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Reversed sequence enhances antimicrobial activity of a synthetic peptide‡

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A class of cationic antimicrobial peptides involved in host defense consists of sequences rich in Lys and Trp. Small peptides, $(WK)_3$ and $(KW)_3$, were designed by the combination of alternating Lys (K) and Trp (W) amino acids, and then their antimicrobial and hemolytic activities were determined. It was noticed that the reversed sequence of $(KW)_3$ showed more activity against all strains than did $(WK)_3$. The non-hemolytic behavior of $(WK)_3$ is identical to that of the reversed analog of $(KW)_3$. CD spectra revealed that these peptides had an unfolded structure in buffer and EYPC : CH (10 : 1, w/w), but adopted folded conformation in the presence of EYPE : EYPG (7 : 3, w/w). The reversed-$(KW)_3$ peptide caused a higher extent of calcein release from EYPE : EYPG (7 : 3, w/w), though the activity was higher than that of the $(WK)_3$. The interaction of the peptides with model lipid vesicles was examined using Trp fluorescence. The reversed-$(KW)_3$ showed higher interaction with EYPE : EYPG (7 : 3, w/w) membrane than did $(WK)_3$. Both the peptides show less affinities while binding to EYPC : CH (10 : 1, w/w). This clearly indicated that the reversal of sequence factors is relevant to increased antimicrobial activity and lipid membrane permeability. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Lys; Trp; reversed peptide; antimicrobial activity; leakage of calcein

Introduction

In general, natural and synthetic AMPs are considered potent antibiotics due to their broad spectrum activity, including antibiotics-resistant bacterial strains and some fungi, parasites and viruses [1–3]. There are many methods used to develop analog peptides with increased activity, based on the changes of all structural motifs, such as amino acid mutation, net charge, total hydrophobicity/hydrophilicity, amphipathicity and helical propensity [4–9]. Many biologically active peptide analogs have been modified in such ways, but apart from antimicrobial activity they are also active against the eukaryotic membrane, thereby having a cytotoxic effect [4–9]. These kinds of analog peptides are thus not selective for microbial membranes. Also, the introduction of the D-enantiomers of amino acid residues in short AMPs, to generate a diastereomer, may result in loss of structure as well as antimicrobial and hemolytic activities [10,11]. All these considered, reversed sequence of antimicrobial peptides would be of considerable help in designing peptides for specific and improved activity against microbial cells, which could be very useful therapeutically. Both reversed and short AMPs, which minimize damage to eukaryotic or host cells, would be the most promising candidates for large-scale antibiotic production.

It is well known that there are two amino acids, Lys and Trp, which share a special importance in the interaction of AMPs with microbial membrane. The antimicrobial peptide database reported that Lys is a typical hydrophilic amino acid residue of natural cationic AMPs [12]. In the literature, synthetic peptides containing Lys as the only cationic elements showed interesting antimicrobial activities [13–15]. Furthermore, systematically designed amphipathic AMPs composed of all Lys side chains placed on the polar face of an amphipathic structure are proposed to interact with anionic lipid headgroups, likely contributing to the specificity of AMPs for microbes [16–18]. Trp is a conserved residue in all natural AMPs and is essential for their antibacterial activity, due to its bulky indole side chain, which strongly interacts with the bacterial phospholipid membranes, thereby promoting prokaryotic cell toxicity [19]. With all of these in mind we designed small peptides [WKWKWK ($(WK)_3$) and KWKWKW ($(KW)_3$)] with repeated sequences that contain balanced numbers of Lys and Trp residues. We synthesized normal-$(WK)_3$ and reversed-$(KW)_3$ peptides and compared their antimicrobial

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Abbreviations used: *AMPs, antimicrobial peptides; CH, cholesterol; EYPC, egg yolk L-α-phosphatidylcholine; EYPE, egg yolk L-α-phosphatidylethanolamine; EYPG, egg yolk L-2-phosphatidylglycerol; hRBCs, human red blood cells; LUVs, large unilamellar vesicles; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide.*

and hemolytic activities, as well as membrane permeability and binding abilities. These comparisons were done in a model lipid membrane. Finally, we examined the peptide secondary structure in the model lipids by CD spectroscopy.

