MAJOR KININASES IN RAT URINE ARE NEUTRAL ENDOPEPTIDASE AND CARBOXYPEPTIDASE Y-LIKE EXOPEPTIDASE

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ABSTRACT

Two types of kininases were separated from rat urine by gel-filtration on a column of Superdex 200 and characterized as a membrane-bound neutral endopeptidase (EC 3.4.24.11) and a carboxypeptidase Y-like exopeptidase. The neutral endopeptidase which was found in a void volume fraction, liberated first Phe⁸-Arg⁹ and then Phe⁵-Ser⁶-Pro⁷ from bradykinin (BK)*. The kininase activity was inhibited by phosphoramidon and thiorphan but not by captopril. Another kininase with an apparent molecular weight of 93,000 cleaved rapidly BK into BK1-8 (des Arg⁹ BK) and BK1-7 (des Phe⁸-Arg⁹ BK) into BK1-6 (des Pro⁷-Phe⁸-Arg⁹ BK). The kininase activity was inhibited by inhibitors for serine proteases and cysteine proteases and by metal ions (Co²⁺, Hg²⁺ or Ag⁺), but not by inhibitors for metal proteases. The kininase activity was also inhibited by a rabbit anti-peptide IgG which was raised against a synthetic peptide with the amino acid sequence of human and mouse carboxypeptidase Y-like peptidases. These results indicate that the exopeptidase is not carboxypeptidases N or M but a member of a family of human and mouse carboxypeptidase Y-like exopeptidases which are identified as protective proteins associated with lysosomal β -galactosidase and neuraminidase or platelet peptidase with deamidase activity. When rat urine was incubated with BK, the generation of either BK1-8 or BK1-7 was inhibited in the presence of either DFP* or phosphoramidon. In the presence of both of DFP and phosphoramidon, degradation of BK was inhibited, whereas captopril had no effect on the degradation of BK. These results demonstrate that major kininases in rat urine are neutral endopeptidase and carboxypeptidase Y-like exopeptidase.

One of the important roles of BK, a vasoactive peptide, is the regulation of the kidney function, including excretion of water and sodium ion and prostaglandin synthesis (for reviews, see 16, 17). Kinin-releasing enzymes (kallikreins) and kinin-degradating enzymes (kininases), are found in the kidney (for review, see 1). The intrarenal concentration of BK depends on the production of

BK by kallikrein and the rapid destruction of BK by kininase. Two kinds of kininases are known to be present in plasma, tissues and urine, i.e. kininase II (angiotensin-converting enzyme and neutral endopeptidase) and kininase I (carboxypeptidases N and M) (6, 19). The former liberates carboxy terminal Phe-Arg from BK, the latter, carboxy terminal Arg from BK. Recent studies indicate that the degradation pathway of BK in urine is different from that in plasma and that kininases in urine are derived from kidney, not from plasma (18, 25).

In our previous work (15), we have identified Arg-Pro-Pro-Gly-Phe-Ser (BK1-6) as a major degradation product of BK in rat urine, which was not

*Abbreviations: BK, bradykinin; NEM, *N*-ethyl maleimide; DFP, diisopropyl fluorophosphate; PMSF, phenylmethylsulfonyl fluoride; TPCK, *N*-tosyl-L-phenylalanine chloromethylketone; MGTA, 2-mercaptomethyl-3-guandinoethyl-thiopropionic acid

60 min at 37°C, the hydrolysate was analyzed by reversed phase HPLC as described above. Inhibition of kininase was expressed as percentages of the activity in the absence of inhibitor.

