Biochimica et Biophysica Acta xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

# Scolopendin 2, a cationic antimicrobial peptide from centipede, and its membrane-active mechanism

Heejeong Lee [a,1], Jae-Sam Hwang [b,1], Jaeho Lee [c], Jae Il Kim [c], Dong Gun Lee [a,*]

[a] School of Life Sciences, BK 21 Plus KNU Creative BioResearch Group, College of Natural Sciences, Kyungpook National University, Daehak-ro 80, Buk-gu, Daegu 702-701, Republic of Korea
[b] Department of Agricultural Biology, National Academy of Agricultural Science, RDA, Jeonju, Republic of Korea
[c] School of Life Sciences, Gwangju Institute of Science and Technology, Oryong-dong, Buk-gu, Gwangju 500-712, Republic of Korea

ARTICLE INFO

*Article history:*
Received 10 June 2014
Received in revised form 4 November 2014
Accepted 17 November 2014
Available online xxxx

*Keywords:*
Scolopendin 2
*Scolopendra subspinipes mutilans*
Antimicrobial peptide
Membrane damage

ABSTRACT

Scolopendin 2 is a 16-mer peptide (AGLQFPVGRIGRLLRK) derived from the centipede *Scolopendra subspinipes mutilans*. We observed that this peptide exhibited antimicrobial activity in a salt-dependent manner against various fungal and bacterial pathogens and showed no hemolytic effect in the range of 1.6 μM to 100 μM. Circular dichroism analysis showed that the peptide has an α-helical properties. Furthermore, we determined the mechanism(s) of action using flow cytometry and by investigating the release of intracellular potassium. The results showed that the peptide permeabilized the membranes of *Escherichia coli* O157 and *Candida albicans*, resulting in loss of intracellular potassium ions. Additionally, bis-(1,3-dibutylbarbituric acid) trimethine oxonol and 3,3′-dipropylthiacarbocyanine iodide assays showed that the peptide caused membrane depolarization. Using giant unilamellar vesicles encapsulating calcein and large unilamellar vesicles containing fluorescein isothiocyanate-dextran, which were similar in composition to typical *E. coli* O157 and *C. albicans* membranes, we demonstrated that scolopendin 2 disrupts membranes, resulting in a pore size between 4.8 nm and 5.0 nm. Thus, we have demonstrated that a cationic antimicrobial peptide, scolopendin 2, exerts its broad-spectrum antimicrobial effects by forming pores in the cell membrane.



## 1. Introduction

Microbial resistance to antibiotics has increased in recent years, resulting in the invalidation of major antimicrobial drugs used in clinical settings [1]. Resistance to various antibiotics may be due to failure of the drug to interact with its target, efflux of the antibiotic from the cell, or direct destruction or modification of the drug [2,3]. The discovery of novel, broad-spectrum antibiotics with rapid antimicrobial effects and the ability to limit the induction of microbial resistance may not keep up with the pace at which pathogens are developing resistance [4,5].

One way to overcome the problem of antibiotic resistance is to use antimicrobial peptides (AMPs), which can be isolated from natural sources and are effective against a broad range of microorganisms, including Gram-positive and Gram-negative bacteria, fungi, and viruses [4]. AMPs play a significant role in the defense systems of higher organisms such as plants, insects, arthropods, amphibians, and mammals [6]. Cell viability is maintained by preserving the structure of the cell wall or cytoplasmic membrane, regardless of DNA/protein inhibition by single or dual mechanism(s). While their antimicrobial properties have only recently been identified, AMPs have received attention as novel chemotherapeutics, intracellular signaling molecules, and antitumor agents [5].

AMPs from venom, such as melittin from bees and hadrurin from scorpions, have been reported previously [6,7]. Centipedes are venomous arthropods that have been used in traditional medicine to treat several ailments, including carbuncles and neoplasms [8]. Extracts from centipedes have been reported to contain antibacterial components and toxins with anticoagulant properties [6]. In a previous study, we identified an antimicrobial peptide, scolopendin 1, from the centipede *Scolopendra subspinipes mutilans* [9]. Other antimicrobial peptides from this species, such as scolopins 1 and 2 and FXa-inhibiting peptide, have also been reported [9–11]. In this study, we identified an antimicrobial peptide from *S. s. mutilans* and investigated its antimicrobial properties.

