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CARBOXYPEPTIDASE-TYPE KININASE OF HUMAN KIDNEY AND URINE, (U) F/O 6/1
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Technical Report No. 24

Carboxypeptidase-type Kininase of Human Kidney and Urine

Prepared by

Ervin G. Erdős

For Publication in
Proceedings of Society of Experimental
Biology

Departments of Pharmacology and Internal Medicine
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→ carboxypeptidase did not cross-react with antiserum to human pancreatic carboxypeptidase B. These properties distinguish carboxypeptidase of human urine and kidney from plasma carboxypeptidase N and from pancreatic carboxypeptidase B. ←

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CARBOXYPEPTIDASE-TYPE KININASE OF HUMAN
KIDNEY AND URINE

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Kinins are inactivated by various enzymes collectively called kininases (1, 2). Two of them, kininase I and II, cleave the C-terminal arginine and phenylalanyl-arginine of kinins respectively. Kininase II also converts angiotensin I to angiotensin II. The major plasma kininase is kininase I (carboxypeptidase N; arginine carboxypeptidase EC 3.4.17.3). Kininase II (dipeptidyl carboxypeptidase EC 3.4.15.1) is concentrated mainly on the plasma membrane of endothelial and epithelial cells. Pancreatic carboxypeptidase B (EC 3.4.17.2) is also a potent inactivator of kinins by the release of C-terminal arginine (2).

Both bradykinin and kallidin are present in the urine (3, 4, 5) although kininase II, located on the brush border of the proximal tubules (6), eliminates plasma kinins that enter the nephron after glomerular filtration (7, 8). Thus kinins that appear in the urine are released intrarenally distal to the proximal tubules. Kinins alter sodium and water reabsorption (9) and release prostaglandins in the kidney (10, 11). Because of these effects of kinins, their inactivation in the kidney appears to be an important step in renal autoregulation. This consideration led us to study the properties of a carboxypeptidase-type kininase in human urine and kidney.

Chemicals. The substrates Bz-glycyl-argininic acid (hippuryl-argininic acid: HLAA), Bz-glycyl-arginine (HLA), Bz-glycyl-lysine (HLL), Ac-Phe-Arg, Ac-Ser-Pro-Phe-Arg and bradykinin were purchased from Vega-Fox, Tucson, AZ. Sephadex G-100, Sepharose 6B and Blue Dextran 200 were obtained from Pharmacia, Uppsala, Sweden. DEAE-cellulose types 40 and 70 were from Carl Schleicher and Schuell Co., Kene, NH; CM-cellulose (type 32) from Whatman, Balston, Ltd., England and hydroxyapatite from Bio-Rad, Richmond, CA. The protein standards for molecular weight determination (such as ribonuclease, alpha-chymotrypsin, pepsin, ovalbumin or bovine serum albumin) were purchased from Sigma Chemical Co., St. Louis, MO. Other reagents were analytical grade. Teprotide (SQ 20881) was obtained from Dr. Z. Horovitz of Squibb Institute, Princeton, NJ.

Tissue preparations. Human kidneys were obtained from sudden death cadavers at the Department of Pathology and the Institute of Forensic Sciences, UTHSCD.

Normal human urine was collected from healthy adult volunteers. Kidneys were washed in cold isotonic saline and stored at -20° . After thawing the tissue was ground twice in an electric grinder and homogenized at high speed for 60 sec in a Waring blender in 50% (w/v) 0.25 M sucrose containing 0.1 M Tris-HCl buffer, pH 7.4. The homogenate was diluted with 1.5 volumes of buffered sucrose and homogenized a second time for 60 sec. The homogenate was centrifuged ($10,000 \times g$ for 30 min) in a Sorvall refrigerated centrifuge. The supernatant was recentrifuged in a Beckman L2 or L5-65 ultracentrifuge

(50,000 x g for two hr). The microsomal precipitate was quickly frozen to -40° . Subsequently it was homogenized in cold acetone for 30 sec in a Waring blender.

The acetone homogenate was immediately filtered in a Buchner funnel and the acetone powder dried in a vacuum desiccator for two hr. The powder was then extracted overnight in 0.1 M phosphate buffer (5% w/v), pH 8.0, containing 20% glycerol and 10^{-4} M CoCl_2 .

Insoluble material was removed by centrifugation (5,000 x g for 20 min) in a refrigerated Sorvall RC-2 centrifuge. The extract was then concentrated in the cold room with an Amicon PM-30 Diaflo membrane under nitrogen pressure.

