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# IDENTIFICATION OF [HYDROXYPROLINE$^3$]-BRADYKININ RELEASED FROM HUMAN PLASMA AND PLASMA PROTEIN COHN'S FRACTION IV-4 BY TRYPSIN

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SUMMARY: Aside from bradykinin (BK), a novel kinin, [Hydroxyproline$^3$]-bradykinin ([Hyp$^3$]-BK), was isolated from the reaction mixture of human plasma and plasma protein Cohn's fraction IV-4 with trypsin. The liberated kinins were isolated based on procedures which we previously described for the isolation of [Hyp$^3$]-lysyl-bradykinin([Hyp$^3$]-Lys-BK) formed by kallikrein. The ratio of the amounts of two kinins thus formed from human plasma protein Cohn's fraction IV-4 were [Hyp$^3$]-BK 25±4% and BK 75±4%, similarly to that of [Hyp$^3$]-Lys-BK and Lys-BK, formed by kallikrein, but it varied by persons. The isolation of [Hyp$^3$]-BK and [Hyp$^3$]-Lys-BK suggests that a novel kininogen containing hydroxyproline in the third position of the bradykinin sequence in human plasma protein, possibly undergone post-translational modifications. 

Previously, we reported on two kinins which were generated by trypsin from human plasma protein(1,2). One of these was bradykinin while the other was a novel kinin or proline-lacking bradykinin(1). We thought the latter was [Des-Pro$^3$]-bradykinin(BK)(2), because it lacked one proline residue and had an identical retention time on HPLC to that of synthetic Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg or [Des-Pro$^3$]-BK. Recently however, we have identified [Hyp$^3$]-lysyl-bradykinin(Lys-BK) and not [Des-Pro$^3$]-lysyl-bradykinin as being released from human plasma protein by kallikrein(3). Moreover, we presented the possibility that a new kininogen containing [Hyp$^3$]-Lys-BK exists in human plasma protein. The recent isolation of [Hyp3]-Lys-BK from human urine(4) and from an incubation mixture of human urinary kallikrein and human plasma or kininogens(5,6), gives credence tc

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our data. Based on these recent findings, therefore, we reexamined the kinins liberated from human plasma and plasma protein Cohn's fraction IV-4 by TPCK-treated trypsin.

## MATERIALS AND METHODS

### Reagents and chemicals

Human plasma protein Cohn's fraction IV-4 was obtained from Midori-Juji (Osaka, Japan). Crystalline bovine trypsin (3,000 NF units) was purchased from Miles Laboratory (Slough, England), and 1-Chloro-4-Phenyl-3-L-tosylamido-butan-2-one (TPCK) from ICN Pharmaceuticals, Inc (Cleveland, USA). Trypsin samples were treated with TPCK to inactivate chymotrypsin by the method of Kostka and Carpenter(7). Synthetic kinins, i.e., bradykinin, lysyl-bradykinin, [Des-Pro$^3$]-bradykinin, [Hyp$^3$]-bradykinin, and [Hyp$^3$]-lysyl-bradykinin were obtained from Peptide Institute (Osaka, Japan). The latter two kinins were newly synthetized. All compounds were of analytical grade.

### Isolation of kinins released from human plasma protein Cohn's fraction IV-4 with trypsin

Human plasma protein, Cohn's fraction IV-4 (5 g) was incubated with 5 mg TPCK-treated trypsin at 37°C for 3 in 50 ml 0.1 M Tris/HCl (pH 8.0) containing 0.1 M NaCl, 30 mM EDTA, and 3 mM 1-10 phenanthroline. In brief, the isolation procedures performed, which we previously described(3) are as follows: After incubation, the mixture was deproteinized by addition of 500 ml hot ethanol and then centrifuged. Thereafter, the supernatant was dried by evaporation under reduced pressure at 45°C. The desiccated residue was dissolved in 0.01 M ammonium acetate (pH 5.0), and applied on Sephadex G-15 (1.5 x 94 cm) equilibrated with the same buffer. Each fraction of the eluates was assayed by radioimmunoassay(RIA) for the kinin(8). The fractions containing the immunoreactive kinins were combined and conductivity was adjusted to 3 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 running 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 at 20 ml/h and 3 ml-fractions were collected. Every three fractions were assayed for the kinin by RIA. We studied oxytocin activity of the sample on rat uterus(9), and vasodepressor effects by i.v. injection of the sample to a rat, as previously described(1,10). The amino acid composition and sequences of the peptides were determined using about 2.0 nmol by a high-speed amino acid analyzer (Hitachi model 835) and a peptide sequencer (Model 477A Sequencer, Applied Biosystems) coupled with a one-line PTH Analyzer (Model 120A Applied Biosystems) per description of Hewick et al.(11). A μBondapak $C_{18}$ (3.9 x 300 mm, Waters) HPLC column was isocratically eluted with 20% acetonitrile in 0.04 M triethylammonium formate (pH 4.2) at a flow rate of 1.0 ml/min, and monitored at an absorbance of 214 nm.

