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DEMONSTRATION OF ARGINYL-BRADYKININ MOIETY IN RAT HMW KININOGEN: DIRECT EVIDENCE FOR LIBERATION OF BRADYKININ BY RAT GLANDULAR KALLIKREINS

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SUMMARY: The amino acid sequence around kinin moiety in rat High-Molecular-Weight (HMW) kininogen was determined by isolating a peptide containing bradykinin after cyanogen bromide treatment of the purified kininogen as follows; NH_2-Thr-Ser-Val-Ile-Arg-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Ala-Pro-Arg-Val-Lys-Lys-. The data indicated that rat HMW kininogen contains the arginyl-bradykinin moiety, instead of lysyl-bradykinin. Kinins liberated from rat HMW kininogen by rat urinary and submaxillary kallikreins were identified to be bradykinin, not arginyl-bradykinin.

It has been generally accepted that there exist two kinds of kininogens, precursor of bradykinin, in mammalian plasmas, which are called as High-Molecular-Weight (HMW) kininogen and Low-Molecular-Weight (LMW) kininogen. Structural and enzymatic studies on bovine and human kininogens have given the unequivocal evidence that plasma kallikrein liberates bradykinin and glandular kallikrein liberates lysyl-bradykinin from two kinds of kininogens, although HMW kininogen is more susceptible to plasma kallikrein (1). In the previous paper (2), we have purified and characterized rat HMW kininogen and have shown that rat plasma kallikrein liberates bradykinin from the kininogen. In the present paper, we have determined the amino acid sequence around the kinin moiety in rat HMW kininogen and found that the kininogen contains arginyl-bradykinin moiety along the polypeptide chain, instead of lysyl-bradykinin. The direct evidence for liberation of bradykinin by rat glandular kallikreins will be presented.

MATERIALS AND METHODS

Rat HMW kininogen was highly purified from Sprague-Dawley rat plasma according to the method of Hayashi et al.(2). Purification of rat urinary and submaxillary kallikreins were made by the methods of Chao and

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Margolius(3), and Brandtzaeg et al.(4), respectively. Human urinary kallikrein was isolated from the urinary kallikrein concentrate, which was kindly supplied by Mochida Pharmaceutical Company, using Arginine-Sepharose, DEAE-Sephadex A-50, CM-Sephadex C-50, Benzamidine-Sepharose and Sephadex G-100. The specific activity of kallikreins prepared was calculated to be 1.1 μmole/min/A280 for rat urinary kallikrein and 41.8 μmole/min/A280 for submaxillary kallikrein, and 2.1 μmole/min/A280 for human urinary kallikrein, respectively, using Pro-Phe-Arg-4-methylcoumaryl-7-amide as substrate. Synthetic bradykinin, lysyl-bradykinin and methionyl-lysyl-bradykinin were purchased from Protein Research Foundation, Minoh, Osaka. Arginyl-bradykinin was kindly supplied from the Foundation. Bovine HMW kininogen was purified as reported previously(1,13).

Separation of kinin-containing peptide from rat HMW kininogen. Rat HMW kininogen (Total A280=15) was reduced and carboxymethylated by the method of Crestfield et al. (5),and then treated with cyanogen bromide in 70 % formic acid for 2 days at room temperature. The resulting peptides were separated by a gel-filtration on a column of Sephadex G-100 and a reversed phase high performance liquid chromatography (HPLC) using a Cosmosil 5C18 column (Nakarai Chemicals, Kyoto). For measurement of kinin-containing peptide, 50 μl aliquots of each fraction were lyophilized and dissolved in 200 μl of 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl, and incubated with 10 μl of trypsin (1 mg/ml) for 1 hr at 37° C. The kinin activity was assayed by the contraction of rat uterus using bradykinin as standard.

Amino acid and sequence analyses. Samples were hydrolysed in evacuated, sealed tubes at 110° C for 24 hr with 5.7 N HCl. After evaporation, the hydrolysates were analyzed on a Hitachi 835 automatic analyser. Amino acid sequence of peptide was performed using a Beckman 890D sequencer as described in detail previously(6).