Human blood samples were taken from healthy volunteers and the plasma was separated and lyophilized. All subsequent procedures with plasma were done in the same manner as the extraction of carboxypeptidase from the acetone powder of human kidney microsomes.

Human pancreatic carboxypeptidase B was purified to homogeneity as described previously (12).

Preparation of urinary enzyme. Twenty-five liters of human urine were concentrated 100-fold by ultrafiltration through a Diaflo type AM-10 membrane and dialyzed for one day against two changes of 2 liter 0.05 M Tris-HCl buffer, pH 7.4, containing 10^{-4} M CoCl_2 .

DEAE-cellulose chromatography. The dialysate (225 ml) was added to a column (2 x 66 cm) of DEAE-cellulose. The column was washed with the buffer used above at a flow rate of 56 ml/hr and eluted with a step-wise-increased NaCl gradient. Urinary carboxypeptidase was eluted with 0.05 M NaCl. The active fraction was then concentrated to 100 ml by

ultrafiltration and dialyzed against three changes of 2 liter of 0.01 M potassium phosphate buffer, pH 6.0.

Second DEAE-cellulose chromatography. The sample was applied to a column (2.2 x 28 cm) of DEAE-cellulose equilibrated with 0.01 M potassium phosphate buffer, pH 6.0. Carboxypeptidase was eluted with 0.075 M KCl, added to the same buffer at a flow rate of 30 ml/hr. After concentration to 5 ml by ultrafiltration, the sample was dialyzed against 0.05 M sodium acetate buffer, pH 7.2 containing 0.1 M NaCl.

Gel filtration. The active fraction was filtered on a column (2.5 x 95 cm) of Sephadex G-100 equilibrated with 0.05 M sodium acetate-acetic acid buffer pH 7.2, containing 0.1 M NaCl. Eluent was collected in the same buffer in 3 ml fractions. The active fractions were combined and concentrated as above to 6 ml and dialyzed against 10^{-3} M potassium phosphate buffer, pH 6.5.

Hydroxyapatite column chromatography. The preparation obtained after gel filtration was passed through a column (2.2 x 10 cm) of hydroxyapatite. Carboxypeptidase was recovered only in the fraction that was not adsorbed on the column. The active fractions were combined, concentrated to 5 ml and dialyzed against 0.005 M Tris-HCl buffer, pH 8.0.

Enzyme assay. The hydrolysis of HLA, HLL, Ac-Phe-Arg, and Ac-Ser-Pro-Phe-Arg was determined in a Beckman 121 automatic amino acid analyzer. The hydrolysis of HLAA was assayed in the UV spectrophotometer (13). The concentration of the substrates was 1×10^{-3} M. The inactivation of bradykinin was measured by bio-assay on the isolated rat uterus (14, 15). In inhibition studies, the inhibitors were preincubated with the enzymes for 20 min at 37° before addition of substrate.

Molecular weight. For the determination of the mol wt. of carboxypeptidases from kidney, plasma and urine, a column (2 x 100 cm) of Sepharose 6B was prepared in 0.05 M phosphate buffer, pH 6.8, containing 0.1 M NaCl. A 10 mg sample was applied to the column and eluted in 3 ml fractions with the equilibrating buffer at a flow rate of 21 ml/hr. The proteins used to standardize the column were: thyroglobulin (660,000), fibrinogen (330,000), gamma globulin (157,000), bovine albumin (68,000) ovalbumin (45,000), pancreatic carboxypeptidase B (35,000;12), and α -chymotrypsin (23,000).

The mol wt. of the partially purified urinary enzyme was also determined on a Sephadex G-100 column (2.5 x 100 cm) which was equilibrated with 0.05 M Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl. Fractions of 3 ml were collected at a flow rate of 15 ml/hr. The proteins used to standardize the column were bovine albumin (68,000), ovalbumin (45,000), pancreatic carboxypeptidase B (35,000;12), α -chymotrypsin (23,000), lysozyme (14,000) and ribonuclease (12,500). The elution volume of the carboxypeptidase was determined and the mol wt. was calculated from a standard profile of elution volume vs. mol wt.

