

Antibacterial Activity of Short Peptides Based on Pleurocidin and Their Interaction with Phospholipid Membranes

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We have studied antibacterial activities of model peptides based on Pleurocidin (Ple). In this study, to investigate the effect of the C-terminal portion of these model peptides on antibacterial activity, we synthesized several short peptides which are devoid of the C-terminal four and more amino acid residues from the corresponding model peptides and examined their biological activities and behaviors against lipid and cell membranes. Removal of the C-terminal four residues resulted in maintained antibacterial activity and reduction of hemolytic activity.

Although it is desirable to obtain antibacterial peptides without hemolytic activity from natural sources or by artificial designs, cationic antibacterial peptides generally have more or less hemolytic activity as an undesirable property. On the other hand, studies of peptides with strong antibacterial activity but negligible or no hemolytic activity have been recently reported.[1]

Pleurocidin (Ple) is a cationic peptide isolated from the skin secretions of winter flounder (*Pleuronectes americanus*). Ple consists of 25 amino acid residues (H–Gly–Trp–Gly–Ser–Phe–Phe–Lys–Lys–Ala–Ala–His–Val–Gly–Lys–His–Val–Gly–Lys–Ala–Ala–Leu–Thr–His–Tyr–Leu–OH) and has antibacterial activity against both Gram-positive and Gram-negative bacteria.[2] We have investigated its structure–activity relationship using Ple itself and related peptides.[3–5] Replacement of two Gly residues by Ala at positions 13 and 17 in Ple resulted in remarkable changes in its properties including biological activities. Comparison of biological activities of [Ala^{13}]Ple–NH_2 and [Ala^{17}]Ple–NH_2 showed that substitution of Gly^{13} by Ala influenced the peptide–lipid membrane interaction more than substitution of Gly^{17} by Ala.[4] In this connection, Lim et al. reported that the hinge region from Gly^{13} to Gly^{17} in Ple may provide its conformational flexibility and bacterial cell selectivity from a study on structure–activity relationship of the Gly→Ala-substituted Ple analogs.[6] Furthermore, we synthesized Ple–NH_2 (C-terminal amidated Ple) and three Ple model peptides, Ple I, II, and III, which have also an amidated C-terminus. In Ple I and II, some hydrophobic amino acids and hydrophilic amino acids in the N-terminal 17 residues of Ple were replaced with Leu or Ala, and Lys, respectively, to simplify amino acid composition. Furthermore, in Ple III, all the hydrophobic amino acids except Gly^{13} and hydrophilic amino acids were substituted with Ala and Lys, respectively. Ple–NH_2, Ple I and II had similar antibacterial activities, and hemolytic activity of Ple II and Ple–NH_2 was greatly lower than that of Ple I. Ple III exhibited negligible biological activity. As a result, Ple II was found to have properties superior to Ple I and III.[5] However, Ple II still had some hemolytic activity, suggesting that lowering hydrophobicity of the peptides might result in decrease or disappearance of hemolytic activity.[5]

In a series of our studies,[3–5] all the synthetic peptides had the same amino acid sequences in the C-terminal portions, whereas the N-terminal and middle portions had been often modified with other amino acids. In the present study, to investigate the effect of the C-terminal portion of the peptides on the antibacterial and hemolytic activities, we synthesized some short peptides (SPs; ΔPle–NH_2 and ΔPle I–IV in Table 1), because removal of the four C-terminal residues from the model peptides leads to slight hydrophilicity. ΔPle–NH_2, ΔPle I, ΔPle II, and ΔPle III are devoid of the four C-terminal residues (Thr–His–Tyr–Leu) from Ple–NH_2, Ple I, Ple II, and Ple III, respectively. ΔPle IV, which has a slightly lower hydrophobicity value ($\langle H \rangle$) of −0.138 than that of Ple II (−0.135), was newly designed, because Ple II showed fairly desirable biological activities.[5] Δ_1Ple IV and Δ_2Ple IV, which are shorter model peptides of ΔPle IV, were synthesized to examine the effect of chain length of the peptides on the biological activities. In the preliminary experiment, ΔPle III was found to show no antibacterial activity, and hence CD[8] and membrane permebilization measurements of Ple III were not carried out. The absence of activity of ΔPle III, like Ple III, may be due to its low hydrophobicity. Removal of the four C-terminal residues from the model peptides resulted in maintained strong or moderate antibacterial activity, whereas negligible hemolytic activity was observed for the C-terminal lacking peptides. On the other hand, removal of more than four residues caused considerable decrease in antibacterial activity.

