Journal of Peptide Science
J. Pept. Sci. 2008; **14**: 876–882
Published online 15 February 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.1019

JOURNAL OF PEPTIDE SCIENCE

Bacterial selectivity and plausible mode of antibacterial action of designed Pro-rich short model antimicrobial peptides

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Received 14 November 2007; *Revised 20 December 2007*; *Accepted 24 December 2007*

Abstract: To develop novel Pro-rich model AMPs with shorter length and higher bacterial selectivity/therapeutic index (TI) than natural AMP, indolicidin, we synthesized a series of undodecapeptides derived from the sequence XXPXXPWXPXX-NH_2 (X indicates Leu or Lys) with different ratios of Lys and Leu residues. Several Pro-rich model peptides (K_7WP_3, $K_6WL_1P_3$, $K_5WL_2P_3$-1, $K_5WL_2P_3$-2, and $K_4WL_3P_3$) had approximate 8- to 11-fold higher bacterial selectivity/TI compared to indolicidin. These peptides selectively bind to negatively charged liposomes (EYPG/EYPG; 7 : 3, w/w) mimicking bacterial membranes. Their high selectivity to negatively charged phospholipids corresponds well with their high bacterial selectivity. Indolicidin showed almost complete depolarization of the cytoplasmic membrane of *Staphylococcus aureus* and dye-leakage from negatively charged liposomes at 10 μM, whereas all of Pro-rich model peptides had very little activity in these assays even at 80 μM, as observed in buforin 2. These results suggest that the ultimate target of our designed Pro-rich model peptides is probably the intracellular components (e.g. protein, DNA or RNA) rather than the cytoplasmic membranes. Collectively, our designed Pro-rich short model peptides appear to be excellent candidates for future development as a novel antimicrobial agent. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Pro-rich short model AMP; indolicidin; bacterial selectivity; therapeutic index

INTRODUCTION

AMPs play important roles in innate immune response of all living organisms including humans [1–5]. They have received attention as alternative classes of antimicrobial agents because of their selectivity for prokaryotes and promise of minimizing antibacterial resistance [4]. Many different families of AMPs (based on their amino acid sequence and secondary structure) have been isolated from insects, plants, animals, and microorganisms [1,2]. Despite the wide variety of AMPs in origination, they can be classified into three groups: α-helical peptides [6,7], Cys-rich peptides with one to several disulfide bridges [8,9], and peptides rich in certain amino acids such as Trp or Pro [10–14].

Indolicidin (ILWPKWPWWPWRR-NH_2) is a Trp and Pro-rich member discovered from the cytoplasmic granules of bovine neutrophils [15]. Because of its small molecular size of 13-amino acid residues, indolicidin was considered as a target molecule for development of new antibiotic by peptide engineering. Although it displays activity against a wide range of targets, such as gram-positive bacteria, gram-negative bacteria and fungi, its relatively high toxicity toward mammalian cells prevents their usage as antibiotics [16]. A key problem in the clinical development of natural or synthetic AMPs is the degree of the selectivity between bacterial and mammalian cells.

In this study, to develop novel Pro-rich model AMPs with shorter length and bacterial selectivity higher than natural indolicidin, we synthesized a series of 11-length Pro-rich peptides with different ratio of Lys and Leu residues, XXPXXPWXPXX-NH_2 (X here represents Leu or Lys). Structure–function relationship studies on indolicidin demonstrated that the hemolytic activity of indolicidin is involved in its multiple Trp residues [10,11,15,16]. Subbalakshmi *et al.* reported that indolicidin analogs with a single tryptophan at 4th, 8th or 11th position and the others replaced by leucine retained antibacterial activity and reduced hemolytic activity [16]. For this reason, our designed model peptides contain a single Trp residue.

