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Scolopendrasin I: a novel antimicrobial peptide isolated from the centipede *Scolopendra subspinipes mutilans*

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Abstract

In a previous report, we identified several candidate antimicrobial peptides through *de novo* RNA sequencing of the centipede *Scolopendra subspinipes mutilans*. Here, we identify and characterize one of these peptides, Scolopendrasin I. We identified the centipede antimicrobial peptide Cecropin from the centipede transcriptome using an SVM algorithm, and subsequently analyzed the amino acid sequence for predicted secondary structure using a GOR algorithm. We identified an alpha helical region of Cecropin and named it Scolopendrasin I. We then assessed antimicrobial and hemolytic activity of Scolopendrasin I. Scolopendrasin I showed antimicrobial activity against various microbes, including antibiotic-resistant Gram-negative bacteria, in a radial diffusion assay. Scolopendrasin I had potent antibacterial activity against acne-associated microbes in a colony count assay and showed no hemolytic activity in a hemolysis assay. In addition, we confirmed that Scolopendrasin I bound to the surface of bacteria via a specific interaction with lipoteichoic acid and lipopolysaccharide, two components of bacterial cell membranes. In conclusion, the results presented here provide evidence that this is an efficient strategy for antimicrobial peptide candidate identification and that Scolopendrasin I has potential for successful antibiotic development.

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Introduction

The centipede *Scolopendra subspinipes mutilans* is widely used in traditional medicine in Asian countries for the treatment of a variety of diseases, including cancer and respiratory and cardiovascular disease (Ding *et al*., 1997, Namba *et al*., 1988, Pemberton, 1999). Nevertheless, only a few reports have been published regarding purification and characterization of therapeutically relevant proteins or peptides from the centipedes. Scolopendrin I and scolopin 1 and 2, purified from the venom of the centipede *Scolopendra subspinipes mutilans,* are among the few antimicrobial peptides (AMPs) that have been reported (Peng *et al*., 2010, Wenhua *et al*., 2006).

AMPs play important roles in the host defense mechanisms of most living organisms, including plants, insects,

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amphibians, and mammals. Important for innate immunity, AMPs possess potent antibiotic activity, protecting organisms against bacteria, fungi, and certain viruses. AMPs are typically small (less than 10 kDa), and their secondary structure consists of an α-helix, β-sheets, and extended and loop-structured peptides (Hancock and Chapple, 1999, Hwang and Vogel, 1998). They show amphipathic characteristics and have a positive net charge, with broad-spectrum antibiotic activity against a diverse array of microbes (Hancock and Chapple, 1999). Recently, AMPs have been recognized as novel antibiotic candidates with potential to overcome the limitations of conventional antibiotics and combat antibiotic-resistant bacteria (Jenssen *et al*., 2006). Some of these peptides have been used to develop novel drugs (Fjell *et al*., 2012). However, few AMPs have been clinically evaluated and screening of additional candidates is required for development of novel and effective antimicrobial compounds.

In this paper, we used next-generation sequencing (NGS) and algorithm analysis to identify and characterize a novel antimicrobial peptide, which we named "Scolopendrasin I" from the transcriptome of *Scolopendra subspinipes mutilans*. This peptide has antimicrobial activities against bacteria, yeast, and antibiotic resistant bacteria, and displays no hemolytic activity. We also determined the specific binding partners of Scolopendrasin I with the microbial surface membrane.

Materials and Methods

Peptide synthesis

The Scolopendrasin I peptide was synthesized using solid-phase peptide synthesis methods by Anygen Co., Ltd. (Gwangju, Korea). The peptide was dissolved in acidified distilled water (0.01% acetic acid) and stored at -20°C.

Antimicrobial activity

The antimicrobial activity of each peptide was analyzed using a radial diffusion assay and colony count assay. For the radial diffusion assay (Steinberg and Lehrer, 1997), serial dilutions of peptide stock solution were prepared in acidified distilled water (0.01% acetic acid) by repeatedly diluting the solution two-fold. Resulting solutions ranged in concentration from 25 to 400 μg of peptide/mL. Dilutions were loaded into wells (3 mm in diameter) in the underlay of a gel, where 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. Plates were incubated overnight to allow surviving microbes to form colonies. The diameters of clearing zones were recorded as an indicator of antimicrobial activity and plotted against peptide concentration.

