Journal of Peptide Science
J. Pept. Sci. 2007; **13**: 798–802
Published online 22 August 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.895

JOURNAL OF PEPTIDE SCIENCE

A novel algesic peptide derived from skin secretions of the frog *Amolops loloensis*

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Received 17 May 2007; Revised 30 May 2007; Accepted 2 June 2007

Abstract: Several algesic agents including bradykinin and tachykinin have been identified from skin secretions of amphibians. They may act in defensive roles against aggressors. In this study, a novel peptide named Amolos with an amino acid sequence of FLPIVGAKL and isolated from skin secretion of the frog *Amolops loloensis*, is shown to strengthen nociceptive responses induced by inflammatory factors and strongly inhibit the contraction of isolated ileum. A synthetic peptide based on the sequence obtained showed characterization data identical to those of the isolated material, confirming its structure. These two types of responses seem to be a part of the defensive functions against predators or aggressors. The current results suggest that pharmacological molecules in amphibian skins not only act as innate defense mechanisms against microorganisms but also exert other defensive physiological functions against other aggressors. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: amphibian; *Amolops loloensis*; algesic; ileum contraction; defence

INTRODUCTION

Amphibian skins, which carry out many physiological functions such as defence, respiration, and water metabolism, are the first line to interact with outer environments. The amphibian skin secretion is a cocktail of bioactive molecules such as antimicrobial peptides, bradykinins, bombesins, serine protease inhibitors, and tachykinins. A large amount of antimicrobial peptides have been identified from the skin secretions of amphibians. To date, about 200 antimicrobial peptides belonging more than 20 different families have been identified from more than 40 amphibians [1–6]. They protect the amphibians from infections of microorganism. In addition to their antimicrobial functions, antimicrobial peptides can exert cytotoxicity against tumor cells and provide spermicidal action, and induce mast cell degranulation, histamine release, and insulin secretion [5–9]. Other possible defensive pharmacological molecules found in amphibian skins are bradykinin and tachykinin since they are algesic agents. More than 20 bradykinin-like peptides with different structures and functions have been identified from the skin secretions of amphibians. More than 30 tachykinins have also been identified from the skin secretions of amphibians. All these suggest that amphibian skin secretions are rich in defensive pharmacological substances.

Recently, a bradykinin-like peptide and several antimicrobial peptides have been purified from the skin secretions of the frog *A. loloensis*. To further investigate the possible defensive substances of the skin secretions of *A. loloensis*, the peptides of the skin secretions were separated and their activities studied. A novel small peptide was found to exert algesic function, which suggested a possible new algesic agent in amphibian skin secretions.

MATERIALS AND METHODS

Collection of Frog Skin Secretions

Adult specimens of *A. loloensisi* of both sexes ($n = 30$; weight range 30–40 g) were collected from the Yunnan Province of China. Skin secretions were collected in the following manner: frogs were put into a cylindrical container. A piece of absorbent cotton immersed with anhydrous ether was put on the top of the container. The container was covered with a lid and permeated with volatilized anhydrous ether. Being stimulated by anhydrous ether for 1–2 min, the frog skin was seen to exude copious secretions. Skin secretions were collected by washing the dorsal region of each frog with 0.1 M NaCl solution containing the protease inhibitor cocktail (Sigma). The collected solutions (500 ml total volume) were quickly centrifuged and the supernatants lyophilized.

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Peptide Purification

Lyophilized skin secretion sample of *A. loloensisi* (1.2 g, total $OD_{280\ nm}$ of 300) was dissolved in 10 ml 0.1 M phosphate buffer, pH 6.0, containing 5 mM EDTA. The sample was applied to a Sephadex G-50 (Superfine, Amersham Biosciences, 2.6 × 100 cm) gel filtration column equilibrated with 0.1 M phosphate buffer, pH 6.0. Elution was performed with the same buffer, collecting fractions of 3.0 ml. The absorbance of the elute was monitored at 280 nm. The algesic activities of the fractions were determined as indicated below. The protein peak containing algesic activity was pooled, lyophilized, and resuspended in 2 ml 0.1 M phosphate buffer solution, pH 6.0, and purified further by a C_{18} reverse-phase, high-performance liquid chromatography (RP-HPLC, Hypersil BDS C_{18}, 30 × 0.46 cm) column.

Structural Analysis

Complete peptide sequencing was undertaken by Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer, model 491. Fast atom bombardment (FAB) mass spectrometry was carried out on an Autospec-3000 spectrometer, equipped with a high-field magnet, using glycerol:3-nitrobenzyl alcohol:dimethyl sulphoxide (1:1:1, v:v:v) as the mixed matrix. The ion gun was operated at 25 kV with a current of 1 μA, using Cs^+ as the collision gas.

