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IDENTIFICATION OF [HYDROXYPROLINE^3]-LYSYL-BRADYKININ RELEASED FROM HUMAN PLASMA PROTEIN BY KALLIKREIN.

Manabu Sasaguri, Masaharu Ikeda, Munehito Ideishi and Kikuo Arakawa

Department of Internal Medicine, Fukuoka University, School of Medicine, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-01, Japan

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[Hydroxyproline^3]-lysyl-bradykinin ([Hyp^3]-Lys-BK), a new kinin was isolated, besides lysyl-bradykinin (Lys-BK), from the reaction mixture of human plasma protein Cohn's fraction IV-4 with hog pancreatic kallikrein. The liberated kinins were isolated by procedures including ethanol extraction, Sephadex G-15, CM cellulose and reverse-phase high performance liquid chromatography (HPLC) and quantitated by radioimmunoassay. On HPLC, two peaks of immunoreactive kinins emerged. Peak 1, an unknown kinin proceeded to Peak 2 which had an identical retention time to that of Lys-BK. The amino acid sequence of the unknown Peak 1 proved to be Lys-Arg-Pro-Hyp-Gly-Phe-Ser-Pro-Phe-Arg, or [Hyp^3]-Lys-BK, and Peak 2 Lys-BK. The ratio of the amounts of two kinins thus formed were [Hyp^3]-Lys-BK 25±4% and Lys-BK 75±4%. The existence of [Hyp^3]-Lys-BK suggests a presence of a new kininogen, containing [Hyp^3]-Lys-BK in human plasma protein, possibly undergone post-translational modifications.

Kallikreins are proteolytic enzymes which liberate kinins from precursor kininogens. Among many biological activities of kinins, vasodilative action is potent perhaps because it also stimulates prostaglandin synthesis. Kallikrein-kinin system is thus thought to play roles in regulating tissue perfusion and in blood coagulation-fibrinolysis.(1)

Different kinins are generated depending on the kininogenases used, i.e., lysyl-bradykinin (Lys-BK) by tissue kallikreins, hog pancreatic, horse and human urinary kallikreins and bradykinin by trypsin, plasma kallikrein, tonin and other trypsin-like serine proteases.(2,3) Okamoto et al. reported the presence of T-kinin or Ile-Ser-bradykinin liberated from rat plasma treated with trypsin.(4,5) Recently Mindroiu T. et al. also isolated a newly identified kinin, [Alanine^3]-lysyl-bradykinin ([Ala^3]-Lys-BK) from human urine.(6)

In the present study we isolated a heretofore unknown kinin from the reaction mixture of human plasma protein and hog pancreatic kallikrein and identified it.

MATERIALS AND METHODS

Hog pancreatic kallikrein here used was a gift from Bayer Co. Ltd. (West Germany, specific activity 1463 KE/mg protein). The purity of the kallikrein

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preparation was confirmed by electrophoretic analysis. Disc-gel electrophoresis revealed five bands, all exhibiting Pro-Phe-Arg naphthylester hydrolytic activity on zymogram.(7) Human plasma protein Cohn's fraction IV-4 was obtained from Midori-Juji (Osaka, Japan). Synthetic kinins, i.e., bradykinin, lysyl-bradykinin, and methionyl-lysyl-bradykinin were obtained from Peptide Institute (Osaka, Japan).

