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ISOZYMES OF RAT URINARY KALLIKREIN*

JULIE CHAO and HARRY S. MARGOLIUS†

Department of Basic and Clinical Pharmacology, Medical University of South Carolina, Charleston, SC 29403, U.S.A.

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Abstract—Two isozymes of rat urinary kallikrein (A and B) have been purified and studied in detail. The enzymes were separated by chromatography on DEAE-cellulose and distinguished by electrophoresis and electrofocusing in polyacrylamide slab gels. The electrophoretic mobilities were not altered by treatment with neuraminidase but were shifted toward the cathode after complexing with aprotinin. Molecular weights of rat urinary kallikreins A and B were estimated to be 36,500 and 35,500, respectively, by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis. The enzymes did not differ in pH optimum, specific activity or substrate specificity, but differed slightly in heat stability. They were not distinguishable biologically, nor immunologically using an antiserum generated in a sheep against kallikrein B. Both kallikreins were activated by deoxycholate and several nonionic detergents. α-*N*-tosyl-L-arginine methyl ester (Tos-L-Arg OMe) hydrolysis by both forms was inhibited by aprotinin and stimulated by ovomucoid and lima bean trypsin inhibitors, whereas Tos-L-Arg-OMe hydrolysis was stimulated by soybean trypsin inhibitor at low concentrations and inhibited at high concentrations. Kinetic measurements of α-*N*-benzoyl-L-arginine ethyl ester (Bz-L-Arg-OEt) hydrolysis by the two enzymes showed no significant differences in substrate affinity or in maximal velocity.

Mammalian urinary kallikrein is considered to be a glandular kallikrein (EC 3.4.21.8) of renal origin [1–3]. A series of studies [4] indicates that the level of urinary kallikrein excretion in normal man is regulated by adrenal sodium-retaining steroid hormone activity and that patients with essential hypertension generally excrete less kallikrein than normal subjects. Recent efforts have shown that the enzyme: (1) appears to gain access to tubular fluid from the distal nephron [5], (2) is concentrated in plasma membrane fractions clearly not of proximal tubular origin but possibly of distal tubular origin [6, 7] and (3) can be localized immunohistochemically to the apical part of distal tubule cells from the glomerulus to the collecting duct [8]. Furthermore, high concentrations of aldosterone increased, whereas similar concentrations of spironolactone decreased, kallikrein levels in the media surrounding isolated renal cortical cells in suspension [9]. The possibility has been raised that the enzyme may have its synthesis regulated by aldosterone [10].

We have isolated and purified large quantities of rat urinary kallikrein in order to continue studies of kallikrein synthesis, localization and release. In the process of purification, we found two forms of rat urinary kallikrein which were separated by DEAE-cellulose chromatography and distinguished by polyacrylamide slab gel electrophoresis and isoelectric focusing. The present study describes the purification and properties of these two isozymes.

MATERIALS AND METHODS

The following materials were obtained from commercial sources: DEAE-cellulose (DE-52) and CM-cellulose (Whatman Chemical Co.); Sephadex G-100 and blue dextran (Pharmacia Fine Chemicals, Inc.); enzyme grade ammonium sulfate, ultra pure sucrose, α-*N*-tosyl-L-arginine methyl ester (Tos-L-Arg-OMe),‡ α-*N*-carbobenzyl-oxy-L-arginine methyl ester (CBZ-L-Arg-OMe), serum albumin, bradykinin, crystalline ovalbumin, chymotrypsinogen A, and crystalline myoglobin (Schwartz-Mann); [^{3}H]Tos-L-Arg-OMe and [^{3}H]CBZ-L-Arg-OMe (Biochemical and Nuclear Corp.); polyacrylamide (Eastman Kodak); specially pure sodium dodecyl sulfate (SDS) (BHA Chemicals Ltd.): lysozyme, aprotinin (identical to Kunitz basic pancreatic inhibitor), and trypsin (from bovine pancreas, Type IV, 2x crystallized), sodium deoxycholate, Triton X-100, Lubrol PX and Brij 58; yeast alcohol dehydrogenase, β-nicotinamide adenine nucleotide, and α-*N*-benzoyl-L-arginine ethyl ester (Bz-L-Arg-OEt) (Sigma Chemical Corp., St. Louis, MO); trypsin inhibitors from soybeam (SBTI), ovomucoid (OMTI), lima bean (LBTI) and carboxypeptidase B (Worthington Biochemical Corp.); *Vibrio cholerae* neuraminidase (CalBiochem); and ampholine, pH 3.5 to 5 (LKB). Purified human plasma kininogen and human urinary kallikrein were gifts from Dr. Jack V. Pierce, and purified uricase was a gift from Dr. David G. Priest.

Collection of rat urine

Thirty-two male Sprague–Dawley rats (200–350 g) were kept in sixteen stainless steel metabolism cages (20 × 24 × 18 cm) and were given water but no food during the 22 hr/day of urine collection. Urine was collected through a stainless steel funnel into plastic

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‡ Abbreviations used are: Tos-L-Arg-OMe: α-*N*-tosyl-L-arginine methyl ester; CBZ-L-Arg-OMe: α-*N*-carbobenzyl-oxy-L-arginine methyl ester; SDS: sodium dodecyl sulfate; Bz-L-Arg-OEt: α-*N*-benzoyl-L-arginine ethyl ester; LBTI: lima bean trypsin inhibitor; OMTI: Ovomucoid trypsin inhibitor; and SBTI: soybean trypsin inhibitor.

tubes. Solid debris was deflected at the curved end of the funnel. Twice daily each lot of urine (100–400 ml) was filtered through several layers of cheesecloth and stored at −20° until used.

Protein determination

The protein concentration of solutions was determined by the method of Lowry *et al.* [11]. Bovine serum albumin was used as the standard. Protein concentrations in the column effluents were monitored at 280 nm with an ISCO Absorbance Monitor model UA-5. An extinction coefficient of $E_{280} = 1.51$ was used for 1.0 mg/ml of kallikrein [12].

