

Isolation and Amino Acid Sequence of a New Dodecapeptide from the Skin of *Oreolalax pingii* †

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Abstract. -A novel dodecapeptide has been isolated by alumina column chromatography and HPLC from methanol extracts of the skin of the Chinese frog *Oreolalax pingii*. The sequence of the peptide is: Gly-Leu-Val-Ser-Asp-Leu-Met-Tyr-Gly-Ile-Gly-Leu-NH₂. This peptide differs from all the other amphibian skin peptides and should be regarded as a member of a new peptide family.

Key Words: Amphibia, Anura, Pelobatidae, *Oreolalax pingii*, China, biochemistry, peptides.

Introduction

Many active peptides have been discovered from amphibian skin during the past 20 years or more. Amphibian skin peptides have proved to be of considerable value not only in pharmacology, but also in taxonomic and evolutionary domains (Erspamer 1984; Cei 1985; Lazarus et al. 1985). In order to discover new active peptides, we have carried out research on amphibian skin peptides from Chinese frogs since 1983 (Hua et al. 1985; Tang et al. 1985). This paper concerns another novel dodecapeptide obtained from the skin of *Oreolalax pingii*.

Methods

The materials and experimental procedures were previously reported (Tang et al. 1985), with the following brief mentions and additions.

Six hundred specimens of *Oreolalax pingii* were collected in May, 1983 from the Daliangshan of Sichuan Province, China. The fresh skins (400 g) were removed and extracted with methanol. The methanol extracts were evaporated until dry. The residue was dissolved in 95% ethanol and the solution distributed on the alumina

columns. The column was eluted with ethanol-water mixtures of descending concentrations of ethanol (also see Montecucchi et al. 1981)

HPLC was performed on a Waters HPLC system. Details of individual chromatographic procedure are shown in the figures. Amino acid analysis of peptides after hydrolysis in HCl were carried out on a LKB 4400 amino acid analyzer. Sequence analysis of the peptides were performed by manual DABITC/PITC procedure (Chang 1983). The complete sequence analysis of the dodecapeptide was carried out on an Applied Biosystems Model 470A gas phase sequencer.

Enzymatic digestions of the peptide with α -Chymotrypsin, carboxypeptidase A (CP-A) and Y (CP-Y) were also as before (Tang et al. 1985). Bioassays of each isolated product were tested on the longitudinal muscle myenteric plexus preparation of the guinea pig ileum (GPI),

Results

The water portion eluted from alumina columns was lyophilized to give 214 mg of residue. The residue was separated by HPLC semi-preparatively as in Fig. 1. Perks 32 and 33 each were single peak by verifying on HPLC (analytical μ Bondapak C₁₈ column, the elution conditions were the same as in Fig. 1), respectively. The

† This publication combines material previously published in Chinese by Tang et al (1985) with additional information.

