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A defensin-like antimicrobial peptide from the venoms of spider, *Ornithoctonus hainana*

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The defensin-like antimicrobial peptides have been characterized from various other arthropods including insects, scorpions, and ticks. But no natural spider defensin-like antimicrobial peptides have ever been isolated from spiders, except couple of cDNA and DNA sequences of five spider species revealed by previous genomic study. In this work, a defensin-like antimicrobial peptide named Oh-defensin was purified and characterized from the venoms of the spider, *Ornithoctonus hainana*. Oh-defensin is composed of 52 amino acid (aa) residues including six Cys residues that possibly form three disulfide bridges. Its aa sequence is MLCKLSMFGAVLGV PACAIDCLPMGKTGGSCEGGVCGCRKLTFKILWDKKFG. By BLAST search, Oh-defensin showed significant sequence similarity to other arthropod antimicrobial peptides of the defensin family. Oh-defensin exerted potent antimicrobial activities against tested microorganisms including Gram-positive bacteria, Gram-negative bacteria, and fungi. The cDNA encoding Oh-defensin precursor was also cloned from the cDNA library of *O. hainana*. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: *Ornithoctonus hainana*; venom; antimicrobial peptide; defensin

Introduction

Spiders use their venoms to kill or paralyze the preys and repel against the aggressors. A large amount of bioactive components have been found from spider venoms, and most of them are low-molecular-weight compounds, peptides, and proteins. These venom components possess multiple functions like induction of paralysis, activation or inhibition of ion channels, and cytotoxicity [1].

Antimicrobial peptides are found from virtually all animals and plants, as important components of the innate immune system, act as the first line to defend microorganism infection and represent an ancient mechanism of host defense [2–5]. Most of antimicrobial peptides are composed of 10–50 amino acid residues (aa) including multiple basic residues, which give antimicrobial peptides net positive charges. Defensins are a unique antimicrobial peptide family that is characterized by multiple Cys residues forming three to four disulfide bridges [6]. As effector molecules of innate immunity, defensins have already been identified from mammals, insects, and plants, providing an efficient initial defense against infectious pathogens [7,8]. Some defensin-like antimicrobial peptides have been identified from arthropods like spiders [9,10], scorpions [11,12], ticks [13], and insects [14]. In this work, a defensin-like antimicrobial peptide was purified and characterized from the venoms of spider, *Ornithoctonus hainana*. The result firstly identified the defensin-like antimicrobial peptide on the protein level, but not only on cDNA level.

Material and Methods

The Collection of Spider Venoms

The spider venoms were collected according to previously published methods [15,16]. Briefly, the venom was obtained by electrical stimulation of female spiders. Then the collected venoms were kept at −20 °C.

Purification of Antimicrobial Peptide

The spider venom sample (0.3 g) was dissolved in 10 ml of 0.1 M phosphate buffer solution (PBS), pH 6.0 containing 5 mM EDTA (total absorbance at 280 nm is 500). The venom sample was applied to a Sephadex G-50 (Superfine, Amersham Biosciences, 2.6 cm × 90 cm, Hong Kong, China) gel filtration column which was equilibrated with 0.1 M PBS. The elution was performed with the same PBS buffer. The absorbance of the elute was monitored at 280 nm. Every elution fraction (3.0 ml) was collected. The antimicrobial activity of fractions was determined as described