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Table 1. Amino Acid Sequences and Hydrophobicities ($\langle H \rangle$) and Analytical Data of the Peptides

Peptide	Amino acid sequence	$\langle H \rangle$[a]	MALDI-TOF-MS	
			Found	Calcd($[M+H]^+$)
Ple–NH_2	H–GWGSFFKKAAHVGKHVGKAALTHYL–NH_2	−0.026	2712.0	2711.2 ($C_{129}H_{194}N_{37}O_{28}$)
ΔPle–NH_2	H–GWGSFFKKAAHVGKHVGKAAL–NH_2	−0.030	2197.8	2197.6 ($C_{104}H_{160}N_{31}O_{22}$)
ΔPle I	H–GAGKFLKKALKAGKKLAKAAL–NH_2	−0.142	2112.0	2112.7 ($C_{100}H_{180}N_{29}O_{21}$)
ΔPle II	H–GAGKALKKALKAGKKLAKAAL–NH_2	−0.160	2036.7	2036.6 ($C_{93}H_{176}N_{29}O_{21}$)
ΔPle III	H–AAAKAAKKAAKAGKKAAKAAL–NH_2	−0.191	1937.7	1937.2 ($C_{86}H_{158}N_{28}O_{21}$)
Ple IV	H–AAAKALKKALKLGKKLAKAALTHYL–NH_2	−0.117	2621.1	2621.3 ($C_{123}H_{220}N_{35}O_{27}$)
ΔPle IV	H–AAAKALKKALKLGKKLAKAAL–NH_2	−0.138	2105.9	2106.7 ($C_{98}H_{186}N_{29}O_{21}$)
Δ_1Ple IV	H–AAAKALKKALKLGKKLA–NH_2	−0.166	1723.5	1723.2 ($C_{80}H_{153}N_{24}O_{17}$)
Δ_2Ple IV	H–AAAKALKKALKLG–NH_2	−0.108	1282.4	1282.7 ($C_{59}H_{113}N_{18}O_{13}$)

a) Hydrophobicity/residue. Hydrophobicity was calculated using the consensus value of hydrophobicity for each amino acid residue.[7]

Table 2. α-Helix Contents and Biological Activities of the Peptides

Peptide	α-Helix content/%[a]			MIC/μg mL^{-1}				Hemolytic activity/%[h]
	TFE	PC[b]	PG[c]	*S.a.*[d]	*B.s.*[e]	*E.c.*[f]	*P.a.*[g]	
Ple–NH_2	44	35	46	3.1	100.0	12.5	6.3	5.8
ΔPle–NH_2	27	20	35	6.3	>100.0	12.5	12.5	no
Ple I				1.6	50.0	6.3	3.1	
ΔPle I	29	26	41	3.1	50.0	6.3	6.3	3.4
Ple II				3.1	50.0	12.5	6.3	
ΔPle II	13	15	18	3.1	50.0	6.3	12.5	negligible
Ple IV	37	39	53	1.6	50.0	6.3	3.1	43.0
ΔPle IV	36	28	49	3.1	25.0	3.1	3.1	5.7
Δ_1Ple IV	29	16	38	12.5	100.0	25.0	25.0	no
Δ_2Ple IV	31	random	32	>100.0	>100.0	>100.0	>100.0	no

a) [Peptide] = 20 μM; 25 °C. b) [egg PC] = 1 mM. c) [egg PC/eggPG (3/1)] = 1 mM. d) *S. aureus* IFO 12732. e) *B. subtilis* IFO 3513. f) *E. coli* IFO 12734. g) *P. aureginosa* IFO 3080. h) Percentage of hemolysis at a peptide concentration of 100 μM.

