Characterization of Peptides Isolated from *Trimeresurus flavoviridis* and *Trimeresurus okinavensis* Venoms

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Synopsis. Three peptides were isolated from both *Trimeresurus flavoviridis* (Habu snake) and *T. okinavensis* (Himehabu snake) venoms. Their structures were determined to be pGlu–Lys–Trp, pGlu–Asn–Trp, and pGlu–Gln–Trp. Only pGlu–Lys–Trp showed weak inhibitory activities against angiotensin converting enzyme and H_2-protease.

Snake venoms contain various kinds of toxins, enzymes, and bioactive peptides. A peptide family named bradykinin potentiating peptide have been found in the venoms from *Agkistrodon halys blomhoffii*,[1] *Bothrops jararaca*,[2,3] and *B. insularis*.[4] They consist of about ten amino acid residues and inhibit angiotensin converting enzyme (ACE)[5] which catalyzes conversion of angiotensin I to angiotensin II.[6] Three tripeptides, pGlu–Asn–Trp, pGlu–Gln–Trp, and pGlu–Lys–Trp, were also isolated from several snake venoms.[4,7] Their physiological roles have not been known though pGlu–Lys–Trp showed some bradykinin potentiating activity.[4]

During fractionation of the crude venoms from *Trimeresurus flavoviridis* (Habu snake) and *T. okinavensis* (Himehabu snake), several oligopeptides were found on gel chromatography. In this paper, the isolation, sequences, and biological activities of three peptides are described.

Results and Discussion

Isolation of Peptides. Low molecular weight fraction (G100-1a) was obtained from *T. flavoviridis* venom as described previously.[8] Gel filtration of G100-1a on a Sephadex G-25 column using 3% AcOH gave two main peaks (G25-1a and G25-2a) as shown in Fig. 1A. Fractions G25-1a and G25-2a were further chromatographed on a Sephadex G-15 column using 3% AcOH (Fig. 2). Fractions G15-1a and G15-2a were obtained from G25-1a and G25-2a, respectively. Fraction G15-1a gave a major single peak on HPLC (Fig. 3A), while G15-2a showed two main peaks (Fig. 3B). Fractions H-1a, H-2a, and H-3a (Fig. 3) were isolated. The final products was confirmed to be pure by HPLC under several conditions.

Another low molecular weight fraction (G100-1b) was obtained from crude *T. okinavensis* venom as a third fraction after Sephadex G-100 column chromatography.[9] Sephadex G-25 column chromatography of G100-1b gave an elution pattern (Fig. 1B) different from that of G100-1a. Fraction G100-1b was separated into three fractions. Peak G25-0b contains several peptides longer than tripeptide, but no separation was made in the present study. The elution volumes of G25-1b and G25-2b were close to those of G25-1a and G25-2a, respectively. Sephadex G-15 column chromatography followed by HPLC gave three peptides, H-1b from G25-1b and H-2b and H-3b from G25-2b. From the retention times on HPLC, H-1b, H-2b, and H-3b were suggested to be identical to H-1a, H-2a, and H-3a, respectively. Table 1 shows the amino acid compositions of the peptides.

Amino Acid Sequences of Peptides. CPase Y

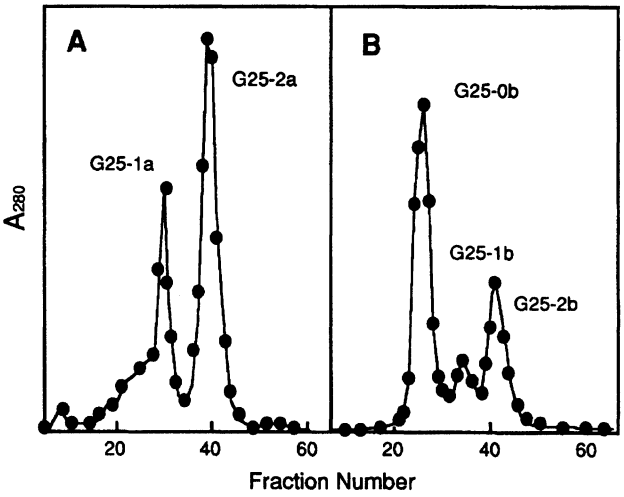

Fig. 1. Chromatograpies of G100-1a (A) and G100-1b (B) on a Sephadex G-25 column (1.7×60 cm). Solvent, 3% AcOH; 3 ml fractions.