Identification of kinin liberated from rat HMW kininogen by rat glandular kallikreins. Fifty μl of rat HMW kininogen (A280=0.53) was mixed with 246 μl of 0.02 M Tris-HCl-0.15 M NaCl, pH 7.8 and 4 μl of rat submaxillary kallikrein (A280=0.0065) or with 245 μl of the same buffer and 5 μl of rat urinary kallikrein (A280=0.05). The mixture was incubated at 37°C and a 50 μl aliquot was taken for bioassay after a definite time of incubation. Fifty μl of bovine HMW kininogen (A280=2.1) was mixed with 150 μl of the same buffer and 5 μl each of rat submaxillary kallikrein (A280=0.65), rat urinary kallikrein (A280=0.05) or human urinary kallikrein (A280=0.61). After 1 hr, a 100 μl aliquot was applied to a reversed phase HPLC column (0.4 X 30 cm) of TSK-Gel LS410-ODS-SIL (Toyo Soda Manufacturing Co., Ltd., Tokyo). Peptides were eluted by a solvent containing 17 % acetonitrile, 0.025 M sodium sulfate, 0.01 M potassium monobasic phosphate and 0.2 % phosphoric acid (pH 2.7) and detected at 210 nm using Toyo Soda UV8-Model II with a full scale range of 0.01. Flow rate was adjusted to 0.8 ml/min with a pump, Toyo Soda HLC-803A.

RESULTS

To isolate a kinin-containing peptide, the S-alkylated kininogen was treated with cyanogen bromide and the resulting peptides were first separated by gel-filtration on a column of Sephadex G-100. Elution profile is shown in Fig. 1. Each fraction was incubated with trypsin and the kinin activity generated was measured using rat uterus. As shown in Fig. 1, a peptide which releases kinin was eluted in a low molecular weight fraction. The

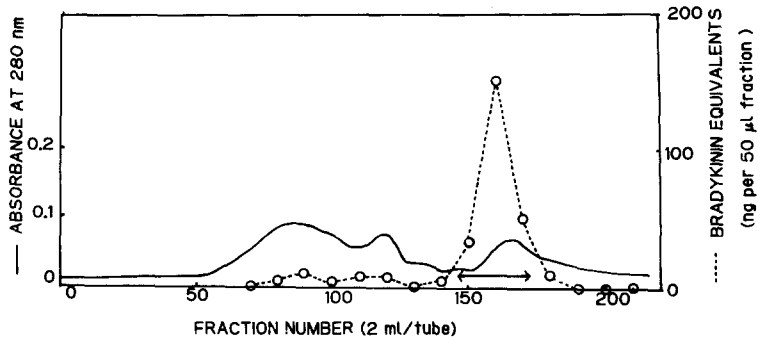

Fig. 1:Gel-filtration of cyanogen bromide-treated HMW kininogen on a column (2 x 144 cm) of Sephadex G-100. Protein was eluted by 10 % acetic acid with a flow rate of 5 ml per hr. A 50 µl aliquot of each fraction was subjected to analyze kinin activity after trypsin treatment.

fractions indicated by an arrow were pooled and subjected to reversed phase HPLC (Fig. 2). The kinin activity which was generated after the treatment with trypsin was found in peaks No. 7, No. 8 and No. 9. The amino acid composition of peptides from these three peaks were almost the same and total amino acid residues of the peptide No. 7 was calculated to be 48, as shown in Table 1. The reason why three peptides with the same amino acid composition were isolated remains uncertain. They are supposed to be the

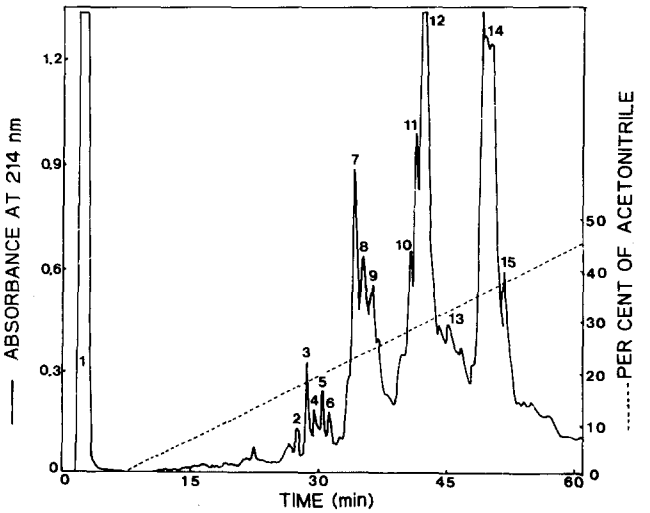

Fig. 2:Separation of kinin-containing peptides by reversed phase HPLC. A part of the sample from a column of Sephadex G-100 was applied to a column of Cosmosil 5C18, equilibrated with 0.1% trifluoroacetic acid. Peptides were eluted at room temperature with a linear gradient from 0 % acetonitrile-0.1% trifluoroacetic acid(v/v) to 50 % acetonitrile-0.1 % trifluoroacetic acid at a flow rate of 1 ml per min and were monitored at 214 nm.

