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Chinese Journal of Natural Medicines 2016, **14**(4): 0294–0298
Chinese Journal of Natural Medicines

Two novel antimicrobial peptides from skin venoms of spadefoot toad *Megophrys minor*

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Available online 20 Apr., 2016

[ABSTRACT] Amphibian skin contains rich bioactive peptides. Especially, a large amount of antimicrobial peptides have been identified from amphibian skin secretions. Antimicrobial peptides display potent cytolytic activities against a range of pathogenic bacteria and fungi and play important defense roles. No antimicrobial peptides have been reported from toads belonging to the family of Pelobatidae. In this work, two novel antimicrobial peptides (Megin 1 and Megin 2) were purified and characterized from the skin venoms of spadefoot toad *Megophrys minor* (Pelobatidae, Anura, Amphibia). Megin 1 had an amino acid sequence of FLKGCWTKWYSLKPKCPF-NH2, which was composed of 18 amino acid residues and contained an intra-molecular disulfide bridge and an amidated C-terminus. Megin 2 had an amino acid sequence of FFVLKFLLKWAGKVGLEHLACKFKNWC, which was composed of 27 amino acid residues and contained an intra-molecular disulfide bridge. Both Megin 1 and Megin 2 showed potential antimicrobial abilities against bacteria and fungi. The MICs of Megin 1 against *Escherichia coli*, *Bacillus dysenteriae*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Candida albicans* were 25, 3, 6.25, 3, and 50 μg·mL^{-1}, respectively. The corresponding MICs for Megin 2 were 6.25, 1.5, 12.5, 1.5, and 12.5 μg·mL^{-1}, respectively. They also exerted strong hemolytic activity against human and rabbit red cells. The results suggested that megin peptides in the toad skin of *M. minor* displayed toxic effects on both eukaryotes and prokaryotes. This was the first report of antimicrobial peptides from amphibians belonging to the family of Pelobatidae.

[KEY WORDS] Antimicrobial peptides; Amphibian; Pelobatidae; Skin venoms

[CLC Number] R964 **[Document code]** A **[Article ID]** 2095-6975(2016)04-0294-05

Introduction

As the first vertebrates living in both water and land, amphibians have to adopt variable environments. Amphibians have naked skin. Their skin directly is exposed on environments and possible noxious factors, such as predators, microorganisms, parasites, and physical injuries. For their surviving, amphibian skin acts as an important defensive mechanism. The chemical components responsible for defense and the functional mechanisms are intensively investigated during last decades. The high level of biochemical diversity in amphibian skin secretions suggests that amphibian skin secretions are attractive subjects for prospecting and screening for compound candidates with potential pharmaceutical applications [1-2]. Especially, many peptides with multiple functions have been found in amphibian skin. One of peptide families, which are the most intensively studied, is antimicrobial peptides. Living environments of amphibians usually are laden with many microorganism pathogens including bacteria, fungi, and parasites. Amphibians have a poor cell-mediated naive immune response against pathogens and have to intensively depend on innate immune defenses, such as antimicrobial peptides, to prevent infection by microorganisms.

Antimicrobial peptides have been found in amphibian skins from species belonging to families of Bombinatoridae, Hylidae, Hyperoliidae, Leiopelmatidae, Leptodactylidae, Myobatrachidae, Pipidae, and Ranidae, but there is no report of

[Received on] 16-Nov.-2015
[Research funding] This work was supported by the National Natural Science Foundation of China (No. 81273375) and Jiangsu Provincial Qing Lan Project.
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These authors have no conflict of interest to declare.

antimicrobial peptides from Pelobatidae families [3]. In order to determine if there is the presence of antimicrobial peptides in Pelobatidae species, we investigated peptide components containing antimicrobial abilities from the skin venoms of spadefoot toad *Megophrys minor* in the present study.

Materials and Methods

Collection of toad skin secretions

Adult specimens of *M. minor* of both sexes (n = 30; weight range 5–10 g) were collected in Yunnan Province of China. Toads were put into a cylinder container. Animals were stimulated by anhydrous ether saturated in absorbent cotton. After the exposure to anhydrous ether for 1–2 min, secretions were secreted from toad skin and washed with 0.1 mol·L^{-1} phosphate buffer, pH 6.0 (PBS, containing protease inhibitor cocktail, Sigma). The collected solutions (200 mL of total volume) were quickly centrifuged and the supernatants were lyophilized. All the animal experiments were approved by the Institutional Animal Care and Use Committee of the Kunming Medical University.

