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Complete *de novo* sequencing of antimicrobial peptides in the venom of the scorpion *Isometrus maculatus*

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ABSTRACT

Scorpion venom contains antimicrobial peptides (AMPs) in addition to neurotoxic peptides. Recent extensive transcriptomic analysis of venom glands of various scorpion species dramatically increased the number of identified AMPs. However, identification of peptides from genetic information requires reference sequences of similar peptides, and moreover, it is often difficult to predict post-translational modifications. In this study, we searched for unknown AMPs contained in the *Isometrus maculatus* scorpion venom based on the structural features, such as the hydrophobic nature and the lack of disulfide bonds, which are commonly observed in the majority of scorpion AMPs. Their primary structures were determined only by a *de novo* sequencing method using two types of MS instruments, which induce peptide fragmentation in a different fashion. Chemical derivatization techniques were also used to facilitate Leu/Ile discrimination. As a result, total 15 AMP candidates were discovered from the *I. maculatus* venom. Among them, three peptides were identified as AMPs by evaluating their biological activity. Other 12 candidate peptides were structurally related to the identified AMPs, possibly generated by enzymatic cleavage of the mature peptides in the venom.

1. Introduction

Scorpion venom is a rich source of bioactive peptides. While many of them have been shown to have neurotoxicity toward mammals, insects or crustaceans presumably playing important roles in the capture of preys or in the defense against predators (de la Vega et al., 2013), substantial number of antimicrobial peptides (AMPs) have been identified from the scorpion venom in recent years (Harrison et al., 2014). It is assumed that their primary role is to protect the venom gland from microbial infection (Gao et al., 2007), but they are likely to make a significant contribution to the insect toxicity of the venom and/or to the enhancement of the actions of other neurotoxins in the venom by their cellular membrane-disrupting capacity (Corzo et al., 2002). Thus, scorpion AMPs attract increasing interest due to their diverse biological functions and potential therapeutic applications.

Recent extensive transcriptomic analysis of venom glands from various scorpion species dramatically increased the number of identified AMPs (Kazemi-Lomedasht et al., 2017; NaderiSoorki et al., 2016; Santibanez-Lopez et al., 2016; Zhong et al., 2017). Particularly, next-generation DNA sequencing technologies have greatly accelerated the identification of the AMPs, where nucleotide sequences encoding the peptides of interest can be obtained by similarity searches with reference sequences in the databases. However, the current databases do not necessarily cover all of the existing peptides. In the cases where similar peptides have never been identified, sequence information must be obtained directly from the peptides. Moreover, many peptides undergo post-translational modifications, and it is difficult to predict mature sequences based only on the gene-based information.

Regarding the peptide sequencing techniques, Edman degradation have long provided the most robust and reliable results. Meanwhile, tandem mass spectrometry (MS)-based sequencing approaches (*de novo* sequencing) are more widely used recently as a primary tool for determination of peptide structures due to its higher sensitivity and higher throughput, compared to Edman degradation (Medzihradszky and Chalkley, 2015). However, MS/MS analysis does not always provide all fragment ions necessary for

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sequence determination. Therefore, *de novo* sequencing is currently applied in combination with Edman degradation and/or genetic analysis in most cases.

To improve the reliability and efficiency of the *de novo* sequencing method, we recently developed a chemical derivatization technique, in which the N-terminus of peptides was modified with a chemical tag having high proton affinity (Miyashita et al., 2011). Accordingly, the fragmentation behavior of peptides under low-energy collision-induced dissociation (LE-CID) conditions was greatly affected to produce more interpretable product ion spectra. This derivatization was also effective for the discrimination between isobaric Leu and Ile residues, by virtue of the enhanced side-chain fragmentation under the high-energy CID (HE-CID) conditions (Kitanaka et al., 2016). Thus, these successful results led us to deal with structural characterization of the peptide components in scorpion venoms by using the MS-based *de novo* sequencing technique alone.

A Buthidae scorpion, *Isometrus maculatus* is found in tropical regions throughout the world including Japan, and have been demonstrated to produce several peptidic insecticidal toxins in the venom (Ichiki et al., 2012; Kawachi et al., 2013). Besides, it has been shown that the venom of *I. maculatus* contains an antimicrobial peptide, imcroporin, as a consequence of cDNA library screening of the venom gland based on the sequence similarity to other known AMPs (Zhao et al., 2009). We also identified Im-1 consisting of 56 amino acid residues from the same venom, which was shown to have antimicrobial activity as well as insecticidal toxicity (Miyashita et al., 2010), and thus, the venom of *I. maculatus* is of considerable interest as an important source of AMPs. In this study, we searched for unknown AMPs in the *I. maculatus* venom based on the structural features that are commonly observed in scorpion AMPs. The primary structures of candidate peptides were determined only by using the *de novo* sequencing method assisted by chemical derivatization. Their biological activity was evaluated after structural determination to confirm their identity as AMPs. As a result, we identified three AMPs from the *I. maculatus* venom. We also demonstrate the existence in the same venom of other peptides composed of the partial sequences of these AMPs, which are possibly generated by enzymatic cleavage of the mature AMPs in the venom.