Peptide Synthesis

The peptide Amolos (FLPIVGKL) was synthesized by solid-phase synthesis on an Applied Biosystems model 433A peptide synthesizer according to the manufacturer's standard protocols. After cleavage and side-chain deprotection, the crude synthetic peptide was purified on a Vydac C_{18} reverse-phase HPLC column (25 × 1 cm) eluted at a flow rate of 1 ml/min by a linear gradient of acetonitrile in 0.1% trifluoroacetic acid in water. Identity of the peptide was confirmed by automated Edman degradation with a protein sequencer and mass spectrometry analysis. FABMS was carried out on an Autospec-3000 spectrometer, equipped with a high-field magnet [9]. The synthetic peptide was then used for evaluating biological activities.

Isolated Ileum Contraction

About 10 cm of the ileum distal of guinea pigs of either sex (150–250 g body weight) was removed immediately after death, and washed with Tyrode solution (137 mM NaCl, 2.7 mM KCl, 1.36 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 5.04 mM D-glucose). Cut segments of 2 cm of the isolated ileum were mounted isotonically, under 1 g load, in a 5 ml muscle bath containing Tyrode solution maintained at 37 °C and bubbled with air. PcLab software package was used for the collection and analysis of biological signal of muscle contraction (Beijing Microsignalstar Technology Development Co. Ltd.). The Animal Care and Experimental Protocol were approved by Heibei Normal University.

Formalin Test

Mice were pretreated with 100 μl test samples resolved in 0.9% salt water by i.p. (40 μmol or 20 μmol per mouse). After the treatment for 30 min, 10 μl of 2.5% formalin was injected under the plantar surface of right hindpaw of the mice. Mice were then placed individually into open polyvinyl cages (20 × 40 × 15 cm). Two different periods of intensive licking and biting responses, i.e. an early phase (0–5 min) and a late phase (15–30 min), were counted separately for each animal in the different groups.

Abdominal Constriction Response Caused by i.p. Injection of Acetic Acid

The abdominal constrictions induced by i.p. injection of 200 μl acetic acid (0.8%), including contraction of the abdominal muscle and stretching of the hind limbs, were performed according to procedures described by Santos *et al.* [10] Mice were pretreated with 100 μl test samples dissolved in 0.9% salt water by i.p. (40 μmol or 20 μmol per mouse) for 30 min prior to the irritant injection. Control animals received the same volume of vehicle. After the challenge, mice were individually placed into open polyvinyl cages (20 × 40 × 15 cm), and the abdominal constrictions were counted cumulatively in three different phases (0–10 min, 10–20 min, and 20–30 min).

Statistical Analysis

All the data were presented as means ± SEM and analyzed by one-way or two-way analysis of variance (ANOVA) followed by Duncan's test or unpaired *t*-test when appropriate. A value of $p < 0.05$ was taken as the level of significance.

RESULTS AND DISCUSSION

Purification of Amolos

The supernatant of *A. loloensisi* skin secretions was fractionated into several peaks by Sephadex G-50, and the result is illustrated in Figure 1(A). Among all peaks obtained, the peak III was collected and subjected to RP-HPLC. More than 50 peaks were obtained (Figure 1(B)). The peak with inhibitory activity on ileum contraction (marked by an arrow) was collected. The purified fraction was named Amolos. Several antimicrobial peptides including temporins-Ala have also been purified.

Structural Characterization

The purified peptide was subject amino acid sequence analysis by automated Edman degradation. Its sequence was FLPIVGAKL. In order to determine whether the sequence was correct, the actual molecular weight of this peptide was analyzed by FAB mass spectrometry, which gave an m/z of 955.38 (Figure 2) and matched sufficiently well with the theoretical molecular weight (956.6). The result confirmed that the sequence of native Amolos is FLPIVGKAL. This sequence shares similarity with the *N*-terminal partial amino acid sequences of some temporin antimicrobial peptides identified from amphibian skin secretions.