Materials and Methods

Peptide Synthesis

The peptides, normal-$(WK)_3$ (WKWKWK) and reserved-$(KW)_3$ (KWKWKW), were synthesized by the solid-phase method, using Fmoc chemistry [20]. The crude peptides were repeatedly extracted with diethyl ether and then purified using reverse phase preparative HPLC on a Vydac C_{18} column (4.6 mm × 250 mm, 300 Å, 5 nm). The molecular masses of the peptides were confirmed with a matrix-assisted laser desorption ionization mass spectrometer (MALDI II, Kratos Analytical Ins, Manchester, U.K.). Finally, the peptide concentration was determined by amino acid analysis.

Antibacterial Activity

The antibacterial activities of the peptides against Gram-negative bacteria [*Escherichia coli*, Korean collection for type culture (KCTC) 1682; *Salmonella typhimurium*, KCTC 1926; and *Pseudomonas aeruginosa*, KCTC 1637], Gram-positive bacteria (*Staphylococcus aureus*, KCTC 1621; *Bacillus subtilis*, KCTC 1918 and *Listeria monocytogenes*, KCTC 3710) and two antibiotic-resistant *E. coli* strains [culture collection of antibiotic-resistant microbes (CCARM) 1229 and CCARM 1238] were examined using the microbroth dilution method [18]. Aliquots of bacterial suspensions (50 µl) in mid-log phase at a concentration of 4×10^5 colony forming units (CFU)/ml in culture medium (1% peptone) were added to each well, containing 50 µl of the peptide solution that had been twofold serially diluted in buffer (10 mM sodium phosphate buffer, pH 7.2). Several wells were kept untreated as a control for monitoring bacterial growth. Inhibition of growth was determined by measuring the absorbance at 620 nm using a Versa-Max microplate Elisa Reader (Molecular Devices, Sunnyvale, CA, USA) after incubation for 18–24 h at 37 °C. The MIC is defined as the minimal peptide concentration that inhibits bacterial growth. All MIC measurements are the average of 3–4 independent experiments. These bacterial strains were procured from KCTC at the Korea Research Institute of Bioscience and Biotechnology. Drug-resistant *E. coli* strains (CCARM 1229 and CCARM 1238) were obtained from the CCARM at Seoul Women's University in Korea.

Antifungal Activity

The fungal strains [*Candida albicans* (KCTC 7270) and *Trichosporon beigelli* (KCTC 7707)] and three antibiotic-resistant strains (CCARM 14001, CCARM 14007 and CCARM 14020) were used to evaluate the antifungal activity of the test peptides by MTT assay [21]. Briefly, fungal cells (4×10^4 CFU/ml) that were grown in 100 µl of yeast peptone dextrose media (yeast extract 0.5%, peptone 1%, dextrose 2% and pH 5.0–5.5) were seeded in each well of a microtitre plate containing 100 µl of twofold serially diluted peptides in buffer (as described earlier). The plate was then incubated for 24 h at 28 °C. After completion of the desired incubation period, 5 µl of MTT solution [5 mg/ml MTT in phosphate buffered saline (PBS), pH 7.4] was added to each well, after which the plates were further incubated at 37 °C for 4 h. Next, 30 µl of 20% SDS (w/v) containing 0.02 M HCl was added, and the plates were subsequently incubated at 37 °C for 16 h to dissolve the formazan crystals that had formed. The optical density of each well was measured at 580 nm using a microplate Elisa Reader (Molecular Devices, Sunnyvale, CA, USA). All activity assays were performed in triplicate.

hRBC Hemolysis

Hemolytic activities of the peptides were assessed using hRBCs collected in heparin from healthy donors. The fresh hRBCs were rinsed three times in PBS via centrifugation at 800 × *g* for 10 min and then re-suspended in PBS. After washing, the peptides were dissolved in PBS and then added to 100 µl of stock hRBCs suspended in PBS (final RBC concentration, 8% v/v). The samples were then incubated with gentle agitation for 60 min at 37 °C, and then centrifuged for 10 min at 800 × *g*. Next, the absorbance of the supernatants was recorded at 414 nm. Controls for zero hemolysis (blank) and 100% hemolysis, composed of hRBCs suspended in PBS and 1% Triton X-100, respectively, were also analyzed. Melittin was used as positive control. Each measurement was conducted in triplicate [22].

