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The effect of charge increase on the specificity and activity of a short antimicrobial peptide

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Abstract

By using short linear antimicrobial peptides as a model system, the effect of peptide charge on the specificity between *Candida albicans* (fungi) and Gram-positive bacteria was investigated. In a present study, we added and/or deleted lysine residue(s) at the C-terminal and/or N-terminal end(s) of an antimicrobial peptide (KKVVFKVKFK-NH_2) and synthesized the peptides that had similar α helical structures in a lipid membrane mimic condition. The increase of peptide charge improved antifungal activity without the change of antibacterial activity. Structure-activity relationship study about the peptides revealed that the net positive charge must play an important role in the specificity between *C. albicans* and Gram-positive bacteria and the increase of the net positive charge without the moderate change of secondary structure could improve activity for *C. albicans* rather than Gram-positive bacteria.

Keywords: Antibacterial peptide; α-helical structure; Hydrophobicity; Net positive charge

1. Introduction

In the past few years, many antibacterial peptides have been isolated from a variety of natural sources [4,20,27]. In spite of the diversity of the amino acid sequence of these peptides, most of them had a high net positive charge and amphipathic secondary structure such as α helical and β sheet structures in lipid membrane conditions. Antibacterial peptides have received attention because of their low toxicity against mammalian cells and their unique biological mechanisms of perturbing the membrane of the pathogen.

Even thought the detailed mechanism of antibacterial peptides was not clearly elucidated, the peptides were believed to act on the lipid membranes of microorganisms [14,21,26,27,30]. First, the positively charged peptides bound to the negatively charged lipid membranes of the pathogen mainly by charge-charge interactions. And then they mostly adapted α helical structure or β sheet structure and increased the permeability of the lipid membranes either by ion channel formation or by the perturbation of the structure of the bilayer, resulting in the death of target cells.

Many structure-activity relationship studies about linear antibacterial peptides [1–3,8–10,18,31] indicated that the net positive charge, hydrophobicity, and α-helical structure were most important factors for the activity and specificity. Recent structure-activity relationship studies [19,24,25,30] revealed that high α helicity and/or high hydrophobicity must be the major factors critically related to the mammalian cell toxicity rather than antibacterial activity.

In the past 10 years, the incidence of fungal infection has increased dramatically. However, despite extensive structure-activity relationship studies about antibacterial peptides, there are few studies about the unique contribution of the net positive charge and hydrophobicity on the specificity between bacteria and fungi. Because most of the peptides had an activity against only bacteria and the relationship between antibacterial activity and mammalian cell toxicity (therapeutic index) had been mostly emphasized in the development for therapeutic agents.

In the present study, we designed and synthesized the peptides with similar α helical structures in a lipid membrane condition and investigated the unique effect of the net positive charge and hydrophobicity on the specificity be-

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tween *Candida albicans* (fungi) and Gram-positive bacteria (*Staphylococcus aureus, Micrococcus luteus*). For this study, we chose the short antimicrobial decapeptide (KSL) identified by combinatorial libraries as a model peptide by following reasons. First, this short decapeptide (KSL, KKV-VFKVKFK-NH_2) acted directly on lipid membranes of microorganisms and shared a common structural feature to many antibacterial peptides, such as an amphipathic α helical structure and high net positive charge [15]. Second, the peptide (KSL) showed potent activity against bacteria and fungi [15]. Third, the structure-activity relationship study about diastereomers (peptide containing D-amino acid) corresponding to an KSL analog (KKVVFKVKFKK-NH_2) indicated that the moderate change of α helicity without the change of the net positive charge and hydrophobicity did not affect activity against bacteria and fungi [16].

To synthesize the peptide (KSL) analogs, which had a different net positive charge without the change of α helicity, we added and/or deleted lysine residue(s) at the N-terminal and/or C-terminal end of the peptide, monitoring the change of the secondary structure. As the synthesized peptide analogs had similar α helicities, we could investigate the effect of the net positive charge and hydrophobicity on the specificity between fungi and Gram-positive bacteria. This study revealed the unique effect of peptide charge on the specificity between fungi and Gram-positive bacteria.