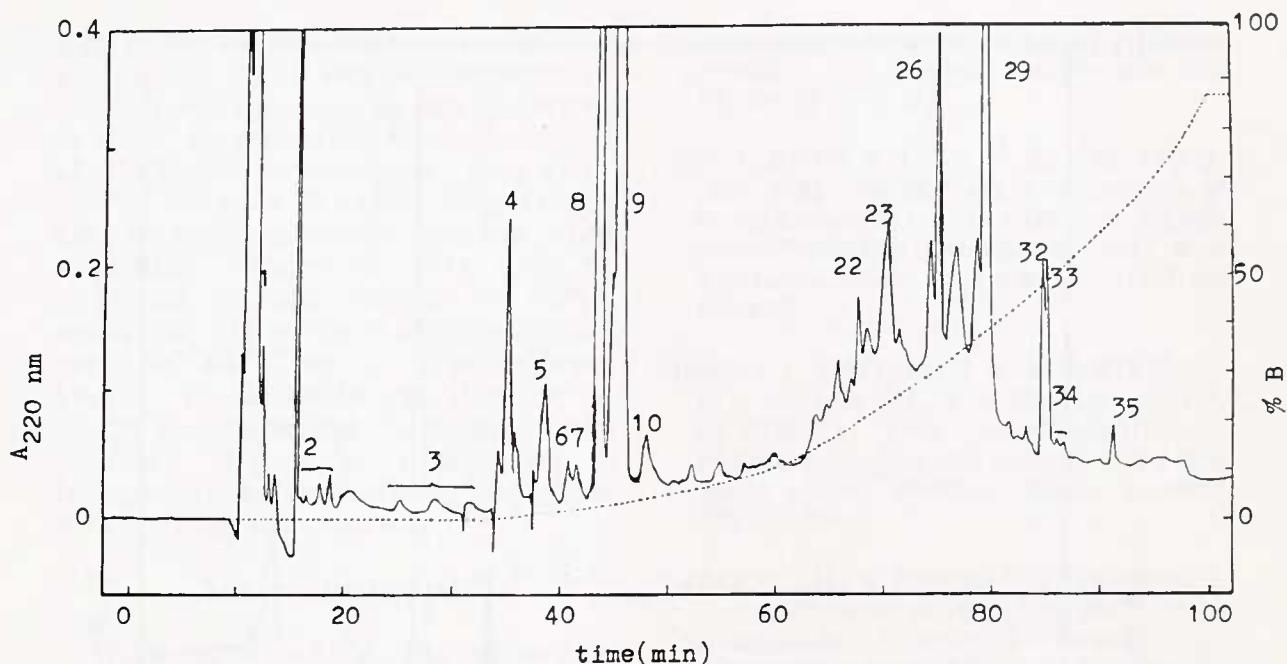


FIG. 1. RP-HPLC of the water eluate from the alumina columns. Column: μ Bondapak C_{18} 7.8X300 mm. Mobile phase: A=0.05% CF_3COOH B=60% CH_3CN in A. Concave gradient elution from 0–85%B (dotted line), 90 min, at 1.0 ml/min. Detected at UV 220 nm, 0.4 aufs.

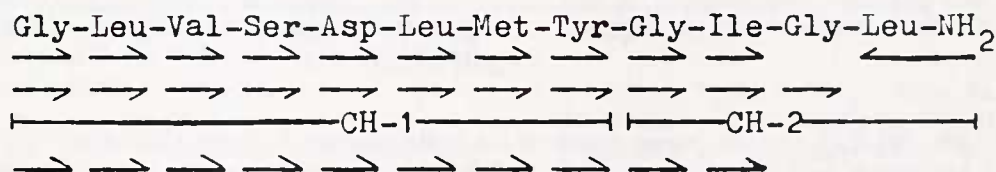


FIG. 2. Profile of sequence analysis of the dodecapeptide. (\rightarrow): DABITC/PITC method. (\rightarrow): gas phase sequencing. (\leftarrow): CP-Y digestion. CH: α -chymotryptic peptides.

difference of retention time of the two peaks was 0.8 min. The peak 32 acted as the representative of the dodecapeptide for further studies and the peak 33 was also investigated simultaneously.

Amino acid composition of the peak 32 was Asp (1), Ser (1), Gly (3), Val (1), Met (1), Ile (1), Leu (3), Tyr (1). Amino acid sequence of the peak 32 was determined by the DABITC/PITC method and gas phase sequencing. The former method proceeded to the tenth step, but the latter to the penultimate residue (Fig. 2). For C-terminal residue analysis, CP-A and CP-Y digestions of the peak 32 were carried out,

and did not release any amino acids by the former. This indicated a blocked C-terminus; upon latter, however, Leu and Gly were obtained. Thus, we deduced that the C-terminal structure of the peak 32 is $Leu-NH_2$. Digestion of the peptide by α -chymotrypsin provided further structural confirmation. The fragment peptides, CH-1 and CH-2 were separated on HPLC as depicted in Fig. 3. Amino acid compositions of the two fragments are in accordance with their sequences (see Fig. 2), respectively.