Results and Discussion

CD Measurement. To examine the secondary structures of the peptides under aqueous and non-aqueous conditions, CD measurements were performed in 20 mM Tris HCl buffer (pH 7.4) (1 M = 1 mol dm^{-3}), TFE, and the buffer in the presence of egg PC or egg PC/egg PG (3/1) vesicles. The α-helical contents calculated according to the method of Scholtz et al.[9] are listed in Table 2. All the peptides were random in the buffer (data was not shown), whereas the α-helix contents of 13–36% were found in TFE. The order of helix contents of SPs was ΔPle IV > ΔPle I > ΔPle–NH_2 > ΔPle II. With respect to ΔPle I, ΔPle II, and ΔPle IV, the order of helix contents is the same as that of their hydrophobicities. However, the α-helix contents of the SPs in TFE were lower than those of the model peptides, indicating that the four C-terminal residues in the original model peptides contribute to formation of α-helical structure. In the presence of egg PC vesicles, α-helix contents of the SPs were somewhat low (15–28%), suggesting that interaction of these peptides with egg PC was slightly insufficient. The order of helix contents was the same as that in TFE. The SPs exhibited α-helix contents of 18–49% in the presence of egg PC/egg PG (3/1) vesicles. Interaction of the cationic peptides and anionic egg PG clearly contributes to α-helix formation. The order of helix contents of the SPs was the same as the order found in TFE and egg PC, and the α-helix contents were lower by 10% or more than those of the model peptides.

In the case of Ple IV series, the order of α-helix contents was Ple IV, ΔPle IV > Δ_1Ple IV, Δ_2Ple IV in TFE and in the presence of PC/PG vesicles. Ple IV and ΔPle IV had fairly high α-helix content of ca. 50% in the presence of PC/PG vesicles. In the presence of PC vesicles, α-helix contents decrease with shortening of the peptide chain and Δ_2Ple IV no longer takes α-helical structure. It seems that chain length of ca. 20 amino acid residues is desirable to form a stable peptide conformation in neutral liposomes.

Antibacterial and Hemolytic Activities. Antibacterial activities of the SPs were examined by microplate culture methods using Muelleri–Hinton broth with Gram-positive bacteria (*S. aureus* and *B. subtilis*) and Gram-negative bacteria (*E. coli* and *P. aeruginosa*) according to the literature.[10] The MICs of the SPs are listed in Table 2. Antibacterial activities of Ple–NH_2, Ple I and Ple II had been examined previously by a method[5] different from the present method.[10] The MICs obtained by the present method are also shown in Table 2. All the SPs had high or moderate activities against *S. aureus* and Gram-negative bacteria, while activities against *B. subtilis* were not strong, especially in the cases of ΔPle–NH_2. It is interesting that the SPs exhibited stronger antibacterial activities against *B. subtilis* and *E. coli* than natural peptide, Ple–NH_2. The result that antibacterial activities of the SPs,