2. Materials and methods

2.1. Peptide synthesis

Peptides were prepared by stepwise solid-phase synthesis on an Applied Biosystems model 431A automatic peptide synthesizer. The peptide chain was assembled on PAL resin by Fmoc chemistry [13]. Side chain protection groups were as follows: Lys, *tert*-butoxylcarbonyl. Deprotection and cleavage were achieved by treatment with a mixture of trifluoroacetic acid (TFA)/water/thioanisole (9/0.5/0.5, v/v/v) at room temperature for 3–4 h. After filtration of resin and washing with TFA, a gentle stream of nitrogen was used to remove the excess TFA. The crude peptide was triturated with diethyl ether chilled at -20° and was centrifuged at 3000 × g for 10 min. Diethyl ether was decanted and crude peptide was dried under nitrogen. The peptides were purified by high-performance liquid chromatography (HPLC) with a Phenomenex C_{18} column (21.2 × 250 mm; Phenomenex, Torrance, CA, USA). The homogeneity of the peptide (>96%) was checked by analytical HPLC with a Waters Delta Pak C_{18} column (3.9 mm × 150 mm). The elution of peptide was monitored by absorbance at 214 nm. Solvent A consisted of 0.1% TFA in water and solvent B of 0.1% TFA in acetonitrile. The peptide was analyzed using linear gradient of 0–60% B in 60 min. Mass spectrometry on Platform II (Micromass, Manchester, United Kingdom) was used to measure the mass of the purified peptide.

2.2. CD measurement

Circular dichroism (CD) spectra were recorded on a Jasco J-715 spectropolarimeter (Jasco, Tokyo, Japan) using a quartz cell of 1 mm path length, at wavelengths ranging from 190 to 245 nm. CD spectra were recorded at room temperature and were obtained with a 0.5 nm bandwidth and a scan speed of 10 nm/min. Two scans were averaged to improve the signal to noise ratio. The concentration of each peptide was determined on the basis of amino acid analysis. CD spectra were measured in the presence of 50% (V/V) TFE. The mean residue ellipticity at 222 nm was analyzed to determine the α-helical content of using the method of Chen et al. [6].

2.3. Antimicrobial assay

In vitro antifungal assays were performed by the broth microdilution method according to the recommendation of the National Committee for Clinical Laboratory Standards [26]. RPMI 1640 (Gibco BRL, Gaithersburg, Md.) was used as the assay medium. *Candida* cells freshly grown on slopes of Sabouraud dextrose agar (logarithmic phase) were suspended in physiological saline, and the cell concentration was adjusted to 10^4 cells per 1 ml of 2×-concentrated medium for use as the inoculum. Sample solution was added to the wells of a 96-well plate (100 μl per well) and serially diluted 2-fold. The final concentration of sample ranged from 0.2 to 250 μg/ml. After inoculation (100 μl per well, 5×10^3 cells per ml), the 96-well plate was incubated at 30° C for 48 h and the absorbance was measured at 620 nm by using an enzyme-linked immunosorbent assay reader (SLT, Salzburg, Austria) to assess cell growth. The MIC was defined as the lowest concentration exhibiting no visible growth compared with the control cell. Each MIC was determined from three independent experiments performed in duplicate. An in vitro antibacterial assay was performed by the aforementioned method for the antifungal assay, with the exception of that the assay media and the incubation temperature were different. In the antibacterial assay, antibiotic medium 3 (pH 7.0 at 25°C; Difco, Detroit, Mich, USA) was used and cells were incubated at 37°C for 24 h.

2.4. Hemolytic assay

The detailed method is described elsewhere [7]. Packed mouse erythrocytes were washed three times with buffer (150 mM KCl, 5 mM Tris-HCl, pH 7.4) and then packed erythrocytes were suspended in 10 volumes of the same buffer (Stock cell suspension). For antibiotic treatment, the cell stock suspension was diluted 25-fold with the same buffer and was preincubated in the waterbath at 37°C for 15 min, and then the test sample was added. After incubation for 1 h, the sample was centrifuged at 4000 × g for 5 min and the absorbance of the supernatant was determined at

Table 1
Sequences, structural parameters, and antimicrobial activities of the peptides