*Abbreviations:* AMPs, antimicrobial peptides; SmAE, *S. s. mutilans* Assembled EST; ATCC, American Type Culture Collection; KCTC, Korean Collection for Type Cultures; MIC, minimum inhibitory concentration; PBS, phosphate-buffered saline; CD, circular dichroism; TFE, trifluoroethanol; DPC, diphosphocholine; $DiBAC_4(3)$, bis-(1,3-dibutylbarbituric acid) trimethine oxonol; $DiSC_3(5)$, 3,3′-dipropylthiacarbocyanine iodide; GUV, giant unilamellar vesicle; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol; LUV, large unilamellar vesicle

* Corresponding author. Tel.: +82 53 950 5373; fax: +82 53 955 5522.
*E-mail address:* dglee222@knu.ac.kr (D.G. Lee).

[1] These authors contributed equally to this work and should be considered co-first authors.

http://dx.doi.org/10.1016/j.bbamem.2014.11.016
0005-2736/

## 2. Materials and methods

### 2.1. Transcriptome sequencing

Centipedes were injected with exponential-phase *Escherichia coli* ($2 \times 10^6$ colony forming units (CFU)/centipede). Eighteen hours later, total RNA was isolated from the centipedes using the RNeasy Total RNA Isolation Kit (Qiagen, USA). The integrity of RNA was verified using an Agilent 2100 Bioanalyzer. Poly(A) mRNA was isolated using oligo(dT) beads. Short mRNA fragments were obtained by adding fragmentation buffer, and first- and second-strand cDNA was synthesized in succession. Short fragments were purified using the QIAquick PCR Purification Kit, end repair was performed, and a poly(A) tail adapter sequence was added. Suitable amplified fragments were selected as templates using the agarose gel electrophoresis method. Finally, the library was sequenced on an Illumina HiSeq™ 2000 System by generating paired-end libraries with an average insert size of 200 bp, following the manufacturer's instructions.

### 2.2. Assembly, screening based on physicochemical properties and selection of an AMP

The *S. s. mutilans* transcriptome was assembled *de novo*, without a reference genome, using the Trinity method [13]. Sequences that could not be extended on either end were acquired using TGICL software [14]. The resulting sequences (contigs and singletons), referred to as *S. s. mutilans* Assembled EST (SmAE) sequences, were translated into amino acid sequences with ESTScan [15]. These amino acid sequences were screened for potential AMPs based on physicochemical properties of known AMPs using EMBOSS PEPSTATS [16]. AMPs were then selected based on sequence similarity with known AMPs, determined using BLASTx. Among the putative AMPs thus obtained, the following AMP was synthesized: scolopendin 2. Peptide synthesis was performed by Anygen Co. (Gwangju, Korea).

### 2.3. Circular dichroism spectroscopy

Circular dichroism (CD) spectra of the peptides were recorded using a Jasco J-710 CD spectrophotometer (Jasco, Tokyo, Japan) with a 1-mm path length cell. CD was measured at wavelengths from 195 nm to 240 nm (bandwidth, 1 nm; step resolution, 0.1 nm; speed, 50 nm/min; response time, 0.5 s). CD spectra were recorded for the peptides in the presence of phosphate-buffered saline (PBS), 50% trifluoroethanol (TFE), 50 mM SDS micelles, and 0–5 mM diphosphocholine (DPC) (pH 7.4) at 20 °C. The spectra were averaged over three scans [17].

### 2.4. Microbial strains

*Enterococcus faecium* (ATCC 19434), *Staphylococcus aureus* (ATCC 25923), *E. coli* O157 (ATCC 43895), *Salmonella typhi* (ATCC 19430), *Pseudomonas aeruginosa* (ATCC 27853), *Candida albicans* (ATCC 90028), and *Candida parapsilosis* (ATCC 22019) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *Trichosporon beigelii* (KCTC 7707) and *Trichophyton rubrum* (KCTC 6345) were obtained from the Korean Collection for Type Cultures (KCTC). Methicillin-resistant *S. aureus* and antibiotic-resistant *P. aeruginosa* were obtained from a tertiary teaching hospital in Daegu, South Korea. The isolates were processed by the MicroScan WalkAway 96 system for genus and species identification. The susceptibility of resistant strains to antimicrobial agents was tested using MicroScan Gram positive MIC/combo (PC1A), Gram negative MIC/combo (NC44) and Gram negative breakpoint combo (NBC39) panels [18].

