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Study of antimicrobial activity and the mode of action of Anal P5 peptide

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Abstract

In a previous study, we showed that Cecropin A (1-8)-Magainin 2 (1-12) hybrid peptide (CA-MA)'s analogue, Anal P5, exhibit broad-spectrum antimicrobial activity. Anal P5, designed by flexible region (positions 9, 10)-substitution, Lys- (positions 4, 8, 14, 15) and Leu- (positions 5, 6, 12, 13, 16, 17, 20) substitutions, showed an enhanced antimicrobial and antitumor activity without hemolysis. The primary objective of the present study was to gain insight into the relevant mechanisms of antimicrobial activities of Anal P5 by using flow cytometric analysis. Anal P5 exhibits strong antifungal activity in a salt concentration independent manner. In addition, Anal P5 causes significant morphological alterations of the bacterial surfaces as shown by scanning electron microscopy, supporting its antibacterial activity. Its potent antibiotic activity suggests that Anal P5 is an excellent candidate as a lead compound for the development of novel antibiotic agents.

Key words : CA (1-8)-MA (1-12) hybrid peptide (CA-MA); CA-MA analogue P5 (Anal P5); Lys- and Leu- substitutions

1. Introduction

Natural antimicrobial peptides are important components of the non-specific host defense system and innate immunity of insects (1), amphibians (2) and mammals (3). These include the cecropins from insects and mammals (1,4), melittin from the honeybee (5) and defensins from insects and various mammalian species (6). Cecropin A (CA), one of the first reported antimicrobial peptides, is found in the hemolymph of *Hyalophora cecropia* pupae and consists of 37 amino acid residues (4). Magainin 2 (MA), a cationic 23-amino acid antimicrobial peptide, was discovered in the skin of the African clawed frog, *Xenopus laevis* (4). Both CA and MA exhibit strong antibacterial activity but no cytotoxicity against normal mammalian cells (7).

Numerous studies using synthetic peptides have been focused on designing analogue peptides with increased antimicrobial activity compared to that of natural peptides without damaging the mammalian cells (8). Several attempts have been made to improve antimicrobial activity simultaneously eliminating cytotoxicity against mammalian cells, such as red blood cells, by changing the structural characteristics including flexible region (9), chain length (7), net charge (10), hydrophobicity (11) and/or a-helicity (12).

In this study, novel analogue peptide Anal P5 (13) with antimicrobial activity was used. To investigate the mechanism of antimicrobial activities of Anal P5, the effect of analogue peptide was measured against bacterial and fungal cells. We will discuss the importance of Leu-Lys rich peptide and the antibiotic effect of Anal P5 using the salts. Finally, in order to investigate the structure-antibiotic activity relationships of the peptides in the cell membrane-mimicking environment such as TFE or SDS micelle were measured using CD spectra.

2. Materials and methods

2.1. Peptide synthesis

The peptides were synthesized by the solid phase method using Fmoc (9-fluorenyl-methoxycarbonyl) chemistry (14,15). Rink Amide 4-methyl benzhydrylamine (MBHA) resin (0.55 mmol/g) was used as the support to obtain a C-terminal amidate peptide. The coupling of Fmoc-L-amino acids was performed with N-hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC). Amino acid side chains were pro-

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tected as follows: *tert*-butyl (Asp and Thr), trityl (Gln), *tert*-butyloxycarbonyl (Lys and Trp), pmc (Arg). Deprotection and cleavage from the resin were carried out using a mixture of trifluoroacetic acid, phenol, water, thioanisole, 1,2-ethandithiol and triisopropylsilane (80.5:5:5:5:2.5:2 v/v) for 3 h at room temperature. The crude peptide was then repeatedly washed with diethylether, and dried in a vacuum. The crude peptides were purified by a reversed-phase preparative HPLC on a Waters 15-mm Deltapak C_{18} column (19×30 cm). The purified peptides were hydrolyzed with 6 N HCl at 110 °C for 22 h, and then dried in a vacuum. The residues were dissolved in 0.02 N HCl and subjected to an amino acid analyzer (Hitachi Model, 8500 A, Japan). The peptide concentration was determined by amino acid analysis. The molecular masses of the peptides were confirmed with MALDI (matrix-assisted laser desorption/ionization) mass spectrometer.