To investigate their usefulness as a therapeutic antimicrobial agent, we compared the bacterial selectivity of the peptides to that of indolicidin. Furthermore, to examine the molecular basis of

Abbreviations: AMPs, antimicrobial peptides; $DiSC_3$-5, 3,3′-dipropylthiadicarbocyanine iodide; EYPC, egg yolk L-α-phosphatidylcholine; EYPE, egg yolk L-α-phosphatidylethanolamine; EYPG, egg yolk L-α-phosphatidyl-DL-glycerol; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); hRBCs, human red blood cells; LUVs, large unilamellar vesicles; MHC, minimal hemolysis concentration; PBS, phosphate buffer saline; SUVs, small unilamellar vesicles

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their bacterial selectivity, we measured their interaction with model phospholipid membranes by measuring tryptophan fluorescence. Furthermore, we assessed their abilities to induce dye-leakage from bacterial membrane-mimicking model liposomes and depolarize membranes of intact *Staphylococcus aureus*.

MATERIALS AND METHODS

Materials

Rink amide 4-methylbenzhydrylamine resin, Fmoc-amino acids and other reagents for the peptide synthesis were purchased from Calibochem-Novabiochem (La Jolla, CA). EYPC, EYPE, EYPG, cholesterol, EDTA, HEPES, calcein and gramicidin D were obtained from Sigma Chemical Co. (St. Louis, MO). $DiSC_3(5)$ was obtained from Molecular Probes (Eugene, OR). All other reagents were of analytical grade. The buffers were prepared in double-glass-distilled water.

Peptide Synthesis

The peptides used in this study were prepared by the standard Fmoc-based solid-phase synthesis technique [17] on a solid support of Rink amide MBHA resin. DCC and HOBt (*N*-hydroxybenzotriazole) were used as coupling reagent, and 10-fold excess Fmoc-amino acids were added during every coupling cycle. After cleavage and deprotection with a mixture of TFA/water/thioanisole/ethanedithiol/triisopropylsilane (81.5 : 5 : 5 : 5 : 2.5 : 1, v/v) for 2 h at room temperature, the crude peptides were repeatedly extracted with diethyl ether and purified by RP-HPLC on a preparative Vydac C_{18} column (15 μm, 20 × 250 mm) using an appropriate 0–80% water/acetonitrile gradient in the presence of 0.05% TFA. The final purity of the peptides (>98%) was assessed by RP-HPLC on an analytical Vydac C_{18} column (4.6 × 250 mm, 300 Å, 5 μm particle size). The molecular mass of the purified peptides was determined by MALDI-TOF MS (Shimadzu, Japan) (Table 1). The concentration of each peptide was determined by amino acid analysis (Hitachi Model, 8500 A, Japan).

Antimicrobial Activity (MIC)

The antibacterial activities of the peptides against two gram-positive and two gram-negative bacteria were examined in sterile 96-well plates using the broth microdilution method as previously reported [18–20]. 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 Microplate autoreader EL 800 (Bio-Tek Instruments). MIC is defined as the minimal peptide concentration that inhibits bacteria growth. Two types of gram-positive bacteria [*Staphylococcus epidermidis* (Korean Collection for Type Cultures, KCTC 1917) and *S. aureus* (KCTC 1621)] and two types of gram-negative bacteria [*Escherichia coli* (KCTC 1682)] and *Salmonella typhimurium* (KCTC 1926)] were procured from the KCTC at the Korea Research Institute of Bioscience and Biotechnology.

Hemolytic Activity (MHC)

Fresh hRBCs were washed three times with PBS by centrifugation for 7 min at 1000 × g and resuspended in PBS. The peptide solutions (serial 2-fold dilutions in PBS) were added to 100 μl suspension of hRBCs [4% (v/v) in final] in PBS to the final volume of 200 μl and were incubated for 1 h at 37 °C. The samples were then centrifuged at 1000 × g for 5 min, and the release of hemoglobin was monitored by measuring the absorbance of the supernatant at 405 nm by Microplate ELISA Reader. For negative and positive controls, hRBCs in PBS (A_{blank}) and in 0.1% Triton X-100 (A_{triton}) were used, respectively. The percentage of hemolysis was calculated according to the equation: % hemolysis = $100 \times [(A_{sample} - A_{blank})/(A_{triton} - A_{blank})]$.