Peptide purification

Lyophilized powder of skin secretions from *M. minor* (2 g) was dissolved in 20 mL of 0.1 mol·L^{-1} phosphate buffer and filtered through a 10-kDa cut-off Centriprep filter (Millipore, Bedford, CA, USA). The filtrate was applied onto a C_8 reversed-phase high performance liquid chromatography (RP-HPLC, Hypersil BDS C_8, 30 cm × 0.46 cm) column. Peptide components in the skin secretions were eluted from the C_8 RP-HPLC column by water/acetonitrile elution system containing 0.1% trifluoroacetic acid. The absorbance of the eluate was monitored at 280 nm. Each eluted fractions were assayed for antimicrobial activity as described below. The fraction containing antimicrobial activity was pooled and lyophilized, and re-suspended in 1 mL PBS, and purified further by a C_{18} reverse phase high performance liquid chromatography (Hypersil BDS C_{18}, 25 cm × 0.46 cm) column by using water/acetonitrile elution system containing 0.1% trifluoroacetic acid.

Structural analysis

The complete amino acid sequences of purified peptides were determined by automated Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer, model 491, according to manufacturer's instruction. Molecular weight analysis was performed by using an AXIMA CFR (Kratos Analytical) MALDI Matrix-Assisted Laser Desorption Ionization Time-of-Flight mass spectrometor (MALDI- TOF-MS). The samples were placed in MALDI plate (Applied Biosystems) and analyzed in a positive ion and liner mode following the manufactory instruction. The following operating parameters were used including ion acceleration voltage 20 kV and accumulating times of single scanning 50 s. Polypeptide mass standard (Kratos Analytical) was served as external standard. The accuracy of mass determinations was within 0.1%.

Antimicrobial assay

Antimicrobial tests were according to the method described by Lai *et al* [4]. Several microorganisms including Gram-positive bacteria *Staphylococcus aureus* (ATCC2592) and *Bacillus cereus*, Gram-negative bacteria *Escherichia coli* (ATCC25922) and *Bacillus dysenteriae*, and fungus *Candida albicans* (ATCC2002) were obtained from Kunming Medical University. Bacteria were first cultured in Luria-Bertani (LB) broth to an absorbance of 0.8 at 600 nm. Ten microliter of aliquot the bacteria culture was then collected and added to 8 ml fresh LB broth with 0.7% agar and poured over a 90 mm Petri dish containing 25 mL 1.5% agar in LB broth. After the top agar hardened, a 20 μL aliquot of the test sample filtered on a 0.22 μm Millipore filter was dropped onto the surface of the top agar and completely dried before being incubated overnight at 37 °C. A clear zone would be observed on the surface of the top agar, which indicated inhibition of bacterial growth, if the sample contains antimicrobial activity. Minimal inhibitory concentration (MIC) was determined in liquid LB medium by incubating the bacteria in LB broth with test sample for 24 h at 37 °C. The sample concentration is based on a 2 × serial dilution. The MIC at which no visible growth occurred was recorded. In order to determine if the MIC of antimicrobial peptide is lethal for sensitive strain, the sensitive strain was treated by antimicrobial peptide with concentration above the corresponding MIC and plated on LB agar plates for 24 h incubation at 37 °C. Resuming growth of the sensitive strain was observed [4].

Hemolysis assay

Hemolysis assay was performed by using rabbit and human red blood cells in liquid medium according to the method reported by Bignami [5]. The samples with different concentration were incubated with red cells at 37 °C for 30 min, and then the red cells were centrifuged. The supernatant was collected for measuring the absorbance at 540 nm. Maximum hemolysis (100%) was determined by adding 1% Triton X-100 to the red cells.

Peptides Synthesis

The peptides Megin 1 (FLKGCWTKWYSLKPKCPF-NH2) and Megin 2 (FFVLKFLLKWAGKVGLEHLACKFKNWC) (both contain an intramolecular disulfide bridge) were synthesized by GL Biochem (Shanghai) Ltd. (Shanghai, China) and analyzed by RP-HPLC and MALDI-TOF mass spectrometry. The purity of the synthesized peptides was determined to be greater than 98% by HPLC.

Results

Purification of antimicrobial peptides

M. minor skin secretion sample was subjected to filter through a 10-kDa cut-off Centriprep filter and separated by C_8 RP-HPLC as illustrated in Fig. 1A. More than 30 fractions were eluted out from the C_8 RP-HPLC. One of them showed antimicrobial activity as indicated by an arrow in Fig. 1A. The interesting fraction was pooled and purified further by C_{18} RP-HPLC as illustrated in Fig. 1B. Two purified peptides