DOI: 10.1002/psc

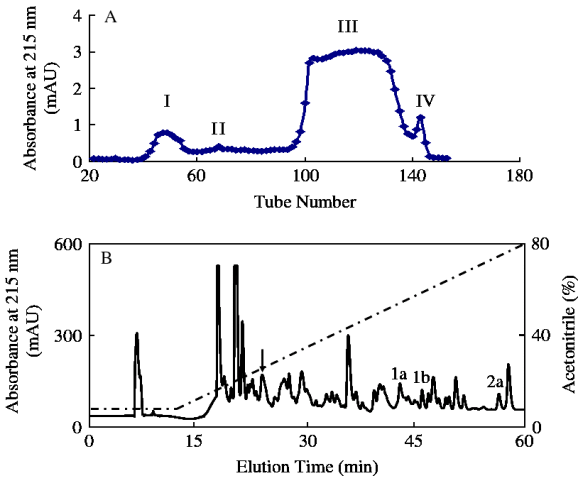

Figure 1 Fractionation of *A. loloensis* skin secretion. Figure 1(A) shows Sephadex G-50 gel filtration of *A. loloensis* skin secretion. *A. loloensis* skin secretion was applied to a Sephadex G-50 (Superfine, Amersham Biosciences, 2.6 × 100 cm) column equilibrated with 0.1 M phosphate buffer, pH 6.0. Elution was performed with the same buffer, collecting fractions of 3.0 ml. The peak III from Sephadex G-50 was further purified on a Hypersil BDS C_{18} RP-HPLC column (30 × 0.46 cm) equilibrated with 0.1% (v/v) trifluoroacetic acid/water. The elution was performed with the indicated gradient of acetonitrile in Figure 1(B) at a flow rate of 0.7 ml/min, and fractions were tested for ileum contractile activity. The purified Amolos is indicated by an arrow in Figure 1(B). This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

Effects on the Isolated Ileum

To test its effect on muscle contraction, 50 ng/ml of Amolos was applied to the isolated ileum. As shown in Figure 3, Amolos clearly inhibited the contraction of the isolated ileum. It appeared that the maximum inhibitory capability was exerted after the administration of Amolos. After washing by Tyrode solution, the muscle contractile capability of the isolated ileum recovered, although the contractile intensity seemed smaller than the untreated one, as illustrated in Figure 3. It suggested that the inhibition of Amolos on muscle contraction was reversible. The contraction was inhibited again when the recovered isolated ileum was treated by Amolos again as indicated

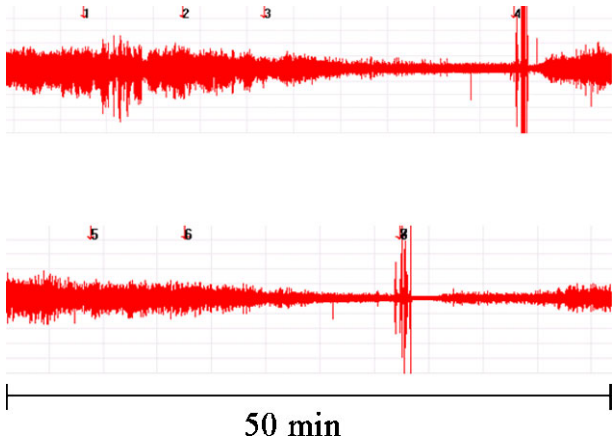

Figure 3 The effects of Amolos on muscle contraction of isolated ileum. Amolos (50 ng/ml) was applied to isolated ileum twice indicated as 2 and 3, the treated isolated ileum by Amolos was washed by Tyrode solution indicated as 4, the washed isolated ileum was treated twice again by 50 ng/ml of Amolos indicated as 5 and 6, and the isolated ileum was washed again indicated as 8. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

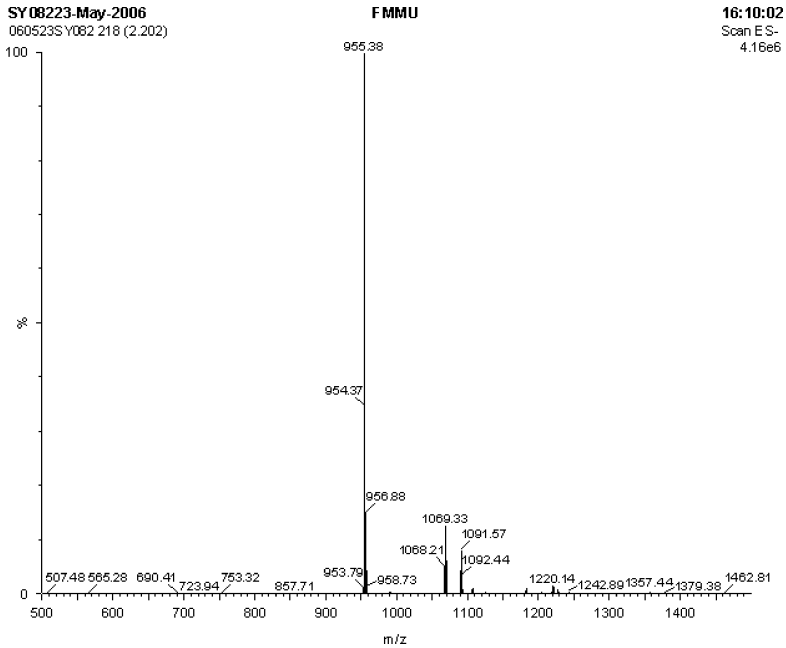

Figure 2 The mass spectrum of Amolos.