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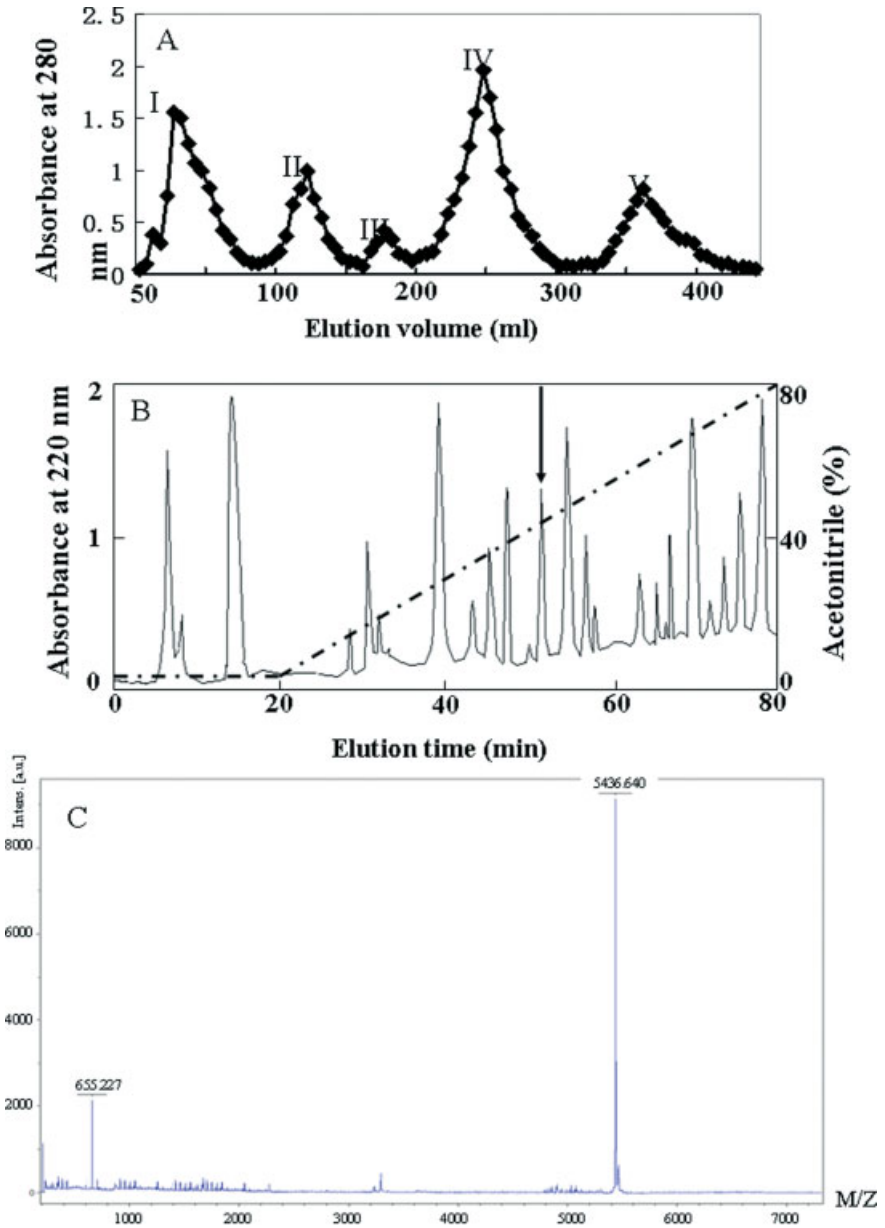

Figure 1. Purification of Oh-defensin from *O. hainana* venoms. (A) Sephadex G-50 gel filtration of *O. hainana* venom. *O. hainana* venom was applied on a Sephadex G-50 column equilibrated with 0.1 M PBS. Elution was performed with the same buffer, collecting fractions of 3.0 ml. Fraction III from Sephadex G-50 exerting antimicrobial activities was further purified on a C_8 RP-HPLC column equilibrated with 0.1% (v/v) trifluoroacetic acid/water. The elution was performed with the indicated gradient of acetonitrile in (B) at a flow rate of 0.7 ml/min, and fractions were tested for antimicrobial activity. The purified peptide is indicated by an arrow. (C) MALDI-TOF-MS analysis of purified peptide.

below. The protein peak containing antimicrobial activity was pooled, lyophilized, and resuspended in 5 ml 0.1 M PBS, and purified further with a C_8 RP-HPLC (Tigerkin C_8, 30 cm × 0.21 cm, Dalian Sipore Co., Ltd, Dalian, China) column. The elution was performed with the indicated gradient of acetonitrile in Figure 1(B) at a flow rate of 0.7 ml/min.