Table 1

Amino acid composition and partial amino acid sequence of a kinin-containing peptide isolated from rat HMW kininogen[a]

Amino acid	Residues per molecule	Amino acid	Residues per molecule
Aspartic acid	2.4 (2)	Isoleucine	2.0 (2)
Threonine	2.9 (3)	Leucine	0.6 (1)
Serine	4.3 (4)	Tyrosine	0.9 (1)
Glutamic acid	4.9 (5)	Phenylalanine	2.0 (2)
Proline	5.9 (6)	Lysine	3.9 (4)
Glycine	3.0 (3)	Histidine	1.0 (1)
Alanine	2.0 (2)	Tryptophan	Not determined
Valine	4.3 (4)	Arginine	5.7 (6)
S-Carboxymethyl cysteine	0.7 (1)	Glucosamine	+
Homoserine	+	Galactosamine	+

Thr-Ser-Val-Ile-Arg-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Ala-Pro-Arg-
0.9 1.2 1.0 1.1 0.5 0.6 0.7 0.8 0.2 0.6 0.4 0.5 0.1 0.3

Val-Lys-Lys-
0.2 0.2 0.4

[a]Amino acid composition was determined after hydrolysis with 5.7 N HCl for 24 hr. Amino acid sequence was determined using a Beckman 890D sequencer. Values under the amino acid residues indicate nanomoles of PTH amino acids recovered in each step.

peptides with homoserine lactone and homoserine at the COOH termini and/or with different carbohydrate. The lower half of Table 1 shows the partial NH_2-terminal sequence of the peptide containing kinin moiety and the result indicates that the peptide includes the arginyl-bradykinin moiety, linked with four amino acid residues (Thr-Ser-Val-Ile) at the NH_2-terminal portion.

In the previous paper (2), we have shown that rat plasma kallikrein liberates bradykinin from rat HMW kininogen. We examined here if rat glandular kallikreins liberate arginyl-bradykinin from rat HMW kininogen. Figure 3 A shows the time course curve of kinin liberation from the kininogen by rat urinary and submaxillary kallikreins, respectively. Kinin

was rapidly liberated and the liberation reached a plateau after 30 min incubation with a substrate to enzyme ratio in terms of absorbance at 280 nm of 100 to 1 for urinary kallikrein and of 1,000 to 1 for submaxillary kallikrein. Moreover, the kinin liberated by both kallikreins was identified to be bradykinin as judged from the retention time on the column (Fig. 3B,1 and 2). A peptide peak which corresponds to arginyl-bradykinin was not observed. In the preparation of rat HMW kininogen and rat urinary and submaxillary kallikreins, an aminopeptidase-like activity was not found, which converts arginyl-bradykinin to bradykinin (data not shown). These results indicate that not only rat plasma kallikrein but also rat glandular kallikreins liberate bradykinin from rat HMW kininogen,cleaving arginylarginyl and arginylalanyl bonds located in the NH_2- and COOH-terminal parts of the kinin moiety (Table 1). On the other hand, rat glandular kallikreins liberated also bradykinin, not lysyl-bradykinin, from bovine HMW kininogen (Fig. 3B ,3 and 4), although human urinary kallikrein used for comparison, liberated lysyl-bradykinin(Fig. 3B,5).