2.2. Antibacterial activity

Bacillus subtilis (KCTC 1918), *Streptococcus epidermidis* (KCTC 3096), *Pseudomonas aeruginosa* (KCTC 1637) and *Salmonella typhimurium* (KCTC 1926) were supplied from the Korean Collection for Type cultures (KCTC), Korean Research Institute of Bioscience & Biotechnology (Taejon, Korea). The bacteria were grown to the mid-phase in a medium (g/l) [10 bactotryptone/5 yeastextract/10 NaCl (pH 7.0)]. The peptides were filtrated through a 0.22 mm filter and stepwise-diluted in a medium of 1% bactopeptone. The tested organism (final bacterial suspension: 2×10^6 colony formation units (CFU)/ml) suspended in growth medium (100 ml) was mixed with 100 ml of the two-fold diluted serial solution of each peptide in a microtiter plate well with three replicates for each test sample. The plates were incubated for 18 h at 37°C. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of peptide which gave no visible growth on the plate (16).

2.3. Antifungal activity assay

The fungal strains, *Candida albicans* (TIMM 1768) and *Trichosporon beigelii* (KCTC 7707) were seeded on 96-well plates (NUNC, USA) at a density of 2×10^4 cells per well in a volume of 100 ml of YPD media (Dextrose 2%, Peptone 1%, Yeast extract 0.5%, pH 5.5), respectively. *T. beigelii* (KCTC 7707) was obtained from the Korean Collection for Type Culture (KCTC), Korea Research of Bioscience & Biotechnology (KRIBB), Taejon, Korea. *C. albicans* (TIMM 1768) was obtained from the Center for Academic Societies, Osaka, Japan. To these fungal cells were added each 100 μl of serially diluted peptides, and the cell suspension was incubated for 24 h at 28°C. After incubation, 5 μl of a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (5 mg/ml MTT in phosphate-buffered saline (PBS), pH 7.4) was added to each well, and the plates were incubated at 37°C for a further 4 h. The optical density of each well was measured at 580 nm by a microtiter ELISA reader (Molecular Devices Emax, USA) (17).

2.4. Salt-dependency test

For analysis of the membrane integrity of treated peptide, *C. albicans* cells (2×10^5 cells in YPD media) were first harvested at log phase. Peptides were added to a final concentration of 20 μM and the potassium chloride (final concentrations; 0 and 150 mM), were added for salt-dependent test. The cells were incubated for a further 30 min at physiological temperature of 28°C under constant shaking (140 rpm). After incubation, the cells were harvested by centrifugation and washed three times with PBS. Permeabilization of the cell membrane was detected by incubation of the peptide treated cells in propidium iodide (PI, 50 μg/ml final concentration) at 4°C for 30 min followed by removal of unbound dye through excessive washing with PBS. The fluorescence of PI was monitored in the FL2-H channel. As a control experiment, the cell suspension was treated with melittin. Flow cytometric analysis was performed by the FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

2.5. Scanning electron microscopy (SEM)

Midlog phase *B. subtillis* were resuspended at 10^8 CFU/ml in Na-phosphate buffer, pH 7.4, supplemented with 100 mM NaCl (buffer A), and incubated at 37°C with CA-MA and Anal P5. Controls were run in the absence of peptide solvent. After 30 min the cells were fixed with an equal volume of 5% glutaraldehyde in 0.2 M Na-cacodylate buffer, pH 7.4. After fixation for 2 h at 4°C, the samples were filtered on Isopore filters (0.2 mm pore size, Millipore, Bedford, MA, USA) and extensively washed with 0.1 M Na-cacodylate buffer,

pH 7.4. The filters were then treated with 1% osmium tetroxide, washed with 5% sucrose in cacodylate buffer and subsequently dehydrated with a graded ethanol series. After lyophilization and gold coating, the samples were examined on a HITHACHI S-2400 instrument (HITHACHI, Japan).

