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The characteristic region of arenicin-1 involved with a bacterial membrane targeting mechanism

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ABSTRACT

The antimicrobial peptide arenicin-1 consists of two antiparallel β-sheets linked by a hydrophilic β-turn. To determine the role of a specific region found in a particular β-sheet structure of the peptide for antibacterial activity, two analogs with N-terminal deletions (RW) and substitutions of Arg to Ala in the β-turn region were designed. In the minimum inhibitory concentration (MIC) test, the antibacterial activities of the analogs were reduced for both Gram-positive and Gram-negative bacteria, when compared to arenicin-1. The influence of the decrease in hydrophobicity on the antibacterial activity was confirmed by a hemolytic assay. Through flow cytometric analysis using propidium iodide (PI) and a 1,6-diphenyl-1,3,5-hexatriene (DPH) assay, it was confirmed that the analogs decreased the degree of plasma membrane permeability compared to arenicin-1. In particular, analog 2 showed a lower permeability in Gram-negative bacteria than in Gram-positive bacteria. The results indicate that a reduction in the net charge weakened the electrostatic interactions between the peptides and the negatively charged membranes. In liposomes, which mimic bacterial membranes, due to a reduced binding affinity to the membranes, the analogs could not deeply penetrate into the hydrocarbon region and induce enough fluorescein isothiocyanate-dextran (FD) leakage compared to that of arenicin-1. It is thought that the Arg residue in the hydrophilic β-turn region is more important to antibacterial activity than the Arg residue in the N-terminal region. This study suggests that the Arg and Trp residues in the N-terminal region and the Arg residue in the β-turn region of arenicin-1 play a key role in antibacterial activity.

1. Introduction

Arenicin-1 (RWCVYAYVRVRGVLVRYRRCW), a 21-residue antibacterial peptide, is a two-stranded-antiparallel β-sheet peptide isolated from the coelomocytes of the marine polychaeta lugworm, *Arenicola marina* [1]. This peptide has been reported to exhibit broad antibacterial activity against the human pathogenic bacterial strains [2]. From the structure of arenicin-1, it has a net positive charge (+6) and hydrophobic characteristics due to its six positively charged Arg residues and hydrophobic residues, which include Trp, Val, Leu, Ala, and Gly residues. A single disulfide bond between Cys3 and Cys10 forms a large ring and contributes to the structural rigidity and antimicrobial activity of arenicin-1 [3]. It is worth noting that the antimicrobial peptides with a β-sheet structure, such as tachyplesin II (RWCFRVCYRGICYRKCR-NH_2) and polyphemusin I (RRWCFRVCYRGFCYRKCR-NH_2) have a conserved Arg and Trp in the N-terminal region [4]. It was reported that the consecutive sequence of Arg and Trp discovered in the antimicrobial peptides plays a key role in their antimicrobial activity [5]. The Arg residue retains a more dispersed positive charge than the Lys residue; this may enable it to increase the electrostatic interactions between the cationic peptides and the negatively charged bacterial membrane surface including the lipopolysaccharides (LPS) [6]. The hydrophobic Trp residue may support a more efficient interaction with the bacterial membrane surfaces allowing the peptides to partition into the bilayer interface, in contrast with other hydrophobic residues [7,8]. To investigate their roles in regard to the antibacterial activity of arenicin-1, analog 1 was designed by truncating the N-terminal (RW) (Table 1). Subsequently, it is known that the number of positively charged residues in the polar face affects the biological activities in prokaryotic and eukaryotic cells. The positively charged Arg residue is able to interact between the cationic peptide and the electronegative cell surface and stabilizes the pore or channel by increasing the hydrophilicity of the pore [9,10]. Based on the fact that arenicin-1 displayed a lower antibacterial activity at high salt concentration [33], its antibacterial activity may be affected by the number of positive residues. Analog 2 was designed by substituting Arg with

Abbreviations: CLSI, Clinical and Laboratory Standards Institute; DPH, 1,6-diphenyl-1,3,5-hexatriene; FD, fluorescein isothiocyanate-dextran; LPS, lipopolysaccharides; PI, propidium iodide.

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Table 1
Amino acid sequences of the synthetic antimicrobial peptide, arenicin-1 and its analogs.

Peptides	Amino acid sequences	Net charge	Retention time (min)	Remarks
Arenicin-1	RWCVYAYVRVRGVLVRYRRCW	+6	20.133	Wild type peptide
Analog 1	CVYAYVRVRGVLVRYRRCW	+5	18.933	N-terminal deletion (RW)
Analog 2	RWCVYAYVRV**A**GVLVRYRRCW	+5	19.687	Substitution of R → A

Ala to probe the influence of positively charged residues in the hydrophilic region of the antimicrobial peptides and their effect on the antibacterial activity.

2. Materials and methods

2.1. Peptide synthesis

Peptides were synthesized by the solid phase method using Fmoc (9-fluorenyl-methoxycarbonyl) chemistry [11,12]. Assembly of the peptides was achieved with a 60-min cycle for each residue at ambient temperature, using a reactor with a specially designed shape. The crude peptide was repeatedly washed with diethyl ether and dissolved in 10 ml 0.1 mM ammonium bicarbonate, 50 ml H_2O, and 50 ml acetonitrile. The mixture was freeze-dried in a lyophilizer after the salts were excluded and purified using reversed-phase preparative HPLC on a Waters 15-μm Delta Pak C_{18} Column (19 × 30 cm). The purity of the peptides was checked by the analytical reversed-phase HPLC on a Shimadzu C_{18} analytical column (5 μm, 0.46 cm × 25 cm) with a flow rate of 8 ml/min for the preparative C_{18} (10 μm, 2.5 cm × 25 cm) column [13].

