Bioorganic & Medicinal Chemistry Letters 19 (2009) 5627–5631

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis of antibacterial pseudopeptides with less hemolytic activity from a cytotoxic peptide and their pH-dependent activity

Sung-Min Kim, Joung-Min Kim, Hyeongjin Cho, Keun-Hyeung Lee *

Bioorganic Chemistry Lab., Department of Chemistry, Inha University, 253 Younghyun-Dong, Nam-Gu, Inchon-City 402-751, Republic of Korea

ARTICLE INFO

Article history:
Received 1 June 2009
Revised 5 August 2009
Accepted 7 August 2009
Available online 12 August 2009

Keywords:
Antibacterial peptide
Hydrophobicity
Non-peptide
Reduced amide bond
Selectivity
Antibacterial pseudopeptide

ABSTRACT

We synthesized antibacterial pseudopeptides with less hemolytic activity by incorporation of reduced amide bond ψ[CH_2NH] into α helical antibacterial peptide with hemolytic activity. As the pK_a value of reduced amide bond is 7–8, it is protonated depending on the pH. We investigated the secondary structure, the binding affinity and the leakage activity for the vesicles, and the antibacterial activity of the peptide and its pseudopeptides at neutral and basic pH. Unlike the peptide, the pseudopeptides showed a more potent leakage activity when pH increased. The peptide exhibited a lower antibacterial activity at basic pH than at neutral pH, whereas the pseudopeptide showed the same antibacterial activity at basic and neutral pH. Overall results indicated that hydrophobicity of backbone of the pseudopeptide plays an important role in the increase of leakage activity and retention of antibacterial activity at basic pH.

© 2009 Elsevier Ltd. All rights reserved.

The increasing infectious diseases caused by antibiotic-resistant bacteria demand the exploration of novel antibacterial molecules with unexploited mechanisms of action.[1] More than 700 cationic antimicrobial peptides (CAPs) have been discovered from various living organisms such as insects, amphibians, and mammals and their properties have been investigated.[2] In spite of different lengths and primary structures of CAPs, they have common features such as high net positive charge at physiological pH and amphipathic α helical or β sheet structure upon association with lipid bilayers of microorganisms.[3] Although the detailed mode of the action of CAPs was not completely understood, the peptides are supposed to act on the cytoplasmic membranes of microorganisms and kill the microorganisms by permeabilizing and/or disrupting their lipid membranes.[3] CAPs have received attention as potential antimicrobial agents because they have properties different from those of current antibiotics, such as fast killing, bactericidal activity, broad antimicrobial spectra, and a great synergism with currently available antibiotics.[1–3]

Several studies done on amino acid replacement revealed that the net positive charge, hydrophobicity, hydrophobic momentum, amphipathicity, and α helicity of CAPs played an important role in the antibacterial activity and lipid membrane perturbation activity.[4–7] Considering the mode of action, amide backbone of CAPs was important for stabilizing amphipathic α helical structures as well as for direct interactions with lipid membranes for penetrating the peptides into lipid membranes of bacteria. However, previous simple amino acid replacement study did not elucidate the role of amide backbone of CAPs for direct interactions with lipid membranes for the activity. Various amide backbone modifications have been developed for increasing the stability of the peptides. However, the amide backbone modifications have not been intensively applied into CAPs because α helical or β sheet structure of the peptides upon lipid membranes has been regarded as a prerequisite factor for the activity and application of backbone modifications generally resulted in the destabilization of α helical or β sheet structure of peptides.[8] However, the studies for developing non-hemolytic antibacterial peptides revealed that high α helicity and/or high hydrophobicity were necessary for mammalian cell toxicity rather than antibacterial activity.[1–3] Previously, we synthesized antibacterial pseudopeptides by replacing amide bonds of a cytotoxic peptide with reduced amide bond ψ[CH_2NH].[9] The pseudopeptides devoid of α helical structure did not have hemolytic activity but exhibited similar antibacterial activity as the reference cytotoxic peptide. Recently, we synthesized the pseudopeptides by introduction of reduced amide bonds into indolicidin (ILPWKWPWWPWRR-NH_2), that is, the smallest known, naturally occurring, linear antimicrobial defense peptide with the highest percentage of Trp residue.[10] The pseudopeptide containing two reduced amide bonds showed less hemolytic activity as well as improved stability without a decrease in its antimicrobial activity.