Calcein Leakage

Calcein-entrapped LUVs composed of EYPE : EYPG (7 : 3, w/w) or EYPC : CH (10 : 1, w/w) were prepared by vortexing the dried lipid in dye buffer solution (70 mM calcein, 10 mM sodium phosphate buffer, pH 7.4). The suspension was freeze-thawed in liquid nitrogen for nine cycles and then extruded 16 times through polycarbonate filters (two stacked 0.2 µM pore size filters), using an *Avanti* Mini-Extruder (Avanti Polar Lipids Inc., Alabaster, AL, USA). Calcein-entrapped vesicles were separated from free calcein by gel filtration chromatography on a Sephadex G-50 column. Entrapped LUVs in a suspension containing 60 µM lipids were then incubated with various concentrations of the peptide (1–12 µM) for 25 min. The fluorescence of the released calcein was assessed using a spectrofluorometer (Perkin-Elmer LS55, Mid Glamorgan, UK) at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. Complete (100%) release was achieved via the addition of 0.1% Triton X-100. Spontaneous leakage was determined to be negligible at this timescale. The experiments were conducted at 25 °C. The apparent percentage of calcein release was calculated according to the following equation [23]:

$$\text{Release (\%)} = 100 \times \frac{F - F_0}{F_t - F_0} \quad (1)$$

in which F and F_t represent the fluorescence intensity prior to and after the addition of the detergent, respectively, and F_0 represents the fluorescence of the intact vesicles.

Trp Fluorescence and Acrylamide Quenching Assay

The fluorescence emission spectrum of Trp of the peptides was monitored in 10 mM sodium phosphate buffer, in the presence of either EYPE : EYPG (7 : 3, w/w) or EYPC : CH (10 : 1, w/w) small unilamellar vesicles (SUVs). In these fluorescence studies, SUVs were used to minimize differential light scattering effects [24]. The Trp fluorescence measurements were taken using a spectrofluorometer. Each peptide was added to 1 ml of 10 mM sodium phosphate buffer, pH 7.2 containing 60 µM liposomes, and the peptide:liposome mixture (a molar ratio of 1 : 30) was allowed to interact at 25 °C for 10 min. The fluorescence was excited at 280 nm, and the emission was scanned from 300 to 400 nm.

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The fluorescence quenching experiments were conducted using acrylamide as the quencher, the concentration of which was between 0.04 and 0.20 M, in the cuvette. The effect of acrylamide on the fluorescence of each peptide was analyzed with a Stern–Volmer equation:

$$\frac{F_0}{F} = 1 + K_{SV}(Q) \quad (2)$$

where F_0 and F represent the fluorescence intensities in the absence and the presence of acrylamide, respectively, K_{SV} is the Stern–Volmer quenching constant, and (Q) is the concentration of acrylamide.

CD Spectroscopy

CD spectra were recorded at 25 °C on a Jasco 810 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a temperature control unit using a 0.1-cm path-length quartz cell. The CD spectra of the 50 μM peptides were obtained in different environments, including 10 mM sodium phosphate, EYPE : EYPG (7 : 3, w/w), and EYPC : CH (10 : 1, w/w). Ten millimoles sodium phosphate buffer was used to prepare the 1 mM EYPE : EYPG (7 : 3, w/w) and 1 mM EYPC : CH (10 : 1, w/w). At least four scans in the 250–190 nm wavelength range were conducted and the average blank spectra were subtracted from the average of the sample spectra.

Results and Discussion

One of the most widely used approaches in the design of synthetic cationic AMPs is reversed analogs of natural peptides. Reversed peptides are generally considered to have similar physiochemical (length, net charge and proportion of hydrophilic and hydrophobic amino acids) and structural properties compared to normal peptides; however, the amide bonds are inverted, i.e. –NHCO– rather than the normal –CONH– bond of peptide structure. Inversion of the amide changes the helical dipole movement and, in particular, it changes the hydrogen bonding pattern. This modification, which does not affect the orientation of side chains, has been introduced in many biologically active peptide analogs in order to reduce hemolytic activity [25,26].