Preparation of Small Unilamellar Vesicles (SUVs)

SUVs were prepared by a standard procedure with required amounts of either EYPE/EYPG (7 : 3, w/w) or EYPC/cholesterol

Table 1 Amino acid sequences, calculated and observed molecular masses and net charge of the peptides used in this study

Peptide	Amino acid sequence	Net charge	Molecular mass (Da)	
			Calculated	Observed
K_7WP_3	KKPKKPWKPKK-NH_2	+8	1391.8	1392.0
$K_6WL_1P_3$	KKPKLPWKPKK-NH_2	+7	1376.8	1375.7
$K_5WL_2P_3$-1	KLPKLPWKPKK-NH_2	+6	1361.8	1362.6
$K_5WL_2P_3$-2	KKPKLPWLPKK-NH_2	+6	1361.8	1362.0
$K_4WL_3P_3$	KKPKLPWLPLK-NH_2	+5	1346.8	1347.7
$K_3WL_4P_3$	KLPLLPWLPKK-NH_2	+4	1331.7	1331.5
$K_2WL_5P_3$	KLPLLPWLPLK-NH_2	+3	1316.7	1316.1
Indolicidin	ILPWKWPWWPWRR-NH_2	+4	1909.3	1909.5

DOI: 10.1002/psc

(10 : 1, w/w) for tryptophan fluorescence. Dry lipid was dissolved in chloroform in a small glass vessel. Solvent was removed by rotary evaporation to form a thin film on the wall of the glass vessel and then lyophilized overnight. Dried thin films, prepared this way, were resuspended in Tris–HCl buffer by vortex mixing. The lipid dispersions were then sonicated in ice water for 10–20 min with a titanium-tip ultrasonicator until the solutions became transparent.

Tryptophan Fluorescence Blue Shift

The fluorescence emission spectrum of tryptophan of peptides was monitored in aqueous Tris-HCl buffer, and in the presence of vesicles composed of either EYPE/EYPG (7 : 3 w/w) SUVs or EYPC/cholesterol (10 : 1 w/w) SUVs. In these fluorometric studies, SUVs were used to minimize differential light scattering effects [21,22]. The tryptophan fluorescence measurements were taken with a model RF-5301PC spectrophotometer (Shimadzu, Japan). Each peptide was added to 3 ml of Tris-HCl buffer (10 mM Tris, 0.1 mM EDTA, 150 mM NaCl, pH 7.4) containing 0.6 mM liposomes (pH 7.4), and the peptide/liposome mixture (molar ratio of 1 : 200) was allowed to interact at 20 °C for 10 min. The fluorescence was excited at 280 nm, and the emission was scanned from 300 to 400 nm.

Dye-Leakage

Calcein-entrapped LUVs composed of negatively charged EYPE/EYPG (7 : 3, w/w) were prepared by vortexing the dried lipid in dye buffer solution (70 mM calcein, 10 mM Tris, 150 mM NaCl, 0.1 mM EDTA, pH 7.4). The suspension was frozen–thawed in liquid nitrogen for 11 cycles and extruded through polycarbonate filters (two stacked filters; pore size, 100 nm) by a LiposoFast extruder (Avestin, Inc., Canada). Untrapped calcein was removed by gel filtration on a Sephadex G-50 column. The leakage of calcein from the LUVs was monitored by measuring fluorescence intensity at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. For the determination of 100% dye-release, 0.1% Triton-X100 in Tris-HCl buffer was added to dissolve the vesicles. The percentage of dye-leakage caused by 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.