2. Method

2.1. Collection of venom

The scorpions *I. maculatus* were collected in Ishigaki Island, located at the southern end of the Ryukyu islands in Japan. They were reared in the laboratory under dry conditions at 25 °C, and fed crickets. For venom collection, each scorpion was placed in a cage, and stimulated to sting a sheet of Parafilm set on a wire frame by gently touching its side of the mesosoma with forceps. The venom secreted on Parafilm was dissolved in aqueous 2% acetic acid and filtered, which was lyophilized and stored at −80 °C. Approximately 1 mg of the venom was obtained by collecting the secretion of fifty stings. The venom obtained from female specimens was used in this study because the relative abundance of each component is known to be different between male and female venoms in several scorpion species (Cid Uribe et al., 2017; Rodriguez-Ravelo et al., 2015). Distilled water was added to the lyophilized venom (8.9 mg), and after vortex the mixture was centrifuged 10,000 × g for 10 min to obtain a water-soluble fraction, which was used for HPLC fractionation.

2.2. Antimicrobial activity

Antimicrobial activity was measured using Gram-negative bacteria, *Escherichia coli* NBRC 3972, and Gram-positive bacteria, *Staphylococcus aureus* NBRC 13276 and *Bacillus subtilis* NBRC 3009 (NITE Biological Resource Center, Chiba, Japan) by a liquid growth inhibition assay. Each bacteria strain was grown in liquid LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl). Minimal inhibitory concentrations (MICs) were determined using a 2-fold microtiter broth dilution assay. Aliquots of each sample (10 μl) were incubated with 90 μl of a suspension of a mid-logarithmic phase culture of bacteria in a 96-well plate at a starting absorbance of A_{595} = 0.001 in LB medium for 20 h at 37 °C with continuous shaking. Inhibition of growth was monitored by measuring the absorbance at 595 nm using a Benchmark microplate reader (Bio-Rad, Hercules, CA). Experiments were performed in duplicate and repeated at least three times. Distilled water or Im-1 (Miyashita et al., 2010) were used as negative and positive controls, respectively. MICs are expressed as the interval of concentrations, [a]-[b], where [a] is the highest concentration at which bacteria still grow, and [b] the lowest concentration causing 100% growth inhibition.

2.3. Insect toxicity test

Insect toxicity was tested using crickets (*Acheta domestica*, ~50 mg body weight) by injection of 1–2 μl of each sample dissolved in distilled water into their abdominal region. Distilled water was injected as a control. For each measurement, 10 animals were used, and the number of dead animals was counted 24 h after injection. The doses required to induce death in half of the test animals (LD_{50}) were determined using a statistical software PRISM (GraphPad Software, La Jolla, CA).

2.4. Insect cell lytic activity

Insect cell lytic activity was evaluated using *Spodoptera frugiperda* (Sf9) cells. Sf9 cells were cultured at 25 °C in EX-CELL™ 420 medium supplemented with 10% fetal bovine serum (SAFC Biosciences, Lenexa, KS, USA). The cells were incubated with the peptides in a microtube for 1 h at 25 °C. The mixture was transferred to a 96-well plate, and cell lytic activity was measured using the CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega, Madison, WI) according to manufacturer's instructions. Luminescent signals were measured using a GLOMAX luminometer system (Promega). Experiments were repeated three times. Distilled water or 0.1% Triton X-100 were used as the negative and positive controls, respectively.

2.5. Hemolytic activity

Hemolytic activity was measured according to the literature (Takei et al., 2013). Fresh sheep red blood cells (sRBCs) were washed three times with PBS (35 mM phosphate buffer and 150 mM NaCl, pH 7.2) by centrifugation at 2000 × g for 5 min and resuspended in PBS. Aliquots of each sample in PBS (50 μl) were added to 50 μl of sRBC suspension [4% (v/v) in final] in a microtube, and incubated for 1 h at 37 °C. The samples were centrifuged at 2000 × g for 5 min, and the supernatant was transferred to a 96-well plate to monitor hemoglobin release by measuring the absorbance of supernatant at 450 nm using a Benchmark microplate reader. PBS or 0.1% Triton X-100 were used as the negative and positive controls, respectively.

2.6. HPLC fractionation

The water-soluble fraction of the *I. maculatus* venom was applied to a C4 semipreparative RP-HPLC column (10 × 250 mm, Grace Vydac, Hesperia, CA, USA). The column was eluted with 0.1%