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Cell selectivity and mechanism of action of short antimicrobial peptides designed from the cell-penetrating peptide Pep-1‡

Wan Long Zhu,[a] Kyung-Soo Hahm[a,b] and Song Yub Shin[a,b]*

Pep-1-K (PK) is a good cell-selective antimicrobial peptide designed from cell-penetrating peptide Pep-1. To develop novel short antimicrobial peptides with higher cell selectivity and shorter length compared with PK, several PK analogs were designed by the deletion, addition and/or substitution of amino acids. Among these analogs, PK-12-KKP (KKPWWKPWWPKWKK) showing the sequence and structure homology with a Trp/Pro-rich natural antimicrobial peptide, indolicidin (IN), displayed a 20-fold higher cell selectivity as compared to IN. Circular dichroism analysis revealed that PK-12-KKP adopts a folded structure combined with some portions of unordered structure. PK-12-KKP selectively binds to negatively charged bacterial membrane–mimetic vesicles, and its high phospholipid selectivity corresponds well with its high cell selectivity. Moreover, it showed very weak potential in depolarization of the cytoplasmic membrane of *Staphylococcus aureus* at 8 μM (4× minimal inhibitory concentration) and dye leakage from negatively charged liposomes. These results suggest that the ultimate target of our designed PK-12-KKP maybe the intracellular components (e.g. protein, DNA or RNA) rather than the cytoplasmic membranes. Collectively, our designed short Trp/Pro-rich peptide, PK-12-KKP, appears to be an excellent candidate for future development as a novel antimicrobial agent.

Keywords: antimicrobial peptide; cell-penetrating peptide; cell selectivity; Pep-1; Pep-1-K; mechanism of action

Introduction

Pep-1 (KETWWETWWTEWSQPKKKRKV) is a cell-penetrating peptide derived from the nuclear localization sequence of simian virus 40 large T antigen and from reverse transcriptase of human immunodeficiency virus [1–3]. It is composed of a hydrophobic tryptophan–rich domain (KETWWETWWTEW), a spacer domain (SQP) and a hydrophilic lysine–rich domain (KKKRKV). Pep-1 is cationic and adopts amphipathic α-helical structure on the membrane. These characteristics that are similar to those of cationic antimicrobial peptides involved in host innate immunity, suggesting that Pep-1 can kill microorganisms [4,5].

In our previous study, the antimicrobial activity of Pep-1 against several selected Gram-negative and Gram-positive bacterial strains was examined. Pep-1 had a broad spectrum but relatively weak antimicrobial activity [6]. Although some anionic peptides, such as dermcidin-derived peptides, have been found to kill microorganisms [7], most of the naturally occurring antimicrobial peptides are cationic and adopts α-helical secondary structure. Many studies revealed that the cationicity and α-helical secondary structure of these antimicrobial peptides are essential for their bacterial killing activity. Therefore, we suspected that the low antimicrobial activity of Pep-1 was due to the presence of anionic Glu residues, which may prevent the association of the peptide with the negatively charged bacterial membrane surface. Therefore, to increase the electrostatic interaction between the peptide and bacterial membranes, an analog of Pep-1, Pep-1-K (PK; KKTWWKTWWTKWSQPKKKRKV), was designed by Glu→Lys substitution of Pep-1 [6]. We found that PK exhibited strong antibacterial activity against Gram-positive and Gram-negative bacteria with no hemolytic activity even at a concentration of 200 μM. Its antimicrobial activity was comparable with that of melittin, known as a hemolytic antimicrobial peptide isolated from bee venom with powerful antimicrobial activity. Interestingly, PK had little ability in dye leakage from negatively charged liposomes, which mimic bacterial membranes but showed a potent ability to cause depolarization of the cytoplasmic membranes potential of intact *Staphylococcus aureus* cells. This result suggested that Pep-1-K kills microbes by the formation of small channels that permit transit of ions or protons but not molecules as large as calcein [6].