Membrane Depolarization

The cytoplasmic membrane depolarization activity of peptides was determined as previously described [23–25], using *S. aureus* strain KCTC 1621 and the membrane potential-sensitive dye, $DiSC_3$-5. Briefly, *S. aureus* was grown at 37 °C with agitation to mid-log phase ($OD_{600} = 0.4$) and harvested by centrifuge. The cells were washed twice with washing buffer (20 mM glucose, 5 mM HEPES, pH 7.4) and resuspended to an OD_{600} of 0.05 in a similar buffer containing 0.1 M KCl. Following, the cells were incubated with 20 nM $DiSC_3$-5 until a stable reduction of fluorescence was achieved, indicating the incorporation of the dye into the bacterial membrane. Membrane depolarization was then monitored by observing the change in the intensity of fluorescence emission of the membrane potential-sensitive dye $DiSC_3$-5 ($\lambda_{ex} = 622$ nm, $\lambda_{em} = 670$ nm) after the addition of peptides. Full dissipation of the membrane potential was obtained by adding gramicidin D (final concentration, 0.2 nM).

RESULTS AND DISCUSSION

Antimicrobial and Hemolytic Activity

To provide an overall measure of antimicrobial activity of the peptides, we calculated the geometric mean (GM) of the minimal peptide concentration that inhibits bacteria growth (MIC) in four selected bacterial strains (Table 2). Compared to indolicidin, the

Table 2 Antimicrobial activity of the peptides used in this study

Peptide	MIC[a] (μM)			
	Gram-negative bacteria		Gram-positive bacteria	
	E. coli (KCTC 1682)	*S. typhimurium* (KCTC 1926)	*S. epidermidis* KCTC 1917)	*S. aureus* (KCTC 1621)
K_7WP_3	10 ± 0	6.7 ± 0.7	12.9 ± 1.6	18.2 ± 3.7
$K_6WL_1P_3$	8 ± 0	6.7 ± 0.7	8.9 ± 0.9	16 ± 0
$K_5WL_2P_3$-1	8 ± 0	4.0 ± 0	13.3 ± 1.3	18.7 ± 3.5
$K_5WL_2P_3$-2	8 ± 0	5.3 ± 0.7	10.7 ± 1.3	13.3 ± 1.3
$K_4WL_3P_3$	8 ± 0	8 ± 0	13.3 ± 1.3	24 ± 4
$K_3WL_4P_3$	64 ± 0	64 ± 0	64 ± 0	64 ± 0
$K_2WL_5P_3$	>64 ± 0	>64 ± 0	>64 ± 0	>64 ± 0
Indolicidin	7.5 ± 0.4	6.7 ± 0.7	6.7 ± 0.7	5.3 ± 0.7

[a] MIC is the mean of three independent experiments performed in triplicate ± S D

DOI: 10.1002/psc

five Pro-rich peptides (K_7WP_3, $K_6WL_1P_3$, $K_5WL_2P_3$-1, $K_5WL_2P_3$-2 and $K_4WL_3P_3$) with +5 to +8 net positive charge showed almost similar or only 2- to 3.5-fold reduced antimicrobial activity. In contrast, $K_3WL_4P_3$ and $K_2WL_5P_3$ with +4 to +3 net positive charge displayed very low antimicrobial activity (MIC 64 μM or >64 μM). To exhibit the hemolytic activity of the peptides, we consider here MHC, which is defined as the minimal peptide concentration that produces 10% hemolysis. Indolicidin induced a significant hemolysis at 50 μM. However, none of Pro-rich model peptides caused hemolysis at concentrations as high as 400 μM (Table 3).