Peptide	Sequence	Net positive charge	H/μH	t_R (min)	α-helicity (%)	Minimal inhibition concentration (μg/ml)		
						S. Aureus (ATCC 6538)	*M. luteus* (ATCC 9341)	*C. albicans* (ATCC 36232)
KSL	$(K)_2$-V-V-F-K-V-K-F-$(K)_1$-NH_2	6	−0.19/0.22	21.3	46	3.13	3.13	12.5
KSL1	$(K)_1$-V-V-F-K-V-K-F-$(K)_1$-NH_2	5	−0.05/0.30	22.8	36	6.25	6.25	25
KSL2	$(K)_3$-V-V-F-K-V-K-F-$(K)_1$-NH_2	7	−0.31/0.07	20.6	41	3.13	3.13	12.5
KSL3	$(K)_1$-V-V-F-K-V-K-F-$(K)_3$-NH_2	7	−0.31/0.11	20.0	47	3.13	3.13	6.25
KSL4	$(K)_2$-V-V-F-K-V-K-F-$(K)_2$-NH_2	7	−0.31/0.07	20.1	43	3.13	3.13	6.25
KSL5	$(K)_3$-V-V-F-K-V-K-F-$(K)_2$-NH_2	8	−0.41/0.06	18.9	44	3.13	3.13	6.25
KSL6	$(K)_2$-V-V-F-K-V-K-F-$(K)_3$-NH_2	8	−0.41/0.16	19.1	46	3.13	3.13	3.13
KSL7	$(K)_3$-V-V-F-K-V-K-F-$(K)_3$-NH_2	9	−0.5/0/10	18.2	47	3.13	3.13	3.13
Magainin II	GIGKFLHSAKKFGKAFVGEI MNS-COOH	3	—	—	—	25	50	>50

Retention times (t_R) were determined on C_{18} reverse phase column. Hydrophobicity (H) and hydrophobic moment (μH) were calculated using Eisenberg method [11]. The percent of α-helical content was calculated using the method of Chen *et al*. [6].

540 nm. The hemolysis effected by 0.1% Triton X-100 was considered 100% hemolysis.

3. Results

3.1. Design, synthesis, and characterization of the peptides

As described in Table 1, seven KSL analogs with different numbers of lysine residue(s) were synthesized to modulate net positive charge and hydrophobicity. To minimize the change of the secondary structure, lysine residue(s) were added at the N- and/or C-terminal end of KSL. According to our previous study [16], the terminal region rather than the middle region of the peptide must be less important for maintaining the α helical structure induced in a lipid membrane condition. The KSL analog (KSL1) with a reduced net positive charge was also prepared by the deletion of the N-terminal lysine residue. Each peptide was synthesized in solid phase peptide synthesis and characterized by mass spectrometer. The purity of each peptide was above 96% as measured by analytical HPLC. The model peptides had different net positive charges, ranging between +5 and +9. The hydrophobicities of the peptides were calculated using the Eisenberg method [11] and compared to retention times on a C_{18} reverse phase column. The retention time of peptides on reverse phase matrix was reported to be parallel to hydrophobicity of peptides [17]. As shown in Table 1, the comparison between retention time and hydrophobicity suggested that hydrophobicity is inversely proportional to the net positive charge of the peptides employed in this study.

The effect of the addition of lysine residue(s) on the secondary structure was monitored by CD spectroscopy. In aqueous solution, all peptides had random coil structures (data not shown). As the secondary structure of antibacterial peptides in lipid membranes correlated with the activity, CD spectrum of the peptides, was measured in the membrane mimic condition [5]. As shown in Fig. 1 (A), CD spectra revealed that all peptides had a predominantly α-helical structure in the presence of 50% TFE (pH 7.4). The α-helical content of the peptide was calculated from the mean residue ellipticity at 222 nm and summarized in Table 1. The α-helical contents of all peptides except KSL1 were similar and the secondary structure of the peptides was not greatly changed by the addition of lysine residue(s) at the N- and/or C-terminal end of KSL. However, the comparison of α helicity between KSL and KSL1 indicated that the deletion of a lysine residue at the N-terminus of KSL affected the α-helical structure. The hydrophobic moment of the peptides was calculated using the Eisenberg method [11] and compared to the α helicity measured in the lipid membrane condition by CD spectroscopy. It was not observed any relationship between the calculated hydrophobic moment and the α helicity.