Aubry and co-workers reported that a reduced amide bond is partially protonated at physiological pH because the pK_a of a reduced amide bond is about 7–8.[11] This suggests the possibility that

* Corresponding author. Tel.: +82 32 860 7674; fax: +82 32 867 5604.
E-mail address: leekh@inha.ac.kr (K.-H. Lee).

0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.08.031

antibacterial pseudopeptides containing reduced amide bond may exhibit an activity depending on the pH. In the present study, we synthesized the pseudopeptides containing reduced amide bond corresponding to α helical antibacterial peptide (**KL1**; Ac-KLLL-KLWL-KLLK-NH_2). We measured the secondary structures, lipid membrane perturbation activities, and antimicrobial activities of the pseudopeptides at neutral and basic pH. The pseudopeptides showed the same antimicrobial activity as the peptide but less hemolytic activity at neutral pH. The pseudopeptides unlike the peptide showed a more potent leakage activity for the vesicles at basic pH than at neutral pH. At basic pH, the peptide exhibited decreased antibacterial activity, whereas the pseudopeptides showed the same antibacterial activity regardless of pH change. The hydrophobicity of backbone of the pseudopeptide plays an important role in the increase of leakage activity and retention of antibacterial activity at basic pH. To the best of our knowledge, we first showed that direct interactions between the backbone of pseudopeptides and lipid membranes were important for the activity. This study showed the important information about the design of novel non-peptide antibacterial agents corresponding to cationic antimicrobial peptides.

We chose **KL1** as a model peptide because **KL1** consisting of Lys and Lue residues with a high α helical propensity may adopt an amphiphilic α helical structure in lipid mimic condition (Table 1). Trp residue was inserted into the middle of the peptide because Trp residue has been used for monitoring the binding of peptides with lipid membranes by a change in the emission spectrum of Trp residue.[12] As the pK_a value of N-terminal amino group of the peptide is around 9.5, α amino group is acetylated to investigate the only effect of the reduced amide bond on the structure and activity at various pH. As shown in Table 1, a reduced amide bond was incorporated into the N-terminal or the middle of the peptide. The peptide and pseudopeptides containing reduced amide bond were synthesized by Fmoc-chemistry in solid phase peptide synthesis according to the literature procedure.[13] Reduced amide bond was incorporated into the resin-bound peptides using the microwave irradiation procedure.[14] The deprotection and cleavage of the peptide and pseudopeptides from resins were achieved by treatment with a mixture of TFA:H_2O (95:5, v/v). The peptide and pseudopeptides were purified by preparative HPLC. The purified peptide and pseudopeptides were analyzed by analytical HPLC and MALDI TOF mass spectrometer to investigate purity and to confirm the successful synthesis. HPLC and mass spectra results revealed the successful synthesis and high purity (>95%) of the peptide and pseudopeptides.

The peptide and its pseudopeptides had different retention times on the C_{18} reverse phase column using CH_3CN–H_2O as an eluent (Table 1). The retention time was reported to be correlated to the global hydrophobicity of the peptides.[15] The retention time difference indicated that the peptide (**KL1**) might be the most hydrophobic. Despite having the same primary structure of the pseudopeptides except for the location of the reduced amide bond, the retention time of **KL3** was much shorter than that of **KL2**. This indicated that **KL2** was more hydrophobic than **KL3**. Generally, a reduced amide bond ψ[CH_2NH] is more flexible than an amide bond and forms weaker hydrogen bond compared to an amide bond due to the loss of the carbonyl group that acted as a hydrogen-bonding acceptor. Thus, the incorporation of reduced amide bond into the peptide may result in destabilization of α helical structure.