Immunodiffusion. Antibody to human pancreatic carboxypeptidase B was elicited in rabbits. Qualitative immunoprecipitation was performed by Ouchterlony double immunodiffusion analysis. Pancreatic and urinary carboxypeptidase were loaded separately onto agar gels. The center well of the plate contained rabbit antiserum elicited against human pancreatic carboxypeptidase B (12).

pH effect. The pH optimum of the urinary enzyme was studied by following the inactivation of bradykinin on the isolated rat uterus in a phosphate buffer. The source of enzyme was the carboxypeptidase obtained after the second DEAE-cellulose column chromatography.

RESULTS

Purification. The partial purification of a carboxypeptidase-type kininase from human urine is summarized in Table 1. The purification of the enzyme was followed by measuring the hydrolysis of HLAa after each step. The urinary enzyme was purified 283-fold from crude urine or 27-fold from urine concentrated by membrane filtration. Table 1

Substrate specificity. Table 2 compares the relative rates of hydrolysis of various substrates by human plasma carboxypeptidase N (16), kidney carboxypeptidase, urinary carboxypeptidase and pancreatic carboxypeptidase B (17). The plasma, kidney and urinary carboxypeptidases cleaved HLL faster than HLA. Pancreatic carboxypeptidase B acted the opposite way. Table 2 indicates that the renal enzyme hydrolyzed HLA and Ac-Phe-Arg relatively faster than the plasma enzyme, but urinary kininase hydrolyzed Ac-Ser-Pro-Phe-Arg and bradykinin more rapidly than either the plasma or the kidney enzyme. None of the preparations had any carboxypeptidase A-type activity since they did not cleave Bz-glycyl-phenylalanine. Table 2

Inhibition. Inhibition of the four human carboxypeptidases was studied with HLL as substrate (Table 3). The compounds used inhibited plasma carboxypeptidase N and pancreatic carboxypeptidase B differently than Table 3

the renal or urinary carboxypeptidases. The inhibition of renal and urinary carboxypeptidases, however, was similar. EDTA and CdSO_4 inhibited pancreatic carboxypeptidase B (16, 17) less than the other three carboxypeptidases. ξ -amino-n-caproic acid was effective only against the plasma enzyme (13, 16). Inhibitors of proteases, SH-enzymes and kininase II, (DFP, p-chloromercuriphenyl sulfonic acid and teprotide) were ineffective.

Molecular weight. Figure 1 shows gel filtration of the plasma, renal and urinary carboxypeptidases on a Sepharose 6B column. The elution volume of plasma carboxypeptidase H was similar to fibrinogen and indicated a mol wt of approximately 280,000 in agreement with published data (2, 16). The elution volumes of both the renal and urinary carboxypeptidases indicated a mol wt of about 40,000. Similar results were obtained on a Sephadex G-100 column. The mol wt of human pancreatic carboxypeptidase B was found to be 35,000 as previously established (12). Fig. 1.

Effect of pH. The effect of pH on the inactivation of bradykinin by urinary carboxypeptidase was studied at pH 6, 7 and 8 in a 0.1 M phosphate buffer. The source of the enzyme was a partially purified fraction collected after the 2nd DEAE-cellulose column chromatography step. (The enzyme was stable when stored at -20° for 14 months). The inactivation of bradykinin, as measured on the isolated rat uterus, was maximal (100%) at pH 7. It was slightly slower at pH 8 (90%) and much slower at pH 6 (53%).

Immunodiffusion. The reaction of rabbit antiserum to purified human pancreatic carboxypeptidase B with human pancreatic and urinary carboxy-

peptidases in Ouchterlony double immunodiffusion plates is shown in Fig. 2.

2. The urinary carboxypeptidase did not cross-react with antiserum to pancreatic carboxypeptidase B indicating that the two enzymes are antigenically different. In the control studies, pancreatic carboxypeptidase formed a line of identity with the antiserum.

DISCUSSION

The current upsurge of interest in urinary kallikrein excretion in various forms of hypertension (9, 18, 19, 20) stresses the importance of urinary kininases as regulators of locally acting kinins. If urinary kallikrein plays a role in the etiology of hypertension, it is probably through the kinin that it releases. Urine contains kinins (3, 4, 5) although plasma kinins normally do not appear in urine (7, 8) unless inactivating enzymes such as kininase II on the brush border of the proximal tubules (6) are inhibited (7). The kininase activity of urine was reported simultaneously with the discovery of urinary kinins (3). Later it was suggested that both kininase I and II - type enzymes are present in urine (14), but only kininase II or (angiotensin I converting enzyme) has previously been purified from human urine (21).

