



Structure-Activity Relationships of Peptide Antibiotics with Improved Bacterial Cell Selectivity of Pseudin

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Abstract Pseudin is a naturally occurring 24 amino-acid-residue antimicrobial peptide derived from the skin of paradoxical frog *Pseudis paradoxa*. It shows potency against the bacteria and antibiotic-resistant bacteria strain, but has high cytotoxicity against mammalian cell. In our previous study, substitution of Pro¹¹ for Gly (Ps-P) increased bacterial cell selectivity but decreased the antibacterial activity of pseudin. In this study, we designed pseudin analogue, Ps-4K-P with increased cationicity up to +7 in Ps-P by substituting Glu14, Gln10, Gln24, and Leu18 with Lys. Ps-4K-P showed improved potent antibacterial activity with high bacterial cell selectivity. We determined the tertiary structure of Ps-4K-P in the presence of DPC micelles by NMR spectroscopy and it has a hinge structure at Pro¹¹ followed by three turn helices from Pro¹¹ to Val²³ at the C-terminus. Amphipathicity with increased cationicity as well as helix-hinge-helix structural motif provided by introduction of a Pro at position Gly¹¹ are the crucial factors which confer antibacterial activity with bacterial cell selectivity to Ps-4K-P.

Keywords Pseudin, Antimicrobial peptide, Structure, NMR, bacterial cell selectivity

Introduction

Up to now, many therapeutic antibiotics are developed and used. Recently, pathogenic bacteria and fungi have resistance to commonly used antibiotics. So, it is important to search for novel types of antimicrobial agent which is not been exposed against microorganisms. The probability of clinical success for host defense peptide-based therapeutics is on the rise as options for a wider range of clinical indications emerge.¹ Antimicrobial peptides are an abundant and diverse group of molecules that are produced by many tissues and cell types in a variety of invertebrate, plant and animal species.² The antimicrobial activities of antimicrobial peptides play important roles in the host defense system, and the innate immunity of all species is related to the ability of the peptides to adopt an amphipathic structure, including an α -helix, β -sheet, or β -turn. Although the detailed mechanisms are not fully understood, antibiotic activity appears to involve transmembrane pore formation or intracellular killing.³

Many kinds of antimicrobial peptides have been isolated from skin extracts and/or skin secretions of frogs belonging to the Ascaphidae, Bombinatoridae, Hylidae, Hyperoliidae Myobatrachidae, Pipidae, and Ranidae families.⁴ Antimicrobial peptides from frog skin comprise between 12 and 48 amino acid residues and a comparison of their amino acid sequences reveals the lack of any consensus amino acid sequences that are associated with biological activity.

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The family Pseudidae comprises four species of semiaquatic frogs, organized in two genera *Pseudis* and *Lysapus*, which are found in tropical lowland areas in the eastern part of South America. Pseudins (1-4), four structurally related peptides with antimicrobial activity were isolated from an extract of the skin of the paradoxical frog *Pseudis paradoxa* (Pseudidae).⁵ We have determined the tertiary structure of Pseudin-2 (GLNALKKVFQGIHEAIKLNHVVQ) and it revealed that pseudin has a linear α -helix from Leu² to Glu²⁴.⁶ Since pseudin has high cytotoxicity against mammalian cells, we tried to design more potent peptides. We designed Ps-P analogues with substitution of Gly¹¹ with Pro as well as Ps-K14-K18-P with further substitution of E14 and L18 with Lys to increase bacterial cell selectivity.⁶ These Ps analogues exhibited reduced cytotoxicity but antimicrobial activity was also decreased. In this study, we designed new analogue, Ps-4K-P with four Lys substitution for Glu, Gln, and Leu, retaining high antibacterial activity with bacterial cell selectivity. We also determined the tertiary structure of Ps-4K-P in 150 mM DPC micelles using NMR spectroscopy to understand the structural requirements of peptides for potent and selective activity against microbial cells. This study would be of considerable help in designing peptides useful for future therapeutic use.

Experimental Methods

Peptide synthesis- Peptides were prepared by solid-phase synthesis using Fmoc (fluorenylmethoxycarbonyl) chemistry and purified by reversed-phase preparative high-performance liquid chromatography (HPLC) on a C18 column (20 × 250 mm; Shim-pack) as described previously.⁶ The molecular mass of peptide was determined by matrix-assisted laser-desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry (Shimadzu, Kyoto, Japan).