Bacterial Selectivity/Therapeutic Index

To assess the bacterial selectivity of Pro-rich model peptides, we calculated their TI, which is a widely accepted measure of the bacterial selectivity of antimicrobial agents [14,26,27]. The TI of each peptide was calculated as the ratio of MHC-to-GM (GM of the MIC values from four bacteria). When no hemolytic activity was detectable at the highest concentration tested (400 μM), an 800 μM MHC value was used for the TI calculation. When no antimicrobial activity was detectable at the highest concentration tested (64 μM), 126 μM MIC value was used for the TI calculation. Larger value of TI corresponds to a greater bacterial selectivity. Compared to indolicidin, the five Pro-rich peptides (K_7WP_3, $K_6WL_1P_3$, $K_5WL_2P_3$-1, $K_5WL_2P_3$-2 and $K_4WL_3P_3$) had approximately 8- to 11-fold higher bacterial selectivity/TI. In particular, $K_5WL_2P_3$-2 had the highest TI (approximately 11-fold increase compared to indolicidin). In contrast, $K_3WL_4P_3$ and $K_2WL_5P_3$ had very low TI of 12.5 and 6.2, respectively.

Table 3 Therapeutic index of the peptides used in this study

Peptide	GM (μM)[a]	MHC (μM)[b]	TI (MHC/GM)
K_7WP_3	11.9	>400	67.2
$K_6WL_1P_3$	9.9	>400	80.8
$K_5WL_2P_3$-1	11.0	>400	72.7
$K_5WL_2P_3$-2	9.3	>400	86.0
$K_4WL_3P_3$	13.3	>400	60.2
$K_3WL_4P_3$	64.0	>400	12.5
$K_2WL_5P_3$	>64.0	>400	6.2
Indolicidin	6.5	50	7.7

[a] The geometric mean (GM) of the MIC values from four bacterial strains described in Table 2.
[b] The MHC (minimal hemolytic concentration) is defined as the peptide concentration that produces 10% hemolysis.

Blue Shift of Trp Residues

Tryptophan residue in each peptide allowed us to monitor the binding specificity of the peptides to artificial liposomes by the fluorescence emission of the tryptophan (Figure 1 and Table 4). In negatively charged phospholipids [EYPE/EYPG (7 : 3, w/w) SUVs], which mimic bacterial membranes, the fluorescence emission maxima of all of Pro-rich model peptides exhibited a significant blue shift compared to that in in Tris-buffer, indicating that all the peptides anchor into the hydrocarbon region of bilayer. In zwitterionic phospholipid membranes [EYPC/cholesterol (10 : 1, w/w) SUVs], which mimic the outer surface of mammalian membranes, indolicidin also exhibited a significant blue shift (7.8 nm) in the fluorescence emission maxima. In contrast, all of Pro-rich model peptides exhibited none or very weak blue shift in zwitterionic phospholipids membranes (Figure 1 and Table 4). These results suggest that indolicidin has good binding affinity to both bacterial and mammalian membranes mimicked artificial liposomes, but all of Pro-rich model peptides can only bind to negatively charged artificial liposome. This lipid selectivity of all of Pro-rich model peptides for artificial liposomes corresponds well with their bacterial selectivity/TI (Table 3).

Peptide-Induced Dye Leakage from Negatively Charged Liposomes

To determine whether the antimicrobial activities of peptides depend on their ability to permeate bacterial membranes, we measured their abilities to induce the fluorescent dye calcein leakage from negatively charged EYPE/EYPG (7 : 3 w/w) LUVs, which mimics bacterial membranes. Indolicidin was able to achieve a significant dye-leakage (more than 70%) at 10 μM (this concentration is similar to its MIC). In contrast, all of Pro-rich model peptides had no or little dye-leakage

Table 4 Tryptophan emission maxima of the peptides (3 μM) in the presence of 0.6 mM EYPE/EYPG (7 : 3, w/w) SUVs and 0.6 mM EYPC/cholesterol (10 : 1, w/w) SUVs