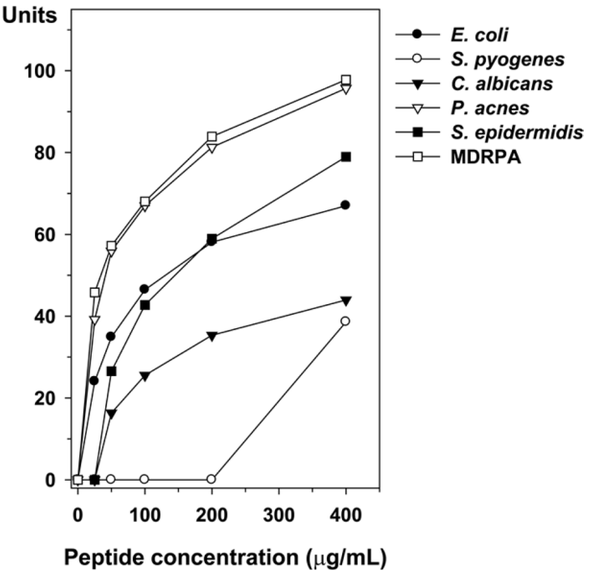

Fig. 1. Radial diffusion assay to determine antimicrobial activity. The novel peptide Scolopendrasin I showed antimicrobial activity against various microorganisms. Clearing zone diameter is expressed in units, where 1 mm = 10 units.

For the colony count assay, Scolopendrasin I was mixed with mid-logarithmic phase *Staphylococcus epidermidis* or *Propionibacterium acnes* in sterile 10 mM sodium phosphate buffer (pH 7.4) according at concentrations indicated in Fig. 2. For control, equivalent volumes of 0.01% acetic acid were added to each tube instead of peptide containing solution. Mixtures were incubated for 1 h at 37°C in a shaking incubator. After incubation, 10 μL aliquots were either directly removed and plated on tryptic soy bacto-agar (1.5% in TSB), or diluted 10 fold with buffer before removal and plating on TSB agar. After an overnight incubation, the resulting colonies were counted. The tested microorganisms in the radial diffusion assay were *Escherichia coli* (KACC13821), *Streptococcus pyogenes* (KACC11956), *Candida albicans* (KCTC7121),

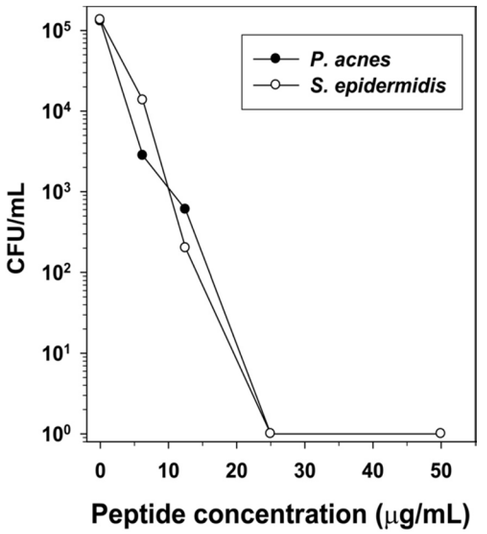

Fig. 2. Colony count assay for antibacterial activity of Scolopendrasin I against *P. acnes* and *S. epidermidis*. The medium contained 10 mM sodium phosphate buffer (pH 7.4) and mid-logarithmic bacteria of the pre-determined colony forming unit (CFU).

Propionibacterium acnes (KCTC3314), *Staphylococcus epidermidis* (KACC13234), and multidrug-resistant *Pseudomonas aeruginosa* (MDRPA) (CCARM2002). All microorganisms were purchased from the Culture Collection of Antibiotic-Resistant Microbes (CCARM, MDRPA) at Seoul Women's University, the Korean Agricultural Culture Collection (KACC, *Escherichia coli*, *Streptococcus pyogenes*, *Staphylococcus epidermidis*), or the Korean Collection for Type Cultures (KCTC, *Candida albicans*, *Propionibacterium acnes*).

Hemolytic activity

For the hemolytic assay, 20 μL of peptide solution at various concentrations (10, 20, 40, 80, 160, and 320 μg/mL) was added to 180 μL of a 2.5% (v/v) suspension of rat erythrocytes in phosphate buffered saline (PBS). Melittin (Sigma, USA), a hemolytic and α-helical peptide isolated from bee venom, was used as the positive control. The mixture was incubated for 30 min at 37°C, and then 600 μL of PBS was added to each tube. After 3 min of centrifugation at 10,000 × *g*, the supernatant was removed and absorbance was measured at 540 nm.