Antibacterial activity- *Escherichia coli* (KCTC 1682), *Pseudomonas aeruginosa* (KCTC 1637),

Staphylococcus aureus (KCTC 1621), *Bacillus subtilis* (KCTC 3068) were purchased from the Korean Collection for Type Cultures, Korea Research Institute of Bioscience & Biotechnology (Daejeon, Korea). Minimum inhibitory concentrations (MICs) of Ps-4K-P peptide against these bacteria were determined using a broth microdilution assay and compared with those of pseudin analogues as described in our previous report.⁶

Hemolytic activity- Hemolytic activity of the peptides was tested against human red blood cells (hRBCs) as described previously.⁷ Controls for no hemolysis (blank) and 100% hemolysis consisted of human red blood cells suspended in PBS and 0.1% Triton-X 100, respectively. The percent hemolysis was calculated using the following equation:

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

NMR experiments and assignment- Perdeuterated DPC was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Ps-4K-P dissolved at 1.0 mM in 0.45 ml of 9:1 (v/v) H₂O/D₂O (pH 4.6) containing 150 mM DPC micelles. We performed phase-sensitive 2D experiments, including total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY) using time-proportional phase incrementation.^{8,9} 50 and 80 ms MLEV-17 spin-lock mixing pulses were used for TOCSY experiments and for NOESY experiments, mixing times of 150 ms and 250 ms were used. The ³J_{HN α} coupling constants were measured from the DQF-COSY spectra. Chemical shifts are expressed relative to the DSS at 0 ppm. All NMR spectra were recorded on a Bruker 800 MHz spectrometer (Bruker, Rheinstetten, Germany) at KBSI, Ochang. NMR spectra were processed with NMRPipe and visualized with Sparky.¹⁰

Structure calculations- Distance constraints were extracted from the NOESY spectra with mixing times

of 150 ms. Using the standard protocol of Cyana program in a LINUX environment, structure of Ps-4K-P was calculated.¹¹ A total of 500 structures were calculated using the torsion angle dynamics protocol. The structures were sorted according to the final value of the target function, and the best 20 structures were analyzed in terms of distance and angle violations.

Results

Cationicity is an integral component of antimicrobial peptide interacting with negatively charged phospholipids membranes of bacteria and other microorganisms. Pseudin-2 (Ps) has very low positively charged (+2) compared to other antimicrobial peptides. In our previous study, we designed Ps-P with substitution of Gly¹¹ with Pro. Since Leu¹⁸ is located on the hydrophilic side of the amphipathic α -helix and substitution of Leu¹⁸ with Lys can remarkable decrease of hydrophobicity and increase of cationicity of pseudin, we designed Ps-K14-K18-P with further substitution of E14 and L18 with Lys to increase bacterial cell selectivity. However, these peptides showed much lower antibacterial activities. Therefore, in this study, we further substituted two Gln with Lys to improve its antibacterial activities. Here, we designed Ps-4K-P with increased cationicity by substitution of Glu14, Gln10, Gln24, Leu18 with Lys, resulting in net charge of +7 as listed in Table 1.

Hemolytic activity- We checked the cytotoxicity of

the peptides against mammalian cells by measuring their abilities to cause lysis of human erythrocytes. Dose-response curves for the hemolytic activity of the peptides are shown in Fig. 1. Ps shows about 23% hemolytic activities at 100 μ M. All Ps analogues with Pro or Lys substitutions did not show hemolytic activity up to 100 μ M. These results imply that Pro in the middle of the helix disrupt the helical structure of the peptide and increases structural flexibility, resulting in decrease in cytotoxicity. Furthermore, substitution of Leu, Glu, and Gln with Lys increased the cationicity and decreased the cytotoxicity.