2.2. Antibacterial activity assay

Staphylococcus aureus (ATCC 25923), *Enterococcus faecium* (ATCC 19434), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Methicillin-resistant *S. aureus* (MRSA), vancomycin resistant *Enterococci* (VRE), antibiotic-resistant *E. coli* (AREC), and antibiotic-resistant *P. aeruginosa* (ARPA) strains were clinically isolated from the nosocomial patients from the Kyungpook National University Hospital, Daegu, Korea. Bacterial cells (2×10^7 cells/ml) were incubated into a Luria–Bertani (LB) broth and 0.1 ml bacterial culture was dispensed per well into 96-well microtiter plates. MIC was determined by a serial twofold dilution of test compounds following the recommendations of the Clinical and Laboratory Standards Institute (CLSI) [14]. After 24 h of incubation at 37 °C, the minimal concentration of the compound to prevent the growth of the given test organism was determined and was defined as MIC. Growth was assayed with a microtiter ELISA Reader (Molecular Devices Emax, California, USA) by monitoring absorption at 620 nm [15].

2.3. Hemolytic activity

The hemolytic activity of the peptides was evaluated by determining the amount of the released hemoglobin from a 4% suspension of fresh human red blood cells (RBCs) at 414 nm [16]. Human RBCs were harvested and washed with phosphate buffered saline (PBS; 0.15 M NaCl in 35 mM phosphate buffer, pH 7.0) several times. One hundred microliters of human RBCs diluted to 8% (v/v) in PBS was seeded into 96-well plates, and 100 μl of the peptide solution was then added to each well. The plates were incubated for 1 h at 37 °C. One hundred microliters of aliquots was transferred to new 96-well plates, and hemolysis was measured by the absorbance at 414 nm with an ELISA plate Reader. A value of zero and 100% hemolysis were determined in PBS and 0.1% Triton X-100, respectively. The hemolysis percentages were calculated by employing the following equation: Percentage 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 [17,18].

2.4. Membrane permeability assay

S. aureus and *E. coli* cells (2×10^6/ml) were first harvested from single grown cultures on agar plates and cultured overnight at 37 °C in LB media. The bacteria were centrifuged at 1000*g* for 5 min and resuspended in 10 mM Na_2HPO_4 buffer (pH 7.4). Cells were treated with and without the peptide and left for 2 h at 37 °C. The bacteria were then stained with PI (10 μg/mL) followed by incubation at 37 °C for 20 min prior to flow cytometric analysis [19]. Flow cytometry was done via a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) [20].

2.5. Measurements of the plasma membrane fluorescence intensity

The anisotropy of fluorescence from the exponential bacterial cells labeled by DPH (Molecular Probes, Eugene, OR, USA) was used to monitor changes in membrane dynamics. The cells (2×10^8 cells in a Mueller–Hinton medium) containing the peptide were incubated at a physiological temperature of 37 °C on a rotary shaker at 140 rpm for 2 h. The control cells were incubated without the peptide. For DPH labeling, the cells were resuspended in a PBS buffer and incubated at 37 °C for 45 min in the presence of 0.6 mM of DPH followed by several washings in a PBS buffer. Steady-state fluorescence anisotropy was measured using a Shimadzu RF-5301PC spectrofluorometer (Kyoto, Japan) at 350 nm excitation and 425 nm emission wavelengths [21].

2.6. Preparation of small unilamellar vesicles (SUV)

SUVs composed of PG:PE 3:1 (w/w) [22] and PG:CL 58:42 (w/w) [23] were dissolved in chloroform (2 mg/ml). Solvents were removed by rotatory evaporation for 2 h to form a thin film. The dried thin films were hydrated with 2 ml of a HEPES/EDTA buffer (5 mM HEPES, 0.1 mM EDTA, pH 7.0). The lipid dispersions were then sonicated in ice water for 40 min until the solution became transparent. The solution was extruded through polycarbonate filters (two stacked 30 nm pore size filters) by an extruder (Avanti Polar Lipids, Inc., USA) [24].

2.7. Tryptophan fluorescence blue shift

The fluorescence emission spectrum of the tryptophan residues in the peptides was monitored in a HEPES/EDTA buffer or in a buffer containing PG:PE (3:1, w/w) and PG:CL (58:42, w/w) SUVs. The SUVs were used to minimize the differential light scattering effects. After 10 min, fluorescence spectra were measured with a RF-5301PC spectrophotometer (Shimazu, Japan). The fluorescence was excited at 280 nm and the emission was scanned from 290 to 450 nm [25].

2.8. Preparation of FD-loaded liposomes and leakage experiments

A buffer I solution (1 ml, 50 mM potassium phosphate, pH 7.4, with 0.1 mM EDTA) containing 2 mg/ml of the FD (FD4, FD10 and