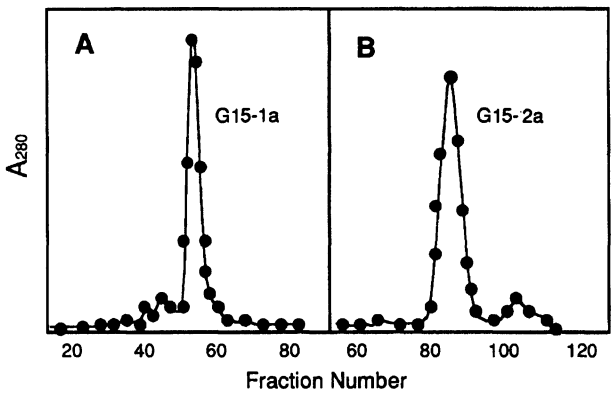

Fig. 2. Chromatographies of G25-1a (A) and G25-1b (B) on a Sephadex G-15 column (1.7×60 cm). Solvent, 3% AcOH; 3 ml fractions.

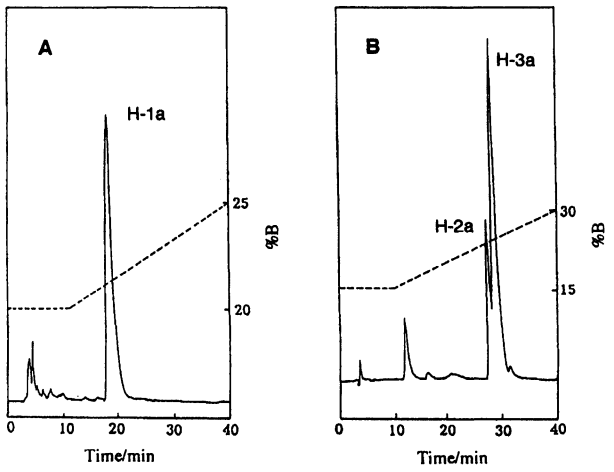

Fig. 3. HPLC of G15-1a (A) and G15-2a (B). Column, TSK-gel ODS-120T (0.46×25 cm); solvent, 0.1 % TFA (A)–CH_3CN containing 20% A (B).

Table 1. Amino Acid Compositions of Hydrolyzates of Isolated and Synthetic Peptides[a)]

Peptide	Asp	Glu	Lys	Trp
H-1a[b)]		1.00	1.04	0.96
H-1b[b)]		1.00	0.97	0.95
pGlu–Lys–Trp[c)]		1.00	1.02	0.94
H-2a[b)]	0.86	1.00		0.87
H-2b[b)]	0.84	1.00		0.93
pGlu–Asn–Trp[c)]	0.90	1.00		0.93
H-3a[b)]		2.00		0.98
H-3b[b)]		2.00		0.98
pGlu–Gln–Trp[c)]		2.00		0.94

a) Samples were hydrolyzed with 3 M mercaptoethanesulfonic acid at 110°C for 24 h. b) Isolated peptides. c) Synthetic peptides.

digestion revealed the C-terminal sequences to be Lys–Trp for H-1a and H-1b, Asx–Trp for H-2a and H-2b, and Glx–Trp for H-3a and H-3b. Manual Edman degradation of the peptides gave no PTH-amino acid, while HPLC of the CPase Y digests indicated the involvement of pGlu. Therefore, the peptides were partially hydrolyzed with 0.1 M NaOH (1 M=1 moldm^{-3}) and subjected to Edman degradation. Peptides H-1a and H-1b gave Glu–Lys–Trp, H-2a and H-2b Glu–Asn–Trp, and H-3a and H-3b Glu–Gln–Trp. Thus H-1a and H-1b were determined to be pGlu–Lys–Trp, H-2a and H-2b pGlu–Asn–Trp, and H-3a and H-3b pGlu–Gln–Trp. Correctness of the structures for the peptides was confirmed by comparing the elution volumes with those of the corresponding synthetic peptides on HPLC. Furthermore, FAB-MS measurement showed that M+1 values of H-1b, H-2b, and H-3b were 444, 430, and 444, respectively, which were in accord with their calculated values.