Preparation of Anti IgG against Synthetic Peptide (CPY-N17) Corresponding to Amino Terminal Portion of Protective Protein Associated with Lysosomal β -Galactosidase and Neuraminidase

A peptide with the amino acid sequence of Leu-Pro-Gly-Leu-Ala-Lys-Gln-Pro-Ser-Phe-Arg-Gln-Tyr-Ser-Gly-Tyr-Leu, designated as CPY-N17, was synthesized using a solid phase peptide synthesizer, Model 430 (ABI). The peptide, CPY-N17, corresponded to the amino terminal identical portion of human and mouse protective proteins associated with lysosomal β -galactosidase and neuraminidase (10, 11). The amino acid sequence of CPY-N17 was confirmed by the amino acid and sequence analyses. CPY-N17 was conjugated with Keyhole Limpets hemocyanin (Sigma Chemical) using glutaraldehyde. A rabbit was immunized by injecting the conjugate with complete Freund's adjuvant subcutaneously three times during a month. IgG was prepared from the antiserum using a column of protein A Sepharose. A specific anti CPY-N17 IgG

was prepared using a column of Affigel 10 (Bio Rad) conjugated with CPY-N17. Non-immunized IgG was prepared from normal rabbit serum using a column of protein A Sepharose.

RESULTS

Separation of BK1-7-Producing Kininase and BK1-8-Producing Kininase in Rat Urine by Gel-Filtration

Fig. 1 shows the elution profiles of rat urine on a column of Superdex 200. Each fraction was incubated with BK and the degradation products were measured by reversed phase HPLC as described under Materials and Methods. Kininases were separated into four fractions and they were pooled as indicated by solid bars in Fig. 1.

BK1-7- and BK1-8-producing kininase activities in four fractions (A-D) were measured and shown in Table 1. About 70% of BK1-7-producing activity was found in fraction A, while a major BK1-8-producing kininase was found in fraction D and minor kininases in fractions B and C.

Identification of BK1-7-Producing Kininase as Neutral Endopeptidase

BK1-7-producing kininase in the void volume frac-

Fig. 1 Gel-filtration of rat urine on a column of Superdex 200. Rat urine (20 ml) was concentrated and applied to a column (1.6 $\times$ 60 cm) of Superdex 200 equilibrated with 0.05 M acetate-0.15 M NaCl buffer, pH 6.5. Each 1 ml fraction was collected. Each 5 μ l aliquot was incubated with BK and the digests were analyzed by reversed phase HPLC as described under Materials and Methods. $\bullet$ —, BK1-7-producing activity; $\blacktriangle$ —, BK1-8-producing activity. Four fractions were pooled as indicated by solid bars.

Table 1 *Distribution of Activities of BK1-7- and BK1-8-Producing Kininases in Four Fractions from Gel-Filtration of Rat Urine*

	Fraction A	Fraction B	Fraction C	Fraction D
Apparent MW ^a	>1,100,000	670,000	240,000	93,000
BK1-7-producing activity (pmol/min)	78 (66)	14 (11)	12 (10)	14 (12)
BK1-8-producing activity (pmol/min)	1 (1)	20 (20)	34 (34)	47 (46)

^aApparent molecular weight of each fraction was calculated from the fraction with peak activity using a calibration curve. Values in parentheses indicate relative distribution of kininase activity in four fractions expressed as percent.

Fig. 2 Gel-filtration of the void volume fraction (Fraction A) on a column of Superdex 200 in the presence of Triton X-100. The fraction A was concentrated and mixed with Triton X-100 to 1%. The solution was applied to a column of Superdex 200 (1.6 × 60 cm) equilibrated with 0.05 M acetate buffer, pH 6.5, containing 0.15 M NaCl and 1% Triton X-100. Each 10 µl aliquot was incubated with BK and generation of BK1-7 was analyzed by reversed phase HPLC as described under Materials and Methods. —●—, BK1-7-producing activity. - - - - - , ABSORBANCE AT 280 nm

tion (fraction A) was gel-filtered in the presence of Triton X-100. As shown in Fig. 2, BK1-7-producing kininase was eluted in a lower molecular weight fraction. BK1-7-producing kininase from the second gel-filtration was incubated with BK for 3 h at 37°C and the degradation products were separated by reversed phase HPLC as shown in Fig. 3. The

amino acid sequences of each fragment were assigned by amino acid and sequence analyses and by the comparison of the retention time with the synthetic peptides. From the results shown in Fig. 3, BK fragments generated by BK1-7-producing kininase were identified as BK1-7, Phe-Ser-Pro, Phe-Arg and BK1-4. Peptide peak 6 was confirmed