To investigate the effect of pH on the secondary structure, CD spectrum of KSL was measured at pH 5 and pH 9. As shown in Fig. 1 (B), α helicity did not change as the pH was increased from 7 to 9 but decreased at pH 5. The other peptides also had a similar secondary structural change depending on pH (data not shown).

3.2. Antibacterial activity, antifungal activity, and hemolytic activity of the peptides

As shown in Table 1, the activities of the peptides against *Candida albicans (*fungi), *Staphylococcus aureus* (Gram-positive bacteria), and *Micrococcus luteus* (Gram-positive bacteria*)* were measured. All KSL analogs except KSL1 had the same minimal inhibition concentration (MIC) value for *Staphylococcus aureus* (ATCC 6538) and *Micrococcus luteus* (ATCC 9341) as KSL. This result indicated that the modulation between net positive charge and hydrophobicity without the alternation of the secondary structure did not affect the activity against Gram-positive bacteria. On the other hand, the antifungal activity was affected by the mod-

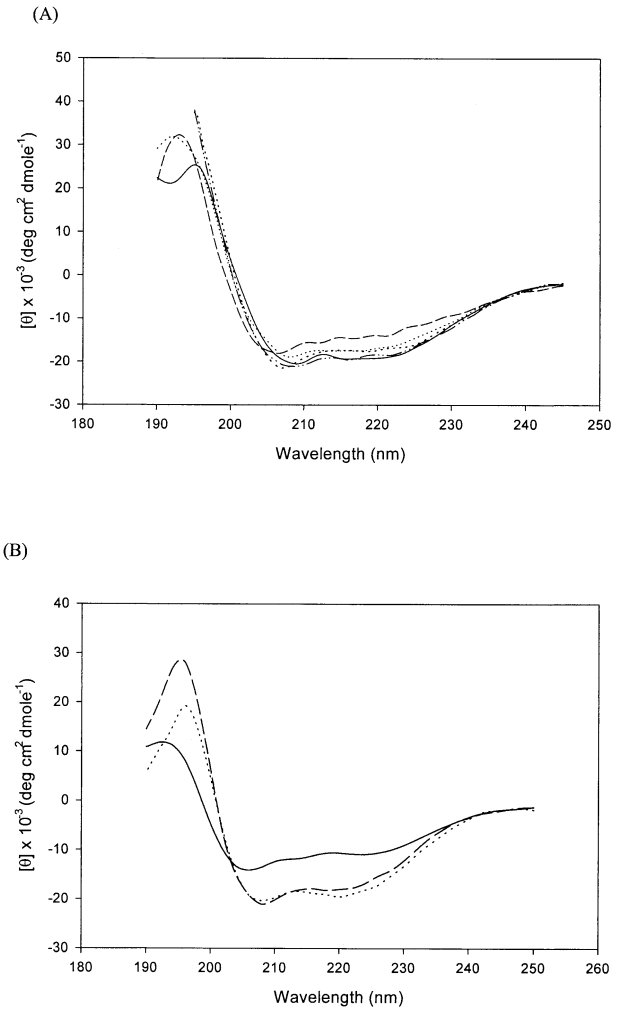

Fig. 1. (A) CD spectra of KSL and its analogs in 10 mM phosphate buffer (pH 7.4) including the 50% TFE (v/v). ———, KSL; — — —, KSL1; ·····, KSL4; - - - - -, KSL5; — · — · —, KSL7. (B) CD spectra of KSL in 10 mM phosphate buffer including 50% TFE (v/v) at different pH: ———, pH 5; ·····, pH 7; — — —, pH 9.

ulation between net positive charge and hydrophobicity without the change of the secondary structure; the increase of net positive charge and decrease of hydrophobicity improved the antifungal activity. However, KSL1, of which α helicity was mostly decreased in the peptide series, showed decreased activity against Gram-positive bacteria and fungi unlike the other analogs.