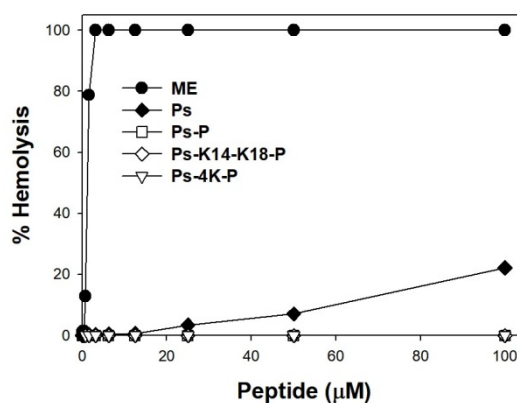


Figure 1. Dose-response curves of hemolytic activity of the peptides against h-RBCs induced by pseudin and its analogues

Antimicrobial activities- Antimicrobial activities of the peptides against a representative set of bacterial strains including two Gram-positive species as well as two Gram-negative bacteria as listed in Table 2. Introduction of Pro¹¹ in Ps resulted in dramatic

Table 1. Amino acid sequences and properties of the peptides

Peptide	Sequence	MW	Net charge
Ps (Native)	GLNALKKVFQGIHEAIKLNHHVQ	2685.5	2
Ps-P	GLNALKKVFQPIHEAIKLNHHVQ	2724.7	2
Ps-K14-K18-P	GLNALKKVFQPIHKAIKKLNHHVQ	2739.3	5
Ps-4K-P	GLNALKKVF K PIHKAIKKLNHHV K	2739.4	7

Table 2. Antimicrobial activity of Ps and its analogues

Peptide	MIC: μM						
	Gram (+)		Gram (-)		GM(μM)	MHC(μM)	Therapeutic Index (MHC/GM)
	<i>S. aureus</i>	<i>B. subtilis</i>	<i>E. coli</i>	<i>P. aeruginosa</i>			
Ps	4	4	1	2	2.8	50	18
Ps-P	32	32	8	8	20	>100	10.
Ps-K14-K18-P	32	32	4	8	19	>100	11
Ps-4K-P	4	4	1	1	2.5	>100	80.
Melittin	4	4	4	4	4	0.78	0.20

decrease of antimicrobial activity.⁶ Ps-K14-K18-P also showed decrease in antibacterial activities.⁶ Remarkably, Ps-4K-P showed potent antibacterial activity comparable to that of parent Ps. Ps-P and Ps-K14-K18-P showed poor antibacterial activity against Gram-positive bacteria. However, Ps-4K-P peptide showed broad spectrum antibacterial activity against not only Gram-negative bacteria but also Gram-positive bacterial strains.

Resonance Assignment- We performed sequence-specific resonance assignments using mainly the DQF-COSY, TOCSY, and NOESY data. Figure 2 shows the NOESY spectra with the sequential assignments for Ps-4K-P in the NH-NH region. This NOESY spectra showed that chemical shifts of Ps-4K-P in 150 mM DPC micelles were clearly assigned and all peaks are well resolved.

The sequential NOE connectivities and the other NMR data are illustrated in Fig. 3. As shown in Fig. 3, many nonsequential NOE connectivities that are characteristic of an α -helix, specifically $d_{\alpha\text{N}}(i, i+3)$ and $d_{\alpha\text{N}}(i, i+4)$, were observed. Ps-4K-P has $d_{\alpha\text{N}}(i, i+3)$ and $d_{\alpha\text{N}}(i, i+4)$ connectivities from Pro¹¹ to Val²³.

The observed values of the $^3J_{\text{HN}\alpha}$ coupling constants for the helical region of all the peptides were generally below 6 Hz and temperature coefficients of

