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Antimicrobial Activity of the Scolopendrasin V Peptide Identified from the Centipede *Scolopendra subspinipes mutilans*

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In a previous study, we analyzed the transcriptome of *Scolopendra subspinipes mutilans* using next-generation sequencing technology and identified several antimicrobial peptide candidates. One of the peptides, scolopendrasin V, was selected based on the physicochemical properties of antimicrobial peptides using a bioinformatics strategy. In this study, we assessed the antimicrobial activities of scolopendrasin V using the radial diffusion assay and colony count assay. We also investigated the mode of action of scolopendrasin V using flow cytometry. We found that scolopendrasin V's mechanism of action involved binding to the surface of microorganisms via a specific interaction with lipopolysaccharides, lipoteichoic acid, and peptidoglycans, which are components of the bacterial membrane. These results provide a basis for developing peptide antibiotics.

Keywords: Antimicrobial peptide, membrane damage, bacterial membrane, scolopendrasin V, *Scolopendra subspinipes mutilans*

Introduction

The centipede *Scolopendra subspinipes mutilans* has been used as an herbal oriental medicine [1–3]. However, bioactive molecules such as antimicrobial peptides (AMPs) of the centipede have not been extensively studied. Earlier, a few AMPs were isolated from the centipede venom [4, 5], and a recent study identified venom proteins and peptides from venom cDNA libraries and extractions [6]. Previously, we analyzed the transcriptome of this centipede via RNA sequencing and identified large numbers of AMP candidates using bioinformatics tools based on the physicochemical properties of AMPs [7]. The selected AMPs showed various biological activities, such as antimicrobial [8, 9], antifungal [10–13], anticancer [14], and immunomodulatory activities [15].

AMPs play an important role in the innate immunity of invertebrates and vertebrates [16]. AMPs have common features, such as low molecular weight, amphipathic structure, and net positive charge [17]. According to their structure, AMPs can be divided into four groups: α-helices, β-sheet, extended, and loop [18, 19]. These diverse AMPs are used to develop novel antibiotics for antibiotic-drug-resistant bacteria because of their rapid action and selectivity [20, 21]. Most of the AMPs are mainly targeting the lipid bilayer of the bacterial membrane. The proposed mechanisms of antimicrobial activity for AMPs are the carpet, barrel-stave, and toroidal mechanisms [22]. These membranolytic activities involve interaction between cationic AMPs and anionic cell surface components, such as phospholipids in the outer membrane (*i.e.*, phosphatidylglycerol and cardiolipin) or lipopolysaccharide (LPS) and lipoteichoic acid (LTA) [23].

We previously identified that the scolopendrasin I, II, and VII peptides derived from the centipede transcripts exhibit antimicrobial and anticancer activities [8, 9, 14]. Here, we investigated the antimicrobial activities of the scolopendrasin V peptide against various bacteria, including acne-associated microbes and yeast. We also determined the binding properties of scolopendrasin V to the components on the surface of the microbial cell membrane. In addition, we found that the antimicrobial activity of scolopendrasin V is attributed to its membranolytic activity, using flow

cytometry analysis. This peptide can be used as a potential AMP candidate to develop antibiotic agents.

Materials and Methods

Peptide

The scolopendrasin V peptide was synthesized using the solid-phase peptide synthesis method by Anygen (Gwangju, Korea). The peptide was dissolved in acidified distilled water (0.01% acetic acid) and stored at −20°C until use.

Antimicrobial Activity

The antimicrobial activity of each peptide was tested by radial diffusion assay and colony count assay. For the radial diffusion assay [24], a stock peptide solution was prepared in acidified distilled water (0.01% acetic acid) and 5 µl samples were introduced as five serial 2-fold dilutions. The concentrations ranged from 6.25 to 200 µg of peptide/ml and were loaded into the wells (3 mm in diameter) in the underlay, in which washed mid-logarithmic phase bacteria were trapped. The underlay agar consisted of 9 mM sodium phosphate, 1 mM sodium citrate buffer, 1% (w/v) agarose (Sigma, USA), and 0.3 mg of tryptic soy broth (TSB) (Difco, USA). After incubation at 37°C for 3 h, a 10 ml overlay agar containing 1% agarose and 6% TSB was poured onto the underlay agar. After the plates were incubated overnight to allow surviving microbes to form colonies, the diameters of clearing zones, indicating antimicrobial activity, were plotted against the peptide concentrations.

