Cell Selectivity of Arenicin-1 and Its Derivative with Two Disulfide Bonds

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Antibiotics has become more important in modern health care system. Antimicrobial peptide is one of the attractive candidates for new mechanism of antibiotics. Arenicin-1 (AR-1) is 21-residue antimicrobial peptide isolated from marine polychaeta *Arenicola marina* with single disulfide bond forming an 18-residue ring [Ovchinnikova, T. V. *et al.*, *FEBS Lett.* **2004**, *577*, 209-214]. Our previous study on AR-1 and its linear derivative showed that disulfide bridge and the amphipathic â-sheet structure of the AR-1 play important roles in their biological activities. AR-1 has high antibacterial activity but it also displays hemolytic activity against human red blood cells. In order to develop more potent and more bacterial cell selective peptide, we designed and synthesized a new derivative, AR-1-C (RW**C**VYAY**C**RVRGVL**C**RYRR**C**W) by substitution of $Val^{8,15}$ with $Cys^{8,15}$. AR-1-C has higher antibacterial activity but displayed less hemolytic activity against human red blood cells than the parent AR-1. AR-1 has a two-stranded antiparallel â-sheet structures forming a large and flexible ring while AR-1-C with two disulfide bonds forming a 12-residue ring has higher structural rigidity and higher hydrophobic-hydrophilic balance than AR-1. Peptide rigidity and optimum balance between the hydrophobic and hydrophilic phase in amphipathic â-sheet structure are important in biological activities of arenicin and its derivative.

Key Words : Arenicin-1, Antimicrobial peptide, Disulfide bond, Structure-activity relationship

Introduction

Antibiotic agents have become essential in the modern health care system, assisting and complementing the natural immune system. However, the appearance of resistant strains and the cytotoxicity of antibiotic agents have necessitated ongoing efforts to identify more potent and safer antibiotic agents.[1-3] In this respect, the endogenous antimicrobial peptides and their derivatives are attractive candidates. Antimicrobial peptides play important roles in the innate host defense mechanisms of most living organisms, including plants, insects, amphibians, and mammals.[4-7]

A 21-residue antimicrobial peptide, Arenicin-1 (AR-1: RWCVYAYVRVRGVLVRYRRCW), a novel 21-residue antimicrobial peptide, was purified from coelomocytes of the marine polychaeta *Arenicola marina* (lugworm).[8] This antimicrobial peptide contains a single disulfide bridge between Cys^3 and Cys^{20}, forming a large 18-residue ring. To determine the role of this disulfide bond, we synthesized AR-1 (RWCVYAYVRVRGVLVRYRRCW) and its linear derivative, arenicin-1-S (AR-1-S: RWSVYAYVRVRGVLVRYRRSW). Activity assays revealed that AR-1-S is somewhat less active against bacterial cells than AR-1. Both peptides were very hydrophobic and displayed cytotoxicity against human red blood cells. Analysis of the tertiary structures of AR-1 and AR-1-S by NMR spectroscopy disclosed that AR-1 has two-stranded antiparallel β-sheet structures with amphipathicity, while AR-1-S displays a random coil structure in DMSO.[9]

In order to design more effective antimicrobial peptide without hemolytic activity, we synthesized a derivative, AR-1-C (RWCVYAYCRVRGCLVRYRRCW) by substitution of valines with cysteines in the middle of β-sheet structure of AR-1 (Table 1). We measured their antimicrobial activities against bacteria and hemolytic activities against human erythrocytes as well as leakage ability from liposomes composed of a mixture of phosphatidycholine (PC) and cholesterol (CH), which mimics the mammalian cytoplasmic membranes. We proposed the structure-activity relationship of AR-1-C based on its activities and the structure of AR-1.