From the above results the complete amino acid sequence of the peak 32 was

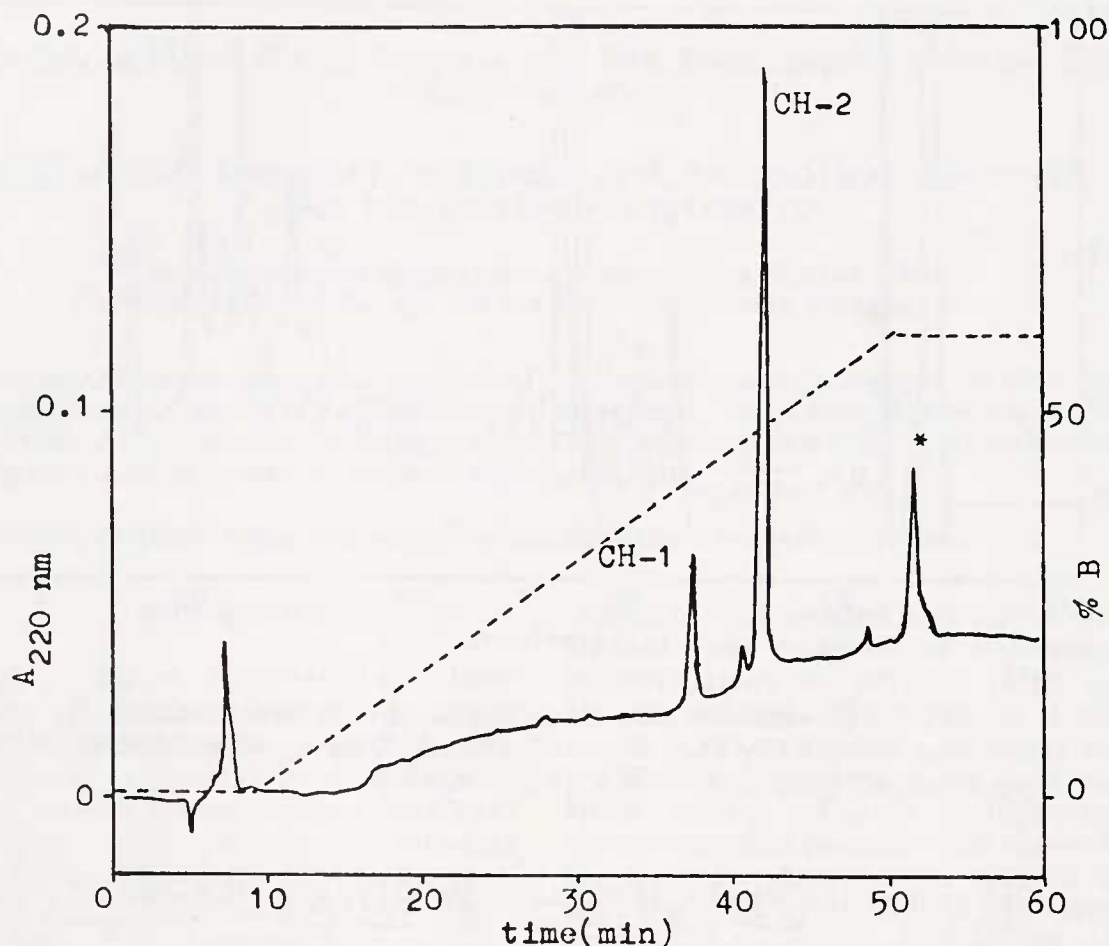


FIG. 3. RP-HPLC of α -chymotryptic digests of the dodecapeptide. Column: μ Bondapak C_{18} 3.9X300 mm. Mobile phase: A=0.1% CF_3COOH B=60% CH_3CN in A. Linear gradient elution from 0–60%B, 40 min, at 0.6 ml/min. Detected at UV 220 nm, 0.2 aufs. Peak (*) is the unreacted dodecapeptide.

established as in Fig. 2.

The peak 33 had the same properties with the peak 32 in the amino acid compositions and sequence analysis, respectively. We do not know the structural differences between the two peaks.

Based on the amino acid analysis, the yield of the pure dodecapeptide (including peak 32 and 33 in Fig. 1) was estimated to be at least 1.0 nmol starting from 1.0 g of fresh skin, according to that the rate of recovery of all above isolation steps was 10%. The dodecapeptide was inactive in GPI test, and its activity awaits to be established by assay methods other than those used in the present screening.

Discussion

The dodecapeptide described above is a completely novel peptide. The peptide may be a member of a new peptide family, and it should be regarded as the biochemical characteristic of *Oreolalax pingii* in taxonomy. Furthermore, the C-terminal portion of the dodecapeptide has some homologies to the mammalian Leu-enkephalin (Hughes et al. 1975) as shown below:

The dodecapeptide: Gly-Leu-Val-Ser-Asp-Leu-Met-Try-Gly-Ile-Gly-Leu-NH₂

Leu-enkephalin: Try-Gly-Gly-Phe-Leu

In amphibian skin peptide research, it

was observed that one peptide can be separated by HPLC into two components--the peak splitting, such as that discovered in HPLC separation of PGL-a (Andreu et al. 1985) and ranamargatin (Tang et al. 1988). The peak 32 and 33 (Fig. 1) both have the same amino acid composition and sequence. Therefore, they may be produced by one peptide in HPLC separation. The reason of the peak splitting mentioned above, however, is presently not known. To our knowledge, there are no similar findings besides the amphibian skin peptides. Hence, it is necessary to understand if the peak splitting is unique to the amphibian skin peptides.

Acknowledgments

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