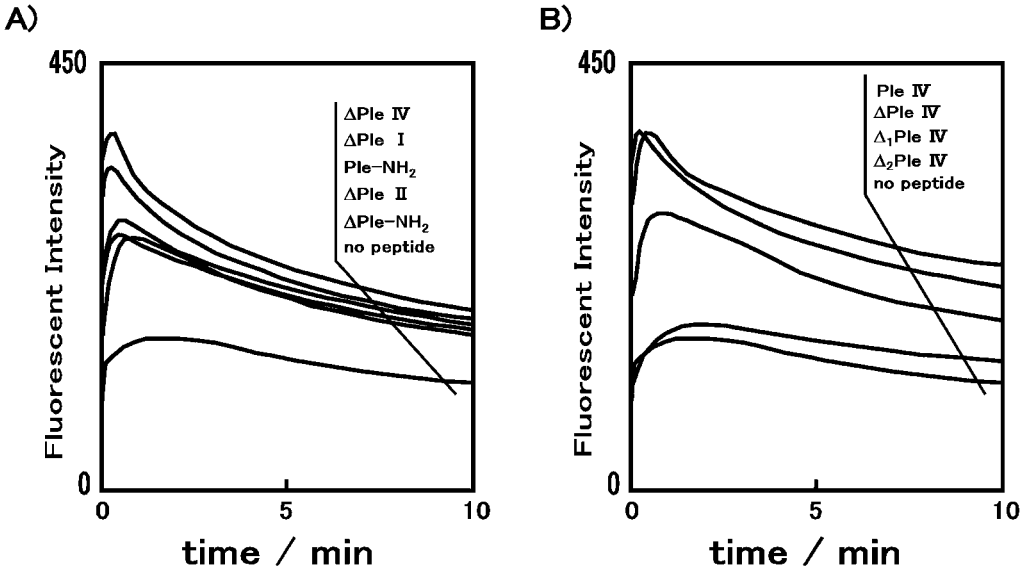

Figure 1. Effect of the short model peptides on permeabilization of the outer membrane of *E. coli*. [Peptide] = 16 μM, [NPN] = 10 μM, $\lambda_{ex} = 350$ nm, $\lambda_{em} = 420$ nm. A) Ple–NH_2, ΔPle–NH_2, ΔPle I, ΔPle II, ΔPle IV, B) Ple IV, ΔPle IV, Δ_1Ple IV, Δ_2Ple IV.

except ΔPle–NH_2, were parallel to their $\langle H \rangle$s means that the $\langle H \rangle$ relates to biological activities. In this connection, the result that ΔPle IV showed strong antibacterial activity is convincing, because the $\langle H \rangle$ of ΔPle IV was almost the same as that of Ple II in spite of their different amino acid sequences and chain lengths. Another noticeable point is that antibacterial activities of SPs were similar to those of the corresponding model peptides, in spite of deletion of the four C-terminal residues. This seems to be due to the number of cationic amino acid residues. The peptides have, in addition to their chain lengths being sufficient to take a certain conformation, the same number of cationic residues, which cause a charge interaction between peptide–anionic cell membrane of similar magnitude. This view may be reasonable, because this is consistent with the result that a designed peptide NK27 and its slightly shorter peptide NK23c, both of which have the same charge and $\langle H \rangle$, showed similar antibacterial activities.[11]

In the case of Ple IV group, the activities of ΔPle IV, Δ_1Ple IV, and Δ_2Ple IV decreased in that order and Δ_2Ple IV no longer showed activity. This may be due to differences in the chain length and/or number of cationic residues of the peptides, because antibacterial activities of the peptides were not entirely in accord with their $\langle H \rangle$s. The membrane permeabilization activities that will be described below were in accord with antibacterial activities.

Hemolytic activities of the SPs were determined using fresh rabbit blood cells according to the literature[2] (Table 2). Ple–NH_2 had hemolytic activity of 5.8% at a peptide concentration of 100 μM, whereas no activity of ΔPle–NH_2 was observed. In the previous study, we found that Ple I and Ple II showed hemolytic activities of 47% and 6%, respectively, at a peptide concentration of 100 μM.[5] Hemolytic activities of ΔPle I and ΔPle II were 3.4% and negligible, respectively. Ple IV showed high hemolytic activity of 43% and ΔPle IV also had activity of 5.7%. Shorter peptides, Δ_1Ple IV and Δ_2Ple IV, had no activity. These results clearly indicated that removal of the four C-terminal residues from natural and model peptides greatly caused decrease in the hemolytic activity. In this connection, from the result of a structure–activity relationship approach using rational designed antibacterial peptides, Chen et al. described that strong hemolytic activity of peptides generally correlated with high hydrophobicity, high amphipathicity, and high helicity.[12] These findings seem to be significant from the view of design of antibacterial peptides without hemolytic activity. Appropriate balance of hydrophobicity and hydrophobic moment of cationic peptides would result the desirable property of strongly interacting with bacterial membranes but not with usual cell membranes.