Human plasma protein, Cohn's fraction IV-4 (5g) was incubated with 5 mg of purified hog pancreatic kallikrein at 37°C for 3 hours in 50 ml of 0.1 M Tris/HCl pH 8.0 containing 0.1 M NaCl, 30 mM of EDTA and 3 mM of 1-10 phenanthroline. After incubation, the mixture was deproteinized by adding 500 ml of hot ethanol and centrifuged. The supernatant was evaporated to dryness under reduced pressure at 45°C. The dried residue was dissolved in 0.01 M ammonium acetate pH 5.0, and applied on Sephadex G-15 column (1.5 x 94 cm) equilibrated with the same buffer. Each fraction of the eluates was assayed by radioimmunoassay for the kinin. The antiserum used showed 53% cross-reactivity with Lys-BK and 77% with Met-Lys-BK when bradykinin was used as the standard, as we previously described.(3) The fractions containing the immunoreactive kinins were combined and the conductivity was adjusted to 3.0 mmhos. The active fractions were pooled and chromatographed on a CM-cellulose column (1.0 x 15 cm) equilibrated with 0.03 M ammonium acetate pH 5.0. After run with 300 ml of the initial buffer, elution of the kinin was carried out with a linear gradient from 0.03 M ammonium acetate pH 5.0, to 0.3 M ammonium acetate pH 7.5 in a total volume of 300 ml. Flow rate was 20 ml/hr and 3 ml-fractions were collected. Each three fraction was assayed by radioimmunoassay for the kinin. Immunoreactive kinins were eluted between 13 and 16 mmhos. Kinins fractions were combined and evaporated. The dried residues were dissolved in 500 µl of deionized and distilled water, and processed to apply to high performance liquid chromatography (HPLC).

Kinin content of each 0.5 ml fraction was quantitated by radioimmunoassay for the kinin. One hundred microliter of the sample was injected to the column (µBondapak C_{18} 3.9 x 300 mm, Waters). The column was isocratically eluted with 15% acetonitrile in 0.04 M triethylammonium formate pH 4.2 at a flow rate of 1.0 ml/min, monitored on an absorbance at 214 nm. Each 0.5 ml-fraction was collected and evaporated to dryness and immunoreactive kinins were measured.

We studied oxytocic activity of the sample using rat uterus.(8)

Amino acid sequences of the peptides were determined using about 2.0 nmol by a peptide sequencer (Model 477A Sequencer, Applied Biosystems) coupled with the one-line PTH Analyzer (Model 120A Applied Biosystems) as described by Hewick et al.(9)

RESULTS

When the purified kinin-containing sample was applied on HPLC column, two peaks emerged. Synthetic kinins, i.e. bradykinin (BK), lysyl-bradykinin (Lys-BK), and methionyl-lysyl-bradykinin (Met-Lys-BK), were also applied on HPLC column for the references as shown by arrows (Fig.1a).

The retention time of the second peak (Peak 2) corresponded to that of Lys-BK, but, the first peak (Peak 1) had a different retention time from those of the three available synthetic kinins i.e., BK, Lys-BK, and Met-Lys-BK (Fig.1a). Two peaks of immunoreactive kinins were also found (Fig.1b).

Furthermore, we investigated the immunochemical identity of Peak 1 with bradykinin. With the available antiserum to bradykinin dose-response curve of Peak 1 paralleled the bradykinin standard curve (Fig.2). Peak 1 is immunochemically similar to bradykinin and showed oxytocic activity.