Conductivity determination

The conductivity of fractions from DEAE-cellulose chromatography was measured at 25° with a Yellow Springs Instruments conductivity bridge model 31 and a conductivity cell type 3403.

Electrophoresis in 15% polyacrylamide slab gels

The method used for vertical slab gel electrophoresis was that described by Studier [13] with slab gels 1 mm thick and solutions and buffers described by Laemmli [14]. The precise details have been described previously [15].

Isoelectric focusing on polyacrylamide slab gels

Isoelectric focusing was carried out in an LKB Multiphor electrophoresis system modified to hold 1 mm thick acrylamide slab gels [16]. Gels were prepared containing 10% acrylamide, 0.27% bisacrylamide, and 1% ampholine, pH 3.5 to 5. Polymerization of the gels was effected with 0.3% ammonium persulfate and 0.3% tetramethylethylenediamine. Kallikreins A and B (*ca.* 4 μg) were applied to filter paper tabs (Whatman 3MM) 1 cm × 0.8 cm, using 20 μl/tab. The cathode wick buffer was 1 M NaOH and the anode wick buffer was 1 M phosphoric acid. Separation was carried out for 1 hr with increasing power settings: 20 W for 20 min, 25 W for 30 min, and 30 W for 10 min. Following completion of focusing, the filter paper tabs and an edge portion of the gel were removed. The edge strip (1 cm width) was cut transversely at 0.5-cm intervals and each section was placed in a tube containing 1 ml of distilled water. After standing about 18 hr, the pH values of the sections were determined. A pH gradient was obtained by plotting pH versus gel distance. The remainder of the slab was fixed in 12.5% trichloroacetic acid for 10 min, and rinsed with water. The gel was then placed in 0.1% Coomassie Blue R250 in methanol–acetic acid–water (3:1:6) for 30 min. The slab gel was destained in methanol–acetic acid–water (3:1:6) solution and then dried.

Molecular weight determination

The molecular weights of kallikreins A and B were estimated by 5–15% SDS discontinuous gradient polyacrylamide slab gel electrophoresis, using lysozyme, myoglobin, chymotrypsinogen A, uricase, ovalbumin and serum albumin as protein markers [17]. The mobilities were measured relative to a number of marker proteins [18].

The protein samples were made to 0.2 mg/ml in 0.0625 M Tris–HCl buffer (pH 6.8), 2.3% sodium dodecyl sulfate, 10% glycerol and 5% mercaptoethanol and heated to 100° for 5 min; 2–4 μg of each protein were applied to each well of the slab gel. Electrophoresis was performed in 0.1% sodium dodecyl sulfate by the method of Webster and Osborn [19] in Tris–glycine buffer, pH 8.8 [14].

Tos-L-Arg-OMe esterase assay

The modified assay of Beaven *et al.* [20] was used routinely to determine Tos-L-Arg-OMe esterase activity, a measure of kallikrein-like enzyme activity, as described previously [15]. One Tos-L-Arg-OMe esterase unit (E.U.) is defined as that amount of enzyme which hydrolyzes 1.0 μmole Tos-L-Arg-OMe/min at pH 8.0 and 30° [20].

Kallikrein biological assay

The kinin-releasing activity of kallikrein was determined by incubating 0.3 ml of 0.2 M Tris–HCl buffer, pH 8.0, 0.1 ml of enzyme solution ($2–6 \times 10^{-2}$ E.U./ml) and 0.1 ml of previously heated dog plasma (60°, 30 min) for 10 min at 37°. Purified human plasma kininogen (0.1 ml containing $0.026\ A_{280}$) was also used as a substrate. The kinin released was measured on the isolated guinea pig ileum [21], using bradykinin as a standard. The contractile response of the isolated rat uterus [22] to kallikreins was also measured.

Immunization with rat urinary kallikrein B

Rat urinary kallikrein B from Sephadex G-100 fractions (0.5 to 1.0 mg, sp. act. 35.2 E.U./A_{280}) was dialyzed against distilled water and lyophilized. The lyophilized powder was dissolved in 2 ml of sterile Dulbecco's phosphate-buffered saline, sterilized by millipore filtration, emulsified with 2 ml of Freund's complete adjuvant (Difco) and injected into a sheep subcutaneously along the spine and intramuscularly at multiple sites. The volume of each injection was 0.2 ml. The first booster was given 1 month later, followed by three additional boosters at 2-week intervals.

Weak precipitating antibody was found after the first booster injection, but an appreciable increase in antibody titer was noted after the second injection, which did not change perceptibly after the third or fourth booster injection.

Immunodiffusion in agarose

Double-diffusion analysis [23] was done at room temperature for 7 hr in 1.0% agarose in 0.01 M phosphate-buffered saline, pH 7.5, containing 0.05% NaN_3.

Kinetic analysis

The initial velocity of the Bz-L-Arg OEt hydrolysis by kallikrein was made in a Cary 15 spectrophotometer using the 0.1 absorbance slide wire. The initial velocities were determined at 25° with a coupled assay system using alcohol dehydrogenase [24, 25]. Reaction mixtures contained: 2.83 ml of 0.2 M Tris–HCl buffer, pH 8.5, 0.1 ml of 0.03 M NAD^+, 0.01 ml of yeast alcohol dehydrogenase (15 mg/ml) and 0.05 ml of Bz-L-Arg-OEt at various concentrations. Reactions were initiated by the addition of 0.01 ml kallikrein (0.28 E.U./ml). Kallikrein activity was measured by following the production of NADH at 340 nm in a cuvette with a 1 cm light path and corrected for the spontaneous hydrolysis of Bz-L-Arg-OEt.