Structural Analysis

The purified peptide was subjected to aa sequencing by Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer, model 491. The Cys sulfhydryl group was alkylated by reaction with 4-vinyl pyridine according to the method described by Friedman *et al*. [17]. The actual molecular mass of the peptide was measured by MALDI-TOF-MS AXIMA CFR (Kratos Analytical) in a positive ion and linear mode. α-Cyano-4-hydroxycinnamic acid (CHCA) was used as matrix. The specific operating parameters were as follows: the ion acceleration voltage was 20 kV; the accumulating time of single scanning was 50 s. The polypeptide mass standard (Kratos Analytical) was used as external standard.

cDNA Library Construction

Forty venom reservoirs of *O. hainana* were dissected and collected. TRIzol reagent (Life Technologies, Ltd.) was used to extract total RNA from the collected venom reservoirs. The cDNA was synthesized using a SMART™ PCR cDNA synthesis

kit (Clontech, Palo Alto, CA, USA). For the first strand synthesis, two primers provided by the kit are 3′ SMART CDS Primer II A (5′-AAGCAGTGGTATCAACGCAGAGTACT (30) N-1N-3′ (N = A, C, G or T; N-1 = A, G or C)), and SMART II A oligonucleotide (5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′). The second strand was amplified using Advantage polymerase by 5′ PCR primer II A (5′-AAGCAGTGGTATCAACGCAGAGT-3′).

Screening of cDNA

The cDNA synthesized was used as template to screen the cDNAs encoding Oh-defensin. Two oligonucleotide primers, S_1 5-atg(t/c)t(a/t//c/g)tg(t/c)aa(a/g) (t/c)t(a/t//c/g)ag(t/c)atg-3′ in the sense direction, a specific primer designed according to the peptide sequence of Oh-defensin and primer II A as mentioned in 'SMART cDNA synthesis' in the antisense direction were used in PCR reactions. The DNA polymerase was advantage polymerase from Clontech. The PCR conditions were: 2 min at 94 °C, followed by 30 cycles of 10 s at 92 °C, 30 s at 50 °C, 40 s at 72 °C. Finally, the PCR products were cloned into pGEM®-T Easy vector (Promega, Madison, WI, USA). DNA sequencing was performed on an Applied Biosystems DNA sequencer, model ABI PRISM 377.

Antimicrobial Assays

Microorganisms used in antimicrobial assays included Gram-positive bacteria *Staphylococcus aureus* (ATCC2592), wild *Bacillus cereus* and *Shigella dysenteriae*, Gram-negative bacteria *Escherichia coli* (ATCC25922) *and Pseudomonas aeruginosa*, and fungus *Candida albicans* (ATCC2002) [18]. All of them were obtained from Nanjing Medical University as mentioned in our previous report. According to the methods described by Li *et al*. [5], microorganisms were first grown in Luria-Bertani (LB) broth to an $OD_{600\ nm}$ of 0.8. A 10 μl aliquot of the bacteria was mixed with 8 ml of fresh LB broth containing 0.7% agar and poured over a 90 mm Petri dish containing 25 ml of 1.5% agar in LB broth. After the top agar hardened, the test sample (20 μl) filtered by 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. If the sample examined had antimicrobial activity, a clear zone would appear on the surface of the top agar, which represents the inhibition of bacterial growth. MIC was determined in liquid LB medium by incubating bacteria in LB broth with variable amounts of the sample tested. The MIC at which no visible growth occurred was recorded.

Hemolysis Assays

According to the method reported by Bignami [19], hemolysis assays were performed using rabbit red blood cells (5×10^{10} cells/ml) in Alsever's solution (75 mM NaCl, 25 mM sodium citrate, 110 mM glucose, pH 6.1). Serial dilutions of the peptide were used, and after incubation at 37 °C for 30 min, the cells were centrifuged and the absorbance in the supernatant was measured at 540 nm. Maximum hemolysis (100%) was determined by adding 1% Triton X-100 to a sample of cells.