### Isolation of kinins from human plasma with trypsin

Venous blood (100 ml) was obtained from five normal volunteers of both sexes and collected into a plastic syringe containing 25 ml of Tris/HCl (pH 8.0) containing 150 mM EDTA and 15 mM 1-10 phenanthroline. Plasma was separated by

centrifugation (1000 g, 15 min). After acidified to pH 3.0 with 1N HCl, plasma was alkalized to pH 8.0 with 10N NaOH. Fifty ml of plasma was incubated with 2 mg of TPCK-treated trypsin in Tris/HCl (pH 8.0) at 37°C for 2 hrs. The kinins released from the incubation mixture were isolated as described above. The ratio of two kinins was determined by peak area of each kinin on HPLC.

## RESULTS

When kinin fractions released from human plasma protein were chromatographed on a CM-cellulose column, immunoreactive kinins were eluted between 3 and 6 mmhos. Kinins fractions were pooled and evaporated. The desiccated residues were dissolved in 500 µl deionized and distilled water, and processed for high performance liquid chromatography (HPLC).

Kinin content of each 0.5 ml fraction was quantitated for the kinin by RIA. One hundred microliters of the sample was injected to the HPLC column. Each 0.5 ml-fraction was collected and evaporated to dryness and immunoreactive kinins were measured.

When the purified kinin containing sample was applied to an HPLC column, two peaks emerged(Fig.1 a). The retention time of Peak 1 corresponded to that of [$Hyp^3$]-BK and [Des-$Pro^3$]-BK. On HPLC, authentic [$Hyp^3$]-BK had the same retention time as [Des-$Pro^3$]-BK(Fig.1 c, d). Two peaks of immunoreactive kinins were also separated(Fig.1 b).

Two peaks were collected and evaporated. Bioassay on rat uterus showed both samples of Peaks 1 and 2 to contain oxytoxic actions. Likewise, i.v. injection of these samples to rats revealed its depressure effects.

Amino acid compositions of Peaks 1 and 2 were determined as shown in Table 1. One molecule of hydroxyproline was incorporated in Peak 1 in lieu of proline.

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

Peak 1: Arg-Pro-Hyp-Gly-Phe-Ser-Pro-Phe-Arg

Peak 2: Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

Each kinin was obtained in the following proportions: [Hydroxyproline$^3$]-bradykinin ([$Hyp^3$]-BK): 25±4%, BK: 75±4% (mean of n=3).

When human plasma (n=5) was incubated with trypsin, the ratio of the two kinins showed individual variations (Table 2).