Table 1. Purification of rat urinary kallikreins

Treatment	Total esterase units	Specific activity (E.U./A_{280})	Purif. factor	Recovery (%)
Rat urine (6 l.)	6500	0.29	1	100
(1) 30–60% Amm. sulfate fractionation	3900	0.62	2	60
(2) DEAE-cellulose chromatography	EsA—1650*	1.1	4	25
twice	RUK_A— 280†	6.3	22	4.3
	RUK_B— 572‡	7.2	25	8.8
(3) CM-cellulose chromatography	RUK_A— 132	9.5	33	2.0
	RUK_B— 286	10.2	35	4.4
(4) Sephadex G-100 chromatography	RUK_A— 91	34.6	120	1.4
	RUK_B— 229	35.2	120	3.5

* EsA, rat urinary esterase A [22].
† RUK_A, rat urinary kallikrein A.
‡ RUK_B, rat urinary kallikrein B.

RESULTS

Purification of rat urinary kallikreins

The following steps in the purification of rat urinary kallikreins were carried out at room temperature and are summarized in Table 1.

Step 1: Ammonium sulfate fractionation. Rat urine (6 l.) was thawed and centrifuged at 5000 *g* for 30 min to remove formed precipitates. Solid ammonium sulfate (176 g/l) was added to the supernatant fraction until the salt concentration reached 30% saturation. The mixture was stirred for an additional 60 min and centrifuged at 5000 *g* for 30 min. The resulting supernatant fraction was brought to 60% saturation by slowly adding ammonium sulfate (198 g/l). Two-tenths ml of 1 M NaOH was used/10 g of solid ammonium sulfate added to adjust the pH to 7.0. The precipitate collected by centrifugation was dissolved in distilled water. The crude protein solution was dialyzed overnight against distilled water at 4°, changed three times, to remove a considerable amount of amber-colored material into the dialysate and then dialyzed overnight at 4° against 0.1 N NaCl–0.01 M sodium phosphate, pH 7.0.

Step 2: DEAE-Cellulose chromatography. A half-portion of the enzyme solution from ammonium sulfate fractionation in 0.1 M NaCl–0.01 M sodium phosphate, pH 7.0, was passed through a DEAE-cellulose column. The column was equilibrated and eluted with the same buffer until absorbance of the effluent at 280 nm dropped to below 0.05 unit. About 50 per cent of the total Tos-L-Arg-OMe esterase activity flowed through the column (rat urine esterase A, Nustad and Pierce, 1974, results not shown). The absorbed enzyme was then eluted with a linear sodium chloride gradient from 0.1 to 0.5 N. Two peaks of kallikrein esterase activity were noted in the gradient (Fig. 1). Peak A eluted at 0.2 M NaCl and Peak B eluted at 0.25 M NaCl. Fractions from Peak A and Peak B were pooled

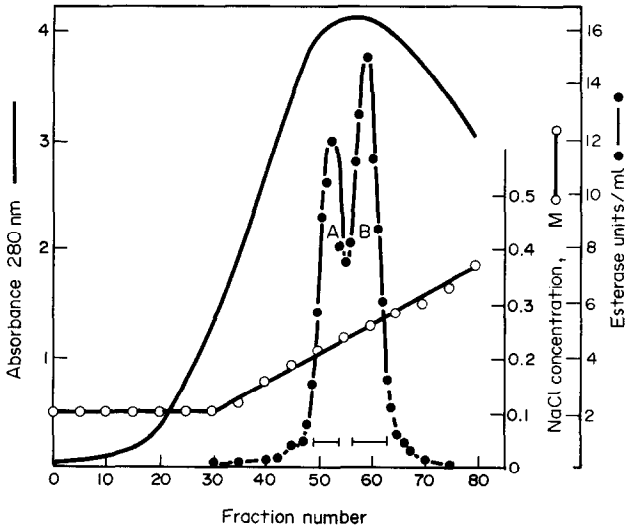

Fig. 1. DEAE-cellulose chromatographic separation of rat urinary kallikreins A and B: column 4.2 × 25 cm; starting sample, 2.5 g protein in 400 ml of 0.1 M NaCl–0.01 M Na phosphate (pH 7.0); 1.0 liter of a linear gradient of 0.1 M NaCl–0.01 M Na phosphate (pH 7.0) to 0.5 M NaCl–0.01 M Na phosphate (pH 7.0); fraction volume, 9 ml; flow rate, 40 ml/hr. Separately pooled fractions of peaks A and B are represented by (|——|).

separately, adjusted to 0.1 N NaCl with the addition of 0.01 M sodium phosphate, pH 7.0, and rechromatographed once or twice on DEAE-cellulose columns under the same conditions for the complete separation of the two peaks. Peak A and Peak B were purified separately by the following procedures.

Step 3: Carboxymethyl-cellulose chromatography. The pooled enzyme fractions from Peak A or B were dialyzed against 0.01 M ammonium acetate, pH 5.0. The enzyme solution was then centrifuged and the supernatant fraction (~120 ml) was chromatographed on a CM-cellulose column (2.2 × 16 cm) equilibrated with the same buffer. The flow rate was 20 ml/hr. Most of the esterase activity was recovered in the flow-through fractions and contaminant proteins retarded in the column. The pooled fractions of the elute were combined and lyophilized.