Because the clinical use of antimicrobial peptides is hampered by issues of cost, short antimicrobial peptides with high cell selectivity have been recently considered. To this aim, in the present study we designed and synthesized several peptides, based on the PK sequence, characterized by the deletion, addition and/or substitution of some residues. Their cell selectivity was investigated by examining their antimicrobial activities against Gram-positive and Gram-negative bacterial strains as well as their abilities to cause hemolysis. The affinity of the peptides to liposomes that mimic bacterial or eukaryotic membranes was

* *Correspondence to: Song Yub Shin, Department of Cellular & Molecular Medicine, School of Medicine, Chosun University, Gwangju 501-759, Korea. E-mail: syshin@chosun.ac.kr*

a *Department of Bio-Materials, Graduate School and Research Center for Proteineous Materials, Chosun University, Gwangju 501-759, Korea*

b *Department of Cellular & Molecular Medicine, School of Medicine, Chosun University, Gwangju 501-759, Korea*

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assayed by tryptophan fluorescence blue shift and tryptophan fluorescence quenching by acrylamide. Furthermore, the plausible bactericidal mechanism by which the peptides kill bacteria was investigated by measuring their abilities to cause the leakage of a fluorescent dye from lipid vesicles mimicking bacterial membranes and the depolarization of the cytoplasmic membrane potential of intact *S. aureus* cells.

Materials and Methods

Materials

Rink amide 4-methylbenzhydrylamine (MBHA) resin, Fmoc amino acid-Wang resin, Fmoc amino acids and other reagents for the peptide synthesis were purchased from Calbiochem-Novabiochem (La Jolla, California, USA). Egg yolk L-phosphatidylcholine (EYPC), egg yolk L-phosphatidyl-DL-glycerol (EYPG), calcein, cholesterol and gramicidin D were supplied from Sigma Chemical Co. (St Louis, Missouri, USA). 3,3′-dipropylthiadicarbocyanine iodide ($diSC_3$-5) was obtained from Molecular Probes (Eugene, Oregon, USA). 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 on a solid support of Rink amide MBHA resin or Wang resin. DCC and HOBt were used as coupling reagent, and tenfold excess Fmoc amino acids were added during every coupling cycle. After cleavage and deprotection with a mixture of trifluoroacetic acid/water/thioanisole/phenol/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 reverse phase 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% trifluoroacetic acid. The final purity of the peptides (>98%) was assessed by reverse phase 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).

Antimicrobial Activity (Minimal Inhibitory Concentration)

The antibacterial activities of the peptides against three Gram-positive bacterial strains, three Gram-negative bacterial strains and four antibiotic-resistant clinical isolates were examined in sterile 96-well plates, using the broth microdilution method as previously described [8,9]. Aliquots (100 μl) of a bacterial suspension at 2×10^6 colony-forming units (CFU)/ml in 1% peptone were added to 10 μl of peptide solution (serial twofold 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, Vermont, USA). The MIC is defined as the minimal peptide concentration that inhibits bacteria growth. Three types of Gram-positive bacteria [*Bacillus subtilis* (KCTC 3068), *Staphylococcus epidermidis* (KCTC 1917) and *S. aureus* (KCTC 1621)] and three types of Gram-negative bacteria [*Escherichia coli* (KCTC 1682), *Pseudomonas aeruginosa* (KCTC 1637) and *Salmonella typhimurium* (KCTC 1926)] were procured from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology.

Minimal Hemolytic Concentration (MHC)

Fresh human red blood cells (hRBCs) were washed three times with phosphate buffered saline (PBS; 35 mM phosphate buffer, 0.15 M NaCl, pH 7.4) by centrifugation for 7 min at 1000× g and resuspended in PBS. The peptide solutions (serial twofold 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. The MHC is defined as the minimal peptide concentration that produces hemolysis. 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 following equation:

$$\%\ \text{hemolysis} = 100 \times [(A_{sample} - A_{blank})/(A_{triton} - A_{blank})]$$

Preparation of Small Unilamellar Vesicles

Small unilamellar vesicles (SUVs) were prepared by a standard procedure with required amounts of either EYPC/EYPG (7 : 3, w/w) or EYPC/cholesterol (10 : 1, w/w) for tryptophan fluorescence. Dry lipids were dissolved in chloroform in small glass vessel. Solvents were removed by rotary evaporation to form a thin film on the wall of a glass vessel and then lyophilized overnight. Dried thin films 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 solution became transparent.