Methods

Peptide synthesis. Peptides were prepared by the standard Fmoc-based solid-phase method.[10] Fmoc(9-fluorenylmethoxycarbonyl)-Trp-Wang-resin, Fmoc-amino acids and other reagents for the peptide synthesis were purchased from Calibiochem-Novabiochem (La Jolla, CA, USA). DCC (dicyclohexylcarbodiimide) and HOBt (*N*-hydroxybenzotriazole) were used as coupling reagent, and ten-fold excess Fmoc-amino acids were added during every coupling cycle. The side protection of Cys was as follows: Cys(Trt) (for AR-

1 synthesis) and Cys[3, 20](Trt) and Cys[8, 15](Acm) (for AR-1-C synthesis). AR-1 containing a single disulfide bond was deprotected with trifluoroacetic acid/triisopropylsilane/water/phenol/ethanedithiol (82.5:5:5:5:2.5, v/v) for 2 h at room temperature. The reduced peptide of AR-1 was air-oxidized in ammonium acetate solution (0.1 M ammonium acetate, 0.1 M NaCl, 20 mM Na_2HPO_4, 1 mM EDTA, pH 8.0) in the presence of reduced and oxidized glutathiones (1:100:10 molar ratio of peptide:GSH:GSSG) for 24 h at room temperature.[11] Oxidized crude peptide was then purified by high performance liquid chromatography (HPLC) on a preparative (15 μm, 20 × 250 mm) C_{18} Vydac column using water-acetonitrile gradient (0-80%) containing 0.05% TFA. AR-1-C containing two disulfide bonds was deprotected with trifluoroacetic acid/triisopropylsilane/water/phenol/ethanedithiol (82.5:5:5:5:2.5, v/v). The partially protected AR-1-C [Cys[8, 15](Acm)] was dissolved in acetic acid solution (acetic acid:water = 1:19, v/v) containing 25% DMSO, and stirred until oxidation (peptide concentration: 30 μM). The solution was then made up to 30% acetic acid and the Acm groups were removed simultaneously with Cys oxidation by dropwise addition of 5 eq of iodine (freshly prepared 5 mM solution in methanol). The reaction mixture was directly loaded onto a preparative (15 μm, 20 × 250 mm) C_{18} Vydac column using water-acetonitrile gradient (0-80%) containing 0.05% TFA. The identity of AR-1 and AR-1-C were confirmed by MALDI-TOF MS (matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry) (Shimadzu, Japan) (Table 1).

MIC test. The antimicrobial activities of the peptides were examined in sterile 96-well plates in a final volume of 100 μL and the procedures are as follows. Briefly, aliquots (100 μL) of a bacterial suspension at 2×10^6 colony-forming units (CFU)/mL in 1% peptone were added to 100 μL of peptide solution (serial 2-fold dilutions in 1% peptone). After incubation for 18-20 h at 37 °C, the inhibition of bacterial growth was determined by measuring the absorbance at 620 nm with a Micro plate auto reader EL 800 (Bio-Tek Instruments). The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the peptide requited to inhibit bacterial growth. Two types of Gram-negative bacteria (*Escherichia coli* [KCTC 1682] and *Salmonella typhimurium* [KCTC 1926]) and two types of Gram-positive bacteria (*Staphylococcus aureus* [KCTC 1621] and *Staphylococcus epidermidis* [KCTC 1917]) were purchased from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology.

Table 1. Amino acid sequences of AR-1 and AR-1-C

Peptide	Sequence	Molecular mass (Da)	
		Calculated	Observed
AR-1	RW**C**VYAYVRVRGVLVRYRR**C**W	2758.3	2758.8
AR-1-C	RW**C**VYAY**C**RVRGVL**C**RYRR**C**W	2768.3	2765.5

Hemolytic activity. Hemolytic activity of the peptides was tested against human red blood cells (h-RBC). Fresh h-RBCs were washed three times with phosphate-buffered saline (PBS; 35 mM phosphate buffer containing 150 mM NaCl, pH 7.4) by centrifugation for 10 min at 1,000 × g and resuspended in PBS. The peptide solutions were then added to 50 μL of h-RBC in PBS to give a final volume of 100 μL and a final erythrocyte concentration of 4% (v/v). The resulting suspension was incubated with agitation for 1 h at 37 °C. The samples were centrifuged at 1,000 × g for 5 min. Release of hemoglobin was monitored by measuring the absorbance at 405 nm of the supernatant Controls for no hemolysis (blank) and 100% hemolysis, respectively. The percent hemolysis was calculated using the following equation:

$$\text{Hemolysis (\%)} = [(OD_{405\,nm}\ \text{sample} - OD_{405\,nm}\ \text{zero lysis}) / (OD_{405\,nm}\ 100\%\ \text{lysis} - OD_{405\,nm}\ \text{zero lysis})] \times 100.$$

