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# Hydroxyprolyl$^3$-bradykinin in high molecular mass kininogen

## Presence in human and monkey kininogens, but not in kininogens from bovine, rat, rabbit, guinea pig and mouse plasmas

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The contents of hydroxyprolyl$^3$-bradykinin in high molecular mass (HMM) kininogens from human and animal plasmas were examined by reversed-phase HPLC following their proteolytic scission by bovine plasma kallikrein. The relative contents of hydroxyprolyl$^3$-bradykinin in kinins from HMM kininogens from pooled plasmas of human and monkey origin were 33 and 73%, respectively. On the other hand, hydroxyprolyl$^3$-bradykinin could not be detected in HMM kininogen preparations from bovine, rat, guinea pig, rabbit and mouse plasmas. Hydroxyproline in hydroxyprolyl$^3$-bradykinin was assigned as *trans*-4-hydroxyproline by comparison of the retention times on reversed-phase HPLC with isomers of hydroxyproline after derivatization with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole chloride.

Kinin; Hydroxyproline; Kininogen; (Human, Monkey)

## 1. INTRODUCTION

In previous reports [1,2], we isolated Hyp analogues of BK, Hyp$^3$-BK and Lys-Hyp$^3$-BK, from ascites of cancer patients and normal human urine. In addition, Sasaguri et al. [3] recently isolated Lys-Hyp$^3$-BK after the digestion of human plasma fraction with hog pancreatic kallikrein. The presence of the Hyp$^3$-BK moiety in human HMM kininogens was confirmed very recently by two independent groups of investigators [4,5]. Their results suggest that individual plasmas contain HMM kininogen with different contents of Hyp$^3$-BK. It has been shown by McGee et al. [6] that the third proline residue in synthetic BK was specifically hydroxylated by proline hydroxylase. Therefore, Hyp$^3$-BK may be derived from BK in vivo by proline hydroxylase during or after biosynthesis of kininogen in liver, or other tissues like Hyp in collagen [7] and/or after liberation from kininogen by kallikreins. However, little is known of the in vivo mechanism of hydroxylation of BK in kininogens. To study the mechanism in vivo and in vitro, it will be helpful to use experimental animals in order to determine whether kininogens from animal plasmas contain Hyp$^3$-BK.

Here, we examined the Hyp$^3$-BK content in HMM kininogens from animal plasmas in comparison with human HMM kininogen. We also studied the stereospecificity of Hyp in Hyp$^3$-BK obtained from human HMM kininogen.

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*Abbreviations:* Hyp, hydroxyproline; HPLC, high-performance liquid chromatography; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole chloride; HMM, high molecular mass; BK, bradykinin

## 2. MATERIALS AND METHODS

Synthetic BK, Hyp$^3$-BK and *trans*-4-Hyp was purchased from the Peptide Institute (Minoh, Osaka). *cis*-4-Hyp was a product of Sigma (St. Louis, MO). *trans*-3-Hyp and *cis*-3-Hyp were kindly provided by Professor B. Witkop (NIH, Bethesda, MD)

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through the courtesy of Dr F. Sakiyama (Institute for Protein Research, Osaka University, Osaka). Chelating Sepharose and Q-Sepharose (fast flow) were purchased from Pharmacia (Japan). Bovine plasma kallikrein with specific activity toward Z-Phe-Arg-methylcoumarin amide of 5.0 μmol aminomethyl coumarin per absorbance unit at 280 nm [8], bovine HMM kininogen [9] and rat HMM kininogen [10] were isolated as reported previously.