Table 1. Amino acid sequences and calculated and observed molecular masses of the peptides

Peptide	Amino acid sequence	Molecular mass (Da)		Net charge	Retention time (min)[a]
		Calculated	Observed		
Pep-1-K (PK)	KKTWWKTWWTKWSQPKKKRKV	2845.4	2845.7	+9	27.8
PK-17	KKTWWKTWWTKWSQPKK	2333.8	2333.3	+6	28.4
PK-15	KKTWWKTWWTKWSQP	2077.4	2076.7	+4	29.4
PK-12	KKTWWKTWWTKW	1765.1	1764.7	+4	30.6
PK-12-KK	KKTWWKTWWTKWKK	2021.4	2020.9	+6	27.9
PK-12-KKP	KKPWWKPWWPKWKK	2009.5	2009.6	+6	27.7
Indolicidin	ILPWKWPWWPWRR-NH_2	1906.3	1906.1	+4	33.7

[a] Retention time was measured using an analytical Vydac C_{18} column (4.6 × 250 mm, 300 Å, 5-μm particle size). The peptides were eluted over 40 min, using a linear gradient of 0% to 60% acetonitrile in water containing 0.05% (v/v) trifluoroacetic acid.

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Tryptophan Fluorescence Blue Shift

The fluorescence emission spectrum of the tryptophan residues in the peptides was monitored in aqueous Tris-HCl buffer, Tris-HCl buffer containing 1.5 M NaCl and in the presence of vesicles composed of either EYPC/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 [10,11]. 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 (a 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.

Tryptophan Fluorescence Quenching by Acrylamide

For fluorescence quenching experiment, acrylamide was used as the quencher. To reduce absorbance by acrylamide, excitation of Trp at 295 nm instead of 280 nm was used [12,13]. Aliquots of the 3.0 M solution of this water-soluble quencher were added to the peptide in the presence of 0.6 mM EYPC/EYPG (7 : 3 w/w) SUVs or EYPC/cholesterol (10 : 1 w/w) SUVs at a peptide/lipid molar ratio of 1 : 200. The effect of acrylamide on the fluorescence of each peptide was analyzed by a Stern–Volmer equation: $F_0/F = 1 + K_{sv}[Q]$ [14,15], where F_0 and F represent the fluorescence intensities in the absence and the presence of acrylamide, respectively, and K_{sv} is the Stern–Volmer quenching constant and [Q] is the concentration of acrylamide.

Dye Leakage

Calcein-entrapped large unilamellar vesicles (LUVs) composed of EYPC/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 ten cycles and extruded 21 times through polycarbonate filters (two stacked 100-nm pore size filters) by a LiposoFast extruder (Avestin, Inc. Canada). Untrapped calcein was removed by gel filtration on a Sephadex G-50 column. Calcein-entrapped LUVs concentration was determined in triplicate by phosphorus analysis [16]. 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 on a model RF-5301PC spectrophotometer at room temperature. For determination of 100% dye release, 20 µl of 10% Triton-X_{100} in Tris-buffer 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 at 5 min after addition of peptides, F_0 and F_t are fluorescence intensities without the peptides and with Triton X-100, respectively.

Membrane Depolarization

The membrane depolarization activity of the peptides was determined using intact *S. aureus* cells and the membrane potential-sensitive fluorescent dye, $diSC_3$-5, based on the methods of Friedrich *et al*. [17,18]. Briefly, *S. aureus* was grown at 37 °C with agitation to mid-log phase ($OD_{600} = 0.4$), and then the cells were harvested by centrifugation. The cells were washed twice with washing buffer [20 mM glucose, 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4] and resuspended to an OD_{600} of 0.05 in a similar buffer containing 0.1 M KCl. Then, 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 (ex. = 622 nm, 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). The membrane potential-dissipating activity of the peptides is expressed as follows:

$$\%\ \text{Membrane depolarization} = 100 \times [(F_p - F_0)/(F_g - F_0)],$$

where F_0 is the stable fluorescence value after addition of the $diSC_3$-5 dye, F_p is the fluorescence value 5 min after addition of the peptides and F_g is the fluorescence signal after the addition of gramicidin D.

