TWO NEW FROG SKIN PEPTIDES, PHYLLOLITORINS, OF THE BOMBESIN-RANATENSIN FAMILY FROM *Phyllomedusa sauvagei*

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ABSTRACT

Two novel peptides, phyllolitorins, of the bombesin-ranatensin family having litorin-like activity were isolated from the skin of *Phyllomedusa sauvagei*, the South American hyliid frog. Both peptides were characterized as nonapeptide amides which differ each other only in the penultimate amino acid residue: Pyr-Leu-Trp-Ala-Val-Gly-Ser-X-Met-NH₂, X=Leu or Phe. The presence of Ser residue, instead of His, in the third position from the C-terminus is a specific feature of the phyllolitorins. Chemical synthesis of the Leu⁸- and Phe⁸-nonapeptide amides confirmed the proposed structures of the new peptides.

As a part of our (T.Y., T.N., V.E. and G.F.E.) continuous study on isolation and structure determination of biologically active peptides from various kinds of amphibian skin, we now succeeded in isolation of two novel peptides of the bombesin-ranatensin family, phyllolitorins, from fresh skin of the South American hyliid frog, *Phyllomedusa sauvagei*. The phyllolitorins were found to be characteristic of having Ser residue in the third position from the C-terminus, instead of His commonly existing in this position in all other peptides of the bombesin-ranatensin family.

The present report describes the isolation, sequence analysis, and chemical synthesis of the phyllolitorins.

MATERIALS AND METHODS

Preparation of Starting Material

Fresh skin of *Phyllomedusa sauvagei* (1 kg) was treated with absolute methanol (1 l) and the solvent was removed at reduced pressure. The residue was dissolved in 95% ethanol (1 l) and the solution was applied to a column of alkaline

alumina (1 kg), which was then eluted with three portions of 95% ethanol (1 l each). All litorin-like activity was recovered in the 95% ethanol eluates (ethanol-fraction), which were used as starting material for further purification.

Enzymatic Digestion of Peptides

Enzymes used were as follows: γ -chymotrypsin (Worthington Biochemicals) 83 units/mg protein; carboxypeptidase A (Worthington Biochemicals) 57 units/mg protein; leucine aminopeptidase (Worthington Biochemicals) 3.5 units/mg protein. The enzymes were dissolved in 0.001 N HCl at concentration of 1 mg/ml and stored at -20°C . Pyrrolidone carboxylpeptidase (4) was a gift from Professor D. Tsuru, Nagasaki University (the enzyme split pyrrolidone carboxyl α -naphthylamide at a rate of 0.23 $\mu\text{mol/ml/min}$).

Peptide (1–10 nmol) was dissolved in 0.1 M triethylamine bicarbonate (pH 8.5) (90 μl) and incubated at 25°C for 16 h (for γ -chymotrypsin) or an appropriate period (for the other enzymes) after adding desired enzyme solution (10 μl).

Dansylation and Edman Degradation

Peptide (1 nmol) in 0.1 M triethylamine bicarbonate (pH 8.5) (10 μ l) was mixed with 0.5% solution of dansylchloride in dioxane (10 μ l) and the mixture was stored in a dark place for 16 h and then lyophilized.

Edman degradation was performed according to the method of Edman (2). An aliquot of the reaction mixture of peptide (10–50 pmol) with phenylisothiocyanate was evaporated and the residue was treated with 6 N HCl at 110°C for 24 h. The hydrolysate was submitted to amino acid analysis.

High Performance Liquid Chromatography (HPLC)

Amino acid analysis was performed by orthophthalaldehyde method (1) and hypochloride oxidation method with a TSK 803A Liquid Chromatograph using an ion exchange column (TSK IEX 215, 8 $\times$ 50 mm).

Purification of peptides and isolation of peptide fragments produced by enzymatic digestion were carried out on a reverse phase column (TSK LS-410, 4 $\times$ 300 mm) with a JASCO Trirotor II Liquid Chromatograph.