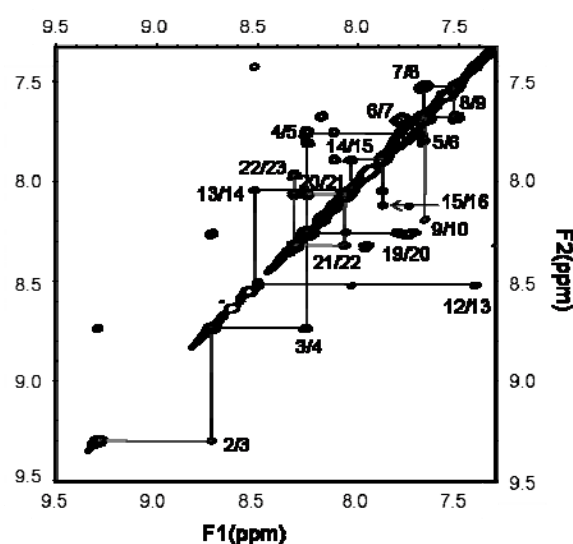


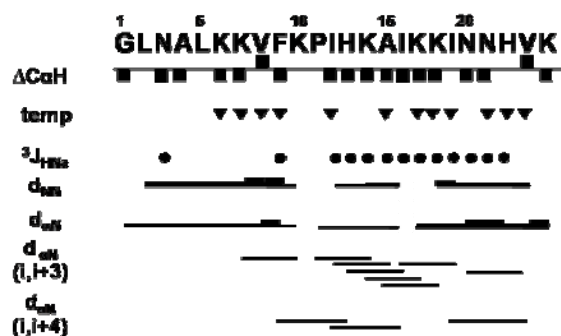
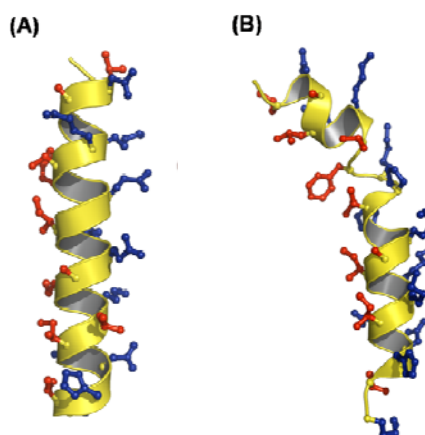
Figure 2. NOESY spectra of the NH-NH region of Ps-4K-P in 150 mM DPC micelles at pH 5.9 and 303 K (mixing time, 250 ms).

the amide protons in the α -helical region of the peptides were generally above -4.5 ppb/K. The ^1H chemical shift deviation was referenced according to the method of Wishart et al.¹² In the case of Ps-4K-P, residues from Ile¹² to the C-terminus have a negative $^1\text{H}\alpha$ Chemical Shift Index (CSI) as well as small $^3J_{\text{HN}\alpha}$ coupling constants which imply that it has a stable α -helix structure in this region.

Table 3. Structural statistics and mean pairwise root mean squared deviations for the 20 lowest energy structures of Ps-4K-P in 150 mM DPC Micelles.

Restraints for structure calculation	Ps-4K-P
Experiment distance restraints	
Total	259
Sequential	80
Medium range	39
Intra residue	115
Hydrogen-bond restraints	10
Dihedral angle restraints	15
Rmsd from experimental geometry	
NOE (Å)	0.020±0.00
ϕ (deg)	0.119±0.13
Rmsd from covalent geometry	
Bonds (Å)	0.002±0.00
Angles (deg)	0.484±0.01
Impropers (deg)	0.348±0.01
Average energies (kcal mol ⁻¹)	
E_{tot}	39.67±2.87
E_{NOE}	4.84±1.27
E_{tor}	0.03±0.04
E_{repe1}	1.72±0.86
Rmsd from the mean structure	
Backbone atoms of all residues	1.899±0.61
All heavy atoms of all residues	2.668±0.96
Backbone atoms of residues (11-24)	0.504±0.21
All heavy atoms of residues (11-24)	1.271±0.20

^a E_{NOE} , E_{tor} and E_{repe1} are the energies related to the NOE violations, the torsion angle violations, and the van der Waals repulsion term, respectively. The value of the square-well NOE (E_{NOE}) and torsion angle (E_{tor}) potentials were calculated with force constants of 50 kcal mol⁻¹ Å⁻² and 200 kcal mol⁻¹ rad⁻², respectively. The values of the quartic van der Waals repulsion term (E_{repe1}) was calculated with a force constant 4 kcal mol⁻¹ Å⁻⁴. The rmsd values were obtained by best fitting the backbone atom (N, C, C', and O) coordinates for all residues of the 20 converged structures. The numbers given for the backbone and all heavy atoms represent the mean values ± standard deviations

**Figure 3.** Summary of NOE connectivities and C_αH chemical shift indices for Ps-4K-P in 150 mM DPC micelles. The thickness of the line for the NOEs reflects the intensity of the NOE connectivities**Figure 4.** The ribbon diagram of the average structure of Ps (2NCX)⁶, (B) The ribbon diagram of the average structure of Ps-4K-P determined in 150 mM DPC micelles.