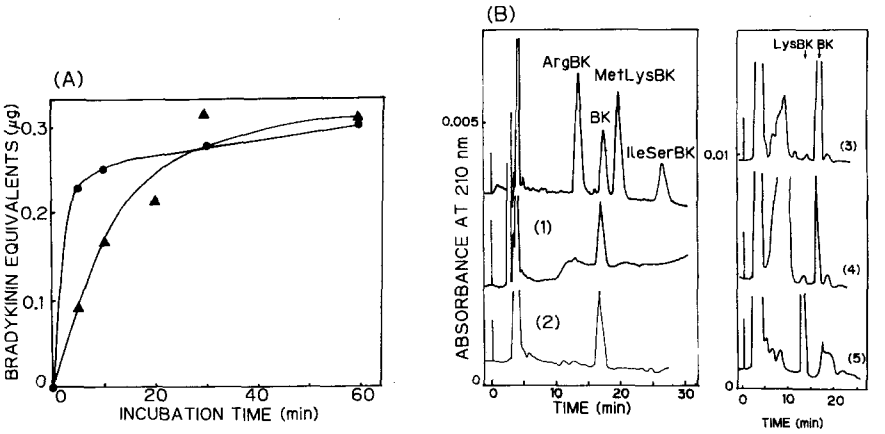

Fig. 3: Identification of kinin liberated from rat and bovine HMW kininogens by rat urinary kallikrein or rat submaxillary kallikrein. (A) Time course curve for the liberation of kinin from rat HMW kininogen by rat glandular kallikreins. HMW kininogen was incubated with kallikreins as described in MATERIALS AND METHODS and the amounts of kinin liberated were assayed using rat uterus. -●-; rat urinary kallikrein, -▲-; rat submaxillary kallikrein. (B) Rat HMW kininogen was incubated with rat urinary kallikrein(1) or rat submaxillary kallikrein(2). Bovine HMW kininogen was incubated with rat urinary kallikrein(3), rat submaxillary kallikrein(4) and human urinary kallikrein(5). After 60 min incubation, a 100 μl aliquot was injected into a reversed phase HPLC column. Top panel of the left column shows an elution profile of synthetic kinins. BK, bradykinin.

DISCUSSION

The present paper demonstrates that rat HMW kininogen includes the arginyl-bradykinin moiety and releases bradykinin by rat glandular kallikreins. The possibility that rat glandular kallikreins liberate first arginyl-bradykinin, then convert to bradykinin, can be excluded, because we could not find the activity to convert arginyl-bradykinin to bradykinin in the substrate and the enzymes used for this study. Another possibility still remains that rat glandular kallikrein liberates first a larger peptide, then the peptide was converted to bradykinin by an enzyme contaminating in our preparation. However, we could not detect any peptide other than bradykinin in the samples on the initial stage of incubations (unpublished data). Therefore, we conclude that rat kallikrein liberate directly bradykinin from rat HMW kininogen. Furthermore, rat glandular kallikreins liberate bradykinin, not lysyl-bradykinin, from bovine HMW kininogen(Fig. 3B, 3 and 4). This substrate specificity seems to be characteristic for rat kallikreins, because other glandular kallikreins so far examined liberate lysyl-bradykinin from HMW and LMW kininogens (1). Although it has been suggested that urine liberates bradykinin from rat plasma (7) and rat pituitary kallikrein liberates bradykinin from canine kininogen (8), this paper provides the direct evidence for the release of bradykinin from HMW kininogen by rat glandular kallikreins.

Recently, a new kinin (T-kinin, Ile-Ser-bradykinin) and its precursor were isolated from rat plasma by several investigators (9-12). The kininogen, so called T-kininogen, is resistant to kallikreins and has not been found in bovine and human plasmas so far. The different feature of the components in rat kallikrein-kinin system is supported in the present paper by the demonstration that rat glandular kallikreins liberate bradykinin from HMW kininogen.

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REFERENCES

1) Kato, H., Iwanaga, S. and Nagasawa, S. (1981) Methods Enzymol. 80,172-197.
2) Hayashi,I., Kato, H., Iwanaga, S. and Oh-ishi, S. (1985) J. Biol. Chem. in press.
3) Chao, J. and Margolius, H. S. (1979) Biochem. Pharmacol. 28, 2071-2079.
4) Brandtzaeg, P., Gautvik, K. M., Nustad, K. and Pierce, J. V. (1976) Br. J. Pharmac. 56, 155-167.
5) Crestfield, A. M., Moore, S. and Stein, W. H. (1963) J. Biol. Chem. 244, 4406-4412.
6) Miyata, T., Usui, K. and Iwanaga, S. (1984) J. Biochem. 95, 1793-1801.
7) Alhenc-Gelas, F., Marchetti, J., Allegrini, J., Corvol, P. and Menard, J. (1981) Biochim. Biophys. Acta 677, 477-488.
8) Powers, C. A. and Nasjletti, A. (1983) Endocrinology, 112, 1194-1200
9) Bedi, G, S. Balwierczack, J. and Back, N. (1983) Biochem. Biophys. Res. Commun. 112, 621-628.
10) Okamoto, H. and Greenbaum, L. M. (1983) Biochem. Biophys. Res. Commun. 112, 701-708.
11) Sakamoto, W., Yoshikawa, K., Uehara, S. Nishikaze, O. and Handa, H. (1984) J. Biochem. 96, 81-88.
12) Enjyoji, K., Kato, H., Iwanaga, S., Hayashi, I. and Oh-ishi, S. (1984) Seikagaku(In Japanese) 56, 759.
13) Shimada, T., Sugo, T., Kato, H. and Iwanaga, S.(1982) J. Biochem. 92, 679-688