### 2.1. *Purification of HMM kininogens from human, monkey, guinea pig, rabbit and mouse plasmas*

Citrated blood was collected from a rabbit and three guinea pigs, five normal volunteers, three monkeys and 45 mice. 15 or 20 ml plasma was applied to a column (0.9 × 20 cm) of Q-Sepharose (fast flow) equilibrated with 0.02 M Tris-HCl, pH 8.0, containing 0.05 M NaCl. Gradient elution was performed using an FPLC system (Pharmacia) with an NaCl gradient of 0.05–0.5 M NaCl during a period of 120 min at a flow rate of 1 ml/min. Kininogen activity was measured by cysteine protease inhibitory activity using papain as in [11]. HMM kininogen was eluted with 0.35–0.45 M NaCl and applied to a column (0.9 × 20 cm) of zinc-chelating Sepharose, equilibrated with 0.02 M Tris-HCl, pH 8.0, containing 0.05 M NaCl. HMM kininogen was eluted with 0.02 M Tris-HCl, pH 8.0, containing 1 M NaCl and 50 mM EDTA. HMM kininogen preparations from each plasma showed a major band on SDS-PAGE in the presence of 2-mercaptoethanol with molecular masses of 104.8, 106.1, 104.3, 108.6 and 97.6 kDa, for human, rabbit, mouse, monkey and guinea pig kininogens, respectively. Their properties as HMM kininogen were also confirmed by correcting the prolonged clotting time of Fitzgerald trait plasma, and by kinin release following plasma kallikrein treatment.

### 2.2. *Kinin assay*

50–200 μl HMM kininogen solution in 0.02 M Tris-HCl, pH 8.0, containing 0.15 M NaCl ($A_{280}$ = 0.2–1.0) was mixed with 10 μl bovine plasma kallikrein ($A_{280}$ = 0.45) and the mixture was incubated for 20 min at 37°C. Quantitative analysis of kinin was performed by reversed-phase HPLC using a column (4.6 mm × 25 cm) of TSK gel ODS-120A (Toso) with isocratic elution using 17% acetonitrile as reported [1]. The area of peptides corresponding to Hyp$^3$-BK and BK was calculated on a Chromatocorder 11 (System Instruments, Tokyo) and converted to the amounts of kinin using a standard curve that was constructed using synthetic kinins.

### 2.3. *Separation of isomers of Hyp*

Isomers of Hyp were separated after derivatization with NBD-Cl according to Lindblad and Diegelmann [12]. NBD-Hyp was subjected to reversed-phase HPLC using a column (4.6 mm × 25 cm) of TSK gel ODS-120A at room temperature and eluted by increasing the concentration of methanol in 1% acetic acid, pH 2.73, at a flow rate of 1 ml/min and with detection at 495 nm.

### 2.4. *Amino acid sequence analysis*

The amino acid sequence was analysed using a model 470A gas-phase protein sequencer equipped with a 120A PTH analyzer on an on-line system (Applied Biosystems, Foster City, CA). PTH-Hyp was assigned and quantitated by its characteristic two-peak profile as in [1].

## 3. RESULTS

The amounts of kinins liberated from HMM kininogens from human, monkey, bovine, rat, mouse, rabbit and guinea pig plasmas were measured by reversed-phase HPLC after incubation with bovine plasma kallikrein as described in section 2. As shown in fig.1B,E, two peptide peaks were detected on human and monkey HMM kininogens from pooled plasmas, which correspond to Hyp$^3$-BK and BK. The relative content of Hyp$^3$-BK in total kinin was calculated to be 33 and 73% in human and monkey HMM kininogens, respectively. The Hyp$^3$-BK content in human HMM kininogens from five individuals ranged from 14 to 64%. On the other hand, a peptide peak that corresponds to Hyp$^3$-BK was not detected from bovine, rat, guinea pig and rabbit HMM kininogen preparations (fig.1C,D,F,G). Two peptide peaks were detected in mouse HMM kininogen, one of which corresponded to BK, and another that did not correspond to Hyp$^3$-BK (fig.1H). When the digest of mouse HMM kininogen with bovine plasma kallikrein was subjected to preparative HPLC using a gradient of acetonitrile, neither peptide was separated. Amino acid sequence analysis of the mixture indicated that a major component in the mixture is BK, which includes a minor sequence of Asp-X-Gly-Leu-X-X-Gly-Gln-Gln-Lys-. From these results, we conclude that mouse HMM kininogen does not contain a Hyp$^3$-BK moiety. The amino acid sequences of Hyp$^3$-BK from human and monkey HMM kininogens were confirmed from their amino acid sequence analyses (fig.2) and amino acid composition (not shown).

These results indicate that HMM kininogens from human and monkey plasmas contain Hyp$^3$-BK, but HMM kininogens from bovine, rat, rabbit, guinea pig and mouse plasmas do not.