As the pK_a of a reduced amide bond is about 7–8,[11] the reduced amide bond might be partially protonated at neutral pH. To investigate the effect of the reduced amide bond on the secondary structure depending on the pH, we measured circular dichroism (CD) spectrum of each compound in buffer solution at pH 7.4 and 9 including SDS micelles (Fig. 1).[16] Table 1 summarizes α helicities of the peptide and its pseudopeptides. The CD spectra measured at pH 7.4 indicated that the introduction of the reduced amide bond in the middle considerably decreased α helicity of the peptide, whereas the incorporation of the reduced amide bond at the N-terminal had a relatively weak destabilizing effect on the α helical structure. The secondary structure changes depending on the pH. The peptide adopted similar secondary structures at pH 7.4 and 9. However, the secondary structures of the pseudopeptides changed depending on the pH. The α helicities of the pseudopeptides decreased slightly as the pH increased. Unexpectedly, the unprotonated form of reduced amide bond may have a more potent destabilizing effect on the α helical structure than the protonated form of reduced amide bond. CD spectra of the compounds were also measured in buffer solution at pH 7.4 and 9 including 50% (V/V) trifluoroethanol. The CD spectra indicated that the secondary structures of the peptide and pseudopeptides were similar regardless of pH change (data not shown). This result indicates that the decrease of α helicity of the pseudopeptides at pH 9 in the presence of SDS micelles is due to the decrease of bound amount of the pseudopeptides on SDS micelles because at pH 9, the net positive charge of the pseudopeptides decreased, resulting in the decrease of charge–charge interactions between the pseudopeptides and SDS micelles.

Table 2 summarizes the minimum inhibitory concentration (MIC) values of the peptide and its pseudopeptides for several *bacteria* at pH 7.4 and 8.4. The peptide exhibited lesser antibacterial activity at basic pH; **KL1** exhibited 4-fold lower activities against *Staphylococcus aureus* and *Escherichia coli* at basic pH. This is consistent with our previous result that the short cationic antibacterial peptide exhibited lower antibacterial activity at basic pH.[17] However, the pseudopeptide, **KL2** exhibited the same activity against bacteria at basic pH, whereas the pseudopeptide, **KL3** exhibited twofold lower activities against *S. aureus* and *E. coli* at basic pH. The peptide and its pseudopeptides were added to human erythrocytes and the level of lysis was measured at pH 7.4 (Fig. 2). **KL1** caused 100% lysis at a concentration greater than 50 μg/mL, whereas **KL2** caused approximately 100% lysis at a concentration of 150 μg/mL and **KL3** showed low hemolytic activity at a concentration of up to 200 μg/mL. The cytotoxic peptide, melittin caused 100% lysis at a concentration greater than 5 μg/mL. This result indicates that the introduction of reduced amide bond into the cytotoxic peptide decreased hemolytic activity without decrease of antibacterial activity.

The membrane permeabilizing ability was investigated by entrapped dye release from LUVs at pH 7.4 and 8.4, respectively. Considering the negatively charged surface of lipid membranes of

Table 1
Structures and α helicities of the peptide and pseudopeptides

Name	Sequence	HPLC (CH_3CN%)	α Helicity (%) (pH 7.4/pH 9)	Calculated mass (measured mass)
KL1	Ac-KLLLKLWLKLLK-NH_2	44.0	37.6/36.1	1550.06 (1550.04)
KL2	Ac-Kψ[CH_2NH]LLLKLWLKLLK-NH_2	43.3	31.6/27.6	1536.06 (1538.06)
KL3	Ac-KLLLKLWLψ[CH_2NH]KLLK-NH_2	40.6	22.4/17.7	1536.06 (1534.53)

The α helicity was determined from the mean residue ellipticity [θ] at 222 nm according to the relation $[\theta]_{222} = -30300\,[\alpha] - 2340$, where [α] is the percent of helix.