Dye leakage from lipid vesicles. Calcein-entrapped SUVs composed of PC/CH (10:1, w/w) were prepared by vortexing the dried lipid in dye buffer solution (70 mM calcein, 10 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4). The suspension was sonicated in water bath and extruded through polycarbonate filters (two stacked 100 nm pore size filters) by an *Avanti* Mini-Extruder (Avanti polar Lipids inc., Alabaster). Untrapped calcein was removed by gel filtration on a Sephadex G-50 column. Usually, lipid vesicles were diluted to approximately 10-fold after passing through a Sephadex G-50 column. The eluted calcein-entrapped vesicles were further diluted to the desired final lipid concentration for the experiment. The leakage of calcein from the SUVs was monitored by measuring fluorescence intensity at an excitation wavelength of 490 nm and an emission wavelength of 520 nm using a spectrophotomer (Perkin-Elmer LS55). For determination of 100% dye-release, 10% Triton-X_{100} in HEPES-buffer (20 μL) was added to dissolve the vesicles. The percentage of dye-leakage caused by the peptides was calculated as follows:

$$\text{Dye-leakage (\%)} = 100 \times (F - F_0)/(F_t - F_0)$$

Where, F is the fluorescence intensity achieved by the peptides, F_0 and F_t are fluorescence intensities without the peptides and with Triton X-100, respectively.

CD analysis. CD experiments were performed using a J-810 spectropolarimeter (Jasco, Tokyo, Japan) with a 1-mm path length cell. The CD spectra of the peptides at 100 μM were recorded at 25 °C in 0.1-nm intervals from 190 to 250 nm. To investigate the conformational changes induced by membrane environments, 2,2,2-trifluoroethanol (TFE)/water solution and sodium dodecyl sulfate (SDS) micelles of defined composition were added to the peptides.[12] For each spectrum, the data from 10 scans was averaged and smoothed using J-810. CD data were expressed as the mean residue ellipticity [θ] in deg·cm^2·dmol^{-1}.

Structure modeling of AR-1-C. Since NMR spectra of AR-1-C suffered from the spectral overlapping caused by the peptide aggregation, the model of AR-1-C was built by

appropriate residue replacements based on NMR structure of AR-1. Two Val residues (Val^8 and Val^{15}) were replaced with Cysteines which enabled to form an additional disulfide bond. Final structure of AR-1-C was obtained by energy minimization. Energy minimization was performed with constrained disulfide bond between Cys^8-Cys^{15}. Conjugated gradient algorithm was used to calculate the model structure with the consistent valence force field. These processes were performed using the program InsightII/Discover (Accelrys Inc., San Diego, CA).[13,14] In order to investigate the structural transition of the our hypothetical structure of AR-1-C molecular dynamics (MD) simulation was performed. In case of MD simulation, AR-1-C was solvated in rectangular boxes with DMSO molecules. All configurations were energy minimized with the steepest descents method for 1000 steps, to remove bad van der Waals contacts. MD simulation was performed during 25ns using CHARMM.

Results and Discussion

In order to discover more potent and safer antimicrobial peptide, we designed and synthesized AR-1-C by substitution of $Val^{8,15}$ with $Cys^{8,15}$ based on the structure of AR-1 determined in our previous study. AR-1 has two-stranded antiparallel β-sheet structures. Since AR-1 contains a single disulfide bond between Cys^3 and Cys^{20} forming a large 18-residue ring, its structure is quite flexible. In order to provide a more rigid β-sheet structure to AR-1, we introduce one more disulfide bond in the middle of the β-sheet structures by substitution of Val^8 and Val^{15} resulting in a 12-resiude ring. Figure 1 shows that skeletal structure of AR-1 determined previously and it shows the position of Val^8 and Val^{15} which protrude toward the same side and are suitable for additional disulfide bond.

The antimicrobial activities of the peptides were tested against two types of Gram-negative bacteria (*Escherichia coli* and *Salmonella typhimurium*) and two types of Gram-positive bacteria (*Staphylococcus aureus* and *Staphylococcus epidermidis*). As listed in Table 2, both peptides displayed good antibacterial activities with MIC range of 2.0-8.0 μM. Generally, AR-1-C showed two fold higher antibacterial activities than AR-1. Then, we checked the hemolytic activity of the peptides against human red blood cell. As shown in Figure 2, AR-1-C displayed less hemolytic activity than AR-1. Therefore, substitution of valines with cysteines reduced the hydrophobic potential and the length of β-sheet strand and resulted in decrease of hemolytic activity of AR-1-C compared to AR-1.