2.6. Circular dichroism (CD) analysis

CD spectra were recorded at 25°C on a Jasco 715 spectropolarimeter (Jasco, MD, USA) equipped with a temperature control unit. A 0.1-cm path-length quartz cell was used for a 12.5 mM protein solution. At least five scans were averaged for each sample and the averaged blank spectra were subtracted. Each spectrum was obtained by averaging five scans in the 250-190 nm wavelength range. All CD spectra are reported in mean residue ellipticity, $[\theta]_{MRW}$, in deg·cm^{2}dmol^{-1}. The α-helical content was determined from the mean residue ellipticities at 222 nm, as indicated in Equation (1) [18].

$$\% \text{ Helix} = ([\theta]_{obs} \times 100)/\{[\theta]_{helix} \times (1 - 2.57/l)\} \quad (1)$$

where $[\theta]_{obs}$ is the mean-residue ellipticity observed experimentally at 222 nm, $[\theta]_{helix}$ is the ellipticity of a peptide of infinite length with 100 % helix population, taken as –39,500 deg · cm^{2}dmol^{-1}, and l is the peptide length or, more precisely, the number of peptide bonds.

3. Results and discussion

3.1. Design and synthesis of the peptides

Generally, the amphipathic feature of a-helical antimicrobial peptides plays an important role against target cells. In addition, a number of parameters, including net positive charge, a-helicity, and overall hydrophobicity have also been shown to modulate the antibiotic activity of the α-helical amphipathic antimicrobial peptides (19).

Previously, we reported the hybrid peptide designed from N-terminal amphipathic region of CA and hydrophobic region of MA (15,20). This hybrid peptide, CA (1-8)-MA (1-12) showed increased antimicrobial activity than CA or MA alone. Also, in order to elucidate

Table 1. Amino acid sequence and molecular masses determined by MALDI-MS of CA-MA hybrid peptide and its analogues

Peptides	Amino acid sequence	Remarks	Calculated values	Observed values
CA-MA	KWKLFKKIGIGKFLHSAKK F- NH_2	Parent peptide	2402.48	2403.01
Anal P5	KWKKLLKKPLLKKLLKKL-NH_2	CA-MA : substitution of ^{9}GIG→Pro L^4→K^4, F^5→L^5, K^6→L^6, I^8→K^8, K^{12}→L^{12}, F^{13}→L^{13}, L^{14}→K^{14}, H^{15}→K^{15}, S^{16}→L^{16}, A^{17}→L^{17}, F^{20}→L^{20}	2244.60	2246.03

The several analogues were designed to increase net positive charge and hydrophobicity by employing Lys- and Leu-substitution, Lys-addition, flexible region (Gly-Ile-Gly→Pro)-substitution or C-terminal region (HSAKKF)-deletion maintaining the tryptophan residue at position 2.

Table 2. Antimicrobial activity of CA-MA hybrid peptide and its analogues

Peptides	MIC : μg/ml					
	Gram-positive bacteria		Gram-negative bacteria		Fungal cells	
	B. subtilis	*S. epidermidis*	*P. aeruginosa*	*S. typhimurium*	*C. albicans*	*T. beigelii*
CA-MA	7.5	3.75-7.5	3.75	0.46	30	15
Anal P5	1.75	3.75	1.75-3.5	0.22	14.1	7.3

The bacteria were grown to the mid-logarithmic phase in a medium (g/l) (10 bactotryptone/5 yeast extract/10 NaCl (pH 7.0)). Microbial growth was determined by the increase in optical density at 620 nm after 10 h incubation at 37°C. The fungal strains were grown at 30°C in YPD media (Dextrose 2%, Peptone 1%, Yeast extract 0.5%, pH 5.5). The fungal cells were seeded on the well of a 96-microtiter plate of YPD media at a density of 2×10^3 cells (100 ml per well). The turbidity of each well was measured at 570 nm using a microtiter ELISA reader (Molecular Devices Emax, California, USA).

the relationship between electrostatic property and hydrophobicity of antibiotic peptides, and to design peptides with increased antibiotic activity than CA-MA without cytotoxicity, certain analogues of CA-MA were designed and synthesized. The amino acid sequences of the peptides used in this study were summarized and the correct molecular weights of the synthetic peptides were confirmed by MALDI mass spectrometry (Table 1).