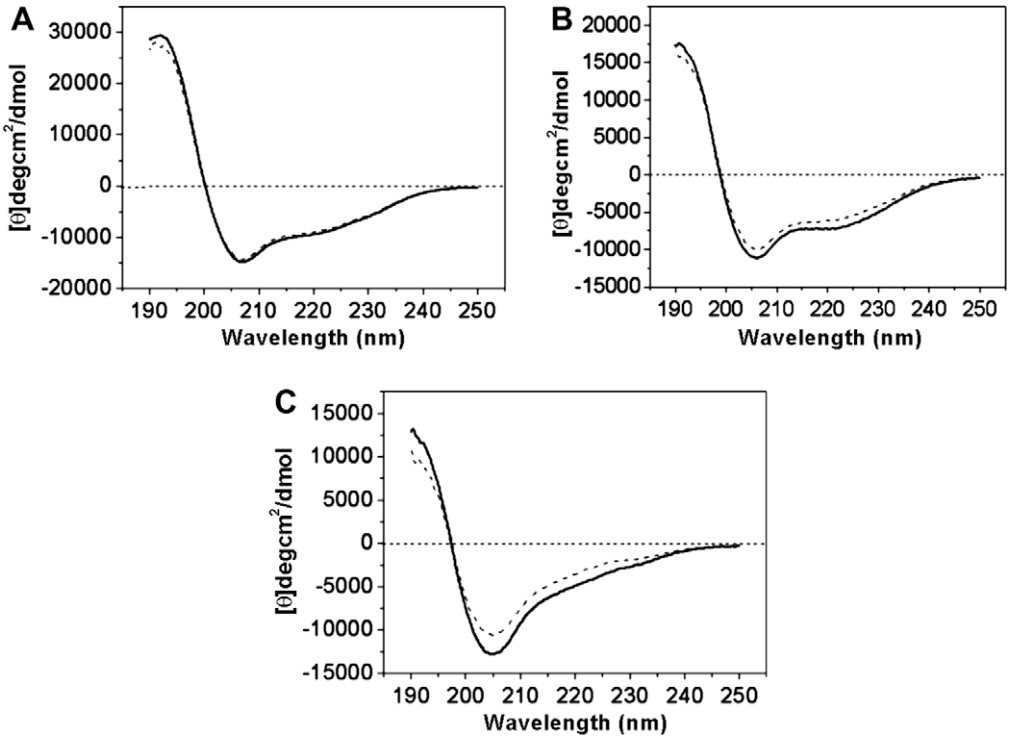

Figure 1. Circular dichroism spectra of (A) **KL**, (B) **KL2**, and (C) **KL3** in 10 mM Tris buffer (pH 7.4 and 9) containing SDS micelles. Samples (100 μM) were dissolved in 10 mM Tris buffer (pH 7.4 and 9) containing 25 mM SDS, respectively. The bold line indicates the CD spectrum measured at pH 7.4 and the broken line indicates the CD spectrum measured at pH 9.

Table 2
Antibacterial activity of the peptide and pseudopeptides at different pH

		Minimum inhibitory concentration (μg/mL)			
		M. luteus ATCC9341	*S. aureus* ATCC6538	*E. coli* ATCC25922	*P. aeruginosa* ATTCC9027
KL1	pH 7.4	6.25	6.25	12.5	50
	pH 8.4	6.25	25	50	50
KL1	pH 7.4	6.25	6.25	12.5	12.5
	pH 8.4	6.25	6.25	12.5	12.5
KL1	pH 7.4	6.25	6.25	12.5	50
	pH 8.4	6.25	12.5	25	50

bacteria,[18] phosphatidylglycerol (PG)/phosphatidylcholine (PC) LUVs containing fluorescence dye calcein, were prepared. Various sample concentrations were mixed with PG/PC (1:1, mole ratio) LUVs and the release of the entrapped dye was measured with an excitation wavelength at 490 nm. All compounds induced a leakage of calcein from the vesicles in a concentration-dependent manner (Fig. 3). **KL1** showed a lower leakage activity for the vesicles at pH 8.4 than at pH 7.4; 7.5 μM of **KL1** induced 90% leakage of dye from the vesicles at pH 7.4, whereas 15 μM of the peptide induced only 80% of dye release at pH 8.4. The pseudopeptides showed a more potent leakage activity for the vesicles at pH 8.4 than at pH 7.4; **KL2** at a concentration of 7.5 μM induced about 80% leakage at pH 7.4, while the same concentration of **KL2** induced 100% leakage at pH 8.4. **KL3** also showed more potent leakage activity at pH 8.4. The leakage activity change depending on the pH indicated that the pseudopeptides exhibited a more potent leakage activity at basic pH, whereas the peptide exhibited less activity at basic pH. However, the leakage activity of **KL3** was significantly weaker than those of **KL1** and **KL2**, whereas the MIC values were the same at pH 7.4. This may be due to the composition difference between the model LUVs and the real lipid membranes of microorganisms. For example, lipid membranes of *E. coli* were