For the colony count assay, scolopendrasin V was mixed with mid-logarithmic phase bacteria or yeast in a sterile 10 mM sodium phosphate buffer (pH 7.4) according to the predetermined concentrations. Mixtures were incubated for 1 h at 37°C in a shaking incubator. After incubation, 10 µl aliquots were directly, or after 10 times dilution with the buffer, removed and plated on tryptic soy bacto-agar (1.5% in TSB). The resulting colonies were counted after an overnight incubation. The tested microorganisms were *Propionibacterium acnes* (KCTC3314), *Staphylococcus epidermidis* (KACC13234), *Pseudomonas aeruginosa* (KCTC1750), *Escherichia coli* (KACC13821), *Staphylococcus aureus* (KACC10768), *Candida albicans* (KCTC7121), and multidrug-resistant *Pseudomonas aeruginosa* (MDRPA) (CCARM2002). All microorganisms were purchased from the Culture Collection of Antibiotic-Resistant Microbes (CCARM) at Seoul Women's University, the Korean Agricultural Culture Collection (KACC), and the Korean Collection for Type Cultures (KCTC).

Flow Cytometry Analysis

The integrity of the membranes after peptide treatment was determined by flow cytometry after nuclear staining with propidium iodide (PI) (Sigma, USA). In brief, microbes were harvested after being cultured overnight or for 3 h, and then washed twice with 10 mM sodium phosphate buffer (pH 7.4). The washed cells (1×10^7 cells) were mixed with peptides at a concentration of 50 µg/ml in 10 mM sodium phosphate buffer, and then incubated at 37°C for 30 min. The peptide-treated cells were incubated in PI solution (50 µg/ml) for 5 min at room temperature. After incubation, the unbound dye was removed via the excessive washing of cells with 10 mM sodium phosphate buffer. The uptake of PI into microbial cells was analyzed with a CytoFLEX flow cytometry analyzer (Beckman Coulter, USA).

Microbial Cell Membrane Binding Assay

The binding of scolopendrasin V to the surface of microbes was assessed as the effect of the cell-membrane components on the anti-*E. coli* activity of scolopendrasin V, using a radial diffusion assay. One microgram of scolopendrasin V was incubated with varying concentrations of LPS, LTA, peptidoglycan (PGN), laminarin, or mannan for 10 min at 37°C in 10 mM sodium phosphate buffer (pH 7.4). Then, 5 µl samples of each mixture were loaded into wells (3 mm diameter) that had been punched into underlay agar containing washed mid-logarithmic *E. coli* (4×10^6 colony-forming units). After incubation at 37°C for 3 h, a 10 ml overlay agar containing 1% agarose and 6% TSB was poured onto the underlay agar. After the plates were incubated overnight to allow surviving microbes to form colonies, the diameters of clearing zones, indicating antimicrobial activity, were plotted.

Results and Discussion

Peptide

Scolopendrasin V (sequence: YYGGGYKYKHWGCR-NH_2) was identified from the transcriptome analysis of *Scolopendra subspinipes mutilans* and had a net charge of +3 with a theoretical isoelectric point (pI) of 9.43. The primary amino acid sequence and molecular mass of the scolopendrasin V are shown in Table 1. Previously, the α-helical peptides scolopendrasin I, II, and VII have shown potent antimicrobial and anticancer activities [8, 9, 14]. Thus, we proposed that scolopendrasin V could be a valuable template for designing an antimicrobial peptide.

Antimicrobial Activity of Scolopendrasin V

We evaluated the antimicrobial activity of scolopendrasin V against acne-associated microbes, including *P. aeruginosa*, in a radial diffusion assay. Scolopendrasin V showed effective antibacterial activity against *P. acnes* and *P. aeruginosa*. However, scolopendrasin V had relatively weak antibacterial

Table 1. Primary sequence and molecular mass of scolopendrasin V.