Step 4: Sephadex G-100 gel filtration. The lyophi-

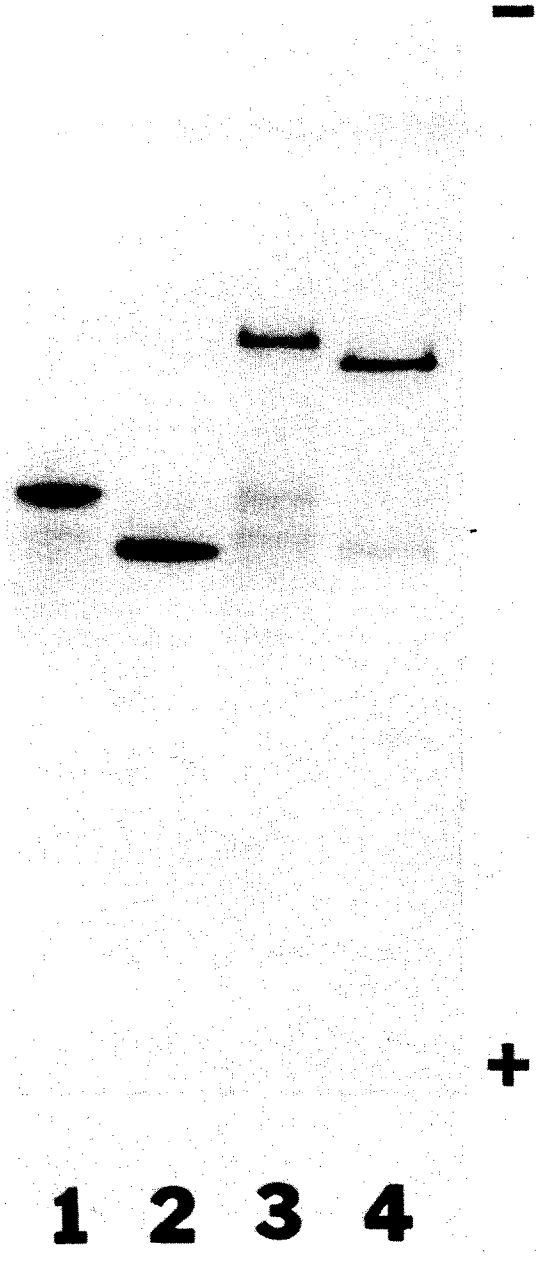

Fig. 2. Polyacrylamide gel electrophoresis of rat urinary kallikreins and kallikrein–aprotinin complexes. Purified rat urinary kallikreins A and B and kallikrein–aprotinin complexes were electrophoresed on a 15% polyacrylamide slab gel in a Tris–glycine buffer system. Kallikrein–aprotinin complexes were formed by incubating *ca.* 7 μg of each kallikrein with 100 units of aprotinin at 37° for 30 min. Experimental details are described in Materials and Methods. Key: (1) kallikrein A; (2) kallikrein B; (3) kallikrein A–aprotinin complex; and (4) kallikrein B–aprotinin complex.

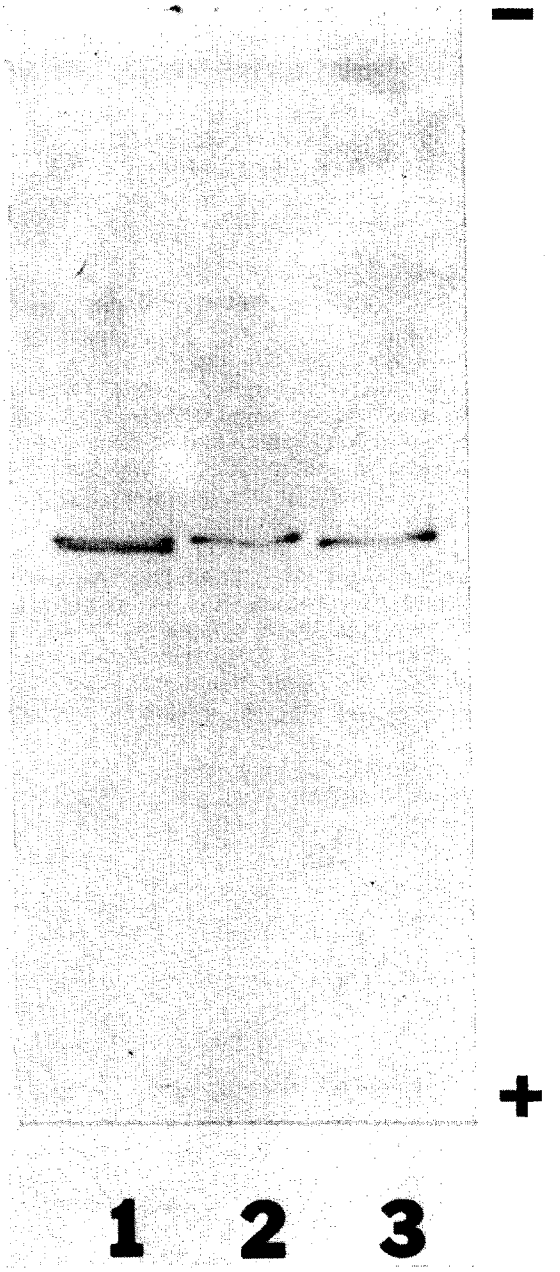

Fig. 3. Isoelectric focusing of rat urinary kallikreins A and B. Purified rat urinary kallikreins A and B were subjected to isoelectric focusing on a 10% polyacrylamide slab gel. The pH range was 3.5 to 5. Each sample contained *ca.* 4 μg proteins. The anode (acidic pH) is at the bottom of the gel. See text for experimental details. Key: kallikreins A and B; (2) kallikrein A; and (3) kallikrein B.

lized powder was dissolved in 3 ml of 0.1 M NaCl–0.01 M sodium phosphate, pH 7.0, and gel filtered through a Sephadex G-100 column (2.6 × 95 cm) equilibrated with the same buffer. The flow rate was 12 ml/hr. The Tos-L-Arg-OMe esterase activity eluted as a single peak between ovalbumin (43,000) and chymotrypsinogen A (23,000) from the precalibrated Sephadex G-100 column.

Polyacrylamide slab gel electrophoresis

Both rat urinary kallikreins A and B from Sephadex G-100 fractions (identified as such by kinin-releasing activity in the guinea pig ileum bioassay) appeared as single protein bands stained with Coomassie Blue in 15% polyacrylamide slab gel electrophoresis (Fig. 2). Kallikrein A may be slightly contaminated with kallikrein B. The electrophoretic mobilities of rat urinary kallikreins A and B were distinctly different and shifted toward the cathode when complexed with aprotinin, the kallikrein–trypsin inhibitor (Fig. 2). These differences in electrophoretic mobility could not be ascribed to a variable number of sialic acid residues, since no mobility changes were observed after neuraminidase treatment [22].