Although *trans*-4-Hyp is most abundant in collagen, four isomers of Hyp are known to be present in nature [13] depending on the stereospecificity of the hydroxyl radical. The stereospecificity of Hyp from human HMM kininogen was examined by comparison with the synthetic isomers, *trans*-4-Hyp, *cis*-4-Hyp, *trans*-3-Hyp and *cis*-3-Hyp. A 6 N HCl hydrolysate of Hyp$^3$-BK and the synthetic isomers were incubated with NBD-Cl. NBD-Hyp derivatives were separated by reversed-

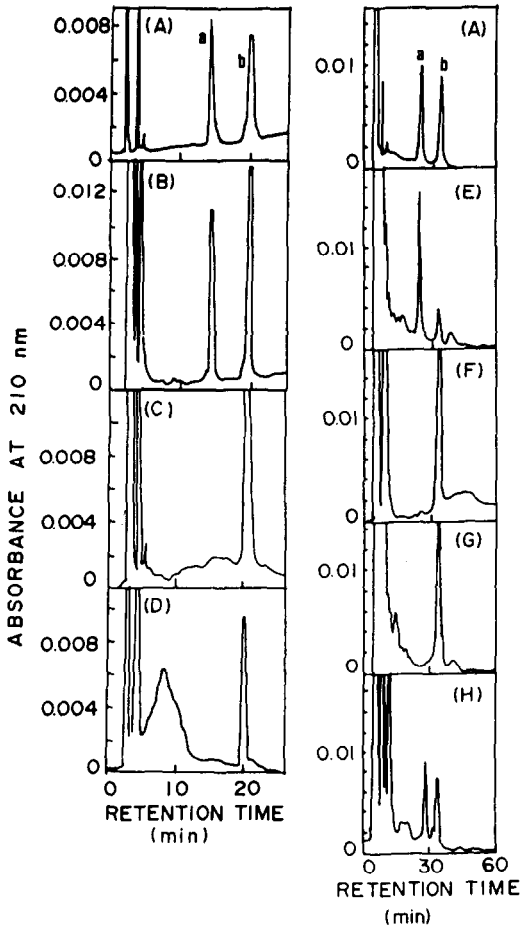


Fig.1. Quantitative analyses of Hyp$^3$-BK and BK in HMM kininogen by reversed-phase HPLC. HMM kininogens from human pooled plasma (B), rat plasma (C), bovine plasma (D), monkey plasma (E), guinea pig plasma (F), rabbit plasma (G) and mouse plasma (H) were incubated with bovine plasma kallikrein and the kinins liberated were separated on a column of TSK gel ODS-120A with isocratic elution with 17% acetonitrile. The amounts of kinin were calculated as described in section 2. (A) Hyp$^3$-BK (a) and BK (b).

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| human | Arg | Pro | Hyp | Gly | Phe | Ser | Pro | Phe | Arg |
| | → | → | → | → | → | → | → | → | → |
| | 48 | 139 | 43 | 126 | 101 | 29 | 64 | 59 | 11 |
| monkey | Arg | Pro | Hyp | Gly | Phe | Ser | Pro | Phe | Arg |
| | → | → | → | → | → | → | → | → | → |
| | 137 | 542 | 185 | 383 | 475 | 107 | 310 | 281 | 24 |

Fig.2. Amino acid sequence of Hyp$^3$-BKs from human and monkey HMM kininogens. Each value under the amino acid residue represents pmol PTH-amino acids.