Bioassay

Rat uteri were suspended in a muscle chamber with de'Jalon's solution and contracting activity of each peptide was measured (3) comparing with synthetic litorin as standard.

RESULTS AND DISCUSSION

Isolation of Active Principles

The ethanol-fraction obtained from 80 g of fresh frog skin was dissolved in H₂O (200 μ l) and centrifuged at 3,000 rpm for 5 min. The supernatant (40 μ l) was submitted to HPLC on a reverse phase column (TSK LS-410) with a linear gradient from 10% CH₃CN in 0.01 M KH₂PO₄ (pH 2.5) to 40% CH₃CN in 0.1 M KH₂PO₄ (pH 2.5) over a period of 30 min. Oxytocic activity was observed in the hatched peak shown in Fig. 1. The active fractions were collected and evaporated and the residue was immediately dissolved in H₂O (1 ml). The solution was passed through a Seppak cartridge (Waters Associates) and the adsorbed material was eluted with absolute methanol containing 0.01% trifluoroacetic acid, which was then hydrolyzed at 110°C for 24 h with 6 N HCl

Fig. 1 Reverse phase HPLC of the crude material (ethanol-fraction). Column: TSK LS-410 (4 $\times$ 300 mm). Elution: a linear gradient from 10% CH₃CN in 0.01 M KH₂PO₄ (pH 2.5) to 40% CH₃CN in 0.1 M KH₂PO₄ (pH 2.5) during 30 min. Flow rate: 1 ml/min

containing 4% thioglycolic acid. The amino acid ratios in the acid hydrolysate were as follows: Ser_{1.0}, Glu_{1.0}, Ala_{1.0}, Val_{1.0}, Met_{1.0}, Leu_{1.7}, Phe_{0.3}, and Trp_{1.0}. The values seemed to indicate that the preparation consists of two kinds of closely related peptides, Leu- and Phe-containing peptides, in the ratio of 7 to 3. A total of 20 nmol of the peptides was isolated and the sequence analysis was carried out with this preparation (preparation A) without further purification.

Sequence Analysis of Active Principles

Preparation A showed negative reaction to fluorescamine and dansyl chloride reagents. Incubation with carboxypeptidase A did not liberate any free amino acids from the preparation. Pyrrolidone carboxylic acid was identified in pyrrolidone carboxypeptidase digest of preparation A by HPLC on a reverse phase column (TSK LS-410) using as eluent 2% CH₃CN in a mixture of 10 mM KH₂PO₄ and 50 mM Na₂SO₄ (pH 2.5). Time course study on leucine aminopeptidase digestion of the pyrrolidone carboxypeptidase digest revealed the sequence of Pyr-Leu-Trp-Ala-Val- for the N-terminal of the peptide principle in prepara-

tion A.

An aliquot of preparation A corresponding to 5 nmol was digested by chymotrypsin and the digest was chromatographed on a reverse phase HPLC (TSK LS-410) with a linear gradient of CH₃CN from 5% to 60% in a mixture of 10 mM KH₂PO₄ and 50 mM Na₂SO₄ (pH 2.5). The digest showed six major peaks (P-1 to P-6) as shown in Fig. 2a. The fractions corresponding to each peak were collected and hydrolyzed with HCl in the usual manner to provide the following amino acid compositions.