Electrofocusing in polyacrylamide gel slabs

Analytical electrofocusing of purified rat urinary kallikreins was performed on polyacrylamide slab gels in a pH gradient of 3.5 to 5 formed with Ampholine. Figure 3 shows that rat urinary kallikreins A or B migrate as single protein bands and the mixture of the enzymes could be separated by electrofocusing. The isoelectric point of kallikrein A was 4.31 and that of kallikrein B, 4.26.

Molecular weight determination

With SDS-polyacrylamide gel electrophoresis, rat urinary kallikreins A and B migrate as single bands with different mobilities between ovalbumin (43,000) and uricase (33,000) (Fig. 4). With a number of marker proteins, the molecular weight of kallikrein A was estimated to be 36,500 and that of kallikrein B to be 35,500. Sephadex G-100 column chromatography also indicated that the molecular weight of kallikrein A is greater than that of B since polyacrylamide slab gel electrophoresis of the sequential fractions from the column showed A eluted before B (results not shown). The agreement in estimated molecular weights of the kallikreins in SDS-polyacrylamide gel electrophoresis and in Sephadex G-100 gel filtration indicated that the enzymes were monomers.

Esterolytic and biological activity

The specific activities of the purified kallikreins A and B were 34.6 and 35.2 Tos-L-Arg-OMe E.U./$A_{280\,nm}$ respectively. [^{3}H]CBZ-L-Arg-OMe and [^{3}H]Tos-L-Arg-OMe were used to compare the activities of kallikreins A and B and other esterases. The ratios of

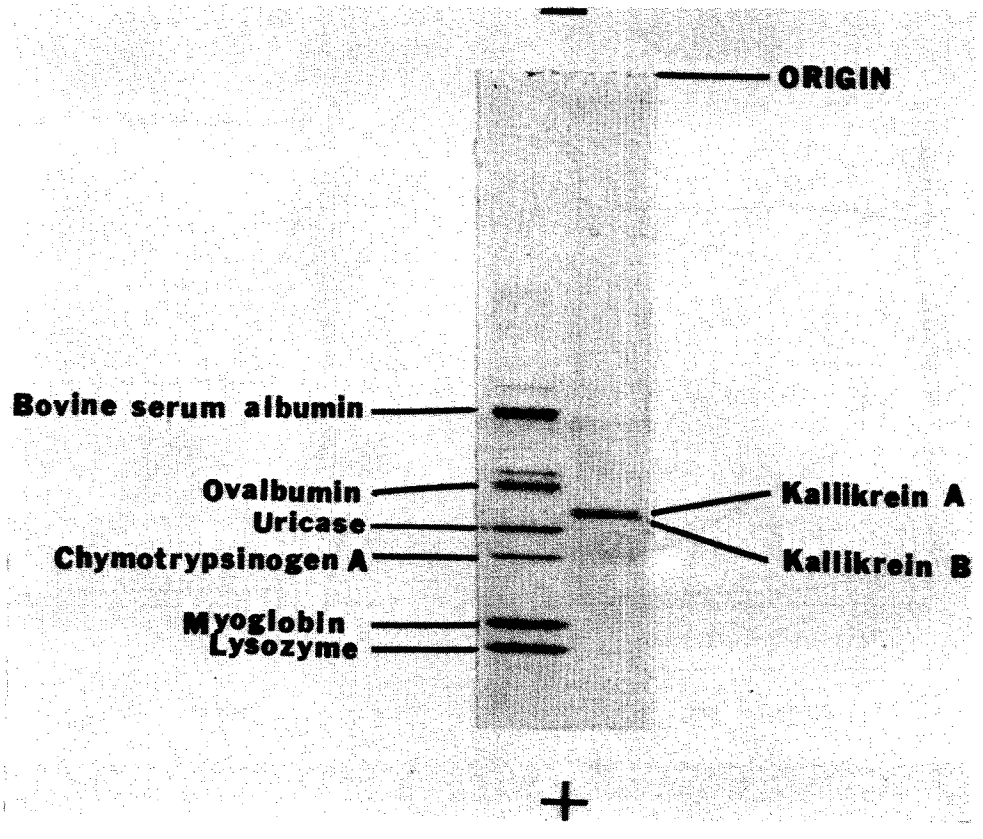

Fig. 4. SDS-polyacrylamide slab gel electrophoresis of kallikreins A and B. Purified rat urinary kallikrein (*ca.* 2 μg each) was dissociated in 2.3% SDS and electrophoresed on a 5–15% discontinuous SDS-polyacrylamide gradient slab gel. Other proteins of known molecular weight were run on the same slab gel as markers. See text for experimental details.

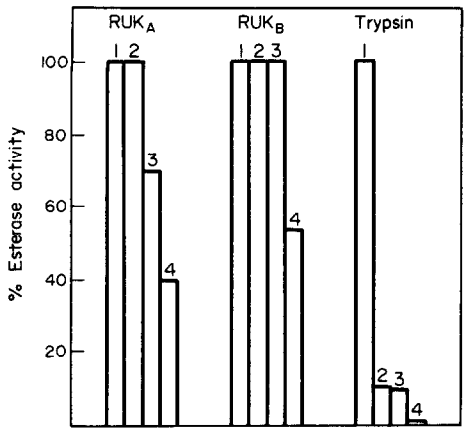

Fig. 5. Heat stability of rat urinary kallikreins A and B and trypsin. One-half ml of enzyme (1.25×10^{-2} E.U.) in 0.01 M sodium phosphate buffer, pH 7.0, was treated as follows: (1) control, 4°, 18 hr; (2) 55°, 30 min; (3) 55°, 18 hr; and (4) 100°, 5 min. The enzyme solutions were then equilibrated at room temperature for 1 hr. Assays were done at room temperature in a 1.5-ml polypropylene tube. Ten μl of the enzyme and 40 μl of 0.2 M Tris–HCl buffer, pH 8.0, were added, mixed and allowed to incubate for 10 min. [^{3}H]Tos-L-Arg-OMe (3.0×10^4 cpm, 10 μl) was added, mixed and allowed to incubate for 30 min. The [^{3}H]methanol released was measured in a Beckman LS-355 liquid scintillation spectrometer. The enzyme activity is expressed as the per cent of esterase activity as compared to control.