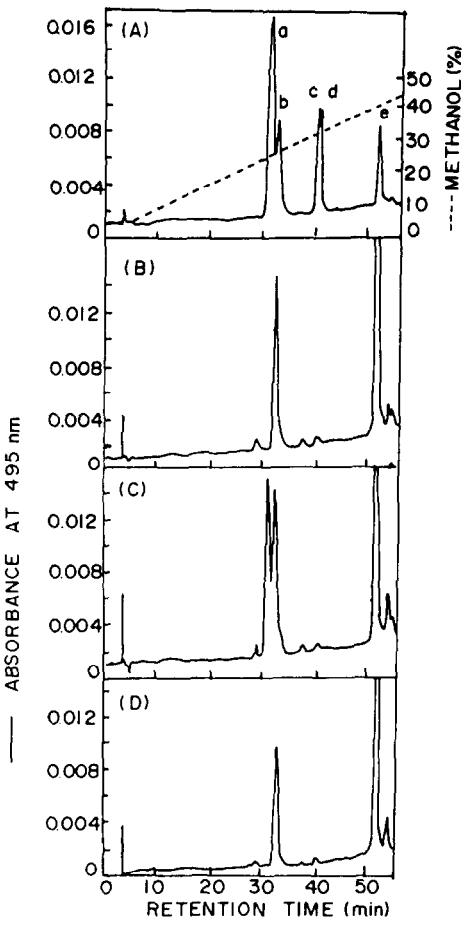


Fig.3. Separation of isomers of Hyp by reversed-phase HPLC. Peptides were hydrolyzed with 6 N HCl for 20 h at 110°C. The hydrolysates and synthetic isomers of Hyp and proline were derivatized with NBD-Cl and subjected to reversed-phase HPLC as described in section 2. (A) Standard Hyps and proline: (a) *trans*-3-Hyp, (b) *trans*-4-Hyp, (c) *cis*-3-Hyp, (d) *cis*-4-Hyp, (e) proline. (B) Hydrolysate of Hyp$^3$-BK from human HMM kininogen (0.6 nmol). (C) B and *trans*-3-Hyp. (D) Hydrolysate of synthetic Hyp$^3$-BK (0.13 nmol).

phase HPLC as described in section 2. As shown in fig.3A, *trans* and *cis* isomers of Hyp and proline were clearly separated under the conditions used. Although the retention times of *trans*-3-Hyp and *trans*-4-Hyp were very close, they were distinguishable. On the other hand, *cis*-3-Hyp and *cis*-4-Hyp were virtually indistinguishable. The retention time of Hyp from Hyp$^3$-BK in human HMM kininogen and from synthetic Hyp$^3$-BK was identical with that of *trans*-4-Hyp (fig.3B,D). The result was con-

firmed by the addition of *trans*-3-Hyp to the hydrolysate of Hyp$^3$-BK from human HMM kininogen (fig.3C).

## 4. DISCUSSION

The present paper demonstrates that Hyp in Hyp$^3$-BK is *trans*-4-Hyp, and therefore that it was derived from the action of proline-4-hydroxylase on kininogen. This article also demonstrates that HMM kininogen from pooled monkey plasmas contained higher amounts of Hyp$^3$-BK than human HMM kininogen. Maier et al. [5] isolated Lys-Hyp$^3$-BK from human individual HMM kininogens after incubation with human urinary kallikrein and estimated the content to be 30–59% from the biological activity of the kinin. Our results confirm that human HMM kininogen from individual plasma contains variable amounts of Hyp$^3$-BK. It is well known that proline-4-hydroxylase is essential for the hydroxylation of proline in procollagen. In particular, the enzymes from human and rat have been well characterized [7]. Hence, it is reasonable to speculate that the BK moiety in HMM kininogen is hydroxylated via the same mechanism as that for collagen.

On the other hand, it is interesting that HMM kininogens from bovine, rat, guinea pig, rabbit and mouse plasmas do not contain Hyp$^3$-BK. We isolated HMM kininogens from pooled plasmas of ox, rat, guinea pig and mice, except rabbit. It may be premature at this point to conclude that only primate HMM kininogens contain Hyp$^3$-BK, as further investigation should be made on other animals. However, our data pose the following questions concerning the mechanism of hydroxylation of the BK moiety in HMM kininogen: (i) is HMM kininogen hydroxylated by proline-4-hydroxylase located on the cisternae of rough endoplastic reticulum in the liver similarly to the hydroxylation of procollagen, or by an enzyme in a different organ? (ii) if we assume the same mechanism in human, monkey and other animals, why then do HMM kininogens from non-primates not contain Hyp$^3$-BK? and (iii) do our data really indicate that experimental animals except monkey cannot be used for studies concerning the mechanism of proline hydroxylation in kininogen? In order to resolve these questions, it may be essential to determine whether HMM kininogen is hydroxylated in vitro by proline hydroxylase, and whether HMM kininogen with Hyp$^3$-BK in animal plasma increases in experimentally induced liver fibrosis and other diseases in which collagen proline hydroxylase activity increases [14,15].

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