(B) Amino acid sequence and amino acid composition of each fragment in (A)

Peak	Amino acid sequence	Amino acid composition
1:	Arg-Pro-Pro-Gly (BK1-4) 113 26 12 14	Arg (1.1), Pro (1.8), Gly (1.2)
2:	Phe 21	Phe
3:	Phe-Arg 1152 173	Phe (1.0), Arg (1.2)
4:	Phe-Ser-Pro 277 52 79	Phe (1.1), Ser (1.1), Pro (1.0)
5:	Arg-Pro-Pro-Gly-Phe- 700 888 642 1241 443 Ser-Pro (BK1-7) 111 69	Arg (1.1), Pro (2.7), Gly (1.1) Phe (1.0), Ser (0.9)

Fig. 3 Identification of BK fragments from the digest of BK with BK1-7-producing kininase. (A) A 3 h digest of BK with BK1-7-producing kininase was separated by reversed phase HPLC. The retention times of the synthetic peptides are shown in the upper part. (B) Each peak in (A) was assigned by the analyzes of its amino acid composition and amino acid sequence. Arrows under the amino acid sequence of each fragment represent the amino acid residues identified by a protein sequencer. The values under the arrows indicate the amount of the amino acid residues identified (pmol).

to be BK undigested. The presence of Phe is supposed to be due to the contamination of peptidase which cleaves Phe-Ser-Pro and Phe-Arg. The presence of the peptidase in the void volume fraction was demonstrated using synthetic Phe-Ser-Pro and Phe-Arg. Although the bulk of the peptidase was separated by the second gel-filtration (data not shown), BK1-7-producing kininase was not completely separated from the peptidase. These fragments in the digests of BK with BK1-7-producing kininase were quantitated from the standard curves which were made using the synthetic peptides by reversed phase HPLC.

Fig. 4 shows the time course of the liberation of these fragments from BK by BK1-7-producing kininase. With the decrease of BK, Phe-Arg and BK1-7 increased rapidly, while BK1-4 and Phe-Ser-Pro slowly increased. These results indicate that BK1-7-producing kininase cleaves BK into BK1-7 and then BK1-4.

As shown in Table 2, the kininase was markedly inhibited by the inhibitors for neutral endopeptidase (EC 3.4.24.11), phosphoramidon and thiorphan (8), but not by angiotensin-converting enzyme inhibitor, captopril (2). It was inhibited by *o*-phenanthroline, but not by carboxypeptidase inhibitor (MGTA), cysteine protease inhibitor (NEM), serine protease inhibitor (DFP) and aspartic protease inhibitor (pepstatin). From the inhibitors spectrum and the cleaved peptide bonds of BK, the BK1-7-producing kininase was identified as neutral endopeptidase, rather than angiotensin-converting enzyme (see Discussion).

Identification of BK1-8-Producing Kininase as Carboxypeptidase Y-Like Exopeptidase

Fig. 5 shows the time course of the degradation of BK, BK1-8 and BK1-7 by the BK1-8-producing kininase (fraction D) separated from gel-filtration of rat urine. The kininase liberated BK1-8 very rapidly from BK and BK1-6 from BK1-7, but liberated slowly BK1-7 from BK1-8. This result indicates that the kininase in fraction D is a carboxypeptidase which prefers to cleave Phe-Arg and Ser-Pro bonds of BK and cleaves Pro-Phe bond slowly. The kininase was inhibited markedly by DFP, PMSF and TPCK and moderately by NEM and E-64, but not by *o*-phenanthroline, phosphoramidon and captopril (Table 3). The kininase is distinguishable from carboxypeptidases N and M since it was not sensitive to MGTA and *o*-phenanthroline and was not enhanced by Co^{2+} . On the contrary, the kini-

