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Antimicrobial Activity of the Synthetic Peptide Scolopendrasin II from the Centipede *Scolopendra subspinipes mutilans*

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The centipede *Scolopendra subpinipes mutilans* is a medicinally important arthropod species. However, its transcriptome is not currently available and transcriptome analysis would be useful in providing insight into a molecular level approach. Hence, we performed *de novo* RNA sequencing of *S. subpinipes mutilans* using next-generation sequencing. We generated a novel peptide (scolopendrasin II) based on a SVM algorithm, and biochemically evaluated the *in vitro* antimicrobial activity of scolopendrasin II against various microbes. Scolopendrasin II showed antibacterial activities against gram-positive and -negative bacterial strains, including the yeast *Candida albicans* and antibiotic-resistant gram-negative bacteria, as determined by a radial diffusion assay and colony count assay without hemolytic activity. In addition, we confirmed that scolopendrasin II bound to the surface of bacteria through a specific interaction with lipoteichoic acid and a lipopolysaccharide, which was one of the bacterial cell-wall components. In conclusion, our results suggest that scolopendrasin II may be useful for developing peptide antibiotics.

Keywords: Antimicrobial peptide, SVM algorithm, α-helical, *Scolopendra subpinipes mutilans*

Introduction

The centipede is a kind of arthropod that belongs to the class Chilopoda, subphylum Myriapoda. There are ~7,000 species of centipedes in the world, and over 3,000 species have been described [1]. Among these centipedes, *Scolopendra subspinipes mutilans* (a relatively large centipede) has been used in oriental medicine for the treatment of various diseases [14]. Nevertheless, the pharmacological basis of the activity of this large centipede has yet to be understood thoroughly, and little is known about the antimicrobial peptides (AMPs) of the centipede. Only a few AMPs (*i.e.*, scolopendrin I and scolopin 1 and -2) from the venom of the centipede *S. subspinipes mutilans* have been reported [15, 20]. Both scolopin-1 and -2 demonstrated strong antimicrobial activities against gram-positive/negative bacteria and fungi [15]. In arthropods, it has been documented that evolutionarily conserved AMPs play a major role in mediating resistance to microbial infections [2].

AMPs are ubiquitous in nature, having been identified and isolated from various sources within living organisms [6, 16]. Over the past two decades, numerous antibiotic peptides have been studied from a wide variety of insects [4], amphibians [5], and mammals, including humans [13]. AMPs are composed of a small number of amino acid residues (below 10 kDa), and their secondary structure consists of an α-helical and/or β-sheet [10]. They show amphipathic characteristics and a positive net charge, with broad-spectrum activity against a diverse array of microbes, such as bacteria, fungi, and viruses [8]. There is an urgent need to develop alternative antimicrobial agents; recently, AMPs have been studied as candidate sources for novel antibiotics [11].

AMPs can be exploited using DAMPD [17] (formerly ANTIMIC [3]), which has recently been updated. Using the

Web server, a variety of AMP families can be predicted *en masse* and subsequently classified. In this study, we characterized a new antimicrobial peptide, scolopendrasin II, from the centipede *S. subpinipes mutilans* and confirmed its antimicrobial activity.

Materials and Methods

Peptide Synthesis

The synthetic peptide, named scolopendrasin II, 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 until use.

Antimicrobial Assays

The antimicrobial activity of each peptide was tested by radial diffusion assay, colony count assay, and the specific binding of scolopendrasin II to the cell-wall components. For the colony count assay, scolopendrasin II was mixed with mid-logarithmic phase *Staphylococcus epidermidis*, *Propionibacterium acnes*, or multidrug-resistant *Pseudomonas aeruginosa* (MDRPA) in a sterile 10 mM sodium phosphate buffer (pH 7.4) according to the pre-determined concentrations (Fig. 2). 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 *Candida albicans* (KCTC7121), *Escherichia coli* (KACC13821), *Propionibacterium acnes* (KCTC3314), *Streptococcus pyogenes* (KACC11956), *Staphylococcus epidermidis* (KACC13234), and 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).