DOI: 10.1002/psc

by 5 and 6 in Figure 3. The synthetic Amolos showed the same activity as the native one. It further confirmed that the amino acid sequence of the native Amolos was correct. Amolos did not show antimicrobial activity in our experiments.

Effects on Nociceptive Responses Induced by Inflammatory Factors

Considering that ileum contraction is a classical response induced by bradykinin-like peptides and they are a type of nociceptive factors [11], we supposed that the inhibition of ileum muscle by Amolos might be related with bradykinin-like peptides or their receptors. Amolos may acts as an inhibitor of bradykinin-like peptides or their receptors to compete with their effects on the ileum muscle. Hence, Amolos was administered into mice to test its effects on nociceptive responses induced by inflammatory factors. Unexpectedly, Amolos strengthened the nociceptive responses induced by acetic acid and formalin, as illustrated in Figures 4 and 5. Amolos had little effect on the response at the early phase in both nociceptive models, while it had significant effects on the response at the late phase. Different from Amolos, bradykinin significantly affected both the first and the second phases as illustrated in Figures 4 and 5. Interestingly, Amolos continuously strengthened the nociceptive responses induced by acetic acid till the third phase, which is different from bradykinin. It suggested that Amolos likely did not interact with bradykinin-like peptides or their receptors to exert ileum contraction. The actual mechanism of action of Amolos on ileum and nociception will be studied further, which will facilitate resolving its physiological significance.

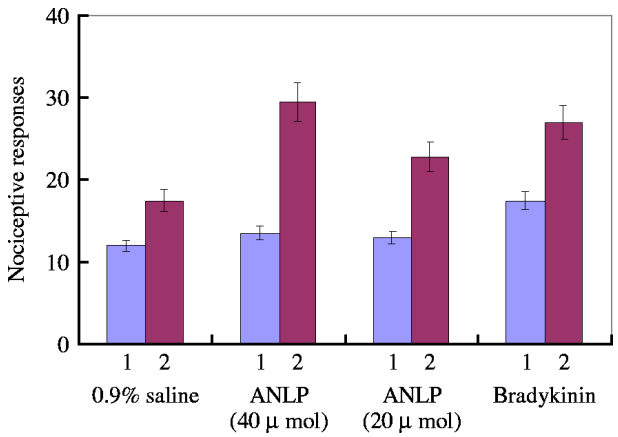

Figure 4 The effects of Amolos on nociceptive responses of mice induced by formalin. Mice were pretreated with test samples by i.p. (40 μmol or 20 μmol in 100 μl 0.9% salt water per mouse). After the treatment for 30 min, 10 μl of 2.5% formalin was injected under the plantar surface of right hindpaw of the animals. Saline (100 μl, 0.9%) and bradykinin (5 ng/ml, Sigma) were used as controls. (1): the early stage (0–5 min), and (2): the late stage (15–30 min). This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

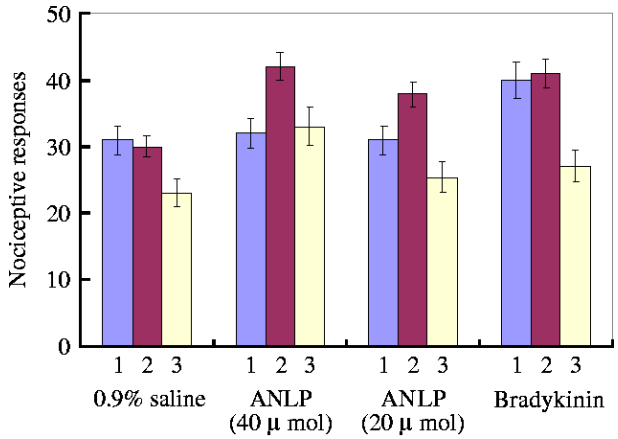

Figure 5 The effects of Amolos on nociceptive responses of mice induced by acetic acid. Saline (100 μl 0.9%) and bradykinin (5 ng/ml, Sigma) were used as controls. Mice were pretreated with 100 μl test samples dissolved in 0.9% salt water by i.p. (40 μmol or 20 μmol per mouse) for 30 min prior to acetic acid injection. (1): the first stage (0–10 min), (2): the second stage (10–20 min), and (3): the third stage (20–30 min). This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

CONCLUSIONS

In this study, a novel peptide named Amolos with an amino acid sequence FLPIVGAKL was identified from skin secretions of the frog *A. loloensis*. It could strengthen the nociceptive responses induced by inflammatory factors and strongly inhibit the contraction of isolated ileum, which are the types of defenses against predators or aggressors. The current results suggest that pharmacological molecules in amphibian skins not only are part of the innate defensive system against microorganisms but also may prevent attack by other aggressors.

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DOI: 10.1002/psc