Fig. 4 Time course of liberation of BK fragments from BK by BK1-7-producing kininase. BK was incubated with the BK1-7-producing kininase for 0.5, 1, 2 and 3 h. The digests were analyzed by reversed phase HPLC and each BK fragment was quantitated as described under Materials and Methods. ●, BK; □, Phe-Arg; ○, BK1-7; ▲, BK1-4; △, Phe-Ser-Pro

nase was inhibited by Co^{2+} , Hg^{2+} and Ag^{+} . The properties of the carboxypeptidase in rat urine is similar to carboxypeptidase Y from yeast (12) because it was inhibited by inhibitors for serine protease and cysteine protease (see Discussion). To confirm that the kininase is a member of a family of carboxypeptidase Y, we synthesized a peptide, CPY-N17, with the amino acid sequence corresponding to the amino terminal identical portion of human and mouse protective proteins associated with lysosomal β -galactosidase and neuraminidase, which are homologous to carboxypeptidase Y (10, 11). The anti CPY-N17 rabbit IgG was prepared as described under Materials and Methods. As shown in Fig. 6, the kininase activity was inhibited with the increasing amount of anti CPY-N17 IgG. Weak inhibition by non-immunized IgG was observed, which may be due to a competitive inhibition of IgG as a substrate for the kininase. As shown in Table 3, BK1-8-producing kininases in fractions B and C showed the similar inhibition spectra, which indicate that these BK1-8-producing kininases in three fractions (B-D) are carboxypeptidase Y-like

Fig. 5 Time course of degradation of BK, BK1-8 or BK1-7 by BK1-8-producing kininase. BK, BK1-8 and BK1-7 were incubated with the BK1-8-producing kininase (fraction D) for 15, 30, 60 and 120 min. The digests were analyzed by reversed phase HPLC as described under Materials and Methods. (A) Degradation of BK: $\square$, BK; $\triangle$, BK1-8; $\circ$, BK1-7. (B) Degradation of BK1-8: $\triangle$, BK1-8; $\circ$, BK1-7. (C) Degradation of BK1-7: $\circ$, BK1-7; $\bullet$, BK1-6; $\blacktriangle$, BK1-5

exopeptidases.

Table 2 Inhibition of BK1-7-Producing Kininase by Various Inhibitors

Inhibitor	Concentration (M)	Inhibition (%)
<i>o</i> -Phenanthroline	10^{-5}	18
	10^{-4}	100
Thiorphan	10^{-8}	46
	10^{-7}	84
	10^{-6}	88
	10^{-5}	88
Phosphoramidon	10^{-8}	52
	10^{-7}	80
	10^{-6}	86
	10^{-5}	86
Captopril	10^{-4}	0
MGTA	10^{-3}	7
NEM	10^{-3}	1
E-64	10^{-3}	3
Pepstatin	10^{-5}	2
DFP	10^{-3}	2
Leupeptin	10^{-4}	1

BK was incubated with BK1-7-producing kininase for 1 h in the presence of various inhibitors at concentrations indicated. The amount of BK1-7 was quantitated by reversed phase HPLC as described under Materials and Methods. The inhibition was expressed as the relative amount of BK1-7 to the amount in the absence of inhibitors.

Effect of DFP, Phosphoramidon and Captopril on the Degradation of BK in Rat Urine

BK was incubated with rat urine in the presence of inhibitors. BK1-7 or BK1-8 generated were measured as described under Materials and Methods. As shown in Fig. 7, generation of BK1-8 was inhibited in the presence of DFP, while generation of BK1-7 was inhibited in the presence of phosphoramidon. In the presence of both of DFP and phosphoramidon, degradation of BK was inhibited. Captopril had no effect of generation of BK1-7 and BK1-8. As described above, BK1-7-producing enzyme was demonstrated to be neutral endopeptidase, sensitive to phosphoramidon, and BK1-8-producing enzyme was demonstrated to be carboxypeptidase Y-like exopeptidase, sensitive to DFP. Therefore, the results shown in this section clearly indicate that major kininases in rat urine are neutral endopeptidase and carboxypeptidase Y-like exopeptidase.

