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Three new antimicrobial peptides from the scorpion *Pandinus imperator*☆

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ABSTRACT

Three novel cysteine-free venom peptides, which were referred to as Pantinin-1, Pantinin-2 and Pantinin-3, respectively, have been identified from the scorpion *Pandinus imperator* by cDNA cloning strategy. The precursor of each peptide consists of a signal peptide, a mature peptide with no disulfide bridges, and an acidic propeptide with a typical processing signal. Each of the three peptides is an α-helical, cationic and amphipathic molecule with 13 or 14 amino acid residues. Their amino acid sequences are homologous to those of some 13-mer antimicrobial peptides isolated from scorpions. Antimicrobial assay showed that all the three peptides possess relatively strong activities against Gram-positive bacteria and a fungus, but have very weak antimicrobial activities against Gram-negative bacteria. Toxicity assay showed that the three peptides exhibit very low or mild hemolytic activities against human red blood cells. It is interesting to see that Pantinin-3 is able to potently inhibit the growth of vancomycin-resistant *Enterococcus* (VRE) S13, a pathogen that can cause a number of human infections; this suggests that Pantinin-3 has great potential to be applied in the treatment of VRE infections. Our findings gain new insights into the structure/function relationships of the small linear cationic antimicrobial peptides from scorpions, and provide new templates for designing of antimicrobial agents targeting antibiotic-resistant pathogenic bacteria.

1. Introduction

Antimicrobial peptides (AMPs) are considered to be important components of innate immunity that fight against invading pathogenic organisms in various unfavorable conditions [8,22,24]. They often show a broad-spectrum, non-specific activity against a wide range of microorganisms, including viruses, Gram-positive and Gram-negative bacteria, protozoa and fungi, and may also exert hemolytic effect on erythrocytes [32]. Because AMPs have a mechanism of action that differs from that of currently available therapeutic antibiotics, antibacterial peptides have become an alternative type of antibiotics for treatment of bacterial infectious diseases [10,23,28].

AMPs are commonly characterized as cationic and amphipathic peptides (13–44 amino acids long), which can be classified into three main groups according to their structures: (i) linear peptides containing amphipathic α-helical structure without cysteines; (ii) peptides including one or more disulfide bridges, forming β-sheet or both α-helix and β-sheet; (iii) peptides with irregular amino acids [13,16,19]. These peptides were derived from prokaryotes, amphibians, mammals and arthropods [1,12,17,33].

Until now, at least thirty AMPs have also been identified from scorpion venoms, including Hadrurin from the scorpion *Hadrurus aztecus* [29], Parabutoporin from *Parabuthus schlechteri* [26], BmKn1, BmKn2, BmKb1 and BmKbpp from *Mesobuthus martensii* Karsch [15,35,36], IsCT and IsCT2 from *Opisthacanthus madagascariensis* [6,7], StCT1 and StCT2 from *Scorpiops tibetanus* [3,31], Cll-dlp from *Centruroides limpidus limpidusin* [27], Pandinin 1, Pandinin 2 from *Pandinus imperator* [4], Opistoporin 1 and Opistoporin 2 from *Opistophtalmus carinatus* [18], Vejovine from *Vaejovis mexicanus* [11], VmCT1 and VmCT2 from *Vaejovis mexicanus smithi* [25], VsCT1 and VsCT2 from *Vaejovis subcristatus* [25], Imcroporin from *Isometrus maculates* [38], Mucroporin from *Lychas mucronatus* [5], Ctriporin from *Chaerilus tricostatus* [9], Hp1090 and Hp1035 from *Heterometrus petersii* [30], HsAp, HsAp2, HsAp3 and HsAp4 from *Heterometrus spinifer* [20], UyCT1, UyCT2 and UyCT5 from

☆ The cDNA sequences of Pantinin-1, Pantinin-2 and Pantinin-3 reported in this paper have been deposited to the GenBank database under the accession numbers of KC538864, KC538865 and KC538866, respectively.

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Urodacus yaschenkoi [14]. Among these scorpion AMPs, Ctriporin, StCT2 and Imcroporin have been shown to have great potentials to treat multidrug-resistant bacterial infections.

Here, we identified three new antimicrobial peptides, which were referred to as Pantinin-1, Pantinin-2, Pantinin-3, respectively, from the scorpion *P. imperator* by a cDNA library screening strategy. These peptides contain 13 or 14 amino acid residues, respectively. They are cationic and amphipathic consisting of α-helical structures. We found that they possess very weak antimicrobial activities against Gram-negative bacteria, but have relatively strong activities against Gram-positive bacteria and a fungus. Their antimicrobial activities and toxicity in human red blood cells were shown to be highly associated with the hydrophobicity of their hydrophilic regions of the molecules. It is noteworthy that Pantinin-3 has great potential to treat the infections caused by vancomycin-resistant *Enterococcus*. Our studies provide new insights into the structure/function relationship of the small linear antimicrobial peptides from scorpions.