In this study, the $(KW)_3$ was noticed to be the reversed sequence of $(WK)_3$. The calculated and observed molecular weights of the synthetic peptides are shown in Table 1, indicating that the product peptides correspond to the desired sequence. We determined the antimicrobial activities of normal-$(WK)_3$ and reversed-$(KW)_3$ against Gram-negative and Gram-positive bacteria as well as fungal strains (Table 2). A twofold increase in antimicrobial activity against more strains was noticed in the reversed sequence of $(KW)_3$ when compared with the normal-$(WK)_3$ peptide. Against *S. typhimurium* $(WK)_3$ was found to be similar to that of reversed-$(KW)_3$ (Table 2). The activity against *E. coli* KCTC 1682 and *E. coli* CCARM 1229 strains, indicated that the $(KW)_3$ showed a fourfold increased activity compared to that of $(WK)_3$. It is also interesting to note that these peptides were highly potent against Gram-negative bacteria, though they were also very effective against the Gram-positive species *L. monocytogenes*. This fact is due to the similarity of the membrane composition and cell wall structure of *Listeria* to Gram-negative bacteria [27]. The reversed peptide displayed twofold greater antifungal activity than did the normal-$(WK)_3$ peptide, suggesting that, in this $(KW)_3$ peptide, the order of amino acid within the repeating motif seems to be more selective than the normal-$(WK)_3$ for microbial membrane. Some studies also reported that reversed peptides are most active against bacteria than are normal peptides [26,28], though a large number of studies on reversed peptides have indicated that reversed peptides and normal peptides had similar structures/antibacterial activities [29–32]. This clearly indicates that the extent of the increasing, decreasing or similar antimicrobial activity depends on the peptide sequence and bacterial or fungal membrane composition.

In the results reported, it has been found that $(KW)_3$ has a strong antibacterial effect in presence of two- or fourfold decrease in MIC compared to the normal peptide. Thus, we propose such effects of $(KW)_3$ potentially act in synergy with antibiotic against *E. coli* strains. Synergy was also observed with $(WR)_3$ and erythromycin

Table 2. MICs of the peptides against microorganisms

Microorganism	MIC (μM)			
	$(WK)_3$	$(KW)_3$	Ampicillin	Fluconazole
Gram negative bacteria				
E. coli	256	64	64	–
S. typhimurium	16	16	32	–
P. aeruginosa	64	32	–	–
Gram positive bacteria				
S. aureus	256	128	–	–
B. subtilis	256	128	–	–
L. monocytogenes	128	64	–	–
Fungal strains				
C. albicans	32	16	–	16
T. beigelli	16	8	–	–
Resistant strains				
E. coli CCARM 1229	256	64	>256	–
E. coli CCARM 1238	256	128	>256	–
C. albicans CCARM 14001	32	16	–	>256
C. albicans CCARM 14007	32	16	–	>256
C. albicans CCARM 14020	32	16	–	>256

Table 1. Amino acid sequences, calculated and observed molecular masses, net charges of peptides

Name	Amino acid sequence	Molecular weight (Da)		Net charge
		Calculated	Observed[a]	
Normal-$(WK)_3$	WKWKWK	961.1	962	+3
Reversed-$(KW)_3$	KWKWKW	961.1	962	+3

[a] Molecular weights were determined by MALDI-TOF-MS.

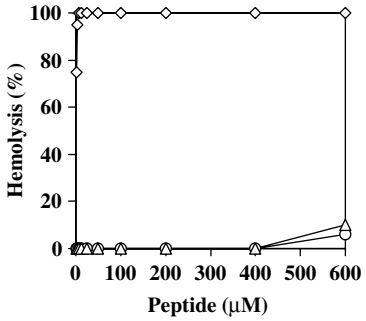

Figure 1. Activity of the peptides against human erythrocytes. $(WK)_3$ (○), $(KW)_3$ (△) and Melittin (◊). The lack of error bars indicates reproducibility.