Results

Purification of Antimicrobial Peptides

The spider venom sample of *O. hainana* (0.3 g) was fractionated into five fractions by Sephadex G-50 as illustrated in Figure 1(A). Among these eluted peaks, fraction III was found to exert antimicrobial activities. This fraction was collected and purified further by RP-HPLC. As showed in Figure 1(B), more than 20 peaks were eluted from the C_8 RP-HPLC purification. Among these eluted peaks, the eluted peak indicated by an arrow in Figure 1(B) was found to exert antimicrobial activities. This peak was collected, pooled, and studied further. The homogeneity of purified peptide was confirmed by MALDI-TOF-MS (Figure 1(C)). About 40 μg pure peptide could be obtained from a single purification step.

N-terminus Partial aa Sequence

The purified antimicrobial peptide was named Oh-defensin and subjected to aa sequence analysis by automated Edman degradation. The N-terminus partial aa sequence of Oh-defensin was determined as MLCKLSMFGAVLGVPACAIDCLPMGKTGGSCEGGVCG. Mass spectrometry gave an observed mass of 5436.64 Da (Figure 1(C)).

cDNA Cloning

By cDNA screening, a cDNA clone encoding the precursor of Oh-defensin was obtained and sequenced. The cDNA sequence and deduced aa sequence of precursor were shown in Figure 2. This precursor is composed of a predicted signal peptide sequence, an N-terminal spacer peptide region containing several Asp and Glu residues, and mature Oh-defensin at the C-terminus. There is a dibasic site (–KR–) for trypsin-like enzymes processing that is positioned between spacer peptide region and mature Oh-defensin (Figure 2). The aa sequence deduced from the cDNA sequence encoding Oh-defensin was identical to the sequence determined by Edman degradation. By BLAST search, Oh-defensin showed similarity with other arthropod defensins. The sequence comparison of Oh-defensin with defensins from wasp, tick, and scorpion was shown in Figure 2(B). There are six half-Cys residues in Oh-defensin which are probable to form three disulfide bridges. The observed mass (5436.64) by mass spectrometry analysis matched well with the theoretical average molecular weight (5436.64) of Oh-defensin with three disulfide bridges and acidic form C-terminus. Defensins were also cloned and sequenced from the spider species: *Cupiennius salei, Phoneutria reidyi, Polybetes pythagoricus, Tegenaria atrica,* and *Meta menardi* [10]. The deduced aa sequences also contain the characteristic six Cys residues forming three disulfide bridges and reveal precursors of 60 or 61 aa residues. Comparatively, their mature peptides consist of only 37 aa residues, whereas the Oh-defensin has 52 residues.

Antimicrobial Activity and Hemolytic Activity

Oh-defensin showed strong antimicrobial activities against all the tested microorganisms including Gram-positive bacteria, Gram-negative bacteria, and fungus (Table 1). Among the tested microorganisms, it showed potent antimicrobial activities against *S. aureus, E. coli, Bacillus dysenteriae,* and *C. albcans* with MIC of 1.25 μg/ml. It had weakest antimicrobial ability against *B. cereus* (MIC 25 μg/ml). The antibiotic activity was proved to be lethal for the sensitive strain. The sensitive strains were not capable of resuming growth on agar plates after a 6-h treatment with concentrations above the corresponding MICs.

Some antimicrobial peptides reportedly have hemolytic activities [21,22]. Rabbit red blood cells were used to examine the sample's hemolytic capability. The result indicated that Oh-defensin had little hemolytic activity, only inducing 7% hemolysis