Peptide	Tris-HCl buffer (nm)	EYPE/EYPG (7 : 3, w/w) (nm)	EYPC/cholesterol (10 : 1, w/w) (nm)
K_7WP_3	350.6	346.6 (4.0)	350.4 (0.2)
$K_6WL_1P_3$	350.4	344.4 (6.0)	350.2 (0.2)
$K_5WL_2P_3$-1	350.4	341.4 (9.0)	350.3 (0.1)
$K_5WL_2P_3$-2	350.4	344.4 (6.0)	350.3 (0.1)
$K_4WL_3P_3$	350.2	343.2 (7.0)	350.2 (0.0)
$K_3WL_4P_3$	351.6	343.8 (7.8)	351.5 (0.1)
$K_2WL_5P_3$	350.4	344.2 (6.2)	350.4 (0.0)
Indolicidin	348.8	341.0 (7.8)	341.0 (7.8)

DOI: 10.1002/psc

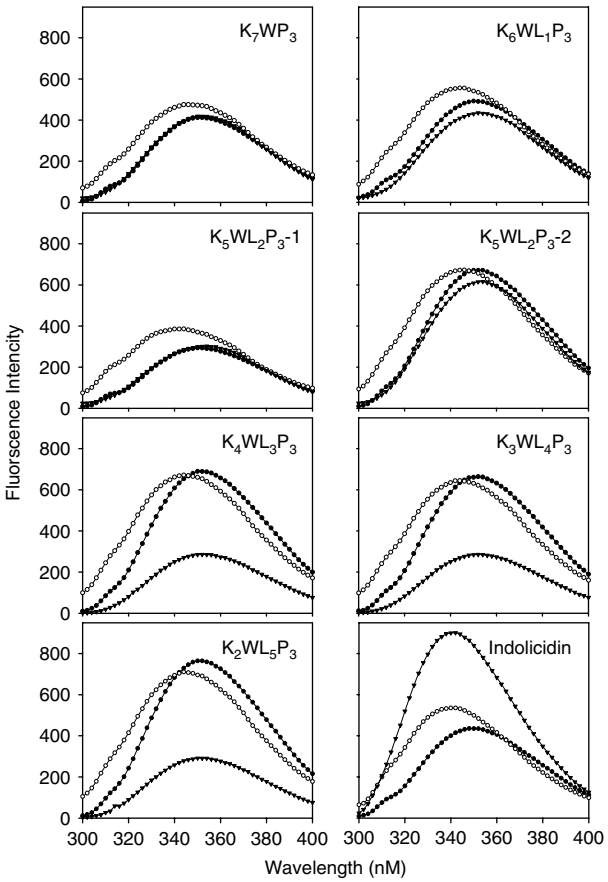

Figure 1 Trp fluorescence emission spectra of the peptides (3.0 μM) in Tris-buffer (pH 7.4) (●), 0.6 mM EYPE/EYPG (7 : 3, w/w) SUVs (○) and 0.6 mM EYPC/cholesterol (10 : 1, w/w) SUVs (▼).

at 10 μM, as observed in buforin 2 discovered in the stomach tissue of the Asian toad (Figure 2). Despite $K_5WL_2P_3$-1 and $K_5WL_2P_3$-2 having potent antimicrobial activity, these peptides induced very little dye-leakage (2.5 ~ 3.9%) even at 80 μM (Figure 2). Buforin 2 has been known to kill microorganisms by penetrating the cell membrane and inhibiting intracellular components such as protein, DNA or RNA [28–30].

Depolarization of the Membrane Potential in Intact Bacteria

To assess bacterial membrane depolarization, the membrane potential-sensitive dye $DiSC_3(5)$ was used. This cationic dye concentrates in the cytoplasmic membrane under the influence of the membrane potential resulting in a self-quenching of fluorescence. Upon disruption of the membrane potential, the dye dissociates into the buffer leading to an increase in fluorescence [23–25]. Depolarization was monitored over a period of 1500 s for the peptides. As shown in Figure 3, indolicidin can completely depolarize cytoplasmic membrane of intact *S. aureus* at 10 μM,