To examine cytotoxicity of the peptides, we measured their abilities to cause the leakage of fluorescent dye from PC/CH (10:1, w/w) liposomes, which mimics mammalian cytoplasmic membranes (Figure 3).[15,16] Melittin known as a strong peptide antibiotics with high hemolytic activity showed a strong dye leakage abilities.[17] AR-1 was also very effective in inducing calcein leakage from PC/CH (10:1, w/w) liposomes while AR-1-C showed very weak vesicle-disrupting activity compared to melittin and AR-1. This result implies that AR-1-C has higher bacterial cell selectivity than AR-1.

To investigate the secondary structures of AR-1-C in membrane-like environments, we measured CD spectra of AR-1-C dissolved in an aqueous solution, 1:1 (v/v) 2,2,2-trifluoroethanol:H_2O and 100 mM SDS micelles. The CD spectra of AR-1-C in Figure 4 showed that AR-1-C has a

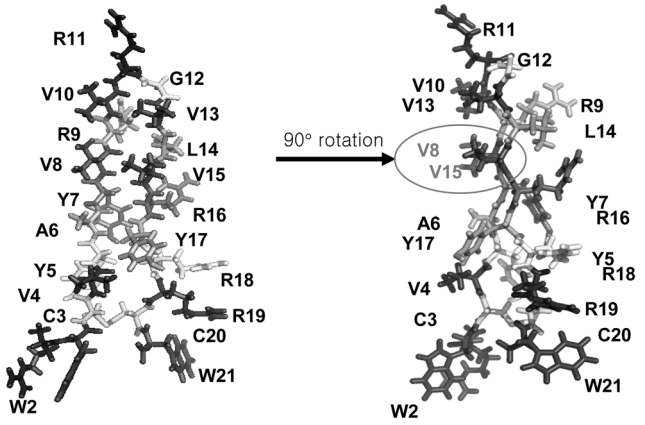

Figure 1. Skeletal structure of AR-1, which shows the position of Val^8 and Val^{15} which were substituted with cysteines in AR-1-C.

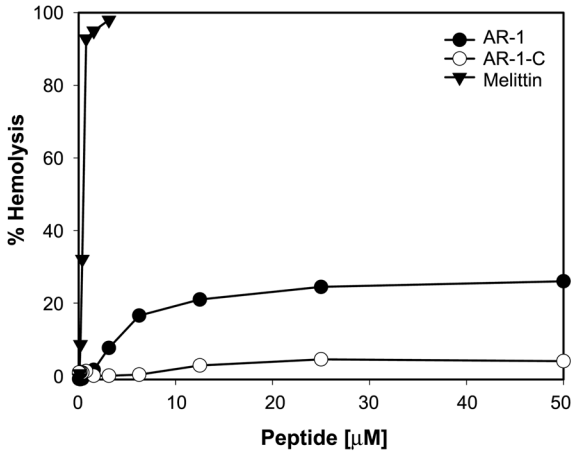

Figure 2. Dose-response curves of the hemolytic activity of the peptides against human red blood cells.

Table 2. Antimicrobial activities of AR-1 and AR-1-C

Peptide	Minimal inhibitory concentration (μM)			
	Escherichia coli [KCTC1682]	*Salmonella typhimurium* [KCTC 1926]	*Staphylococcus aureus* [KCTC 1621]	*Staphylococcus epidermidis* [KCTC 1917]
AR-1	4	4	4-8	4-8
AR-1-C	2-4	4	2-4	4