P-1: Met

P-2: Ser_{1.0}, Gly_{1.0}, Ala_{1.0}, Val_{1.0}, Phe_{1.0}

P-3: Ser_{1.0}, Gly_{1.0}, Ala_{1.0}, Val_{1.0}, Leu_{1.0}

P-4: Glu_{1.0}, Leu_{1.0}, Trp_{1.0}

P-5: Ser_{1.0}, Glu_{1.0}, Gly_{1.0}, Ala_{1.0}, Val_{1.0},
Leu_{1.0}, Phe_{1.0}, Trp_{1.0}

P-6: Ser_{1.0}, Glu_{1.0}, Gly_{1.0}, Ala_{1.0}, Val_{1.0},
Leu_{1.0}, Trp_{1.0}

P-1 was identified as methionine amide by dansylation and co-chromatography with an authentic sample in thin layer chromatography. P-2 and P-3 were identified by subtractive Edman degradation as Ala-Val-Gly-Ser-Phe and Ala-Val-Gly-Ser-Leu, respectively. From the amino acid compositions P-4, P-5, and P-6 were judged to be Pyr-Leu-Trp, Pyr-Leu-Trp-Ala-Val-Gly-Ser-Phe, and Pyr-Leu-Trp-Ala-Val-Gly-Ser-Leu, respectively.

The combined data led us to propose the following structures for the two peptide principles in preparation A.

Synthesis of Leu⁸- and Phe⁸-Nonapeptide Amides

The two nonapeptide amides of the proposed structures were prepared by the conventional method for peptide synthesis according to the route illustrated in Fig. 3. Benzyloxycarbonyl (Z) or *t*-butyloxycarbonyl (Boc) group was used for α -amino group protection. Peptide chains were elongated by combination of the *N*-hydroxysuccinimide active ester method and azide fragment condensation method. The Boc-groups on the intermediate peptides were removed by trifluoroacetic acid and the Z-groups by catalytic hydrogenation over Pd. The final reaction product of the Leu⁸-nonapeptide amide was purified by washing with water followed by precipitation from a mixture of

Fig. 2 Reverse phase HPLC of the chymotryptic fragments of the natural and synthetic peptides. Column: TSK LS-410 (4×300 mm). Elution: 1) isocratic elution with 5% CH₃CN in a mixture of 10 mM KH₂PO₄ and 50 mM Na₂SO₄ (pH 2.5) during 10 min; 2) a linear gradient elution from 5% CH₃CN to 60% CH₃CN in the same mixed buffer during 30 min. Flow rate: 1 ml/min. a) Chymotryptic fragments of natural peptides (preparation A), b) Chymotryptic fragments of synthetic Leu⁸-nonapeptide amide, and c) Chymotryptic fragments of synthetic Phe⁸-nonapeptide amide

dimethylformamide and methanol with ethyl acetate: mp 235°C (dec); Rf^I (solvent system: 1-butanol: water: acetic acid=4: 5: 1) 0.79, Rf^{II} (solvent system: 1-butanol: pyridine: acetic acid: water=30: 20: 6: 24) 0.89; [α]_D²⁰ -17.6° (c 0.74, dimethylformamide). Anal. Calcd. for C₄₆H₇₁N₁₁O₁₁S·2H₂O: C, 54.05; H, 7.10; N,

four components from the Leu⁸-peptide amide were found to correspond to P-1, P-3, P-4 and P-6 from preparation A in terms of not only retention time in HPLC but also amino acid composition. Similarly, the four chymotryptic fragments of the Phe⁸-peptide amide were assigned to P-1, P-2, P-4 and P-5.

These results confirmed the primary structures, Leu⁸- and Phe⁸-nonapeptide amides, of the two novel peptides in preparation A.

The newly isolated two peptides were named phyllolitorins. They differ each other only in the penultimate amino acid residue: one having Leu-like peptides of the bombesin-alytesin sub-family and the other Phe-like those of the litorin-ranatensin sub-family. The presence of Ser residue in the third position from the C-terminus is a specific feature of these peptides. All other known peptides of the bombesin-ranatensin family contain His residue in that position, which has been considered crucially important for biological activity.

The spectra of biological activity of the two phyllolitorins were almost identical, while they were distinctly different, at least quantitatively, from that of litorin. The phyllolitorins were found to be as active as litorin in the rat urinary

bladder contraction but less active, sometimes considerably, in contracting activity on other various smooth muscle preparations. A thorough pharmacological study on the phyllolitorins is in progress.

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