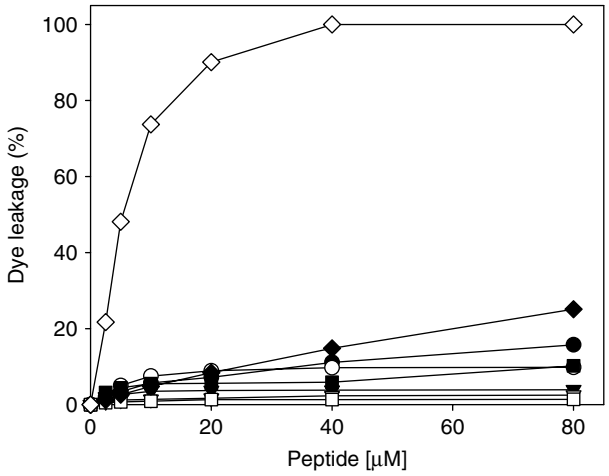

Figure 2 The concentration-dependent % leakage of calcein from negatively charged EYPE/EYPG (7 : 3, w/w) LUVs at pH 7.4 measured at 2 min after the addition of the peptides. The concentration of EYPE/EYPG (7 : 3, w/w) LUVs was 68 μM. Peptides are indicated as follows: K_7WP_3 (●), $K_6WL_1P_3$ (○), $K_5WL_2P_3$-1 (▼), $K_5WL_2P_3$-2 (▽), $K_4WL_3P_3$ (■), $K_3WL_4P_3$ (□), $K_2WL_5P_3$ (♦), Indolicidin (◊) and Buforin 2 (▲).

whereas all of Pro-rich model peptides caused no or little membrane potential depolarization. Furthermore, indolicidin caused a complete membrane depolarization at 2.5 μM. In addition, all of Pro-rich model peptides had little effect on membrane depolarization even at 80 μM, as observed in buforin 2 (Figure 4). Collectively, our results suggest that the bacterial-killing mechanism of our Pro-rich model peptides is probably by the inhibition of intracellular components rather than the depolarization and/or disruption of bacterial cytoplasmic membranes, as observed in buforin 2.

CONCLUSIONS

In this study, we have developed five Pro-rich model AMPs with shorter length and higher bacterial selectivity (TI) than a natural Pro-rich AMP, indolicidin, by synthesizing a set of undodecapeptides derived from the sequence XXPXXPWXPXX-NH_2 (X indicates Leu or Lys). Furthermore, our findings suggest that the ultimate target of our Pro-rich model peptides is probably not cytoplasmic membranes but intracellular components (e.g. protein, DNA or RNA). Finally, our Pro-rich short model peptides with high bacterial selectivity appear to be excellent candidates for future development as a novel antimicrobial agent.

Acknowledgements

This study was supported by the research fund from Chosun University, 2007.