Outer and Inner Membrane Permeabilizations. Membrane permeabilization activity of peptides is essentially related to their antibacterial activity.[5] Gram-negative bacteria have lipopolysaccharide as a main component of the outer membrane, whereas phosphatidylethanolamine and phosphatidylglycerol are main inner membrane components.[13] *N*-Phenyl-1-naphthylamine (NPN), a hydrophobic fluorescent probe, is taken up into the membrane interior when the membrane is disturbed. Since the fluorescence intensity increases in hydrophobic environment, it is applicable to the permeabilization measurement.[14] The permeabilization activity of the SPs was examined using the outer membrane of *E. coli* (Figure 1A). Although both the SPs and Ple–NH_2 had permeabilization activities, there was no meaningful difference between their activities. On the contrary, a well-defined difference was observed for the Ple IV series peptides (Figure 1B). The order of their abilities was Ple IV > ΔPle IV > Δ_1Ple IV > Δ_2Ple IV and was in accord with their order of α-helix contents in TFE and in the presence of PC/PG vesicles.

The inner membrane permeabilization was evaluated by measuring β-galactosidase activity of *E. coli* using *o*-nitrophenyl β-galactopyranoside (ONPG) as a substrate (Figure 2A).[15] Absorbance at 420 nm, which is attributable to *o*-nitro-

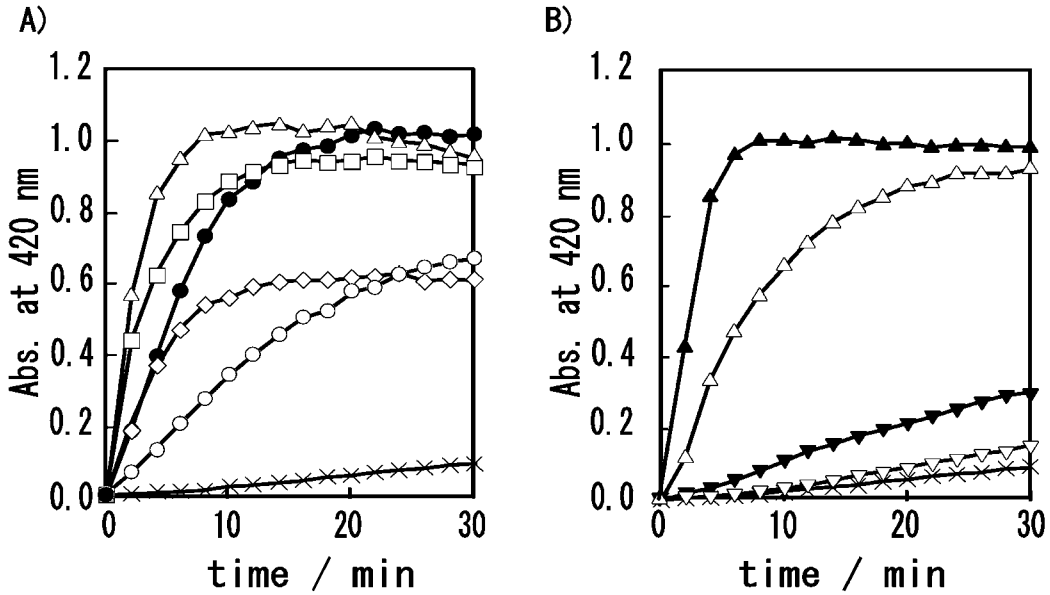

Figure 2. Effect of the short peptides on permeabilization of the inner membrane of *E. coli*. [Peptide] = 32 μM, [ONPG] = 50 μM. A) Ple–NH_2 (●), ΔPle–NH_2 (○), ΔPle I (□), ΔPle II (◇), ΔPle IV (△), no peptide (×). B) Ple IV (▲), ΔPle IV (△), Δ_1Ple IV (▼), Δ_2Ple IV (▽), no peptide (×).