CD Spectra

CD spectra of the peptides were recorded using a J-720 spectropolarimeter (JASCO Corporation, Tokyo, Japan). All peptide samples were maintained at 25 °C during analysis. Four scans per sample were performed over the wavelength range 190–250 nm at 0.1 nm intervals. The spectra were measured in 30 mM SDS in the presence of 10 mM sodium phosphate buffer (pH 7.2) at 25 °C using a 1-mm pathlength cell. The peptide concentrations were 100 µg/ml. The mean residue ellipticity, $[\theta]$, is given in deg ·cm2·dmol−1: $[\theta] = [\theta]$obs (MRW/10lc), where $[\theta]$obs is the ellipticity measured in millidegree, MRW is the mean residue molecular weight of the peptide, c is the concentration of the sample in milligram per milliliter and l is the optical path length of the cell in centimeter. The spectra are expressed as molar ellipticity $[\theta]$ versus wavelength.

Results

Peptide Design

To design the novel short antimicrobial peptides based on PK, three strategies were used in the present study: (i) Delete the *C*-terminal amino acids of PK to form shorter peptides. PK-17, PK-15 and PK-12 were derived from the deletion of $K^{18}RKV^{21}$, $K^{16}KKRKV^{21}$ and $S^{13}QPKKKRKV^{21}$ at the *C*-terminus of PK, respectively. (ii) Add lysine residues at *C*-terminus of PK to increase the net positive charges of peptide. PK-12-KK was designed by KK addition at the *C*-terminus of PK-12. (iii) Substitute amino acids to form a peptide similar in sequence to indolicidin. PK-12-KKP was synthesized by $Thr^{3,7,10} \rightarrow$ Pro-substitution of PK-12-KK (Table 1).

Antimicrobial and Hemolytic Activities

As shown in Table 2, PK-17, PK-12-KK and PK-12-KKP exhibited a comparable antimicrobial activity to PK and displayed no hemolytic activity up to 200 µM. In contrast to PK-17 and PK-12-KK, the antimicrobial activities of PK-15 and PK-12 decreased about twofold, and they induced a visible hemolysis (5% hemolysis) at

Table 2. Antimicrobial and hemolytic activities and cell selectivity of the peptides

Peptide	MIC[a] (μM) *Escherichia coli*	*Pseudomonas aeruginosa*	*Salmonella typhimurium*	*Bacillus subtilis*	*Staphylococcus epidermidis*	*Staphylococcus aureus*	GM[b] (μM)	MHC[c] (μM)	Therapeutic index[d] (MHC/GM)
Pep-1-K (PK)	2	4	2	2	2	2	2.3	>200	173.9
PK-17	2	4	2	2	2	2	2.3	>200	173.9
PK-15	4	8	4	2	4	4	4.3	200	46.5
PK-12	4	8	4	2	4	4	4.3	200	46.5
PK-12-KK	2	4	2	2	2	2	2.3	>200	173.9
PK-12-KKP	2	4	2	1	1	2	2.0	>200	200.0
Indolicidin	8	8	4	2	4	4	5.0	50	10.0

[a] MIC was determined in three independent experiments performed in triplicate.
[b] The geometric mean (GM) of the MIC values from all six bacterial strains in this table.
[c] The minimal peptide concentration (MHC) that produces 5% hemolysis. When no detectable hemolysis was observed at 200 μM, we used a value of 400 μM to calculate the therapeutic index.
[d] The ratio of the MHC (μM) over the GM of the MIC (μM).