To investigate the effect of pH, antifungal activities of the peptides were measured at various pH and summarized in Table 2. Magainin II [20] and amphotericin B [12], as a control for a membrane-active compound, were also tested against microorganisms at various pH. Antifungal activity of the peptides was increased when the pH was lowered below 7, while the activity was not changed when the pH was raised. At pH 5, almost all peptides showed similar antifungal activity (MIC 1.56 ~ 3.12 μg/ml), which indicated that the effect of peptide charge on antifungal activity was decreased at low pH. Magainin II, a membrane-active

Table 2
Activities of the peptides against *C. Albicans* at different pH

Peptide	Minimal inhibition concentration (μg/ml) at the following pH				
	5	6	7	8	9
KSL	3.13	6.25	12.5	12.5	12.5
KSL1	6.25	12.5	25	25	25
KSL2	3.13/6.25	6.25	12.5	12.5	12.5
KSL3	3.13	3.13/6.25	6.25	6.25	6.25
KSL4	3.13	3.13/6.25	6.25	6.25	6.25
KSL5	3.13	3.13	6.25	6.25	6.25
KSL6	1.56/3.13	1.56/3.13	3.13	6.25	6.25
KSL7	1.56/3.13	3.13	3.13	3.13	3.13
Magainin II	50	50	>50	>50	>50
Amphotericin B	0.125	0.03	0.03	0.03	0.015

peptide, also showed the increase of antifungal activity at low pH like KSL analogs. However, the change of antifungal activity of amphotericin B depending on pH was totally different from those of the peptides; antifungal activity was decreased when pH was decreased and increased when the pH was raised.

As shown in Fig 2, hemolytic activity of KSL, KSL2, and KSL7 was measured. All tested peptides did not show hemolytic activity up to 500 μg/ml, whereas a cytotoxic peptide, melittin [24], caused the lysis of whole erythrocytes below 10 μg/ml. This result suggested that the addition of lysine residue(s) might not affect hemolytic activity. However, since the model peptide, KSL, had no hemolytic activity below 500 μg/ml, it was not sure that the addition of lysine residue(s) did not affect hemolytic activity.

4. Discussion

Many structure parameters such as net positive charge, hydrophobicity, peptide helicity, hydrophobic moment, and

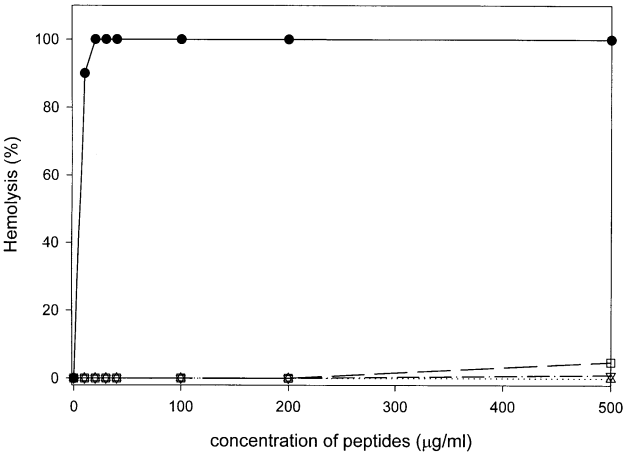

Fig. 2. Hemolytic activity of the peptides. Erythrocytes were incubated in Tris buffer (150 mM KCl, 5 mM Tris-HCl, pH 7.4) with various concentration of the peptide for 1 h at 37°C. Symbol: △, KSL; □, KSL2; ▽, KSL7; ●, melittin.

the size (angle) of hydrophobic/hydrophilic domain were reported to influence on the activity and selectivity of membrane-active peptides [1–3,8–10,18,31]. Among the structure parameters, net positive charge, helicity, and hydrophobicity were reported to be most fundamental factors for activity and selectivity [2,9,19,24,25,31].

Generally, the change of net positive charge and hydrophobicity must result in the change of α-helical structure regarded as a most critical factor for the specificity to neutrally charged membrane. Thus, it was thought to be difficult to investigate the sole effect of net positive charge and hydrophobicity on the specificity between bacteria and fungi.

In this study, we chose KSL as a model peptide and synthesized KSL analogs with similar α-helical structures, which had a different net positive charge and hydrophobicity. Our previous research [16] using diastereomers (peptide containing D amino acids) of a KSL analog revealed that moderate change of α helicity without the change of the net positive charge and hydrophobicity did not affect antifungal activity and antibacterial activity. Thus, the peptide analogs made it possible for investigating the relationship between the specificity and the modulation between net positive charge and hydrophobicity. According to our result, the increase of the net positive charge and the decrease of hydrophobicity improved antifungal activity but did not affect activity against Gram-positive bacteria.