We compared the properties of human carboxypeptidases which inactivate bradykinin by cleaving its C-terminal Arg. We found that human urine and kidney contain a carboxypeptidase which is different from plasma carboxypeptidase N and pancreatic carboxypeptidase B. Plasma carboxypeptidase N (2, 13, 16, 22, 23) and pancreatic carboxypeptidase B (12, 17, 24) are well characterized enzymes and the differences between the two enzymes are firmly established. The renal and the urinary enzymes have a much lower mol wt than plasma carboxypeptidase N (40,000 v. 280,000).

Plasma carboxypeptidase N (or kininase I) also differs from the urinary/renal carboxypeptidase in the ratio of the rates of hydrolysis of substrates and its sensitivity to inhibitors. For example, ϵ -amino-n-caproic acid is effective only against plasma carboxypeptidase N.

Human pancreatic carboxypeptidase B has a mol wt of about 35,000 (12), similar to that of the renal/urinary carboxypeptidase. Although injected porcine pancreatic carboxypeptidase B appears in the kidney and urine of laboratory animals (25), experiments with various substrates, inhibitors and antisera to the pancreatic enzymes proved that the pancreatic and urinary/renal carboxypeptidases are also different.

The renal carboxypeptidase described here appears to be similar to the enzyme found in acetone-dried hog kidney powder (25) in that it cleaves C-terminal basic arginine faster if the penultimate amino acid is phenylalanine instead of glycine. Although there appears to be some differences between renal and urinary carboxypeptidases, for example, in the relative rates of hydrolysis of substrates, the two enzymes have many properties in common when compared to the plasma or pancreatic carboxypeptidases. Based on this, we hypothesize that the urinary kininase I-type enzyme originates in the kidney. A contribution of the low, 45,000 mol wt subunit of plasma carboxypeptidase N (16, 22, 23) to the kininase activity in urine, however, cannot be excluded.

The activity of urinary carboxypeptidase is lower at an acid pH, therefore below neutrality, less urinary kinins are inactivated. The effect of the pH of tubular fluid on the release and inactivation of

kinins will be settled definitely only when the precise site of interaction of renal kallikrein with kininogen substrate is established.

SUMMARY

A carboxypeptidase was partially purified from human urine and a similar or identical enzyme was extracted from a particulate fraction of the human kidney. The enzyme cleaved basic C-terminal amino acids of peptides including bradykinin. The inhibition of the human renal/urinary carboxypeptidase was different from that of plasma carboxypeptidase N (kininase I) or pancreatic carboxypeptidase B. The urinary enzyme was not affected by inhibitors of catheptic enzymes or kininase II and has no carboxypeptidase A activity. The urinary/renal carboxypeptidase had a mol wt of 40,000. The urinary carboxypeptidase did not cross-react with antiserum to human pancreatic carboxypeptidase B. These properties distinguish carboxypeptidase of human urine and kidney from plasma carboxypeptidase II and from pancreatic carboxypeptidase B.

TABLE I
PURIFICATION OF CARBOXYPEPTIDASE FROM HUMAN URINE

Procedure	Total Protein (mg)	Volume (ml)	Total Units ^a	Units Per mg Protb	Yield (%)	Purification
Membrane Filtration	2025	225	112.5	0.055	100.0	1.0
DEAE-Cellulose Column Chromatography pH (pH 7.7)	324	102	40.0	0.123	35.7	2.2
DEAE-Cellulose (pH 6.0)	50	50	18.0	0.36	16.0	6.5
Sephadex G-100 Filtration	20	20	10.0	0.5	8.9	9.0
Hydroxyapatite Column Chromatography	0.6	30	0.9	1.500	0.8	27.0

^aU = 1nmol HLAA cleaved per min

^bCrude urine = 0.0053

TABLE II
Ratio of Hydrolysis of Substrates by Human Carboxypeptidases

<u>Substrate (10^{-3} M)</u>	<u>Source of Enzyme</u>			
	<u>Plasma</u>	<u>Kidney</u>	<u>Urine</u>	<u>Pancreas</u>
Bz-glycyl-lysine(HLL)	100	100	100	100
Bz-glycyl-arginine (HLA)	40	80	60	212
Ac-Phe-Arg ^a	18	80	55	-
Ac-Ser-Pro-Phe-Arg ^a	16	22	42	-
Bradykinin ^b	20	25	45	-
Bz-glycyl-phenylalanine	0	0	0	-