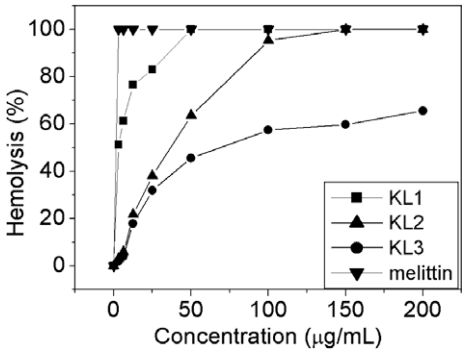

Figure 2. Hemolytic activity of the peptide and its pseudopeptides. Human erythrocytes stock suspension was diluted 250-fold with the buffer (150 mM KCl, 5 mM Tris–HCl, pH 7.4) to a final erythrocyte concentration of 0.4% (v/v). After incubation of the test sample in the erythrocyte solution for 1 h at 37 °C, the solution was centrifuged and the supernatant absorbance was determined at 540 nm. Hemolysis affected by 0.1% Triton X-100 was considered as 100%.

more complicated than the model LUVs. Generally, model LUVs consisting of two or three lipids cannot reproduce exactly the behavior of the *E. coli* whole lipid LUVs.[18,19] The model vesicles consisting of PG/PC were prepared to just mimic the surface charge of lipid membranes of bacteria. Thus, the model vesicles may be suitable to investigate the leakage activity change of the compounds depending on the pH.

The binding affinities of the compounds for PG/PC (1:1, mole ratio) LUVs were investigated by measurement of emission spectrum of Trp residue. Figure 4 shows that the addition of the vesicles into the compounds resulted in a blue shift of emission maxima of Trp residue. The blue shift in the presence of the vesicles indicated that the Trp residue of all compounds moved to a less polar environment upon association with the vesicles. The blue shift in

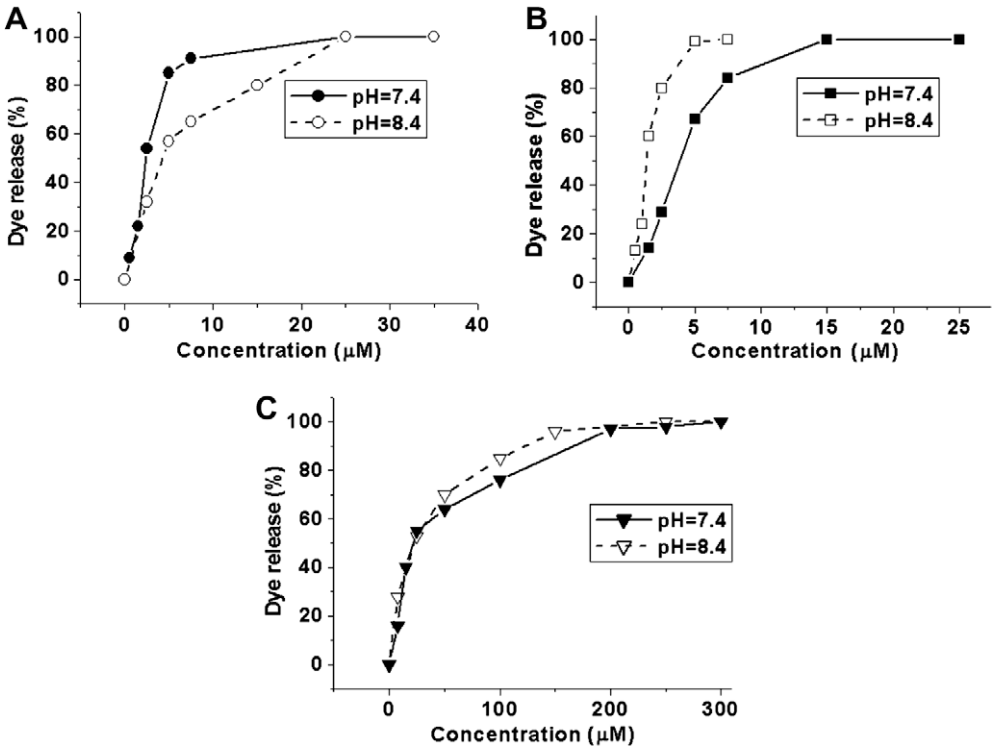

Figure 3. Percent leakage of entrapped dye from PG/PC (1:1, mole ratio) LUVs by (A) **KL**, (B) **KL2**, and (C) **KL3**. Sample was added to 40 μM LUVs solution and the maximum level of fluorescence, scaled to a value of 100%, was determined by complete lysis of a vesicle with TX-100.