Binding assay for Scolopendrasin I to microbial cell membrane components

Potential binding of Scolopendrasin I to the surface of microbes was examined by assessing the effect of bacterial cell-membrane components on the anti-MDRPA activity of Scolopendrasin I using a radial diffusion assay. One microgram of Scolopendrasin I was incubated for 10 min at 37°C in 10 mM sodium phosphate buffer (pH 7.4) with varying concentrations of the following candidate bacterial membrane components: laminarin, mannan, lipopolysaccharide (LPS), or lipoteichoic acid (LTA). Subsequently, 5 μL samples of each mixture were loaded into wells (3 mm diameter) that had been punched into underlay agar containing washed mid-logarithmic MDRPA (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. The plates were incubated overnight to allow surviving microbes to form colonies and the diameters of clearing zones, indicating antimicrobial activity, were plotted.

Results and Discussion

Peptide identification and synthesis

We utilized a support vector machine (SVM) algorithm to identify a novel antimicrobial peptide. The "AMP predictor" tool (http://apps.sanbi.ac.za/dampd/) has been optimized to predict AMPs in various organisms including mammals, amphibians, and insects. We searched for novel AMPs using the AMP predictor and insecta taxonomy after searching homologs with

Table 1. Novel antimicrobial peptide identified by the SVM algorithm.

Unigene ID	AMP name	Length (nt)	NCBI ID	Description	E-value	Fold change	p-value
Unigene11177	Cecropin	300	XP_969514.1	15-hydroxyprostaglandin dehydrogenase [Tribolium castaneum]	4e-06	n/a[1)]	n/a[1)]

[1)] n/a:not available

Table 2. Primary sequence and molecular mass of Scolopendrasin I.

Amino acid sequence	Mass (Da)	
	Measured	Theoretical
KAVVKEFLKRKIKV-NH_2	1685.1	1685.2

several kinds of AMPs in the UniProtKB. The tool was based on SVM, which can classify a peptide into one of 27 AMP families. Cecropin was identified as a novel AMP (Table 1). Cecropin is an antimicrobial peptide that was originally identified in the moth, *Hyalophora cecropia* (Steiner *et al*., 1981). A template search from BLASTP showed 65% sequence identity with "15-hydroxyprostaglandin dehydrogenase" from *Tribolium castaneum* (Tribolium Genome Sequencing Consortium, 2008).

For peptide synthesis, we sought to select an α-helical region of the Cecropin amino acid sequence based on GOR algorithm prediction (version IV), a method for secondary structure prediction (Garnier *et al*., 1996). The GOR algorithm prediction revealed that Cecropin contained two α-helices at residues 10–20(KLHHIRKKIRM-NH_2) and residues 39–52(KAVVKEFLKRKIKV-NH_2). We prepared two synthetic peptides corresponding to these regions and tested their antibacterial activity using a radial diffusion assay (data not shown). Based on the results, we selected the longer, C-terminal α-helical sequence (14mer) and named it Scolopendrasin I. Scolopendrasin I had a net charge of +5 with theoretical p*I* of 10.58. The primary amino acid sequence and molecular mass of Scolopendrasin I are shown in Table 2.

In a previous study, our group identified an α-helical peptide named Scolopendrasin II and demonstrated its strong antibacterial activity (Kwon *et al*., 2013). In addition, we previously generated several antimicrobial peptides via transcriptome analysis using RNA sequencing and bioinformatics tools (Yoo *et al*., 2014). In this context, Scolopendrasin I might be a candidate for antimicrobial peptide design as a valuable template.

Antimicrobial and hemolytic activity of Scolopendrasin I

We evaluated the antimicrobial activity of Scolopendrasin I against Gram-positive and Gram-negative bacteria, including antibiotic-resistant Gram-negative bacteria, in a radial diffusion assay. Scolopendrasin I was effective against a broad range of microbes including Gram-positive and Gram-negative bacteria, yeast, and antibiotic-resistant bacteria, but had relatively weak antibacterial activity against *S. pyogenes* (Fig. 1). Notably, Scolopendrasin I displayed potent antibacterial activity against *P. acnes* and MDRPA. We also assessed antibacterial activity of Scolopendrasin I against mid-logarithmic phase *P. acnes* and *S. epdermidis* using colony count assay (Fig. 2). The data showed that Scolopendrasin I had strong antibiotic activity against these bacteria.

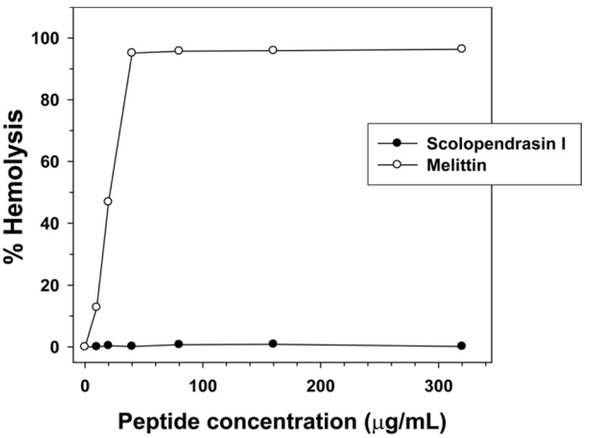

Fig. 3. Hemolytic activity of Scolopendrasin I in rat erythrocytes. Melittin was used as a control. Percentage hemolysis was calculated follows: % Hemolysis = (A_{540} of sample – A_{540} of peptide-free control)/(A_{540} of 100% control – A_{540} of peptide-free control) × 100.