Table 3. Tryptophan emission maxima of 3 μM peptides and K_{sv} in Tris-buffer (pH 7.4) or in the presence of 0.6 mM EYPC/EYPG (7 : 3, w/w) SUVs and 0.6 mM EYPC/cholesterol (10 : 1, w/w) SUVs

Peptide	Tris-buffer (nm)	EYPC/EYPG (7 : 3, w/w) (nm)	EYPC/cholesterol (10 : 1, w/w) (nm)	K_{sv} (M^{-1})[b] EYPC/EYPG (7 : 3, w/w)	EYPC/cholesterol (10 : 1, w/w)
Pep-1-K (PK)	350	338 (12)[a]	348 (2)	1.75	5.08
PK-17	350	341 (9)	347 (3)	1.71	4.18
PK-15	350	340 (10)	347 (3)	1.78	4.25
PK-12	350	343 (7)	348 (2)	1.82	5.08
PK-12-KK	350	341 (9)	347 (2)	1.45	4.75
PK-12-KKP	350	342 (8)	350 (0)	1.75	7.01
Indolicidin	350	341 (9)	340 (10)	1.18	1.85

[a] Blue shift in emission maximum in parentheses.
[b] K_{sv} is the Stern–Volmer quenching constant.

200 μM, respectively. These data implied that the two *C*-terminal lysine residues may play a key role in enhancing antimicrobial activity and reducing hemolytic activity of the peptides (Table 2). In addition, indolicidin displayed a broad-spectrum activity against bacteria and high hemolytic activity as previously reported [19,20].

Cell Selectivity/Therapeutic Index

The therapeutic index (TI), which is a widely accepted measure of the cell selectivity of antimicrobial agents, was determined to assess the cell selectivity of our designed peptides [19,21–23]. The TI was calculated as the ratio of the minimal peptide concentration that produces 5% hemolysis (MHC) to the geometric mean (GM) of MIC against tested bacterial strains (Table 2). When no hemolytic activity was detectable at the highest concentration tested (200 μM), the twofold tested concentration (400 μM) as MHC value was used to calculate TI. Larger values of TI correspond to greater cell selectivity. PK, PK-17, PK-12-KK and PK-12-KKP showed almost the same TI values, indicating that these peptides show nearly similar cell selectivity. In contrast, PK-15 and PK-12 displayed approximately a fourfold lower therapeutic index as compared with PK. In particular, the designed Trp/Pro-rich peptide PK-12-KKP displayed 20-fold higher cell selectivity as compared with indolicidin, a Trp/Pro-rich naturally occurring antimicrobial peptide.

Tryptophan Fluorescence Blue Shift in Model Membranes

When excited at 280 nm, Trp residues of the peptides in aqueous buffer gave rise to an emission peak centered at 350 nm. The fluorescence emission maxima (λ_{max}) of all of the peptides was shifted (7–12 nm) significantly to shorter wavelength (blue shift) when peptides bound to negatively charged EYPC/EYPG (7 : 3, w/w) vesicles, which mimic bacterial membranes. However, these peptides showed less blue shift (1–3 nm) in the presence of zwitterionic EYPC/cholesterol (10 : 1, w/w) vesicles, which mimic the outer surface of mammalian membranes. These results indicated that these peptides more deeply penetrated into the hydrocarbon region of negatively charged lipid bilayers than zwitterionic lipid bilayers. In addition, indolicidin displayed a big blue shift (10 nm) in zwitterionic EYPC/cholesterol (10 : 1, w/w) vesicles as well as negatively charged EYPC/EYPG (7 : 3, w/w) vesicles (Table 3).

Tryptophan Fluorescence Quenching by Acrylamide

To investigate the relative extent of peptide burial in vesicles and the lipid specificity of the peptide–vesicle interaction, we examined the effect of acrylamide, a water-soluble neutral quencher of Trp fluorescence. Acrylamide is useful as a quenching agent because it does not interact with the head group of