activity against CBZ-L-Arg-OMe with respect to Tos-L-Arg-OMe for rat urinary kallikreins A and B were 3.1 and 3.3, respectively, and 1.4 and 0.9 for rat urinary esterase A and trypsin, respectively. A purified human urinary kallikrein showed a ratio of 3.2. The data confirm that CBZ-L-Arg-OMe is a better substrate for kallikreins than for trypsin [22]. Both kallikreins ($2–4 \times 10^{-3}$ E.U.) released kinin from heated dog plasma or purified human plasma kininogen equivalent to 3–6 ng bradykinin/min and caused a slow contraction of the guinea pig ileum. The kinins released by kallikreins A and B were inactivated by the presence of carboxypeptidase B (2 units/10 ml) in the tissue bath.

pH Optimum

The pH profiles of both kallikreins A and B were determined as described previously [9] and are similar to that seen with rat kidney kallikrein or renal cortical cell kallikrein [9]. They follow a Gaussian distribution with an optimum of 9.0.

Heat stability

Rat urinary kallikreins were heat stable compared to trypsin (Fig. 5). Both retained 100 per cent Tos-L-Arg-OMe esterase activity after a 30-min incubation at 55°. At 55° for 18hr, kallikrein A retained 70 per cent and kallikrein B, 100 per cent of initial Tos-L-Arg-OMe esterase activity; at 100° for 5 min, kallikrein A retained 40 per cent and B, 54 per cent of activity. Both

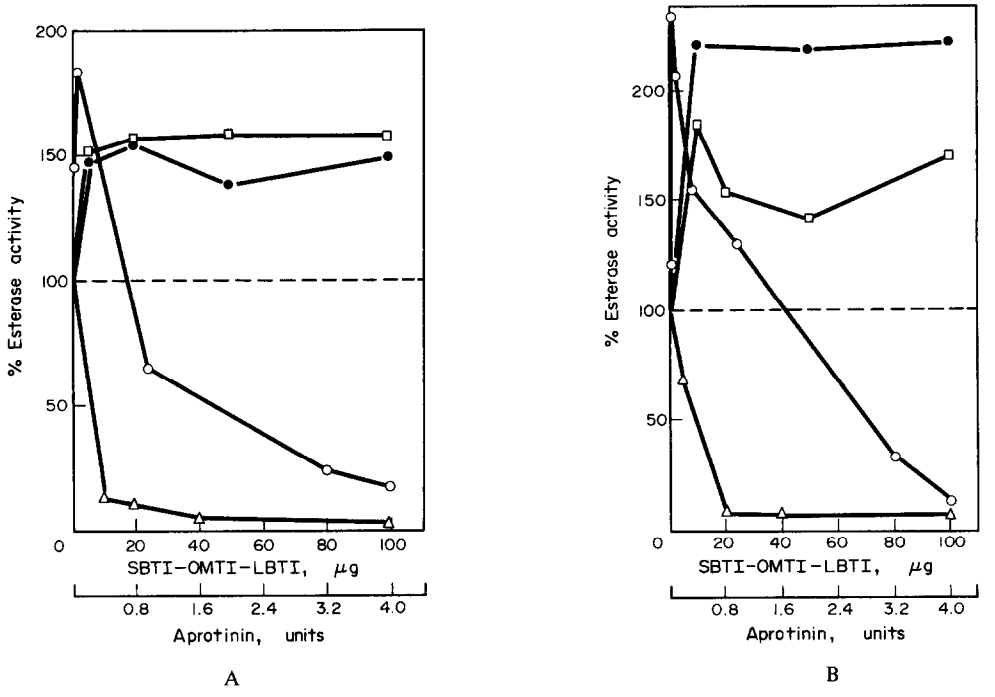

Fig. 6. Panel A: Effects of trypsin inhibitors on rat urinary kallikrein A activity. Ten μl of the enzyme (2×10^{-2} E.U./ml), 10 μl of each inhibitor at the indicated amount and 30 μl of 0.2 M Tris–HCl buffer, pH 8.0, were mixed and allowed to stand for 20 min. [^{3}H]Tos-L-Arg-OMe hydrolysis was determined as described in the legend for Fig. 5. Key: (O——O) SBTI; (●——●) OMTI; (□——□) LBTI; and (△——△) aprotinin. Panel B: Effects of trypsin inhibitors on rat urinary kallikrein B activity. Experimental conditions were as described in the legend for panel A. Key: (O——O) SBTI; (●——●) OMTI; (□——□) LBTI; and (△——△) aprotinin.

purified kallikreins showed a 2 to 3-fold increase in specific activity after 1 year of storage at 4° in 0.1 N NaCl–0.01 M sodium phosphate, pH 7.0. This finding suggested the possible conformational changes of the enzyme molecules during the storage [15].

Inhibition and activation studies

The effects of several trypsin inhibitors on rat urinary kallikrein A and B esterase activity are shown in Fig. 6 (panels A and B). The esterase activity of both kallikreins A and B was stimulated by LBTI and OMTI but inhibited by aprotinin. At low concentrations, SBTI stimulated both kallikrein A and B esterase activity whereas inhibition was observed at higher concentrations. The hydrolysis of Tos-L-Arg-OMe by purified kallikreins A and B was increased in the presence of 0.02% of the nondenaturing detergents, sodium deoxycholate, Triton X-100, Brij 58 and Lubrol PX. Stimulation ranged between 243 and 314 per cent of control activities (Table 2). Activation was not diminished when the enzymes were diluted in polyethylene glycol 6000, 8 mg/ml, or in bovine serum albumin, 1 mg/ml. In addition, both kallikrein A and B were inhibited between 60 and 80 per cent by 0.02% of the cationic detergents benzyltriphenylphosphonium chloride and cetyltrimethylammonium bromide, and guanidium chloride.