In the hemolytic assay, Scolopendrasin I peptide had no evident hemolytic activity against rat red blood cells, even at the highest concentrations, while 40 μg/mL of the positive control Melittin lysed over 90% of the erythrocytes (Fig. 3). These results suggest that Scolopendrasin I may not be detrimental to normal eukaryotic cells.

Specific binding of Scolopendrasin I to components of bacterial cell membranes

Antibacterial peptides are the effector molecules of innate immunity and primarily bind to bacterial membranes (Boman, 2003). The specific binding capacity of Scolopendrasin I to the microbial surface was assessed by evaluating antibacterial activity of Scolopendrasin I in the presence of several microbial cell membrane components. One μg of Scolopendrasin I was incubated with varying concentrations of laminarin, mannan, LPS, or LTA, and the mixture was examined for anti-MDRPA activity using the radial diffusion assay (Fig. 4). The ability of Scolopendrasin I to inhibit MDRPA was clearly dependent on the concentration of LPS or LTA. These results indicated

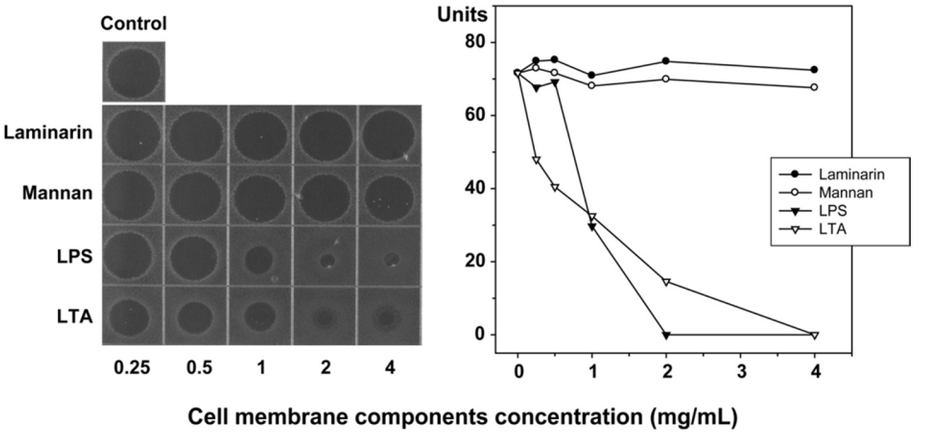

Fig. 4. Specific binding of Scolopendrasin I to lipopolysaccharide (LPS) and lipoteichoic acid (LTA). Binding/radial diffusion assay was conducted after the mixing of various amounts of laminarin, mannan, LPS, or LTA with Scolopendrasin I. The left panel shows images of gels from the radial diffusion assay. In the right panel, the anti-MDRPA activity of Scolopendrasin I in the mixture was graphed against concentration of indicated cell membrane components. Diameters of clearing zone have been expressed in units (1 mm = 10 units).

that LPS and LTA could interfere in the interaction between Scolopendrasin I and the MDRPA cell surface. In contrast, laminarin and mannan did not affect the antibacterial activity of Scolopendrasin I. Thus, we conclude that Scolopendrasin I binds to bacteria by specifically binding to LPS and/or LTA. Our previous study revealed that Scolopendrasin II also interacted with LPS and LTA (Kwon *et al*., 2013). Thus, anionic cell-surface membrane molecules are important for the initial process of electrostatic interaction (Yount and Yeaman, 2013). This indicates that it may exert antibacterial activities by causing the disintegration of the cell membrane.

In this study, we isolated the Scolopendrasin I peptide from centipede and demonstrated its antimicrobial activity against various microbes. Scolopendrasin I showed broad-spectrum antimicrobial activity, with potent activity against acne-associated microbes and antibiotic-resistant Gram-negative bacteria. Moreover, Scolopendrasin I had no hemolytic activity, and it interacted with bacterial membrane components LPS and LTA. These results identify and analyze a therapeutically relevant antimicrobial peptide candidate. Additionally, the method used is an efficient strategy for the development of novel antimicrobial peptides.

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