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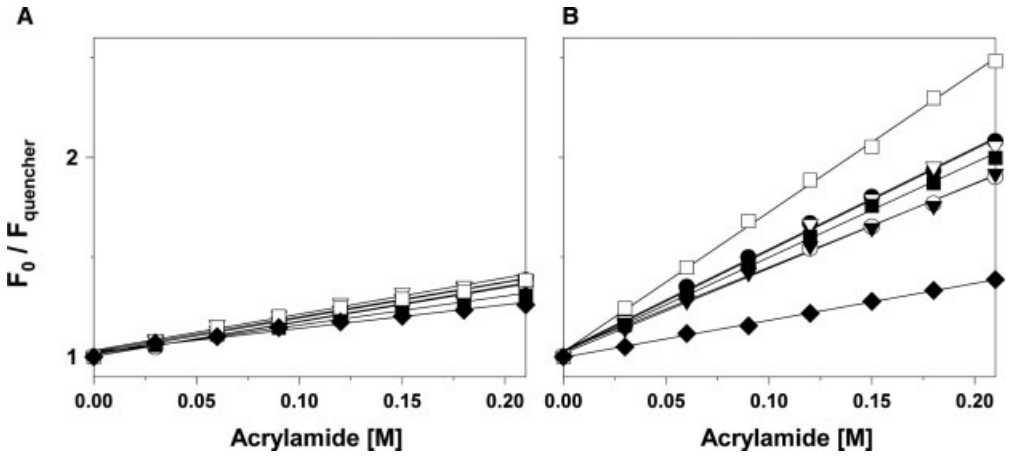

Figure 1. Stern–Volmer plots for the quenching of Trp fluorescence of 3 μM peptides by an aqueous quencher, acrylamide, in the presence of 0.6 mM EYPC/EYPG (7 : 3, w/w) SUVs (a) or 0.6 mM EYPC/cholesterol (10 : 1, w/w) SUVs (b). Peptides are indicated as follows: PK (●), PK-17 (○), PK-15 (▼), PK-12 (▽), PK-12-KK (■), PK-12-KKP (□) and indolicidin (♦).

negatively charged phospholipids. Stern–Volmer plots for the quenching of Trp by acrylamide, recorded in the presence of negatively charged or zwitterionic vesicles, are depicted in Figure 1, and the Stern–Volmer quenching constant (K_{sv}) values are presented in Table 3. The smaller the value for K_{sv} presents, the more inaccessible of the peptides to acrylamide. In the presence of negatively charged EYPC/EYPG (7 : 3, w/w) vesicles, the K_{sv} values of all peptides except indolicidin were much smaller than in the presence of zwitterionic EYPC/cholesterol (10 : 1, w/w) vesicles, indicating that the Trp residue of these peptides was more deeply buried in the negatively charged lipid bilayers and became inaccessible for quenching by acrylamide than in zwitterionic lipid bilayers. This fluorescence data revealed that PK analogs more effectively inserted into the negatively charged phospholipids than zwitterionic lipid bilayers. The K_{sv} of indolicidin was similar in the presence of negatively charged and zwitterionic vesicles, suggesting that its tryptophan residues were buried in the hydrophobic lipid phase of the bilayer in both types of vesicles. Collectively, the lipid specificity of peptides corresponded well with their cell selectivity (Tables 2 and 3; Figure 1).

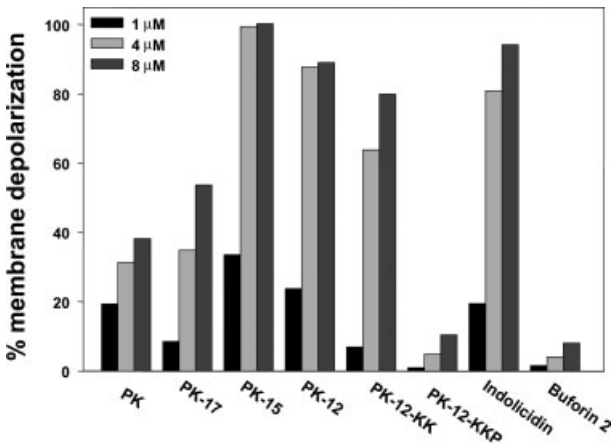

Figure 2. Dose-dependence membrane depolarization of *Staphylococcus aureus* (OD_{600} = 0.05) by the peptides using the membrane potential-sensitive dye, $diSC_3$-5. Dye release was monitored at an excitation wavelength of 622 nm and an emission wavelength of 670 nm.