Table 2. Kallikrein isozyme activation by detergents*

Detergent	% Activity RUK_A	RUK_B
	100	100
Deoxycholate	244	243
Triton X-100	270	305
Brij 58	287	314
Lubrol PX	291	312

* Assays were done at room temperature. Ten μl of the enzyme (2.0×10^{-2} E.U./ml), 10 μl of 0.1% detergent, and 30 μl of 0.2 M Tris–HCl buffer, pH 8.0, were mixed and allowed to stand for 20 min. [^{3}H]Tos-L-Arg-OMe hydrolysis was determined as described in the legend for Fig. 5. Spontaneous Tos-L-Arg-OMe hydrolysis was not altered by detergents alone.

Immunological characterization

The double-diffusion analysis showed that an undiluted sheep antiserum to rat urinary kallikrein B gave a single line of identity with purified rat urinary kallikrein A (30 E.U./ml) and B (28 E.U./ml) (Fig. 7). No precipitin line appeared between either kallikrein A or B and normal sheep serum. The kinin-releasing activities of kallikreins A and B in the guinea pig ileum

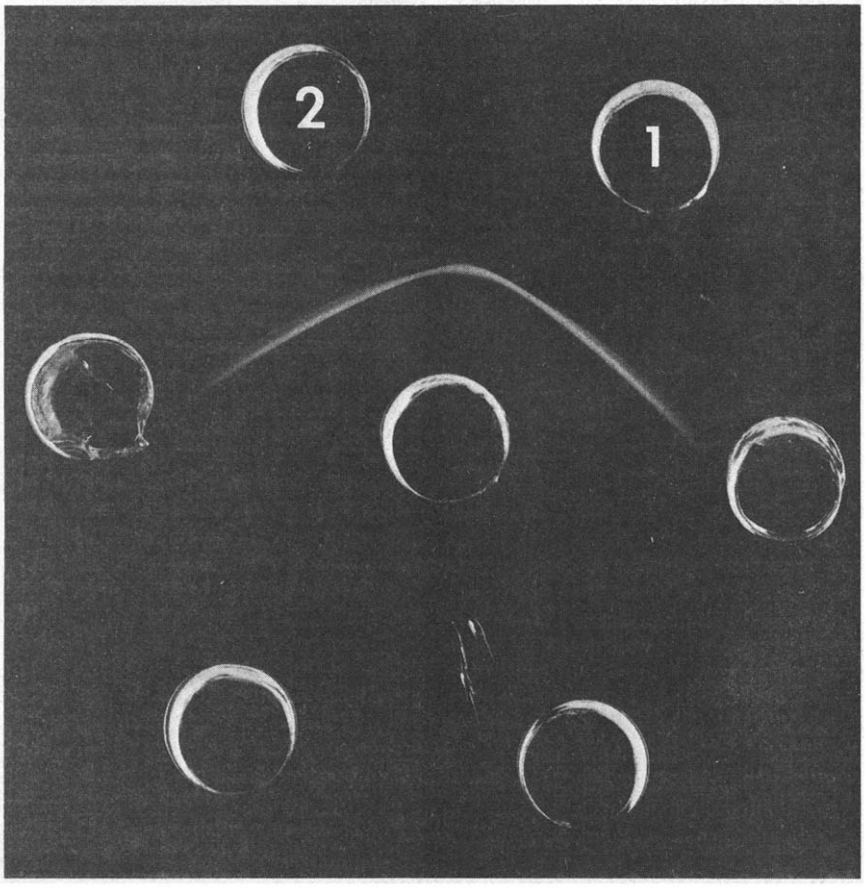

Fig. 7. Double-diffusion analysis of rat urinary kallikreins A and B. Center well: undiluted sheep antiserum to rat urinary kallikrein B; peripheral well: (1) rat urinary kallikrein A (30 E.U./ml × 4), and (2) rat urinary kallikrein B (28 E.U./ml × 4).

bioassay or the contractile response of the rat uterus to kallikrein could be inhibited completely by antiserum but not by normal serum (data not shown). The maximal inhibition of Tos-L-Arg-OMe hydrolysis by rat urinary kallikrein A or B was observed after preincubation with a 5000-fold dilution of antiserum and ranged between 50 and 70 per cent.

Kinetic analysis

The initial rate of Bz-L-Arg-OEt hydrolysis was proportional to kallikrein concentration with saturating amounts of substrate (1 mM). The initial velocity was maximal at pH 8.5 to 9.0 and was inhibited by the addition of NaCl at concentrations of 0.3 M and above. The rate of hydrolysis varied as a function of substrate concentration according to the Michaelis–Menten model. The K_m of kallikrein A was estimated to be 8.3×10^{-5} M and that of kallikrein B, 7.3×10^{-5} M. The V_{max} values for kallikreins A and B were 200 μmoles/min/mg and 213 μmoles/min/mg of protein respectively.

DISCUSSION

The heterogeneity of urinary kallikrein has been reported previously by several investigators [12, 22, 26]. In the present report, evidence is presented to show that two kallikrein isozymes exist in rat urine. Two separate peaks of kallikrein-like activity were resolved with DEAE-cellulose chromatography of crude enzyme fractions. This new observation of separable activities via anion-exchange chromatography, as well as by polyacrylamide gel electrophoresis, is consistent with the original criteria for the identification of isozymes [27]. In addition, these two forms have been separated with electrofocusing in polyacrylamide slab gels.