Amino acid sequence	Mass (Da)	
	Measured	Theoretical
YYGGGYKYKHWGCR-NH_2	1,737.6	1,737.9

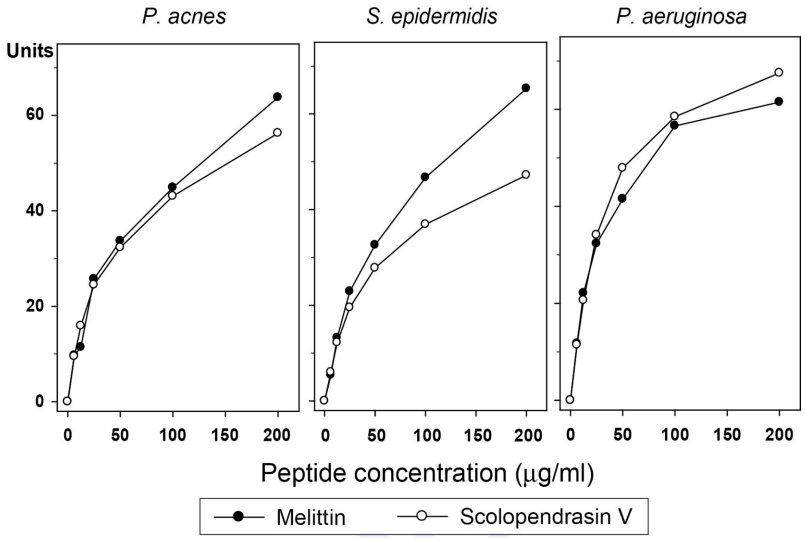

Fig. 1. Antimicrobial activities determined by radial diffusion assay.
The new peptide scolopendrasin V showed antimicrobial activity against microorganisms. Diameters of clearing zone are expressed in units (1 mm = 10 units).

activity compared with melittin against *S. epidermidis* (Fig. 1). In particular, scolopendrasin V displayed more potent antibacterial activity against gram-negative *P. aeruginosa*. Additionally, we assessed the antimicrobial activity of scolopendrasin V against mid-logarithmic phase *E. coli*, *S. aureus*, *C. albicans*, and MDRPA using the colony count assay (Fig. 2). The result showed that scolopendrasin V had broad antimicrobial activities against various microbes. The tendency of antimicrobial activities is consistent with our previous works [8, 9]. Scolopendrasin peptides revealed strong antimicrobial activities against gram-negative bacteria, including antibiotic-drug-resistant gram-negative bacteria, but the peptides had less potent antimicrobial activities against gram-positive *Staphylococcus* species. Interestingly, the scolopendrasin peptides displayed more potent antimicrobial activity against *P. acnes* that causes *P. acnes*-induced dermatitis. Thus, scolopendrasin peptides, including scolopendrasin V, could be applied to *P. acnes*-induced dermatitis, although further studies using animal models are needed to clarify this.

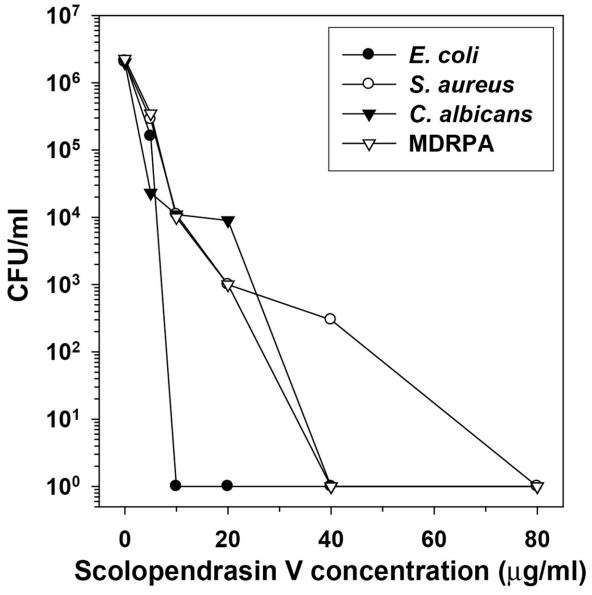

Fig. 2. Colony count assay for antimicrobial activities of scolopendrasin V against microorganisms.
The medium contained 10 mM sodium phosphate buffer (pH 7.4) and mid-logarithmic bacteria or yeast of the predetermined colony-forming unit (CFU). Instead of peptide, an equivalent volume of 0.01% acetic acid was added to each sample tube for the control. MDRPA, Multidrug-resistant *Pseudomonas aeruginosa*.