the Trp emission spectra of all compounds saturated in the presence of 40 μM vesicles at pH 7.4. This indicates that all compounds have similar binding affinities for the vesicles at pH 7.4. The maximum blue shift indicated that the membrane penetration depth of all compounds was similar at pH 7.4. At pH 8.4, the binding affinities of the pseudopeptides increased, whereas the binding affinity of the peptide did not change considerably. The maximum blue shift in the Trp emission spectra of the pseudopeptides saturated in the presence of 20 μM vesicles, whereas the blue shift in the Trp emission spectra of the peptide saturated in the presence of

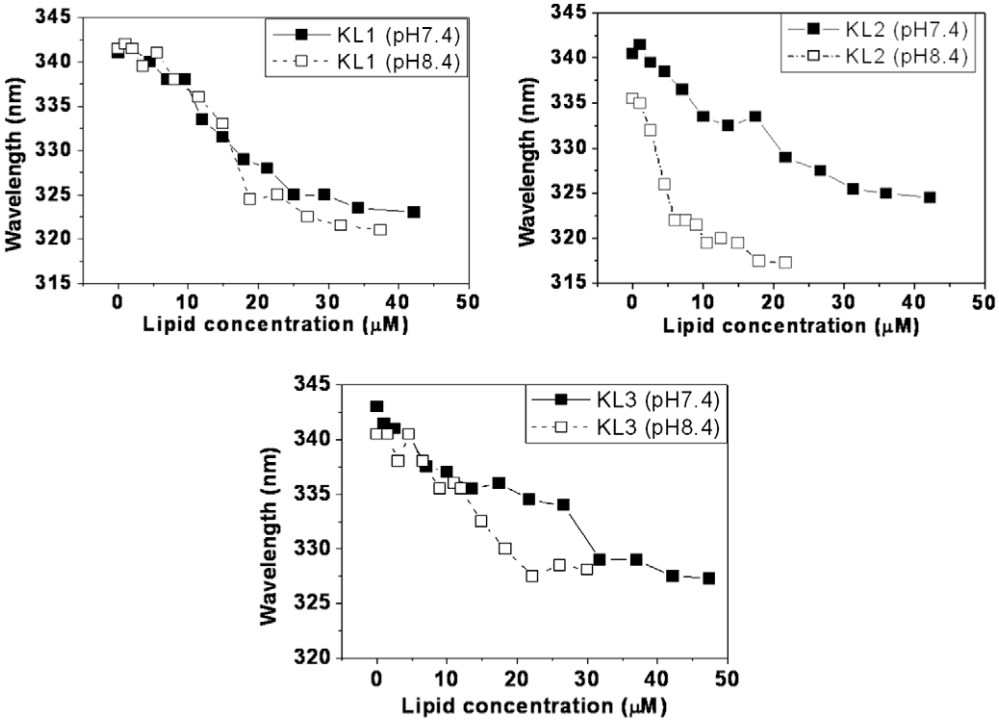

Figure 4. The blue shifts of maximum emission wavelength as function of the concentration of PG/PC (1:1, mole ratio) LUVs. The tryptophan of sample (1 μM) was excited at 280 nm and emission spectra were recorded from 300 to 450 nm.

30 μM vesicles. The blue shift indicated that the pseudopeptide **KL2** (λ_{max} = 317 nm) penetrated the vesicles more deeply than the peptide (λ_{max} = 322 nm) at basic pH. The membrane penetration depths of cationic antibacterial peptides were reported to be correlated with the leakage activities for the vesicles.[20] **KL2** that penetrated the lipid membranes most deeply is the most potent in inducing calcein release at basic pH.