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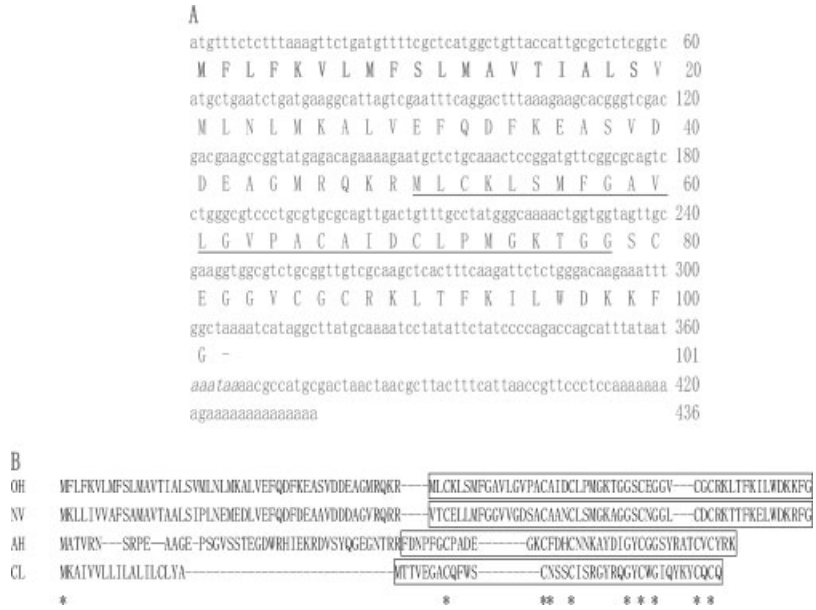

Figure 2. The nucleotide sequence encoding Oh-defensin from *O. hainana* and the deduced aa sequence of the precursor polypeptide, and its sequence comparison defensin precursors from wasp, tick, and scorpion. (A) The nucleotide sequence encoding Oh-defensin, the bar (—) indicates stop condon; the aa sequence determined by Edman degradation is underlined; the predicted signal peptide is bolded; the polyadenylation signal is italic. (B) The precursor sequence comparison of Oh-defensin and other defensins. The star (*) indicates the identical aa residues. The mature peptides are boxed. OH, NV, AH, and CL means the defensin precursor from *O. hainana, Nasonia vitripennis* [17], *Amblyomma hebraeum* [13], and *Centruroides limpidus limpidus* [20], respectively.

Table 1. Antimicrobial activity of Oh-defensin

Microorganism	MIC (μg/ml)[a]
	Oh-defensin
Gram-positive bacteria	
S. aureus	1.25
B. cereus	25.0
Gram-negative bacteria	
E. coli	1.25
B. dysenteriae	1.25
P. aeruginosa	5.0
Fungus	
C. albicans	1.25

[a] MIC, minimal peptide concentration required for total inhibition of cell growth in liquid medium. These concentrations represent mean values of three independent experiments performed in duplicates.

at the concentration up to 200 μg/ml (about 36 μM) compared with 1% Triton X-100 which could induce 100% hemolysis.

Discussion

Currently, approximately 500 venom peptides from 60 spider species with molecular weight of less than 10 kDa are characterized and divided into 20 families [1]. Most of spider venom peptides contain six or eight half-Cys residues to form three to four disulfide bonds but they have different disulfide bond motifs. Most of these disulfide-containing peptides exhibit neurotoxic properties.

As one of the most important antimicrobial peptide families, defensins are widely expressed in animals, plants, and fungi. Previous works have reported the antimicrobial activities of the spider venom components, such as the linear peptides free of disulfide bonds, lycotoxins [20]. The current work identified and characterized a novel defensin-like antimicrobial peptide (Oh-defensin) from venoms of the spider, *O. hainana*. Oh-defensin showed strong antimicrobial activities against all tested microorganisms (Table 1), with MIC as low as 1.25 μg/ml toward four out of the total six tested microorganisms. Therefore, Oh-defensin may provide an excellent candidate to develop anti-infective agents.

Interestingly, Oh-defensin shows significant sequence similarity with defensin from wasp venoms, but a bit similarity with tick or scorpion defensins (Figure 2(B)). It appears that the Oh-defensin is structurally much closer to insect defensin although it is identified from arachnid. To explain this unusual phenomenon, more spider defensins needs to be identified in order to reveal the evolution relationship between spider defensin and other arthropod defensins.

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