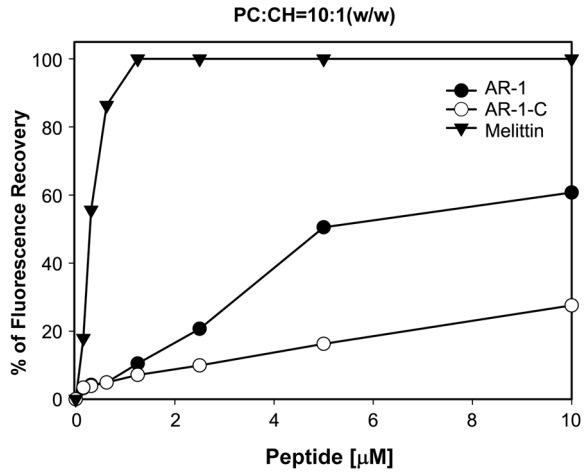

Figure 3. Leakage of the fluorescent dye, calcein from PC/CH (10:1, w/w) SUV. The percentage of dye-leakage caused by the peptides was measured.

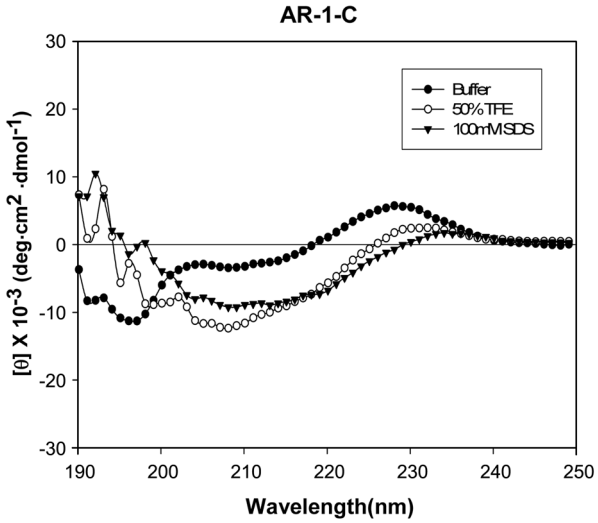

Figure 4. CD spectra of AR-1-C in membrane-mimetic environments.

random coil structures in aqueous solution while it has a minimum at 215 nm in SDS micelles which is a characteristics of β-sheet structure.[18]

Because of the peptide aggregation, NMR spectra of AR-1-C suffered from the peak overlapping and gave bad spectral resolutions. Therefore, tertiary structure of AR-1-C was not able to be solved by NMR spectroscopy. Based on the structure of AR-1 determined by NMR spectroscopy previously,[9] we calculated a hypothetical structure of AR-1-C by energy minimization. In order to investigate the structural transition, MD simulation in DMSO box was performed.[19] During 25ns MD simulation, there was no structural transition from our starting structure and no other local conformer was observed. Three dimensional structure of AR-1 has more hydrophobic residues than hydrophilic residues resulting in high hemolytic activity. Since AR-1 has two-strand antiparallel β-sheet structure from Ala^6 to Ile^{10} and Val^{13} to Tyr^{17} with only one disulfide bond forming a flexible large ring, the structure is not rigid compared to other

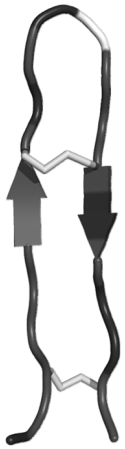

Figure 5. Structure of AR-1-C calculated based on the NMR structure of AR-1. Hydrophobic residues are colored red, while hydrophilic residues are blue. Cysteine residues and disulfide bonds are colored yellow.

peptides with β-sheet structures including two or more disulfide bonds.[20-22] Since AR-1-C had improved antimicrobial activities but showed much less hemolytic activity than AR-1, replacement of hydrophobic valine residues with cysteines decreased the hydrophobic potentials and cytotoxicity of AR-1-C compared to AR-1. As shown in hypothetical structure in Figure 5, addition of one more disulfide bond to AR-1 may result in a shorter amphipathic β-sheet from Cys^8 to Ile^{10} and Cys^{15} to Tyr^{17} forming a 12-residue ring with increased structural rigidity and balance between the hydrophobic and hydrophilic residues. Further study should be carried out to solve the peptide aggregation problems and to determine the accurate tertiary structure of AR-1-C.

We can conclude that peptide rigidity and optimum balance between the hydrophobic and hydrophilic phase of amphipathic β-sheet structure are important in biological activities of AR-1 and AR-1-C. Structure-antibiotic activity relationships of AR-1 studied in this study should facilitate the design of novel non-cytotoxic peptide antibiotics with potent antibacterial activities.

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