^aC-terminal fragment of kinins

^bDetermined by bio-assay

TABLE III
Inhibition of the Activity
of Human Carboxypeptidases
(%)

<u>Inhibitor (M)</u>	<u>Source of Enzyme^a</u>			
	<u>Plasma</u>	<u>Kidney</u>	<u>Urine</u>	<u>Pancreas</u>
EDTA (2×10^{-3})	95	88	86	9
CdSO ₄ (1.5×10^{-5})	80	77	82	31
ξ-Amino-n-caproic acid (1×10^{-2})	92	20	35	38
Argininic Acid (5×10^{-4})	--	30	37	--
p-Chloromercuriphenyl sulfonic acid, DFP (10^{-3}), Teprotide (SQ 20881; 10^{-4})	0	0	0	--

^aSubstrate = Bz-glycyl-lysine

LEGENDS

Fig. 1

Gel filtration on a Sepharose 6B column of human plasma carboxypeptidase N (a—c), human renal carboxypeptidase (d—f), and human urinary carboxypeptidase (g—i).

The estimated mol wt of carboxypeptidase N is 280,000 and that of renal and urinary carboxypeptidase, 40,000.

Fig. 2

Human urinary carboxypeptidase does not cross-react with anti-serum. Ouchterlony double diffusion plates. Center well: rabbit antiserum to human pancreatic carboxypeptidase B. 1-4 = 100 - 900 $\mu\text{g/ml}$ human urinary carboxypeptidase B; 5 = 100 $\mu\text{g/ml}$ human pancreatic carboxypeptidase B.

FIGURE 1

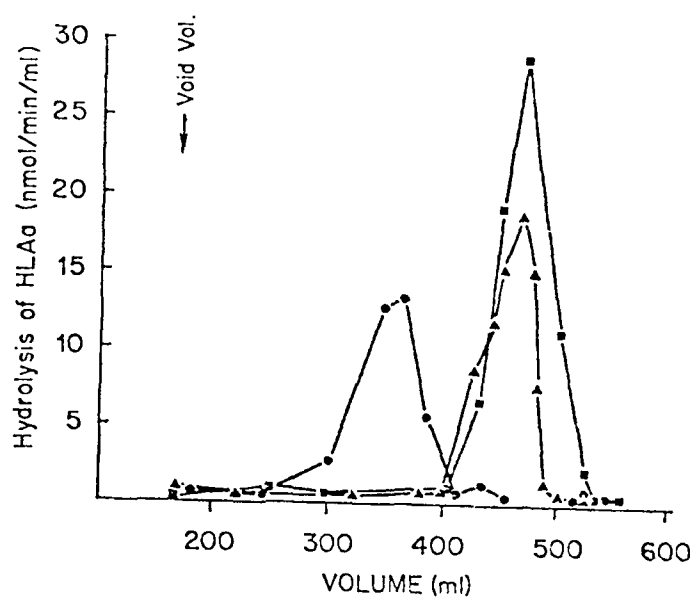
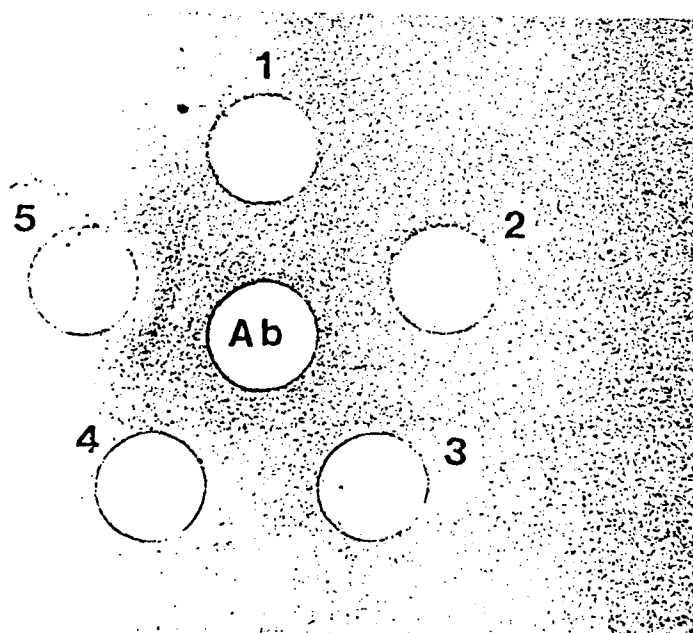


FIGURE 2



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