Nustad and Pierce [22] isolated four forms of rat urinary kallikrein (B_1–B_4) with pI values of 3.50, 3.68, 3.73 and 3.80 by preparative isoelectric focusing. However, their kallikreins were not separable by DEAE-Sephadex chromatography and appear to have identical or slightly different electrophoretic mobilities in polyacrylamide disc gels. However, our kallikreins A and B showed distinctly different mobilities in polyacrylamide slab gel electrophoresis. Silva *et al.* [26] also found two major components with pI = 3.97 and 4.18 and three minor components with pI = 4.50, 4.87 and 5.30 of rat urinary kallikrein in a preparative electrofocusing column. Since they did not characterize these components further, we cannot relate our rat urinary kallikreins A and B to their five forms. It should be noted that the pI values of our purified kallikreins A (4.31) and B (4.26) are somewhat higher than the majority of the previously observed forms [22, 26]. It is possible that pI values obtained from gel electrofocusing are less accurate than those reported using preparative electrofocusing in sucrose density columns but other explanations such as varying carbohydrate content could be responsible for the wide range (3.50 to 5.30) of observed isoelectric points.

Oza *et al.* [12] used an aprotinin-Sepharose affinity column to purify rat urinary kallikrein and showed that there seemed to be one major and one minor protein peak of absorbance at 280 nm in a polyacrylamide gel. These two components when eluted from gel slices both had esterase and kininogenase activities but further studies were not done. Therefore, the exact relation to our kallikreins A and B is uncertain. Porcelli *et al.* [28] reported that 30 per cent of the esterase activity adsorbed to DEAE-cellulose was eluted in a broad peak preceding the sharp main activity peak that was purified further, while the former peak was discarded. Porcine pancreatic kallikrein has been resolved by DEAE-cellulose chromatography into two isozymes called A and B which are distinguishable by polyacrylamide gel electrophoresis at neutral or slightly alkaline pH [29–31]. Our findings with rat urinary kallikrein are similar, except that pancreatic kallikrein isozymes could not be distinguished by polyacrylamide gel electrofocusing. Our separation of urinary kallikreins A and B may be attributed to the improved resolution of electrofocusing in slab gels.

Rat urinary kallikreins A and B do not appear to be artifacts of the purification procedures since kallikreins, isolated from an immunoprecipitate of concentrated rat urine and antikallikrein B antiserum by the method of Nustad and Pierce [22], showed two bands of protein and esterase activity staining [32] in polyacrylamide slab gel electrophoresis (data not shown). The two components eluted from Gel slices both have Tos-L-Arg-OMe esterase and kininogenase activities. These data indicate that both kallikreins A and B are present in rat urine.

The molecular weight of urinary kallikrein B is smaller than that of kallikrein A based on SDS-polyacrylamide gel electrophoresis, and Sephadex G-100 gel filtration. Therefore, the possibility that kallikrein B is a degradation product of A cannot be excluded completely. It seems unlikely for the following reasons. First, the kallikreins have equal biological, as well as esterase activities. Second, urinary kallikrein B behaves as a mono-dispersive species in both gel electrophoresis and isoelectric focusing analyses. These results appear inconsistent with the notion that kallikrein B occurs through random degradation of kallikrein A. Third, we have found two kallikrein-like esterase activities in renal cortical cell homogenates separated by a 0.1 to 0.5 M NaCl gradient on DEAE-cellulose chromatography (unpublished results). Therefore, conversion of rat urinary kallikrein A to rat urinary kallikrein B, if it exists at all, would appear to occur in the kidney, not in urine. Preliminary amino acid analyses suggested differences in amino acid composition between A and B [33]. However, further work is still required to support or negate this finding.

The activity of rat urinary kallikreins A and B is strongly inhibited by aprotinin, and both enzymes form complexes with aprotinin which show altered electrophoretic mobilities on polyacrylamide gels. Previous studies [1, 9, 22, 34] have shown that rat urinary kallikreins are strongly inhibited by approtinin, weakly inhibited by SBTI, but not inhibited by OMTI or LBTI. We found that both kallikreins A and B are activated by various concentrations of OMTI and LBTI. Further, both are activated by low concentrations of SBTI but inhibited by high concentrations. Some activation of kallikrein by SBTI in animal tissues has been reported and the preferred interpretation for this observation was removal of heavy metal contamination [35]. This possibility is unlikely in the present study since purification would have excluded heavy metals and, furthermore,

EDTA (10^{-3} to 10^{-5} M) did not affect the activity of the purified enzymes. Silva *et al.* [26] observed similar phenomena in that several substituted guanidines activated rat urinary kallikrein at low concentrations but inhibited it at high concentrations. Based on the use of competitive inhibitors or of specific active center labeling agents, it has been proposed that the active centers of glandular kallikreins contain an anionic binding site, a hydrophobic site, and an auxiliary site in addition to the catalytic site [36, 37]. Our observations of activition of rat urinary kallikrein by trypsin inhibitors may also suggest binding of the inhibitors to an auxiliary or other site. Despite their ability to activate kallikrein, SBTI, OMTI or LBTI did not alter the electrophoretic mobility of kallikrein (data not shown). It seems that these trypsin inhibitors weakly interact with the enzyme.

Our data show that both rat urinary kallikreins A and B are activated by nondenaturing detergents. Recently, we found that deoxycholate induces conformational changes in purified kallikrein B as measured by circular dichroism. Further, the activation of kallikrein by SBTI, OMTI or LBTI can also be abolished by deoxycholate [15]. These findings support the notion that the activation of urinary kallikreins A and B by trypsin inhibitors is due to conformational changes of the enzyme molecule.

The rat kidney can synthesize kallikrein indistinguishable from the urinary enzyme [3]. All presently available data seem to indicate that the enzyme is enriched in plasma membranes [6, 7], and is localized within the distal nephron [5, 8]. The role of kallikreins and kinins in renal tubular cell function is unclear. Further investigation of kallikrein isozymes in renal cells [38] may help define that role.

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