DISCUSSION

Although kininases in human urine have been isolated (20, 21), the properties of kininases in rat urine are still poorly understood. Ura *et al.* reported that major kininase in rat urine is a neutral endo-

Table 3 *Inhibition of BK1-8-Producing Kininase by Various Inhibitors*

Inhibitor	Concentration (M)	Inhibition (%)		
		Fraction D	Fraction C	Fraction B
DFP	10 ⁻⁶	20		
	10 ⁻⁵	92		
	10 ⁻⁴	100	100	97
PMSF	10 ⁻⁵	13		
	10 ⁻⁴	63		
TPCK	10 ⁻⁶	13		
	10 ⁻⁵	30		
	10 ⁻⁴	70		
NEM	10 ⁻³	40	38	42
E-64	10 ⁻³	20		
MGTA	10 ⁻³	21	35	37
Leupeptin	10 ⁻⁴	20		
Captopril	10 ⁻⁴	0	0	0
<i>o</i> -Phenanthroline	10 ⁻³	0	0	0
Phosphoramidon	10 ⁻⁴	1	0	0
Pepstatin	10 ⁻⁵	4		
Co ²⁺	10 ⁻⁴	32	29	30
Hg ²⁺	10 ⁻⁴	100		
Ag ⁺	10 ⁻⁴	100		

BK was incubated with BK1-8-producing kininase for 2 h in the presence of various inhibitors. The amount of BK1-8 was quantitated by reversed phase HPLC as described under Materials and Methods. The inhibition was expressed as the relative amount of BK1-8 to the amount in the absence of inhibitors.

peptidase from the study on the inhibition of the degradation of BK by rat urine (25). However, further investigation on the properties of each kininase in rat urine is required to understand the degradation pathway of BK in rat urine, hence, in kidney. In the present work, we have demonstrated that two types of kininases can be separated by a gel-filtration of rat urine which are characterized as a neutral endopeptidase and carboxypeptidase Y-like exopeptidases. This is evidenced as follows. A BK1-7-producing kininase in a void volume fraction was eluted in a lower molecular weight fraction by a gel-filtration in the presence of Triton X-100. The kininase liberated first Phe-Arg and then Phe-Ser-Pro from BK. The result is consistent with the specificity of neutral endopeptidase (7, 9). The kininase activity was inhibited by phosphoramidon and thiorphan, but not by captopril. It has been shown that angiotensin-converting enzyme liberates Phe-Arg and then Ser-Pro from BK and is inhibited by captopril (2). BK1-7-producing kininase is also distinct from rat endopeptidase-2 which cleaves BK into BK1-5 and Ser-Pro-Phe-Arg (22). Human kidney neutral endopeptidase has been

solubilized in the presence of Triton X-100 (9). These results indicate that the BK1-7-producing kininase is membrane-bound neutral endopeptidase, but not angiotensin-converting enzyme.

Major BK1-8-producing kininase was eluted in a fraction with a molecular weight of 93,000 (fraction D) and cleaved rapidly BK into BK1-8, and BK1-7 into BK1-6. The rate of the degradation of BK1-8 into BK1-7 by the kininase was about one eighth of those of BK and BK1-7. The kininase is distinct from carboxypeptidases M and N (3, 4, 23, 24), since it is not inhibited by *o*-phenanthroline, only partially inhibited by MGTA and not enhanced by Co²⁺. On the contrary, the BK1-8-producing kininase was inhibited by inhibitors for serine protease and cysteine protease and by metal ions. These properties of the kininase share with the properties of carboxypeptidase Y from *Saccharomyces cerevisiae* (12). The kininase belongs to a family of carboxypeptidase Y which includes human and mouse protective proteins associated with lysosomal β -galactosidase and neuraminidase (10, 11) and human platelet peptidase (13), because the kininase activity was inhibited by anti-rabbit IgG raised