Hemolytic Activity

For the hemolytic assay, 20 μl of the peptide sample at a predetermined concentration 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 600 μl of PBS was added to each tube. After 3 min of centrifugation at 10,000 ×*g*, the supernatant was removed, and the absorbance was measured at 540 nm.

Assay for Binding of Scolopendrasin II to the Components of the Bacterial Cell Wall

The binding of scolopendrasin II to the surface of bacteria was examined by assessing the effect of the cell-wall components on the anti-MDRPA activity of scolopendrasin II, using a radial diffusion assay [19]. One microgram of scolopendrasin II was incubated with varying concentrations of laminarin, mannan, lipopolysaccharide (LPS), or lipoteichoic acid (LTA) for 10 min at 37°C in 10 mM sodium phosphate buffer (pH 7.4). Then, 5 μl samples of each mixture was loaded into wells (3 mm diameter) that had been punched into the underlying agar containing washed mid-logarithmic MDRPA (4×10^6 colony-forming units). The underlay agar consisted of 9 mM sodium phosphate, 1 mM sodium citrate buffer, 1% (w/v) agarose (A6013, Sigma), and 0.3 mg of tryptic soy broth (TSB; Difco). 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.

Results and Discussion

Peptide

In order to discover a novel antimicrobial peptide, we used a support vector machine (SVM) algorithm operating on a database of 1,232 experimentally validated AMPs. The "AMP predictor" tool was optimized to predict AMPs in various organisms, including mammals, amphibians, and insects (http://apps.sanbi.ac.za/dampd/). In the present study, we sought to identify novel AMPs using an "AMP predictor" with a default threshold value using insecta taxonomy after searching for homologs of several kinds of AMPs in the UniProtKB. The tool was based on SVM, which can very faithfully classify a peptide into 1 out of 27 possible AMP families. Among 242 Unigenes, with no matches in the UniProtKB, a new AMP was identified as MCD mastoparan (Unigene70841) (Table 1). Mastoparan is

Table 1. Novel antimicrobial peptide identified by the SVM algorithm.[a]

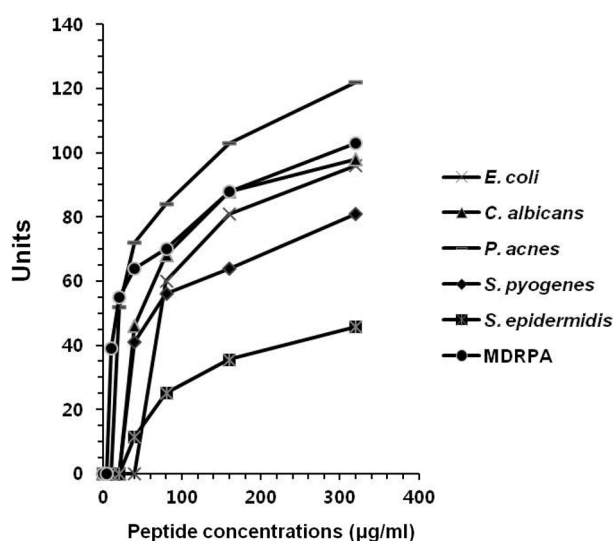| Unigene ID	AMP name	Length (nt)	NCBI ID	Description	E-value	Fold change	*p*-Value[b]	Instability index	p*I*
Unigene70841	MCD_mastoparan	417	XP_002787747.1	Signal recognition particle 19 kda protein [*Perkinsus marinus* ATCC 50983]	3e-22	-2.4529	0.0003	41.90	10.36

[a]Antimicrobial activity was predicted by models built through SVM using the "AMP Prediction" tool of DAMPD (http://apps.sanbi.ac.za/dampd/).
[b]*p*-value < 0.05.