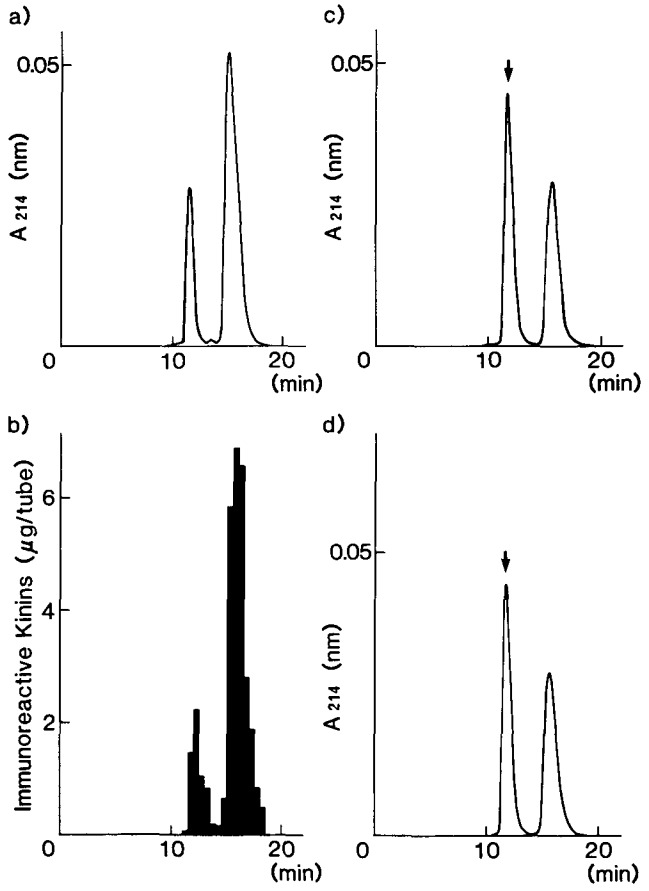


Fig.1 Active kinins fractions were subjected to HPLC. Two peaks emerged at an absorbance of 214 nm (a). Kinin content of each 0.5 ml fraction was quantitated for the kinin by radioimmunoassay. Two peaks of immunoreactive kinins were also revealed (b). One microgram of authentic [Hyp$^3$]-BK and 2 μg of BK were injected to HPLC. Arrow shows [Hyp$^3$]-BK (c). The same doses of authentic [Des-Pro$^3$]-BK and BK were also subjected to HPCL. Arrow represents [Des-Pro$^3$]-BK (d). On HPLC, authentic [Hyp$^3$]-BK had the same retention time as [Des-Pro$^3$]-BK (c,d).

## DISCUSSION

Previously, we identified one proline residue-lacking BK from human plasma protein together with BK when incubated with trypsin(1). Since its retention time in HPLC was identical to that of synthetic [Des-Pro3]-BK, we ostensibly identified it to be [Des-Pro3]-BK(2). Later, we concentrated on purifying a new kininogen containing [Des-Pro$^3$]-BK, although this effort proved to be futile. Instead, we were able to identify [Hyp$^3$]-Lys-BK from human plasma protein when treated with kallikrein(3). We also demonstrated the possibility that a new kininogen containing [Hyp$^3$]-Lys-BK exists in human plasma protein, which has undergone

Table.1 Amino acid compositions analysis of peak 1 and peak 2

| Amino acid | Peak 1 | Peak 2 |
|---|---|---|
| Serine | 2.7 nmol(1) | 2.8(1) |
| Glycine | 2.8 (1) | 3.1(1) |
| Phenylalanine | 5.7 (2) | 4.9(2) |
| Arginine | 5.8 (2) | 4.6(2) |
| Proline | 5.7 (2) | 7.0(3) |
| Hydroxyproline | 2.7 (1) | (-) |

post-translational modification(3). In the present work, we demonstrated that two kinins were formed by trypsin from human plasma protein, of which one was BK, and the other a novel kinin, [Hyp$^3$]-BK. In brief, we had inadvertently regarded [Hyp$^3$]-BK as [Des-Pro$^3$]-BK on amino acid analysis. The reasons for the previous incorrect results are as follows: (a) [Hyp$^3$]-BK had an identical retention time to that of synthetic [Des-Pro$^3$]-BK on HPLC; (b), we had missed the peak of hydroxyproline since amounts of the sample were minuscule for amino acid composition analysis; (c), hydroxyproline was not included as the standard amino acid on amino acid composition analysis. From this vantage point, amino acid sequence analysis is deemed to be superior to amino acid composition analysis when analysing an uncommon amino acid such as hydroxyproline. When we determined the amino acid sequences of [Hyp$^3$]-Lys-BK, we found an unfamiliar peak, i.e. two peaks of hydroxyproline-phenylthiohydantoin derivatives. Recently, Mindroiu et al. had made a similar error. They had reported the presence of [Ala$^3$]-Lys-BK in human urine or as being released from semipurified human low molecular weight kininogen when incubated with human urinary kallikrein(12). More recently,