In addition to its antimicrobial activity, scolopendrasin V does do not induce hemolysis against rat red blood cells, even at the highest concentration (100 μg/ml) from our previous study [7]. Thus, these results suggest that scolopendrasin V may not be detrimental to normal eukaryotic cells.

Effects of Scolopendrasin V on the Integrity of the Microbial Cell Membrane

We characterized the effects of scolopendrasin V on the integrity of cell membranes by detecting the PI staining of the cells, using flow cytometry (Fig. 3). Most of cells were labeled with PI, and the fluorescence intensity was

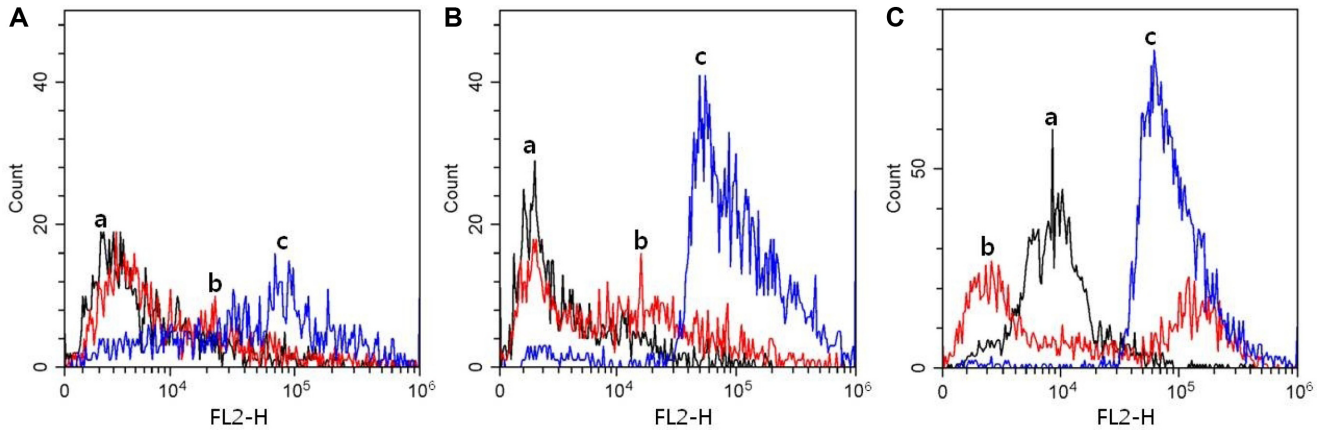

Fig. 3. Flow cytometry analysis.
Membrane permeability of (**A**) multidrug-resistant *Pseudomonas aeruginosa*, (**B**) *Staphylococcus epidermidis*, and (**C**) *Candida albicans*, detected by propidium iodide (PI) staining. Bacteria and yeast cells were treated with scolopendrasin V at 50 μg/ml or melittin at 50 μg/ml. The increments of the fluorescence intensity represent the uptake of PI by the cells. (a) Control, (b) scolopendrasin V, (c) melittin.

increased after treating the cells with scolopendrasin V (50 μg/ml). These results imply that the antimicrobial activity of scolopendrasin V is due to the disintegration of the cell membrane. The action mechanism of AMPs could be divided into membranolytic and non-membranolytic modes of action [25]. Scolopendin and scolopendin 2 peptides showed membrane-active mechanism against *C. albicans* [11–13] and scolopendrasin VII showed membranolytic activity against human leukemia cancer cell lines [14]. On the other hand, scolopendin 1 induced apoptosis without membrane damage against *C. albicans* [10]. These peptides were derived from *Scolopendra subspinipes mutilans* and it seems that their different mechanism of action may be due to their amino acid sequence and structure. Further structural analysis needs to be performed to better understand the precise mechanism of action.