Depolarization of the Membrane Potential in Intact *S. aureus*

To assess bacterial membrane depolarization in the presence of peptides, 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. On disruption of the membrane potential, the dye dissociates into the buffer leading to an increase in fluorescence. PK-15 and PK-12 can almost completely depolarize (>80%) cytoplasmic membrane of intact *S. aureus* at 4 μM (their MIC), as observed in indolicidin. In contrast, PK, PK-17, PK-12KK, PK-12-KKP and buforin 2 caused a partial membrane depolarization of 38.2%, 53.6%, 79.0, 10.4% and 8.1% even at 8 μM, respectively (Figure 2). Buforin 2 used as the control peptide in this assay was known as an intracellular-targeting antimicrobial peptide that penetrates microbial cell membranes and causes bacterial cell death by inhibiting DNA/RNA or protein synthesis [24–26].

Calcein Leakage from Model Membranes

To evaluate the ability of peptides to permeabilize bacterial membranes, we measured their abilities to induce the fluorescent dye calcein leakage from negatively charged EYPC/EYPG (7 : 3 w/w) LUVs, which mimics bacterial membranes (Figure 3). PK-15 and PK-12 induced a partial dye leakage of 60% and 40% at 6 μM, respectively. In contrast, PK, PK-17, PK-12-KK and PK-12-KKP showed no or little dye leakage at 6 μM. Indolicidin was able to achieve a complete dye leakage (>95%) at 2 μM.

CD spectroscopy

As PK-12-KKP has a high sequence homology (43%) with indolicidin, we expect that the secondary structure of PK-12-KKP is similar to that of indolicidin. CD spectroscopy was used to investigate the secondary structure of PK-12-KKP in SDS micelle (membrane-mimetic environment). As expected, the CD spectrum of PK-12-KKP in 30 mM SDS micelle was similar to that of indolicidin, which adopts a folded structure in membrane-mimetic environment (Figure 4). A strong negative band at 227 nm coupled with the positive peak at 216 nm represented the interaction of the aromatic rings of Trp with the peptide backbone and stacking of the Trp rings [27,28]. Furthermore, the strong negative band at 195 nm indicated significant amounts of unordered structure of PK-12-KKP

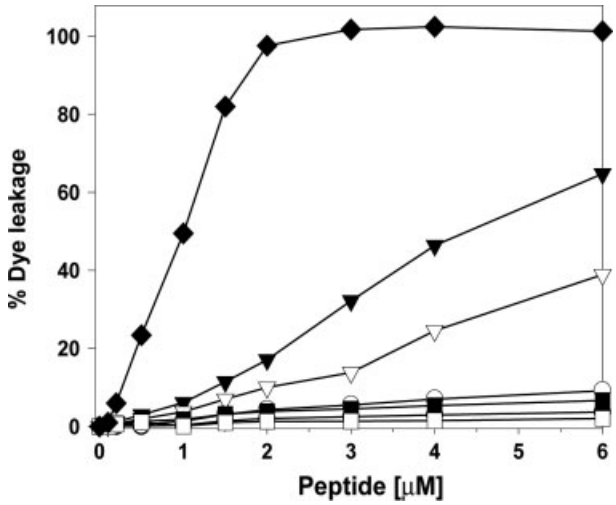

Figure 3. Dose-dependence percent leakage of calcein from negatively charged EYPC/EYPG (7 : 3, w/w) LUVs at pH 7.4 measured at 2 min after the addition the peptides. The concentration of EYPC/EYPG (7 : 3, w/w) LUVs was 68 μM. Peptides are indicated as follows: PK (●), PK-17 (○), PK-15 (▼), PK-12 (▽), PK-12-KK (■), PK-12-KKP (□) and indolicidin (♦).