a toxin peptide from wasp venom [12]. Unigene70841 encoded 93 amino acid residues. There were 3 cysteine residues in the sequence, but disulfide bonds could not be detected. It has a theoretical pI of 10.36 and 26 cationic amino acids. The instability index value was 41.90. A template search from BLASTP showed a 58% sequence identity with the "signal recognition particle 19 kDa protein" from *Perkinsus marinus* [18].
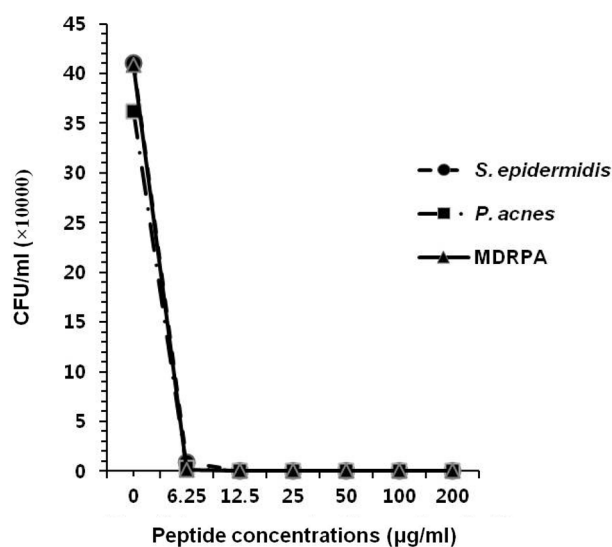
For peptide synthesis, we selected the α-helical region (because of the large molecular mass and instability), using the GOR algorithm (version IV [7]), which is a method used for secondary structure prediction. The results revealed that the sequence had two α-helical regions, at residues 10–17 (MTEVCAML-NH$_2$) and 57–77 (KYALMKKIAELI PNLKSRQVK-NH$_2$). Then, we prepared the two synthetic peptides and tested their antibacterial activity using a radial diffusion assay (data not shown). Finally, we chose the longer sequence of the C-terminal region (21 mer) and named it scolopendrasin II. Scolopendrasin II had a net charge of +5 with a theoretical pI of 10.30, and its calculated molecular mass was 2,472.07 Da.

Previously, it was reported that a defensin-like peptide, named Coprisin, was isolated from the dung beetle *Copris tripartitus*, and the α-helical region of Coprisin was identified to possess strong antibacterial activity [9]. Thus, it suggested that scolopendrasin II could be used as a valuable template for antimicrobial peptide design.

Fig. 2. Assessment of the antibacterial activities of scolopendrasin II against *S. epidermidis, P. acnes*, and MDRPA, by the colony count assay.

The medium contained 10 mM sodium phosphate buffer (pH 7.4) and predetermined CFU of mid-logarithmic phase bacteria. Instead of a peptide, an equivalent volume of 0.01% acetic acid was added to each tube in the control.

Antimicrobial and Hemolytic Activity of Scolopendrasin II

We assessed the effects of scolopendrasin II on various microbes using a radial diffusion assay. Whereas scolopendrasin II had relatively weak antibacterial activity

Fig. 1. Antimicrobial activities determined by radial diffusion assay.