Generally, CAPs bound to lipid membranes with negatively charged surface by charge–charge interactions and then penetrated the lipid membranes mainly by hydrophobic interactions.[3] Thus, electrostatic interactions and hydrophobic interactions between the CAPs and lipid membranes play an important role in the antibacterial activity and hemolytic activity. Structure–activity studies revealed that several structure parameters such as net charge, hydrophobicity, hydrophobic moment, amphiphilicity, α helicity, and the angle subtended by the hydrophobic face have been regarded as critical parameters for antibacterial activity of CAPs.[4–7] These parameters were related to the electrostatic and hydrophobic interactions between the peptides and lipid membranes. As pK_a value of reduced amide bond is about 7–8, the secondary structure, net charge, and hydrophobicity of the pseudopeptides will change depending on the pH. CD spectra indicate that the peptide and the pseudopeptides have similar secondary structures at basic and neutral pH. The protonated and unprotonated forms of reduced amide bond might have similar destabilizing effect on the α helicity of the peptide. As pH increased, the net charge of pseudopeptide backbone decreased, whereas the hydrophobicity of pseudopeptide backbone increased. Interestingly, the pseudopeptides more deeply penetrated the lipid membranes and exhibited more potent leakage activity for the vesicles at basic pH. This result indicated that the hydrophobicity of pseudopeptide backbone played an important role in the increase of leakage activity and the retention of antibacterial activity at basic pH, while the decrease of the net positive charge of the backbone did not considerably affect the activity. To the best of our knowledge, our research first revealed that the direct interactions between the peptide backbone and lipid membranes were important for the activity, and hydrophobicity of the backbone played an important role in the leakage activity and antibacterial activity.

CAPs have several unique properties for the development of antibacterial agents, such as fast killing, bactericidal activity, broad antimicrobial spectra, and a great synergism with currently available antibiotics.[1,5] However, it seems to be difficult to use CAPs themselves as novel antibacterial agents because of their poor bioavailability. Several attempts have been made to develop non-peptide analogs on the basis of the primary structures of CAPs. It was reported that antibacterial cyclic peptides, antibacterial pseudopeptides containing various amide bond surrogates, antibacterial peptide-lipid hybrid compounds, antibacterial unnatural biopolymers, and antibacterial dendrimers were synthesized on the basis of the structures of CAPs.[21] However, the development of non-peptide analogs from CAPs required a difficult synthesis for maintaining secondary structures such as α helical or β sheet structures. Our research revealed that high α helicity or high hydrophobicity of CAPs is not required for antibacterial activity, but is required for hemolytic activity. Furthermore, our result suggests that the increase of hydrophobicity of pseudopeptide backbone may compensate for the decrease of hydrophobicity of CAPs caused by decrease of α helicity, resulting in the retention of antibacterial activity and increase of selectivity between bacteria and erythrocytes. We expect that our result would help in developing novel non-peptide analogs that have great selectivity to bacteria and potent antibacterial activity at neutral and basic pH by application of backbone modification including reduced amide bond into CAPs.

Acknowledgments

This work was supported by grants from the Korea Research Foundation (KRF-2005-C00216). S. Kim, and J. Kim were the recipients of a BK21 (II) fellowship.