pGlu–Asn–Trp and pGlu–Gln–Trp have been isolated from the venoms of *Agkistrodon halys blomhoffi*[1)] and *Bothrops jararaca*.[2,3)] *Vipera russelli* venom contains pGlu–Gln–Trp but not pGlu–Asn–Trp.[7)] Recently, the presence of pGlu–Lys–Trp and pGlu–Gln–Trp in *Bothrops insularis* venom was reported.[4)] Kato et al. found pGlu–Asn–Trp and pGlu–Gln–Trp in *T. flavoviridis* venom,[7)] while in the present study, three tripeptides were isolated from the same venom and from *T. okinavensis* venom. The discrepancy in *T. fravoviridis* venom may be due to the difference in the isolation procedures.

Biological Activity. Inhibitory activities of peptides for ACE and H_2-protease, a protease isolated from *T. flavoviridis* venom,[10,11)] was examined. H_2-protease was used in order to examine whether the peptides can inhibit proteases involved in the same venom.

The activity of ACE was assayed by spectrophotomeric determination of the rate of release of hippuric acid from Hip–His–Leu. pGlu–Asn–Trp and pGlu–Gln–Trp (1 mM) showed no effect for enzyme activity. However, when ACE was incubated with 1 mM pGlu–Lys–Trp, its activity decreased to 50—60% that with no additive, indicating that pGlu–Lys–Trp is a weak inhibitor for ACE.

Effects of the peptides on H_2-protease was investigated using casein as a substrate by the method of Takahashi and Ohsaka.[10)] H_2-protease was not sensitive against pGlu–Asn–Trp and pGlu–Gln–Trp, while its activity dropped to 60% in the presence of 1 mM pGlu–Lys–Trp. However, it is unclear whether these peptides play any physiological role *in vivo*, because their inhibitory activities were weak or not found.

Cintra et al. reported that bradykinin potentiating activity of pGlu–Lys–Trp was 5-fold less than that of pGlu–Lys–Trp–Ala–Pro, while pGlu–Asn–Trp and pGlu–Gln–Trp showed no activity,[4)] being in accord with the present data and suggesting that the presence of basic amino acid residue(s) is necessary for the activity. However, pGlu–Ser–Trp–Pro–Gly–Pro–Asn–Ile–Pro–Pro also showed bradykinin potentiating activity comparable to that of pGlu–Lys–Trp–Ala–Pro.[4)] These results suggest that a certain partial structure is crucial for inhibition of ACE rather than the presence of a basic residue.

Anomalous Behavior of pGlu–Lys–Trp in Gel Filtration. As seen in Fig. 1, pGlu–Lys–Trp was eluted fairly earlier than pGlu–Asn–Trp and pGlu–Gln–Trp on a Sephadex G-25 column in spite of comparable molecular weights. When the synthetic amides corresponding to the three peptides were chromatographed on a Sephadex G-25 column with 3% AcOH, the elution profiles were very similar to those of the free acids. However, synthetic pGlu–Lys(Ac)–Trp was eluted almost at the same elution volume as pGlu–Asn–Trp and pGlu–Gln–Trp (data not shown), indicating that ε-amino group of Lys takes part in anomalous behavior of pGlu–Lys–Trp in chromatography on a dextran-based matrix.

Experimental

Isolation of Peptides. Fraction G100-1a was obtained from crude *T. flavoviridis* venom by Sephadex G-100 column chromatography as described previously.[8] Fraction G100-1a (114 mg but containing a large amount of salts) was chromatographed on a Sephadex G-25 column (1.7×60 cm) with 3% AcOH. Two fractions (G25-1a and G25-2a) (see Fig. 1A) were separately pooled and lyophilized, and then chromatographed on a Sephadex G-15 column (1.7×60 cm) using 3% AcOH to give G15-1a from G25-1a and G15-2a from G25-2a (Fig. 2). HPLC was carried out for each fraction on a TSK-gel ODS-120T column. Solvent system was 0.1% TFA (A) and CH_3CN containing 20% A (B) and elution was done with a linear concentration gradient of B (Fig. 3). Fraction H-1a from G15-1a and fractions H-2a and H-3a from G15-2a were separately pooled and lyophilized. Their weights were 2 mg, 300 μg, and 800 μg, respectively. The same procedure was applied for isolation of three tripeptides (H-1b, H-2b, and H-3b) from G100-1b fraction of *T. okinavensis* venom. The peptides obtained were hydrolyzed with 3 M 2-mercaptoethanesulfonic acid at 110°C for 24 h, the results of amino acid analysis being shown in Table 1.