A new peptide, scolopendrasin II, exhibited antimicrobial activity against select microorganisms. Diameters of the clearing zone are expressed in units (1 mm = 10 units).
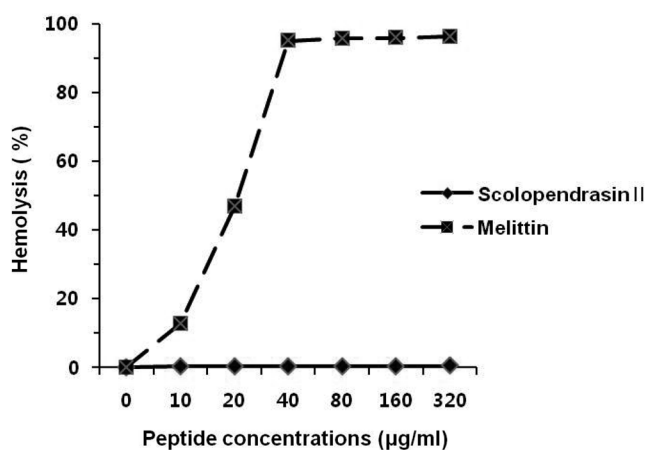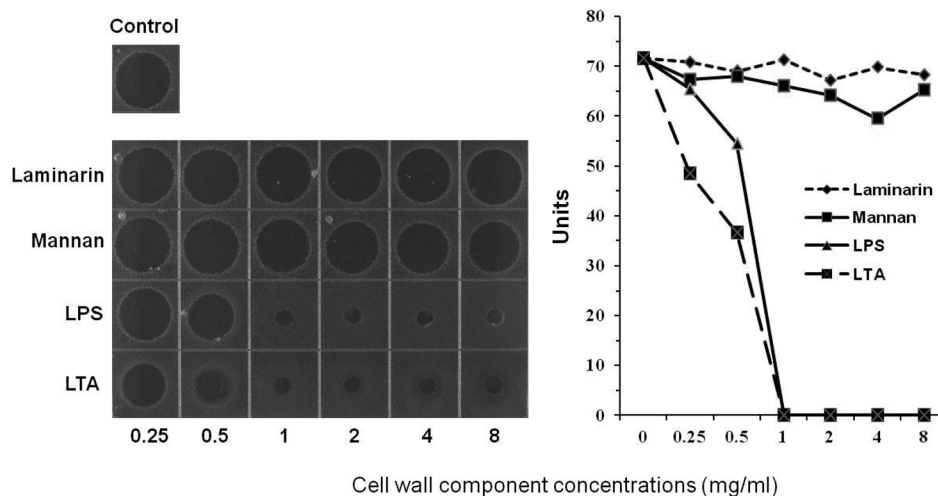

Fig. 3. Hemolytic activity for scolopendrasin II, tested against rat erythrocytes.

Melittin was used as the standard peptide. Percentage hemolysis was calculated by the following equation: Hemolysis (%) = [(A$_{540}$ of sample - A$_{540}$ of peptide-free control)/(A$_{540}$ of 100% control – A$_{540}$ of peptide-free control)] × 100.

Fig. 4. Specific binding of scolopendrasin II to LPS and LTA.

The binding/radial diffusion assay was conducted by mixing various amounts of LPS, LTA, mannan, or laminarin with scolopendrasin II.

against *S. epidermidis*, it was effective against a broad range of microbes, including gram-positive and gram-negative bacteria, yeasts, and antibiotic-resistant bacteria (Fig. 1). In particular, scolopendrasin II showed the strongest antibacterial activity against *P. acnes*. Additionally, we tested the antibacterial activity of scolopendrasin II against mid-logarithmic phase *S. epidermidis*, *P. acnes*, and MDRPA using a colony count assay (Fig. 2). The results indicated that scolopendrasin II exhibits strong activities against the tested bacteria. In the case of the *S. epidermidis* strain, scolopendrasin II appeared to exhibit weak activity according to the radial diffusion assay, but the peptide expressed strong activity as determined by the colony count assay, even at its lowest concentration.

In addition, scolopendrasin II exerted no hemolytic effects on rat erythrocytes, even at the highest concentrations tested, while 40 μg/ml of melittin, a cytolytic bee venom peptide, lysed >90% of erythrocytes (Fig. 3). Therefore, we assume that scolopendrasin II may not affect normal eukaryotic cells.

Specific Binding of Scolopendrasin II to the Bacterial Cell-Wall Components

The effects of several microbial cell-wall polysaccharides on the antibacterial activity of scolopendrasin II were tested to observe whether scolopendrasin II specifically binds to the microbial surface (Fig. 4). One microgram of scolopendrasin II was incubated with varying concentrations of laminarin, mannan, LPS, or LTA, and the mixture was examined for anti-MDRPA activity by the radial diffusion

assay. The anti-MDRPA activity of scolopendrasin II clearly declined with the elevation in the LPS or LTA level. In contrast, laminarin and mannan did not affect the antibacterial activity of scolopendrasin II. The results indicate that LPS and LTA can modulate the interaction between scolopendrasin II and the MDRPA cell surface. Thus, we conclude that scolopendrasin II binds to bacteria by specifically binding to LPS or LTA. This suggests that it may exert antibacterial effects by causing the disintegration of the cell membrane.

Acknowledgments

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