References and notes

1. Zasloff, M. *Nature* **2002**, *415*, 389.
2. (a) Epand, R. M.; Vogel, H. J. *Biochim. Biophys. Acta* **1999**, *1462*, 11; (b) Tossi, A.; Sandri, L.; Giangaspero, A. *Curr. Pharm. Design* **2002**, *8*, 743.
3. (a) Matsuzaki, K. *Biochim. Biophys. Acta* **1999**, *1462*, 1; (b) Shai, Y. *Curr. Pharm. Design* **2002**, *8*, 715.
4. Frecer, V. *Bioorg. Med. Chem.* **2006**, *14*, 6065.
5. Lee, K. H. *Curr. Pharm. Design* **2002**, *8*, 795.
6. Dathe, M.; Wieprecht, T.; Nikolenko, H.; Handel, L.; Maloy, W. L.; Macdonald, D. L.; Beyermann, M.; Bienert, M. *FEBS Lett.* **1997**, *403*, 208.
7. Kiyota, T.; Lee, S.; Sugihara, G. *Biochemistry* **1996**, *35*, 13196.
8. (a) Dathe, M.; Wieparecht, T. *Biochim. Biophys. Acta* **1999**, *1462*, 71; (b) Papo, N.; Oren, Z.; Pag, U.; Sahl, H. G.; Shai, Y. *J. Biol. Chem.* **2002**, *277*, 33913.
9. Oh, J. E.; Lee, K. H. *Biochem. J.* **2000**, *352*, 659.
10. Kim, S. M.; Kim, J. M.; Joshi, B. P.; Cho, H.; Lee, K. H. *Biochim. Biophys. Acta* **2009**, *1794*, 185.
11. Vanderesse, R.; Grand, V.; Limal, D.; Vicherat, A.; Marraud, M.; Didierjean, C.; Aubry, A. *J. Am. Chem. Soc.* **1998**, *120*, 9444.
12. (a) Breukink, E.; van Kraaij, C.; van Dalen, A.; Demel, R. A.; Siezen, R. J.; de Kruijff, B.; Kuipers, O. P. *Biochemistry* **1998**, *37*, 8153; (b) Ren, J.; Lew, S.; Wang, J.; London, E. *Biochemistry* **1999**, *38*, 5905.
13. Fields, G. B.; Noble, R. L. *Int. J. Pept. Protein Res.* **1990**, *35*, 161.
14. Park, M. S.; Oh, H. S.; Cho, H. J.; Lee, K. H. *Tetrahedron Lett.* **2007**, *48*, 1053.
15. (a) Krause, E.; Beyermann, M.; Dathe, M.; Rothemund, S.; Bienert, M. *Anal. Chem.* **1995**, *67*, 252; (b) Buszewski, B.; Kowalska, S.; Kowalkowski, T.; Rozpedowska, K.; Michel, M.; Jonsson, T. *J. Chromatogr. B* **2007**, *845*, 253.
16. Wu, C. C.; Ikeda, K.; Yang, J. T. *Biochemistry* **1981**, *20*, 566.
17. Hong, S. Y.; Park, T. G.; Lee, K. H. *Peptides* **2001**, *22*, 1669.
18. Hristova, K.; Selsted, M. E.; White, S. H. *J. Biol. Chem.* **1997**, *272*, 24224.
19. Blazyk, J.; Wiegand, R.; Klein, J.; Hammer, J.; Epand, R. M.; Epand, R. F.; Maloy, W. L.; Kari, U. P. *J. Biol. Chem.* **2001**, *276*, 27889.
20. (a) Glukhov, E.; Stark, M.; Burrows, L. L.; Deber, C. M. *J. Biol. Chem.* **2005**, *280*, 33960; (b) Halevy, R.; Rozek, A.; Kolusheva, S.; Hancock, R. E. W.; Jelinek, R. *Peptides* **2003**, *24*, 1753.
21. (a) Fernandez, S. L.; Kim, H. S.; Choi, E. C.; Delgado, M.; Granja, J. R.; Khasanov, A.; Kraehenbuehl, K.; Long, G.; Weinberger, D. A.; Wilcoxen, K. M.; Ghadiri, M. R. *Nature* **2001**, *412*, 452; (b) Chu-Kung, A. F.; Bozzelli, K. N.; Lockwood, N. A.; Haseman, J. R.; Mayo, K. H.; Tirrell, M. V. *Bioconjugate Chem.* **2004**, *15*, 530; (c) Oh, H. S.; Kim, S.; Cho, H.; Lee, K. H. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1109; (d) Raguse, T. L.; Porter, E. A.; Weisblum, B.; Gellman, S. H. *J. Am. Chem. Soc.* **2002**, *124*, 12774; (e) Porter, E. A.; Weisblum, B.; Gellman, S. H. *J. Am. Chem. Soc.* **2002**, *124*, 7324; (i) Patch, J. A.; Barron, A. E. *J. Am. Chem. Soc.* **2003**, *125*, 12092; (h) Janiszewska, J.; Swieton, J.; Lipkowski, J. W.; Urbanczyk Lipkowska, Z. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3711.