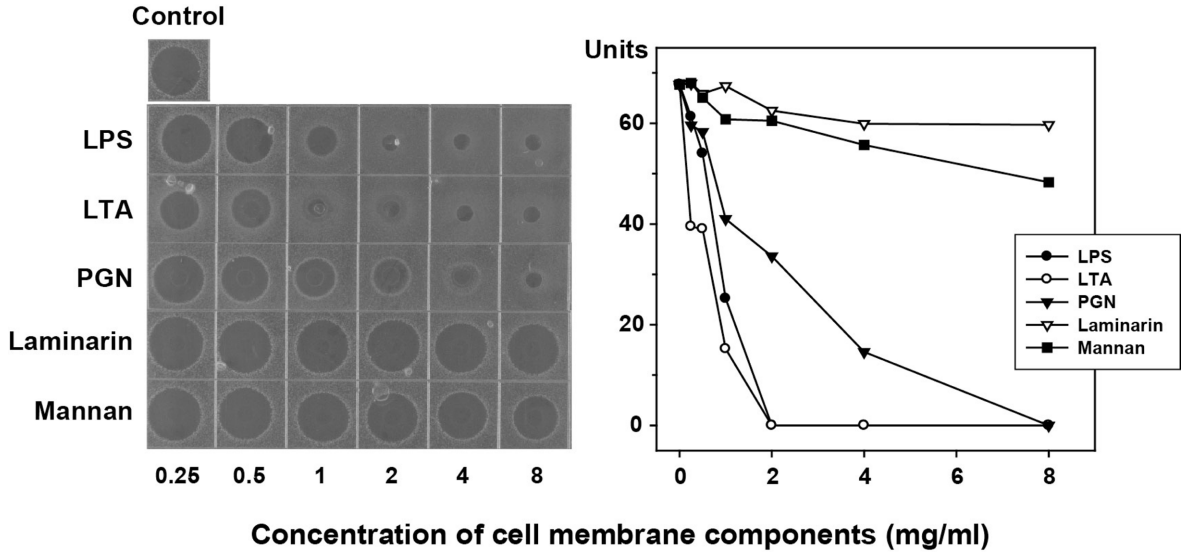

Fig. 4. Specific binding of scolopendrasin V to lipopolysaccharide (LPS), lipoteichoic acid (LTA), and peptidoglycan (PGN).
The binding/radial diffusion assay was conducted via the mixing of various amounts of LPS, LTA, PGN, laminarin, or mannan or with scolopendrasin V. The left panel shows a photograph of the gel in the radial diffusion assay. In the right panel, the anti-*E. coli* activities of scolopendrasin V in the mixtures were graphed against the concentration of cell membrane components. Diameters of clearing zone are expressed in units (1 mm = 10 units).

Specific Binding to a Component of the Bacterial Cell Membrane

The primary targets of AMPs are bacterial membranes, and this interaction is an essential step for antimicrobial activity with insertion and/or penetration [26, 27]. The interaction is induced by electrostatic attraction between cationic AMPs and negatively charged membrane components. These membrane components are also known as pathogen-associated molecular patterns (PAMPs). PAMPs consist of small molecules or repeat units with conserved motifs.

Thus, the specific binding of scolopendrasin V to the microbial surface was confirmed using several microbial membrane components. One microgram of scolopendrasin V was incubated with varying concentrations of LPS, LTA, PGN, laminarin, or mannan and the mixture was tested for anti-*E. coli* activity by the radial diffusion assay (Fig. 4). The anti-*E. coli* activity reduction of scolopendrasin V occurred in a concentration-dependent manner for LPS, LTA, and PGN. On the other hand, laminarin and mannan had little or no effect on the antibacterial activity of scolopendrasin V. Therefore, scolopendrasin V binds to bacteria by specifically binding to LPS, LTA, or PGN. These results indicate that anionic cell-surface membrane molecules are important for the initial process of electrostatic interaction.

We have demonstrated the antimicrobial activity of the scolopendrasin V peptide against various gram-negative and gram-positive bacteria, including acne-associated microbes and yeast. Scolopendrasin V showed broad-spectrum antimicrobial activity, and the peptide had more potent activity against the gram-negative bacteria. In addition, scolopendrasin V could interact with bacterial membrane components such as LPS, LTA, and PGN. These results provide a useful antimicrobial peptide candidate and an efficient strategy to develop new antimicrobial peptides.

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