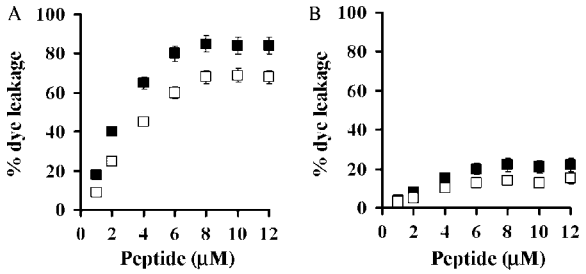

Figure 2. Calcein (dye) leakage from EYPE : EYPG (7 : 3, w/w) (A), and EYPC : CH (10 : 1, w/w) (B) at pH 7.4 was measured for 15 min after the addition of the peptides: $(WK)_3$ (□), $(KW)_3$ (■). The mean values obtained from three individual experiments. Error bar represents the standard deviation and some of error bars were too narrow for this display.

against *E. coli* [33]. We believe that the synergistic combination of antibacterial agents will facilitate the development of novel peptide-based strategies for the treatment of antibiotic-resistant bacteria infection in cornea or skin.

The hemolytic activities of these peptides were assessed against hRBCs to determine the toxicity toward eukaryotic cells (Figure 1). Hemolytic activity did not occur at higher concentration of 400 μM, clearly showing that the reversed peptides maintain their non-hemolytic activity as the normal peptide. Therefore, this modification cannot affect the specificity of AMPs against microbial membrane.

It is well known that most antimicrobial peptides disrupt the bacterial membrane via transmembrane pore formation and/or membrane destabilization [34,35]. Here, we likely assumed that the presence of Lys and Trp residues in these short peptides may attack bacteria via membrane disruption rather than transmembrane pore assembly. The interaction of these peptides with bacterial membranes mainly involve two binding properties: (i) electrostatic interactions among the Lys side chains of the peptides with the phospholipid headgroups and (ii) hydrophobic interaction between the lipid acyl chain and the Trp residues of the peptides. Therefore, the interaction of these peptides to the bacterial lipid membrane was examined by using calcein leakage assay. Calcein leakage behaviors of these peptides toward negatively charged EYPE : EYPG (7 : 3, w/w) vesicles, and induced dye leakage from these vesicles (Figure 2), suggests that the antibacterial activity of $(WK)_3$ and $(KW)_3$ peptides are due to disruption of the lipid bilayer. Treatment of liposomes composed of EYPE : EYPG (7 : 3, w/w) vesicles with $(KW)_3$ at 6 μM was found to disrupt 80% of the vesicles. Treatment with the same concentration of the $(WK)_3$ resulted in the release of 60% of the entrapped calcein (Figure 2A). The differences observed in the activities between $(WK)_3$ and $(KW)_3$ could be associated with different peptide flexibilities (amide

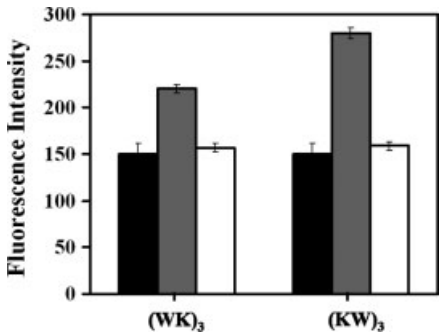

Figure 3. Fluorescence intensity of peptides in aqueous solution and in the presence of lipids (buffer, blank bars; EYPE : EYPG (7 : 3, w/w), gray bars; EYPC : CH (10 : 1, w/w, white bars). The peptide and lipid concentrations were 2 and 60 μM, respectively. Each data point is an average of three independent experiments and error bar represents the standard deviation.

bonds orientation) in the membrane interface of the region of the lipid bilayer, resulting in different peptide–lipid clusters and causing a disruption in the packing of the lipid acyl chains. As well, the differences is likely related to the degree of peptides binding and their depth of insertion into the lipid matrix [16,36].

