	500	92.9	100
Soybean trypsin inhibitor	10 μg	N.D.[a]	N.D.
	100	N.D.	N.D.
	500	N.D.	N.D.
Ovomucoid	10 μg	20.0	N.D.
	100	24.0	N.D.
	500	31.2	N.D.

a) Not detectable.

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Inhibition Studies

In addition to molecular weight and pH optimum, a difference was found between HRK and HPK in behavior towards proteinase inhibitors. As shown in Table IV, HRK was sensitive to Trasylol and ovomucoid, but the activity was inhibited incompletely even by 500 KIU of Trasylol. On the other hand, HPK was so sensitive to Trasylol that 1.0 KIU of the inhibitor caused 80% inhibition and the activity was inhibited completely by 10 KIU. However, it was hardly inhibited by ovomucoid. Soybean trypsin inhibitor had no inhibitory effect on either kallikrein.

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