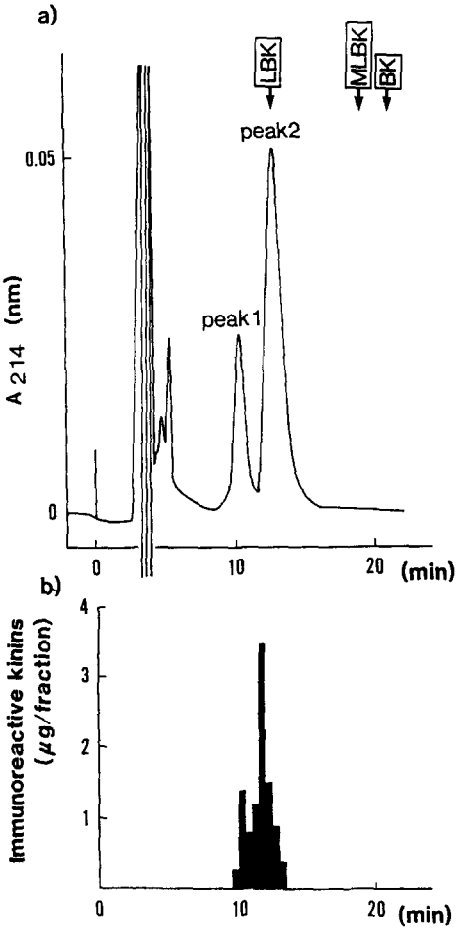

Fig.1 HPLC elution profile of the kinin fractions. Kinin content of each 0.5 ml fraction was quantitated by radioimmunoassay for the kinin. One hundred microliter of the sample was injected and the column (μBondapak C_{18} 3.9 x 300 mm, Waters) was isocratically eluted with 15% acetonitrile in 0.04 M triethylammonium formate pH 4.2 at a flow rate of 1.0 ml/min, monitored on an absorbance at 214 nm. Each 0.5 ml-fraction was collected and evaporated to dryness and immunoreactive kinins were measured (Fig.1b). Three synthetic kinins, i.e. bradykinin (BK), lysyl-bradykinin (LBK), methionyl-lysyl bradykinin (MLBK), were also applied on HPLC column for the references as shown by arrows (Fig.1a).

Amino acid sequences of the two peaks were determined as follows:

Peak 1; Lys-Arg-Pro-Hyp-Gly-Phe-Ser-Pro-Phe-Arg

Peak 2; Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

Each kinin was obtained in the following proportions: [Hydroxyproline^3]-lysyl-bradykinin ([Hyp^3]-Lys-BK) 25±4%, Lys-BK 75±4% (mean of 3 experiments ± S.D.).

In order to exclude a possibility of the hydroxylation of the proline residue during incubation a following experiment was done. When synthetic Lys-BK (500 μg) was incubated with Cohn's fraction IV-4 (5 g) or hog pancreatic kallikrein (5 mg) in buffer containing EDTA and 1-10 phenanthroline. Only

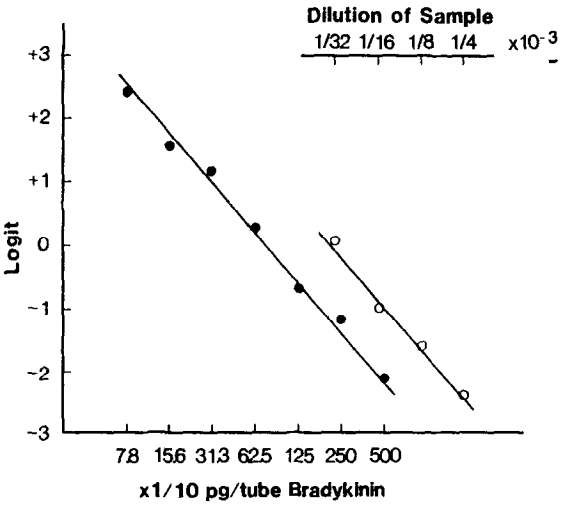

Fig.2 Standard curve of bradykinin and the displacement curve of diluted Peak 1 sample. Closed circle represents bradykinin, open circle Peak 1 sample. The lines were obtained by using the logit-log method. The curve of Peak 1 sample was parralel with the standard curve of bradykinin.

Lys-BK was recovered through the same isolation procedure conducted as described above (Table 1). Hydroxylation of proline by prolyl hydroxylase is reported to be inhibited by EDTA and 1-10 phenanthroline.(10) Theorefore the conversion of proline to hydroxyproline is unlikely to occur during incubation.

Authentic [Hyp^3]-Lys-BK was then newly synthesized and obtained from Peptide Institute (Osaka, Japan). Retention time of the synthetic [Hyp^3]-Lys-BK was completely identical to that of Peak 1. Even when synthetic [Hyp^3]-Lys-BK and Peak 1 were mixed and chromatographed by HPLC, only a single peak was observed (Fig.3).

DISCUSSION

In the last few years a couple of new kinins have been reported. Okamoto et al. found the presence of a kininogen in rat plasma which liberated T-kinin.(4,5) They subsequently demonstrated that T-kinin was Ile-Ser-

Table 1 Generation of lysyl-bradykinin (LBK) and [Hydroxyproline^3]-lysyl-bradykinin from the reaction mixture of synthetic LBK plus hog pancreatic kallikrein or Cohn's fraction IV-4

	Materials			Products
	Fr IV-4	Kallikrein	Synthetic LBK	
Exp 1	(+)	(+)	(-)	LBK+[Hyp^3]LBK
2	(+)	(-)	(+)	LBK only
3	(-)	(+)	(+)	LBK only