Sequence Analysis and Mass Spectrometry. Each peptide was digested with CPase Y (enzyme : peptide=1 : 60 100 w/w) in 0.2 M $AcONH_4$ (pH 6.0) at 37°C for 48 h. At intervals, aliquots were withdrawn, acidified with 10% TCA, and subjected to amino acid analysis. In order to identify the N-terminal residue, CPase Y-digested samples were analyzed on a TSK ODS-120T column (0.46×25 cm) with 0.05 M phosphate buffer (pH 4.0) and the retention times of the eluted materials were compared with that of authentic pGlu. For ring-opening of pGlu residue, peptides were treated with 0.1 M NaOH at 25°C for 72 h.[12] The solution was charged on a Dowex 50W column (H^+ form, 0.5×3 cm), eluted with 0.3 M aqueous NH_4OH, and lyophilized. Manual Edman degradation was carried out for resulting peptides according to the method of Sauer et al.[13] PTH derivatives of amino acids were compared with standard PTH-amino acids using a Dupont Zorbax ODS column (0.46×15 cm) at 45°C. Solvent system used was 0.01 M HCOOH–Et_3N (pH 4.3) (A)–80% MeOH (B) and elution was performed with a linear concentration gradient of B. FAB-MS measurement was conducted using glycerol as matrix, H_2O as solvent, and xenon as fast neutral atom.

Peptide Synthesis. Peptide synthesis was carried out by the solution method. Boc–Lys(Z)–Trp(HCO)–OBzl was prepared by the active ester method.[14] The Boc group was removed with 4 M HCl in dioxane and Lys(Z)–Trp(HCO)–OBzl·HCl obtained was coupled with Z–pGlu–ONSu to give Z–pGlu–Lys(Z)–Trp(HCO)–OBzl. Boc-tripeptide-OBzl was then treated with HF–anisole–1,2-ethanedithiol (17:2:1 v/v) at 0°C for 30 min. Crude tripeptide, pGlu–Lys–Trp, was purified on a Sephadex G-15 column using 3% AcOH. The fraction containing the desired product was lyophilized. pGlu–Asn–Trp and pGlu–Gln–Trp were obtained similarly except for application of the EDC–HOBt method[15] to prepare Boc-dipeptide-OBzl. Their purity was confirmed by HPLC and amino acid analysis (Table 1).

Biological Assay. Assay of ACE activity was carried out according to the method of Cushman and Cheung.[16] A solution (0.25 ml) of 5 mM Hip–His–Leu and 5 mU ACE in 0.1 M potassium phosphate buffer (pH 8.3) containing 0.3 M NaCl was shaken in the absence and presence of 1 mM peptide at 37°C for 30 min and then 0.1 M HCl (0.25 ml) was added to terminate the reaction. Hippuric acid formed was extracted with EtOAc (1.5 ml) by vortex-mixing for 15s. After a brief centrifugation, an aliquot (1.0 ml) of EtOAc layer was evaporated in vacuo. The residue was dissolved in water (1.0 ml) and the absorbance was measured at 280 nm.

Assay of H_2-protease activity was conducted by the method of Takahashi and Ohsaka.[10] A solution (0.7 ml) of H_2-protease (1 mM) and peptide (1.0 mM) in 20 mM $AcONH_4$ buffer (pH 6.8) containing 0.1 mM $CaCl_2$ was incubated for 10 min and added to a solution of casein (2%) dissolved in 0.2 M Tris-HCl (pH 8.5). The solution was shaken at 37°C for 10 min. After addition of 0.44 M TCA (1.5 ml), the precipitate formed was filtered off. The absorbance of the filtrate was measured at 280 nm.

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5) Abbreviations used: ACE, angiotensin-converting enzyme; CPase, carboxypeptidase; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; Et_3N, triethylamine; FAB-MS, fast atom bombardment mass spectrometry; Hip, hippuric acid; HPLC, high-performance liquid chromatography; HOBt, 1-hydroxybenzotriazole; PTH, phenylthiohydatoin; TCA, trichloroacetic acid. Amino acid symbols denote the L-configuration.
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