Structure of Ps-4K-P- Structure of Ps-4K-P has been calculated using NOE constraints. To calculate the tertiary structure of Ps-4K-P, we used experimental restraints such as sequential ($|i-j|=1$), medium-range ($1 < |i-j| \leq 5$), long-range ($|i-j| > 5$), intraresidual distance, hydrogen bonding restraints, and torsion angle restraints. Of the structures that were accepted with small deviations from the idealized covalent geometry and the experimental restraints (≤ 0.05 Å for bonds, $\leq 5^\circ$ for angles, $\leq 5^\circ$ for chirality, ≤ 0.3 Å from NOE restraints, and $\leq 3^\circ$ from torsion angle

restraints), we analyzed 20 output structures with the lowest energy for peptide. Fig. 4 shows the ribbon diagram of average structure of Ps (PDB ID 2NCX)⁶ and that of Ps-4K-P in 150 mM DPC micelles determined in this study. According to Procheck analysis Ps-4K-P has a bent structure at Pro¹¹, an α -helix from Leu² to Val⁸ and Pro¹¹ to Val²³ while β -turn from Phe⁹ to Ile¹². Pro forms a flexible hinge and that this hinge at the central region of a helical antimicrobial peptide is important for conferring high selectivity against bacterial cells.

Discussion

In this study, we attempted to develop novel peptide antibiotics with selectivity for bacterial cells by designing Pro and Lys-substituted analogue of pseudin-2. We then evaluated the antimicrobial activity, cytotoxicity against mammalian cells, and structure-activity relationships.

Cationicity is a crucial factor on peptides because of interactions of antimicrobial peptides to negatively charged phospholipids membranes of bacteria and other microorganisms. Thus, having the higher cationicity, peptides have the higher antimicrobial activity against bacteria but also increase of cytotoxicity against mammalian cells, too. Substitution of Pro for Gly¹¹ in linear alpha helical pseudin resulted in a decrease in both α -helical contents and a dramatically decreased cytotoxicity against human erythrocytes when compared with its linear structure analogues. In this study, Ps-4K-P with Pro¹¹ substitution and Lys-substitution for Gln¹⁰, Glu¹⁴, Leu¹⁸, and Gln²⁴ had higher antibacterial activity compared to those of the analogues of Ps designed previously such as Ps-P and Ps-K14-K18-P. All analogues with Pro¹¹ substitution showed no cytotoxicity against human erythrocytes at 100 μ M. Table 2 lists the geometric mean values (GM) for the MIC values against of 4 bacterial strains and minimal concentration (MHC) that produces hemolysis

against human red blood cells (hRBCs), and the therapeutic index (MHC/GM), which is the ratio of MHC to the average MIC. Thus, a high therapeutic index is an indication of the 2 preferred characteristics of the peptide: a high MHC (low hemolysis) and a low MIC (high antimicrobial activity). If peptide does not show hemolytic activity up to 100 μ M, MHC values set to 200 μ M. Since control peptide, melittin has high antibacterial activities but also has high cytotoxicity, therapeutic index was 0.2. Since Ps-P and Ps-K14-K18-P have not only high bacterial cell selectivity but also poor antibacterial activity, therapeutic activity was about 10. Therapeutic index of parent peptide Ps was 18 while that of Ps-4K-P was 80. Therefore, Ps-4K-P is the most potent peptide among all Ps analogues. Especially, Ps-4K-P also had a good antimicrobial activity against Gram-positive bacteria.

Investigation of the structure-activity relationship of peptides provides useful information for the development of potent peptides.^{12,13} We analyzed the tertiary structure of Ps-4K-P and compared it with that of parent peptide Ps. Ps in DPC micelles has amphipathic α -helical structure from Leu² to Val²⁴ orientating hydrophobic side chains toward one side, and the hydrophilic side chains toward the other side (Fig. 4A).⁶ This result revealed that Ps may enhance the formation of ion channel where hydrophilic residues face inward to contact the solvent and the hydrophobic side chains face toward the acyl chains of the hydrophobic lipid in bacterial cell membrane. The tertiary structure of Ps-4K-P shown in Fig. 4B revealed that Pro¹¹ disrupts α -helical structure and Ps-4K-P forms a flexible bent structure at Pro¹¹. Structural flexibility induced by Pro in Ps-4K-P and Lys substitution provides high amphipathic properties with high cationicity. These might be the key factors for the bacterial cell selectivity of Ps-4K-P. Ps-4K-P could be a candidate of potent peptide antibiotics and this study will give insight for the design of potent peptide therapeutics.

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