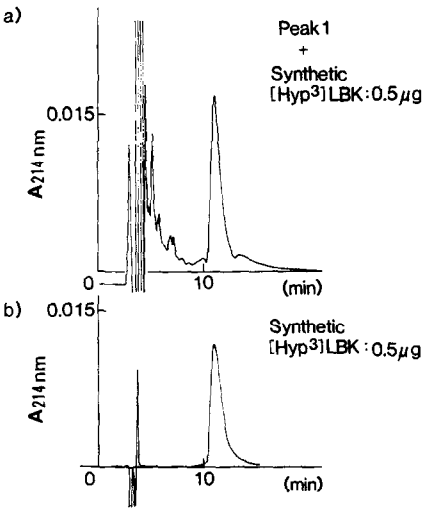

Fig.3 Identity of Peak 1 with synthetic [Hyp^3]-Lys-BK on HPLC. Five hundred nano gram of synthetic [Hyp^3]-Lys-BK and Peak 1 were mixed and injected to the column (μBondapak C_{18}, 3.9 x 300mm). The column was isocratically eluted with 15% acetonitrile in 0.04 M triethyl ammonium formate pH 4.2 at a flow rate of 1.0 ml/min monitored on an absorbance at 214 nm. When the mixture of synthetic [Hyp^3]-Lys-BK and Peak 1 was chromatographed by HPLC, only a single peak was observed (Fig.3.a)). The retention time of the mixture was identical to that of synthetic [Hyp^3]-Lys-BK (Fib.3.b)) and the area under curve of the peak showed summation of the amounts of applied samples of Peak 1 and [Hyp^3]-Lys-BK.

bradykinin. Recent study showed that rat plasma contains T-kininogen different from low and high molecular weight kininogens and T-kinin was liberated only when incubated with trypsin but not with tissue kallikrein.(11) Mindroiu T et al. reported the presence of [Ala^3]-Lys-BK, which was found in human urine or released from semipurified human low molecular weight kininogen when incubated with human uninary kallikrein.(6) More recently they have doubted the presence of [Ala^3]-Lys-BK (in personal communication).

The occurrence of [Hyp^3]-Lys-BK is of special interest since hydroxyproline has been found only in a few proteins, i.e., collagen, and other proteins containing collagen-like sequences such as C_{1q} subcomponent of the complement system(12), 18S asymmetrical form of acetylcholinesterase(13), and pulmonary surfactant apoptoreins.(14)

Hydroxyproline was also found at the third position of bradykinin analogue, or [Hydroxyproline^3]-bradykinin of the venom from Vespa mandarinia Smith, the Japanese hornet(15) and in the skin of the south-african frog, Heleophyryne purcelli.(16)

The isolation of [Hyp^3]-Lys-BK suggests that prolyl residue in the fourth position in Lys-BK sequence is selectively hydroxylated by prolyl hydroxylase. Prolyl hydroxylase is one of the enzymes involved in post-translational modifications of procollagen polypeptide in the process of collagen biosynthesis. This enzyme, located in the rough endoplasmic reticulum

membrane, catalyzes the synthesis of hydroxyproline by the hydroxylation of prolyl residues in unassembled chains of procollagen.(10) It also hydroxylates prolyl residues in synthetic collagen-like polypeptide of the general structure $(-X-Pro-Gly-)_n$, where X is proline or alanine and n is greater than 2. It was also shown in vitro that bradykinin which is structurally different from collagen, can serve as a substrate and the prolyl residue at the third position is susceptible to hydroxylation.(17) Lys-BK was also shown to be hydroxylated by prolyl hydroxylase.(18)

In conclusion, we found a new kinin, [Hyp^3]-Lys-BK besides Lys-BK from human plasma protein when incubated with hog pancreatic kallikrein. Our study suggests that kininogen containing [Hyp^3]-Lys-BK is present in human plasma protein. We have demonstrated the possibility that prolyl residue in kininogen was hydroxylated before the liberation of kinin at the intracellular site, like the process of collagen biosynthesis. It remains to be investigated how the selective hydroxylation of prolyl residue at the fourth position of Lys-BK by prolyl hydroxylase occured in the kininogen molecule.

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