3.2. Antimicrobial activity

Antibacterial activities of the synthetic peptides against Gram-positive and Gram-negative bacterial strains were determined as the minimal inhibitory concentration (MIC) by the microdilution method (16)

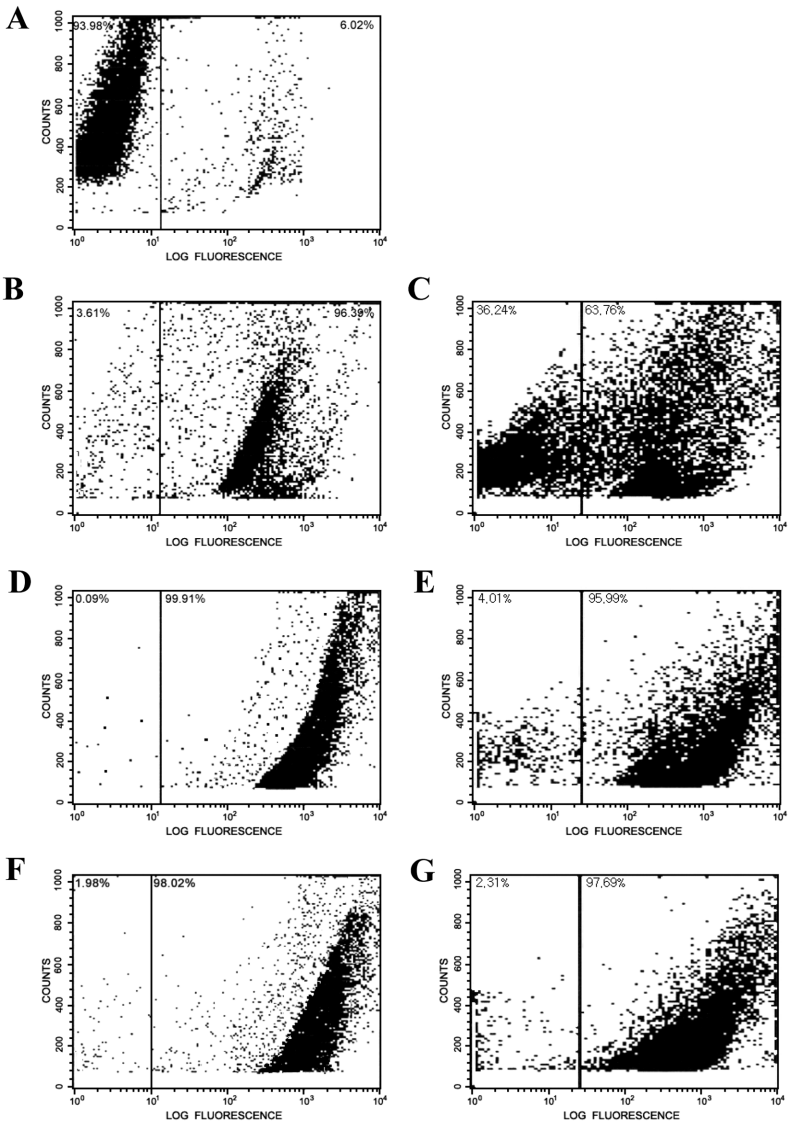

Fig. 1. Flow cytometric measurement of the effects of CA-MA and Anal P5 in a different salt concentrations. The fluorescence of PI was monitored in the FL2-H channel. Flow cytometric analysis was performed by the FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). (A) Control, (B) CA-MA treated cells, (C) CA-MA and 150 mM KCl-treated cells, (D) Anal P5 treated cells, (E) Anal P5 and 150 mM KCl-treated cells, (F) melittin treated cells, (G) melittin and 150 mM KCl-treated cells.

(Table 2). This result indicates that the positive charge and hydrophobicity of synthetic peptide are important in determining the bactericidal rate and activity. The antifungal activity of the peptides against the pathogenic fungi was measured as MIC by MTT assay (17). The results indicated that Anal P5 displayed approximately 2-fold greater antifungal activity than CA-MA against fungal cells (Table 2). This result is similar to antibacterial activity.

In summary, the Anal P5, which showed the most potent antibiotic activity among all the analogues, selectively kills bacterial and fungal cells with no hemolytic activity against human erythrocyte cells (data not shown). These results indicate that the positive charge and hydrophobicity play important roles in antibiotic activity without hemolytic activity.