The lower dye leakage activity of $(WK)_3$ and $(KW)_3$ toward the EYPC : CH (10 : 1, w/w) bilayers can be associated with a lower amount of bound peptides, compared to EYPE : EYPG (7 : 3, w/w) that have been shown in Trp fluorescence and quenching assay (Table 3). An increase in fluorescence intensity is observed for the fluorescence emission when these peptides bind to model membrane containing the negatively charged headgroup phosphatidylglycerol (Figure 3). The observations of larger blue shifts and emission intensities for $(KW)_3$ binding to EYPE : EYPG (7 : 3, w/w) suggest that the Trp side chain partitions preferentially

Table 3. Trp emission maxima of 2 μM peptides and *K*sv in 10 mM sodium phosphate buffer (pH 7.2) or in the presence of 60 μM EYPE : EYPG (7 : 3, w/w) SUVs or 60 μM EYPC : CH (10 : 1, w/w) SUVs

Peptides	λ_{max} buffer (nm)	Blue shift (nm) EYPE : EYPG (7 : 3, w/w)	Blue shift (nm) EYPC : CH (10 : 1, w/w)	*K*sv (M^{-1})[a] Buffer	*K*sv (M^{-1})[a] EYPE : EYPG (7 : 3, w/w)	*K*sv (M^{-1})[a] EYPC : CH (10 : 1, w/w)
$(WK)_3$	355	8	1	13	2.8	5.6
$(KW)_3$	355	11	2	12	2.3	5.3

[a] K_{SV} is the Stern–Volmer constant. K_{SV} (M^{-1}) was determined from the Stern–Volmer equation $F_0/F_1 = 1 + K_{SV}(Q)$, where Q is the concentration of quencher (acrylamide). Concentration of the quencher varied from 0.04 to 0.20 M.

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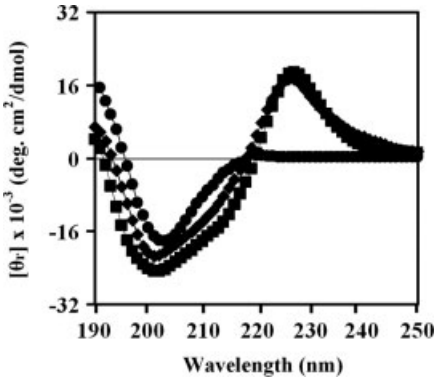

Figure 4. CD spectra of $(KW)_3$ in an aqueous solution (▲) and in the presence of model lipid systems EYPE : EYPG (7 : 3, w/w; ●) and EYPC : CH (10 : 1, w/w; ■) in 10 mM sodium phosphate buffer. CD spectra of $(WK)_3$ was similar to that of $(KW)_3$ in all conditions (data not shown).

into a more rigid, hydrophobic environment in EYPE : EYPG (7 : 3, w/w) lipid bilayers when compared to $(WK)_3$, a tendency consistent with its potent antibacterial activity.

The CD spectra of the peptides in buffer, EYPE : EYPG (7 : 3, w/w) and EYPC : CH (10 : 1, w/w) were analyzed (Figure 4). In sodium phosphate buffer, Trp-containing peptides show negative band at 200 nm region. The negative band around 200 nm is characteristic of random coil, while the band at 225 nm is obtained that is related to the Trp side chain in $(WK)_3$ or $(KW)_3$, which contributed to the CD signal in this spectral region [37–39]. The $(WK)_3$ and $(KW)_3$, did not adopt any secondary structure in both liposomes. However, their Trp residues show folded conformation in the EYPE : EYPG (7 : 3, w/w) but not in EYPC : CH (10 : 1, w/w). Therefore, we suggested that the peptides $(WK)_3$ and $(KW)_3$ have apparent conformation changes in the negatively charged lipid membrane. This is consistent with their respective abilities to disrupt lipid bilayers.

Conclusions

In conclusion, these studies found that a reversed peptide showed increased antimicrobial and membrane permeability activity than did normal peptide, while simultaneously maintaining their non-hemolytic activity. Our finding suggests that the amino acid sequence order in the reversed peptide might play important role in creating flexibility in peptide activity, which in turn could affect the lipid membrane. This reversed peptide analog might therefore be useful in the development of novel antimicrobial peptides. Their simple composition microbial selective properties make them economically viable antibacterial and antifungal compounds for many applications.

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