We could address the question why the same change of the structural parameters (the net positive charge and hydrophobicity) only affected activity against fungi. As not only the structural parameters of antimicrobial peptides but also the lipid membrane composition of target cells were reported to be related to biological activity [8,9,14,18,26, 29], we must consider the lipid membrane composition of target cells and the biological mechanism of antimicrobial peptides.

Among the structure parameters, hydrophobicity was proportion to the permeable activity for neutrally charged membranes. However, hydrophobicity of the peptides employed in this study was inversely proportion to antifungal activity. Therefore, we expected that the net positive charge must be the main factor for the increase of the antifungal activity.

Considering the general biological mechanism of membrane-active peptides, peptides must bind to lipid membranes of microorganisms for their biological action. In this step, charge-charge interactions between the positively charged peptides and the negatively charged lipid membranes were the most critical force for binding. Thus, the increase of the net positive charge of the peptides could enhance its binding to lipid membranes. However, if sufficient charge-charge interactions have already existed, the increase of the net positive charge must have little effect on binding. The lipid membranes of Gram-positive bacteria, which have a high content of phosphatidylglycerol, are more negatively charged [22] than those of fungi consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and ergosterol [23]. Thus, the binding of the peptide to lipid membranes of Gram-positive bacteria must be less affected by the increase of a net positive charge. However, the binding of the peptides to fungal membranes, which were relatively less negatively charged, could be enhanced by the increase of the net positive charge in the peptide. Sequentially, the increase of the binding of the peptide must result in the increase of the activity against fungi. This explanation must be consistent with the other research results [1,8,21], in which the increase of the net positive charge of membrane-active peptides increased its binding to the liposomes with various surface charges, resulting in the improvement of the perturbation-activity of the peptides.

We contemplated the reason why the change of hydrophobicity of the peptides did not affect antibacterial and antifungal activity. We could suggest two possible explanations. In the first explanation, there is a threshold of hydrophobicity for the insertion of the peptide into the lipid membranes. Hydrophobicity of the peptides employed in this study must be over this threshold. In the second explanation, the retained α-helicity compensated for the decrease of hydrophobicity, which maintained hydrophobic interactions of the peptide for the insertion of the peptide into lipid membranes. At present, it is difficult to decide which explanation is more correct.

In this study, we investigated antifungal activity at different pH. All peptides employed in this study seemed to have increased antifungal activity at low pH. To find out the reason, we investigated the pH-sensitive factors related to the activity such as the growth of cells and the secondary structure. The growth of cells seemed to be unaffected by pH because the final number of colony forming unit (CFU) of fungi was not changed at various pH. The secondary structure of the peptides was changed when the pH was changed. However, this structural change could not explain the increase of the activity at low pH because α helicity was rather decreased at low pH.

Considering the above result, we expected that there must be other factor(s) except the secondary structure for increasing antifungal activity at low pH. When pH was decreased, generally, hydrophobic interactions between peptides and lipid membranes decreased while charge-charge interactions increased. Thus, we expected that the increase of charge-charge interactions at low pH might be the main factor for the increase of the activity. This suggestion also explained the fact that most of KSL analogs with a different net positive charge showed a similar activity against fungi at low pH. Interestingly, magainin II as a control antimicrobial peptide also showed an improved activity at low pH whereas amphotericin B did not. Even though both compounds were reported to be act on the lipid membranes of microorganisms, magainin II bound lipid membranes by charge-charge interactions whereas amphotericin B, a hydrophobic polyene compound, [12] bound to

lipid membranes by hydrophobic interactions. The different characteristic and inverse change of the activity depending on the pH suggested that increase of the activity at low pH is a unique character of membrane-active peptide and may relate to the increase of charge-charge interactions.

In conclusion, the modulation between the net positive charge and hydrophobicity of short antimicrobial peptides without the change of α-helical structure improved antifungal activity without the decrease of antibacterial activity. This result showed the unique effect of the net positive charge of antimicrobial peptides on the selectivity between fungi and Gram-positive bacteria.

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