Table 2. Percent distribution of [Hyp$^3$]-BK in normal volunteers plasma

| Donors | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| [Hyp$^3$]-BK | 29.0 | 12.5 | 20.0 | 48.2 | 29.7 |
| BK | 71.0 | 87.5 | 80.0 | 51.8 | 70.3 |

The content of two kinins were determined by peak area of each kinin on HPLC.

they themselves amended the amino acid sequence to [$Hyp^3$]-Lys-BK(6). Corrections have also been done by Kato et al. who have clarified the presence of [$Hyp^3$]-Lys-BK but not [$Ala^3$]-Lys-BK(4). Moreover, Maier et al. also simultaneously demonstrated that [$Hyp^3$]-Lys-BK was released from human low and high molecular weight kininogens by human urinary kallikrein(5).

Conversely, a couple of novel kinins have been reported in recent years. Okamoto et al. found the presence of a kininogen in rat plasma which liberated T-kinin(13). Subsequently, they demonstrated the T-kinin to be Ile-Ser-bradykinin(14). A recent study showed that rat plasma contains T-kininogen different from low and high molecular weight kininogen and that T-kinin was liberated only when incubated with trypsin but not with tissue kallikrein(15). In this aspect, a novel kininogen containing hydroxyproline in the third position of bradykinin sequence is different. In other words, it has susceptibility to both tissue kallikrein such as pancreatic kallikrein, human urinary kallikrein(3,5) and trypsin.

The isolation of [$Hyp^3$]-BK suggests that a prolyl residue in the third position in BK is selectively hydroxylated by prolyl hydroxylase, as is the case with [$Hyp^3$]-Lys-BK(3). Prolyl hydroxylase is one of the enzymes involved in post-translational modifications of procollagen polypeptide in the process of collagen biosynthesis. It was shown in vitro that bradykinin which is structurally different from collagen, can serve as a substrate whilst the prolyl residue at the third position is susceptible to hydroxylation(16).

The ratio of kininogen containing hydroxyproline in the third position of BK (Hyp-kininogen) and the hitherto known kininogen is 25 ± 4% to 75 ± 4% in human plasma protein Cohn's fraction IV-4, which is semipurified from the pooled plasma as previously described(10,17). Kininogen is reportedly rich in Cohn's fraction IV(18) and IV-4(19), although the content of Hyp-kininogen is controversial. Mindroui et al. reported 23 ± 3% Hyp-kininogen in pooled plasma and 40-42% in both low and high molecular weight kininogen(6). Similarly, Maier et al. indicated individual variations in both low and high molecular weight kininogens(5). We also demonstrated the differences of the content of Hyp-kininogen among donors. It remains unknown how the kininogens undergoes post-translational modifications.

To recapitulate, we isolated BK aside from a novel kinin, [$Hyp^3$]-BK from human plasma protein when incubated with trypsin.

The newly confirmed sequence coincides with our previously identified [Hyp$^3$]-Lys-BK from human plasma protein treated with kallikrein. As concerns the structure of BK, confusions have been generated by a couple of papers(20,21,22). Firstly, in 1960, Elliot et al. reported that the sequence Arg-Pro-Pro-Gly-Phe-Ser-Phe-Arg was proposed for the structure(20,21) of BK. In the light of synthetic work of peptides related to BK carried out by Boissonas et al., the above structure of BK was demonstrated to be incorrect. In a later communication, the structure was amended by Elliot as follows: Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg(22). Recently [Des-Pro$^3$] was reported as a novel kinin in our previous communication(1,2), [Ala$^3$]-Lys-BK by others(12). We could put an end to these confusions concerning the structure of kinin since we clarified the presence of [Hyp$^3$]-BK.

Our studies suggest that a new kininogen containing hydroxyproline in the third position of the BK sequence exists in human plasma. We have demonstrated the possibility that the prolyl residue of the third position of the BK sequence in kininogen is hydroxylated prior to liberation of the kinin at the intracelluar site, much similar to the process of collagen biosynthesis. Future studies will focus on the mechanism of selective hydroxylation of prolyl residue at the third position of BK by prolyl hydroxylase occurring in the kininogen molecule and the functional roles of novel kinins in biological systems.

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