Fig. 6 Inhibition of BK1-8-producing activity by anti CPY-N17 rabbit IgG. BK1-8-producing kininase (fraction D) was incubated with various amounts of anti CPY-N17 rabbit IgG or non-immune rabbit IgG in 65 μ l of 0.05 M acetate buffer, pH 6.5, containing 0.15 M NaCl for 1 h at 37°C. After the addition of BK, BK1-8 generated was quantitated by reversed phase HPLC as described under Materials and Methods. Inhibition was expressed as percent relative to the amount of BK1-8 generated in the absence of IgG. The amount of IgG was calculated assuming that one absorbance unit at 280 nm corresponds to 1.0 mg/ml. $\circ$, anti CPY-N17 rabbit IgG; $\bullet$, non-immune rabbit IgG

against a synthetic peptide with the amino acid sequence of these proteins. Studies on BK1-8-producing kininases in other two fractions (fractions B and C) indicated that they were also carboxypeptidase Y-like exopeptidases. They are supposed to be homopolymer of carboxypeptidase Y-like exopeptidase or heteropolymer of the exopeptidase with other proteins. The heterogeneity was not artificially caused by concentration or freezing-thawing of urine, because the elution profile of urine without the above process was indistinguishable with that in Fig. 1 (data not shown). The presence of carboxypeptidase Y-like exopeptidase in rat urine has not been reported so far. However, Galjart *et al.* demonstrated the expression of mRNA for mouse protective protein associated with lysosomal β -galactosidase and neuraminidase in mouse kidney and other tissues (11). These results suggest that carboxypeptidase Y-like exopeptidase may be synthesized also in rat kidney and excreted in in urine.

Effect of inhibitors on the degradation BK by rat urine supports the conclusion that the major kininases in rat urine are neutral endopeptidase and

Fig. 7 Effect of inhibitors on the generation of BK1-7 and BK1-8 from BK in the presence of rat urine. Sixty-fold diluted urine was incubated with BK for 1 h at 37°C and BK1-7 and BK1-8 were measured as described under Materials and Methods. Their amounts were expressed as percent, compared to the amount produced in the absence of inhibitors. (1), control; (2), DFP (10^{-4} M); (3), phosphoramidon (10^{-4} M); (4), captopril (10^{-5} M); (5), DFP and phosphoramidon (10^{-4} M). $\square$, BK1-7; ▨ , BK1-8

carboxypeptidase Y-like exopeptidase. From the results shown in the present work, we can presume the degradation pathway of BK in rat urine as shown in Fig. 8. BK1-8 and BK1-7 are produced from BK by the action of carboxypeptidase Y-like exopeptidases and neutral endopeptidase, respectively. BK1-7 will be then degraded into BK1-6 by carboxypeptidase Y-like exopeptidases and into BK1-4 by neutral endopeptidase. BK1-4 can be directly generated from BK by the action of neutral endopeptidase. However, it will be a minor pathway because we could not detect Phe-Ser-Pro-Phe-Arg. BK1-5 is not produced from BK1-8 unless angiotensin-converting enzyme is involved. These presumption is supported by the previous finding that BK1-6 is a BK degradation product in rat urine but not BK1-5 (15). The degradation pathway of BK in urine is supposed to reflect the pathway in the kidney. It has been shown that membrane-bound forms of angiotensin-converting enzyme, neutral endopeptidase and carboxypeptidase M are synthesized in the kidney and excreted into urine (4, 5, 7, 24), although the mechanism for the excretion of these membrane-bound enzymes into urine is not known. The present work demonstrates that major kininases in rat urine, hence in rat kidney, are clearly different from the major kininases in rat

Fig. 8 Degradation pathways of BK by neutral endopeptidase and carboxypeptidase-Y like exopeptidase from rat urine. NEP, neutral endopeptidase; "Cpase Y", carboxypeptidase Y-like exopeptidase

plasma, which are angiotensin-converting enzyme and carboxypeptidase N.

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