3.3. Effects of salts on antimicrobial activity

Recently, it has been proposed that the ion channel raises the concentration of salt on the epithelial surface, and this high salt concentration inhibits the activity of antimicrobial peptides (18). The salt influences to the antifungal activity of synthetic peptides were investigated using a FACSCalibur flow cytometer.

While the antifungal activity of CA-MA was repressed by the 150 mM potassium chloride treatment, the interaction of Anal P5 or melittin with *C. albicans* cells was not salt dependent at the same concentration (Fig. 1).

3.4. Analysis of the peptide under bacteria morphology by scanning electron microscopy

We also examined morphological changes of bacterial cells induced by the Anal P5 on SEM. Untreated *B. subtilis* had a normal smooth surface (Fig. 2A). In contrast, cells treated for 4 h with Anal P5 and melittin showed cell surface disrupting (Fig. 2B and C, respectively). The SEM observations provide morphological evidence

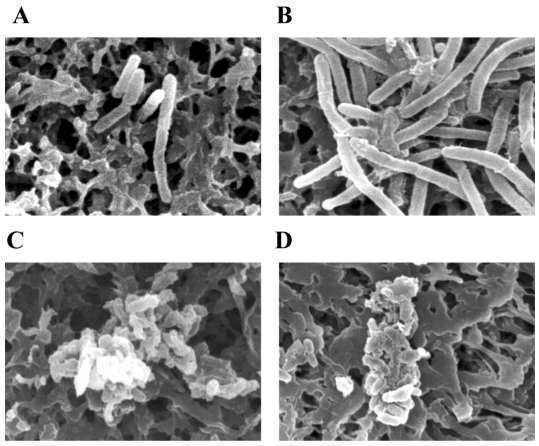

Fig. 2. Scanning electron micrographs of untreated (A) and after treatment of 1.56 μM peptides of *B subtilis* for 30 min at 37°C. Midlog phase *B. subtillis* was resuspended at 10^8 CFU/ml in Na-phosphate buffer, pH 7.4, supplemented with 100 mM NaCl (buffer A), and incubated at 37°C with CA-MA or Ana P5. A; untreated peptide, B; CA (1-8)-MA (1-12), C; Anal P5, D; Melittin (Please insert scale bars on each SEM photos)

of the potent permeabilizing activity of the Anal P5. The cell membrane alterations are similar to those induced by melittin used as a comparison.

3.5. Structural analysis of peptides by CD measurements

In order to investigate the relationship of the structure and the antibiotic activity of the peptides on lipids, the CD spectra of the peptides in phosphate buffer and TFE solution or SDS micelles was measured. CA-MA showed higher a-helical contents than the Anal P5 peptides in 50% TFE solution. Although Anal P5 has more potent antibiotic activity in bacterial and fungal cells than CA-MA, it displayed lower a-helicity than CA-

Table 3. The percentage a-helicity of the peptides in various media deduced from CD spectra and its mean hydrophobicity

Peptides	% α-helicity*		
	Phosphate buffer	50% TFE	30 mM SDS
CA-MA	1.1	6.1	2.4
Anal P5	1.5	5.4	1.98

*The percentage a-helicity of the peptides was calculated with the following equation: % α-helicity = $([\theta]_{obs} \times 100)/\{[\theta]_{helix} \times (1\text{-}2.57/l)\}$ where $([\theta]_{obs}$ is the mean-residue ellipticity observed experimentally at 222 nm, $[\theta]_{helix}$ is the ellipticity of a peptide of infinite length with 100% helix population, taken as -39,500 deg·cm^2·dmol^{-1}, and l is the peptide length or, more precisely, the number of peptide bonds.

MA (Table 3). These results suggest that the α-helical content is not directly correlated with the enhanced antibiotic activity even though it may play an important role in killing bacterial and fungal cells.

4. Conclusions

In order to obtain antibiotic peptides useful for pharmaceutical applications, a strong antibiotic activity is required against bacterial or fungal cells. In the present study, the analogue was synthesized to increase net positive charge and hydrophobicity by introducing Lys- and Leu-substitution. This Anal P5, which exhibits potent antimicrobial activity, may have a potential as a specific pharmacological agent, and the understanding of its mode of action provides a valuable model for the study of the relationship between net positive charge, or hydrophobicity and antibiotic activity of peptides, as well as in the development of a novel therapeutic agent.

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