2. Materials and methods

2.1. Animals

Scorpions of *P. imperator* were purchased from the Guangdong province, southern China. The animals were kept in an escape-proof tank, which was provided with some cricket foods and a wet sponge.

2.2. Construction of a cDNA library from the venom glands of the scorpion

Ten specimens of the scorpion were stimulated using electricity. This allows the toxin-producing cells of the venom glands to enter a secretory phase. Twenty four hours later, the telsons of the scorpions were cut off and immediately placed into a mortar containing liquid nitrogen. The telson tissues were thoroughly ground into fine powders with a pestle. Total RNA was extracted from the homogenized tissues with the TRIZOL reagent. Poly(A) mRNA was purified using a TaKaRa mRNA purification kit. A cDNA library was constructed using the SuperScript™ cDNA library construction kit according to the manufacturer's instructions. The purified cDNA was ligated into the pMD®-19 T plasmid. To generate a cDNA library, the ligated cDNAs were transformed into the *Escherichia coli* DH5α competent cells.

2.3. Screening of the cDNA library

The cDNA library was screened using a "size-selective sequencing strategy" as described previously [34]. The insert sizes of the clones from the cDNA library were determined by PCR using the universal primers M13F and M13R. The cDNA clones that are 350–650 base pairs long were sequenced by Nanjing GenScript Biotechnology Corporation.

2.4. Bioinformatics analysis

Searches for open reading frames (ORFs) of nucleotide sequences were performed using a Translate tool (http://web.expasy.org/translate/). Protein amino acid sequence homology search was performed using the BLASTP server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Signal peptide was predicted with the SignalP 4.0 program (http://www.cbs.dtu.dk/services/SignalP/). Multiple sequence alignment was performed using the ClustalX program in the BioEdit software. Secondary structure was predicted using the NPS@ sever (www.bioinf.manchester.ac.uk/dbbrowser/bioactivity/NPS2.html). The helical wheel diagram of an α-helical peptide was generated using the HeliQuest server (http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParams.py). Biochemical properties were analyzed with the ProtParam tool (http://web.expasy.org/protparam/).

2.5. Peptide synthesis

Peptides were synthesized by GL Biochem Ltd. (Shanghai, China), using the solid phase method on the CS Bio automated multiple peptide synthesizer, and modified by C-terminal amidation. Synthesized peptides were purified using the reverse-phase high performance liquid chromatograph (RP-HPLC), and verified by the mass spectrometry and amino acid composition analysis.

2.6. Antimicrobial assay

The antimicrobial activity of the synthesized peptides was tested against some representative strains of Gram-positive, Gram-negative bacteria and a fungus. Bacteria were grown in the Bacto™ Tryptic Soy Broth (TSB) medium at 37 °C or 30 °C until the OD_{600} value of the culture reached around 0.4. The bacterial culture was then diluted with the fresh TSB medium into a final OD_{600} of 0.002. Twenty microliters of each peptide solution prepared by serial dilution was added to a 96-well plate containing 180 microliters of the diluted bacterial culture. The plate was incubated at 37 °C or 30 °C with vigorous shaking. Bacterial proliferation was determined by measuring the absorbance at 620 nm, and the minimal inhibitory concentration (MIC) of the peptide, expressed as the lowest concentration that causes 100% inhibition of bacterial growth, was thus achieved.

2.7. Hemolytic assay for the human red blood cells

Hemolytic activity of the peptides was determined by measuring the release of hemoglobin from the human red blood cells as described previously [35].

3. Results

3.1. Precursors of three novel cysteine-free peptides from the scorpion P. imperator

Three novel full-length cDNAs, which are 371, 392 and 359 base pairs long, respectively, were obtained from the cDNA library made from the venom glands of the scorpion *P. imperator*. The cDNAs code for the precursors of three novel cysteine-free peptides that were referred to as Pantinin-1, Pantinin-2, and Pantinin-3, respectively. Each of the three transcripts consists of a 5′-untranslated region (5′-UTR), an open reading frame (ORF), and a 3′-untranslated region (3′-UTR) with single or double putative polyadenylation signal (AATAAA or AACAAA) and a typical eukaryotic polyA tail (Fig. 1A–C). The ORFs of the transcripts of Pantinin-1, Pantinin-2 and Pantinin-3 are 210, 207 and 207 nucleotides long, respectively, and display high homology (61–81%) with each other. However, the nucleotide sequences of their 5′-UTRs, which are 113, 65 and 44 nucleotides long, respectively, show little similarity to each other. The 3′-UTR sequences of the three transcripts, which are 48, 120 and 108 nucleotides long, respectively, also show little homology with each other. It has been established that 5′-UTR and 3′-UTR play key roles in the control of amount and timing of gene expression. Therefore, the high level of sequence divergence in the 5′-UTRs and 3′-UTRs of the three transcripts would lead to highly different abundances of the three peptides in the scorpion venom. Our findings also suggest that the genes of the three peptides were originated from a common ancestor by gene duplication, and subsequently

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