### 2.5. Antimicrobial activity

The bacterial strains were cultured in Luria-Bertani (LB) broth (Difco) with aeration at 37 °C and the fungal strains were cultured in yeast extract–peptone–dextrose (YPD) broth (Difco) with aeration at 28 °C. Optical density was measured with a spectrophotometer (DU530, Beckman, Fullerton, CA, USA) and cell cultures were adjusted to obtain standardized populations. Cells in the exponential phase ($2 \times 10^6$ cells/mL) were dispensed into wells of microtiter plates (0.1 mL/well). Minimum inhibitory concentration (MIC) was determined using two-fold serial dilutions of the test peptides, based on the Clinical and Laboratory Standards Institute (CLSI) method [19]. The MIC values were determined from three independent tests.

### 2.6. Hemolytic activity

The hemolytic activity of the peptides was evaluated by determining the release of hemoglobin from a 4% suspension of human erythrocytes, measured at 414 nm with an ELISA reader. Hemolytic levels of 0 and 100% were determined in PBS (35 mM phosphate buffer, 150 mM NaCl, pH 7.4) and with 0.1% Triton X-100, respectively. Percent hemolysis was calculated as follows: hemolysis (%) = [($Abs_{414\ nm}$ in the peptide solution − $Abs_{414\ nm}$ in PBS) / ($Abs_{414\ nm}$ in 0.1% Triton X-100 − $Abs_{414\ nm}$ in PBS)] × 100 [20].

### 2.7. Permeability of microbial cells

*E. coli* O157 and *C. albicans* cells in the log phase ($2 \times 10^6$ cells/mL) were suspended in PBS and treated with the MICs of the peptides. After incubation for 4 h at 37 °C and 28 °C, respectively, the cells were harvested by centrifugation and resuspended in PBS. Subsequently, the cells were treated with 1 μM SYTOX Green. The cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) [21].

### 2.8. Membrane depolarization

Log-phase cells of *E. coli* O157 and *C. albicans* ($2 \times 10^6$ cells/mL) were harvested and resuspended in PBS. After incubation with the MICs of the peptides for 4 h at 37 °C and 28 °C, respectively, the cells were again harvested by centrifugation and resuspended in PBS. Subsequently, the cells were treated with 50 μg/mL bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] (Molecular Probes, Eugene, OR, USA). Flow cytometric analysis was performed using a FACSCalibur flow cytometer [22].

*E. coli* O157 cells were harvested by centrifugation (3500 rpm, 7 min), washed in 5 mM HEPES buffer (pH 7.2) containing 20 mM glucose, and resuspended in buffer (5 mM HEPES buffer, 20 mM glucose, 100 mM KCl, pH 7.2) to an $OD_{600}$ of 0.05. Harvested *C. albicans* cells ($2 \times 10^6$ cells/mL) were washed with $Ca^{2+}$- and $Mg^{2+}$-free PBS. Changes in the membrane potential were measured using a membrane-potential-sensitive probe, 3,3′-dipropylthiacarbocyanine iodide [$DiSC_3(5)$]. To determine the effect of salt, the cells were treated with peptide (at the MIC) under various salt conditions (final concentrations: 150 mM NaCl and 1 mM $MgCl_2$). Changes in fluorescence due to the collapse of the cytoplasmic membrane potential were monitored continuously using a spectrofluorophotometer (Shimadzu, RF-5301PC, Shimadzu, Kyoto, Japan) at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. The experiment was repeated three times under each condition to ensure reproducibility [23,24].

### 2.9. Leakage of potassium

The antimicrobial activity of the peptides was analyzed by measuring the efflux of potassium ions from *E. coli* O157 and *C. albicans* cells using an ISE meter (Orion Star A214, Thermo Scientific, Singapore).



Please cite this article as: H. Lee, et al., Scolopendin 2, a cationic antimicrobial peptide from centipede, and its membrane-active mechanism, Biochim. Biophys. Acta (2014), http://dx.doi.org/10.1016/j.bbamem.2014.11.016