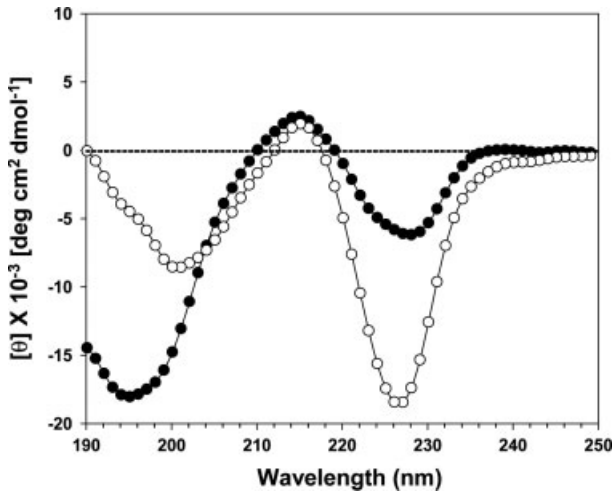

Figure 4. CD spectra of the peptide in 30 mM SDS micelles in the presence of 10 mM sodium phosphate buffer (pH 7.2). Peptides are indicated as follows: PK-12-KKP (●-●-●) and Indolicidin (○-○-○).

in SDS micelle. Taken together, our CD analysis indicates that PK-12-KKP forms a folded structure combined with some portions of unordered structures when it binds to membrane-mimetic SDS micelle.

Discussion

Indolicidin, a Trp/Pro-rich peptide, isolated from the cytoplasmic granules of bovine neutrophils is one of the smallest natural cationic antimicrobial peptides known to date [29–31]. Owing to its relatively short length (13-mer) and broad spectrum of antimicrobial activity, indolicidin is a promising candidate for the development of antimicrobial drugs [32]. However, its toxicity against mammalian cells prevents its successful clinical application [32]. Our present study reveals a very poor selectivity for indolicidin with TI value of 10.0 (Table 2).

The newly designed peptide PK-12-KKP has relatively short length (14-mer), and it contains the same high percentage of Trp (36%) and Pro (21%) as indolicidin. Interestingly, although PK-12-KKP shares high homologies in the sequence with indolicidin, it displayed a 20-fold higher cell selectivity as compared with indolicidin (Tables 1 and 2). This result can be explained from two aspects: (i) PK-12-KKP possesses 2.5-fold higher antimicrobial activity as compared with indolicidin. Its high activity against bacteria is probably because of the fact that it contains two more net positive charges than indolicidin. (ii) PK-12-KKP displayed at least fourfold lower hemolytic activity with respect to indolicidin. The peptides' retention time determined by HPLC indicated that PK-12-KKP's hydrophobicity was lower than that of indolicidin (Table 1). We speculated that PK-12-KKP's low hemolytic activity may be related to its low hydrophobicity. These results suggest that PK-12-KKP is a promising candidate for novel therapeutic agents, complementing conventional antibiotic therapies to combat pathogenic microorganisms.

Currently, several antimicrobial peptides have been extensively studied to elucidate their mode of action. The bactericidal effect of the peptides is considered to be due to the action on the lipid matrix of the bacterial cell membranes, either by forming pores, thinning the membrane or destabilizing the bilayer [33–35]. These cause lysis of the bacterial cell as a result of increased permeability. However, some antimicrobial peptides have also been reported to have intracellular targets. Buforin 2 kills bacteria without cell lysis and has strong affinity for DNA and RNA, suggesting that the target of buforin 2 is not the cytoplasmic membranes of bacteria but intracellular nucleic acids [24–26]. Furthermore, a proline–arginine (PR)–rich antimicrobial peptide, PR-39, has been demonstrated to inhibit DNA and/or protein synthesis [36]. In addition, our recent studies revealed that buforin 2 and PR-39 induced no or little ability in membrane depolarization of bacterial cells and dye leakage from bacterial membrane–mimicking lipid vesicles and effectively penetrated bacterial cell membranes [9,23,37].

Like buforin 2, PK-12-KKP showed very weak ability in the depolarization of membrane potential of intact *S. aureus* cells even at 8 μM (4× MIC) and the fluorescent dye leakage of calcein-entrapped negatively charged bacterial membrane–mimicking lipid vesicles. These results suggested that the target of PK-12-KKP might not be the cytoplasmic membranes of bacteria but intracellular components (e.g. DNA, RNA or protein). In contrast, indolicidin as a membrane disrupting antimicrobial peptide, significantly induces membrane depolarization and dye leakage at 4 μM (MIC). It will be interesting in the future to study the reasons why the bactericidal mechanisms of PK-12-KKP and indolicidin are different, although the two peptides share high homologies in both sequence and structure.

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