DOI: 10.1002/psc

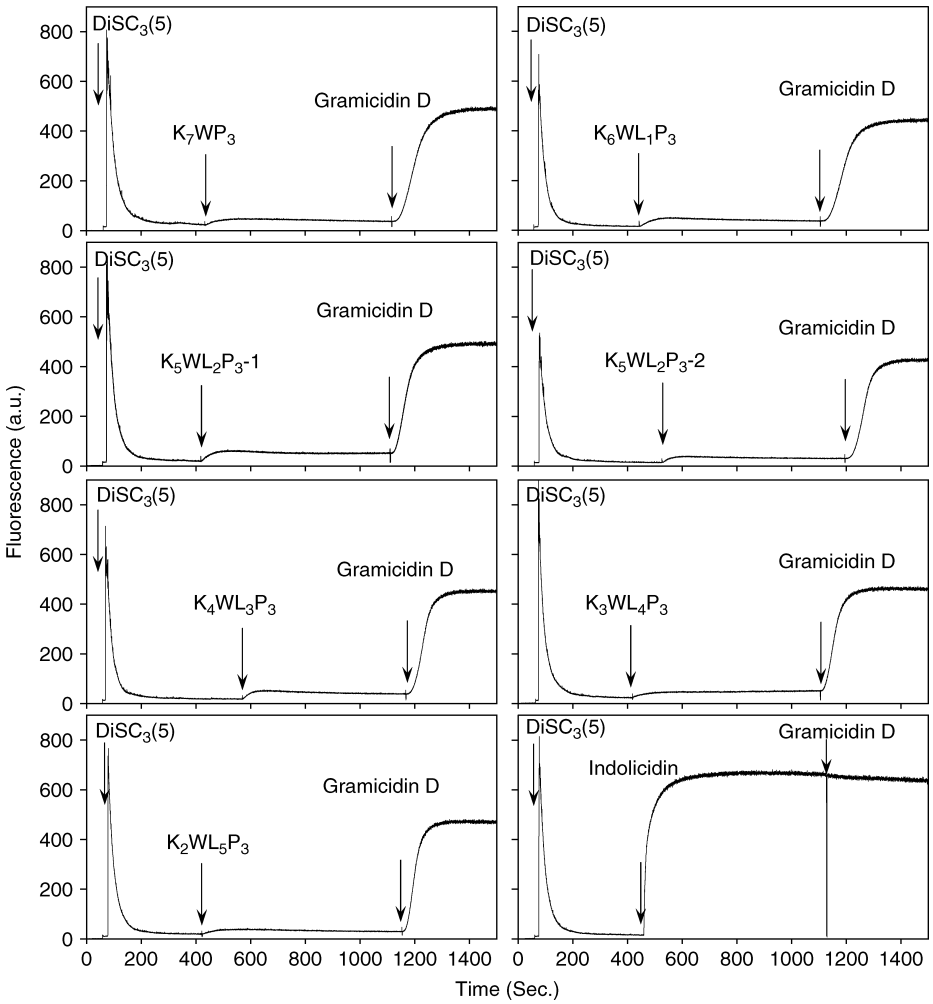

Figure 3 Cytoplasmic membrane depolarization of *S. aureus* (OD_{600} = 0.05) by the peptides (the peptide concentration: 10 μM) using the membrane potential-sensitive dye, $diSC_3$-5. Dye release was monitored at an excitation wavelength of 622 nm and an emission wavelength of 670 nm.

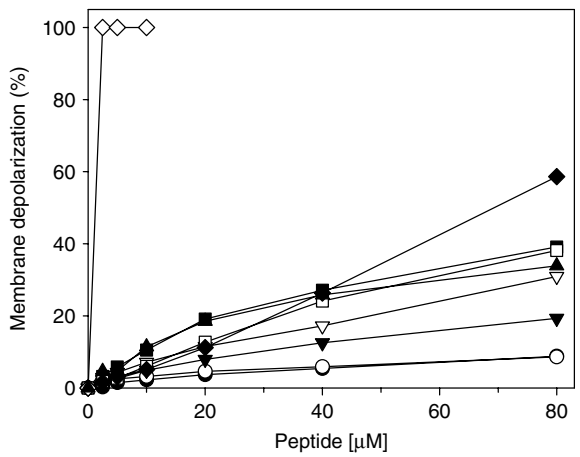

Figure 4 The concentration-dependent membrane depolarization of *S. aureus* (OD_{600} = 0.05) by the peptides using the membrane potential-sensitive dye, $diSC_3$-5. Dye-release was monitored at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. Peptides are indicated as follows: K_7WP_3 (●), $K_6WL_1P_3$ (○), $K_5WL_2P_3$-1 (▼), $K_5WL_2P_3$-2 (▽), $K_4WL_3P_3$ (■), $K_3WL_4P_3$ (□), $K_2WL_5P_3$ (♦), Indolicidin (◊) and Buforin 2 (▲).

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DOI: 10.1002/psc