phenol produced by hydrolysis of the substrate, increased with the passage of time. Permeabilization mode of the peptides was divided into two groups. One is a group of Ple–NH_2, ΔPle IV, and ΔPle I, and another is ΔPle–NH_2 and ΔPle II. Increase in fluorescence intensity of the former was more rapid in early time than that of the latter, and as a result, the former peptides showed higher intensities. The fluorescence intensities of the peptides reached a plateau over 10 to 15 min. On the other hand, the order of permeabilities of the Ple IV series was in accord with their antibacterial activities (Figure 2B). The results in the calcein leakage and membrane permeability measurements are fairly consistent with the previous conclusion that antibacterial activities of the Ple-related peptides are attributable to perturbation and permeabilization of bacterial cell membranes by peptide–lipid hydrophobic interaction in addition to charge interaction.[3] A noteworthy study was recently reported that an enantiomeric analog of Ple, all-D-Ple, showed two-fold more potent antifungal activity than that of Ple.[16] Since it is resistant to proteases and shows low cytotoxicity, possible use of the enantiomer in therapeutic application may be anticipated.

In conclusion, we found that the removal of the four C-terminal residues (Thr–His–Tyr–Leu) from the Ple model peptides resulted in maintained antibacterial activity, whereas hemolytic activity was greatly decreased. The result indicates that hemolytic activity is more subject to change of properties of peptides than antibacterial activity, because decrease in hydrophobicity of the peptides by removal of the four residues was small. In the study on a natural antibacterial peptide, maculatin 1.1, we reached the conclusion that cationic residues mainly participate in the antibacterial activity, and hydrophobic residues affect both antibacterial and hemolytic activities.[17] The present results together with the previous findings[3–5] will give helpful information to design desirable antibacterial peptides without hemolytic activity.

Experimental

Peptide Synthesis. Peptides were synthesized with Fmoc-amino acids as *N*-amino-protecting group and Novasyn TGR resin according to the procedure of Fukuoka et al.[5] The crude products were purified by gel chromatography on a Sephadex G-10 column (10% AcOH) followed by RP-HPLC on a YMC-Pack ODS column (0–100% acetonitrile/0.1% TFE). The purified peptides were identified by amino acid analysis and MALDI-TOF-MS.

CD Measurement. Egg PC and egg PC/egg PG (3:1) small unilamellar vesicles were prepared for CD and calcein-leakage measurements. Phospholipid (3.93 mg) was dissolved in $CHCl_3$/MeOH (2:1, 0.59 mL), and then the mixture was dried by a stream of N_2 gas. The dried lipid was hydrated in 20 mM Tris HCl buffer (pH 7.4, 5 mL) using a Branson bath-type sonicator. The suspension was sonicated for 10 min at 50 °C using a Taitec Ultrasonic Processor VP-ST. The vesicles were left for 30 min at 25 °C before measurements. The lipid concentration was 1 mM. CD spectra were recorded on a JASCO J-720W spectrometer with a thermostatted cell holder using a quartz cell of 1.0-mm path length. The peptides were dissolved in 20 mM Tris HCl buffer (pH 7.4), TFE, and the buffer in the presence of 1 mM phospholipids vesicles. The peptide concentration was 20 μM. Measurements were done at 25 °C. The helix contents were calculated according to the method of Scholtz et al.,[9] and the mean residue ellipticity is given in deg dm^2 mol^{-1}.

Antibacterial and Hemolytic Assays. The microplate dilution method[10] was used to determine the MIC values. Aliquots of 100 μL of each serially diluted peptide in sterilized distilled-water were added to the mixture of 10 μL of bacterial cell suspension (approximately 10^6 colony forming units/mL) and 90 μL of Mueller–Hinton broth in each well of a flat-bottomed microplate. After incubation overnight at 37 °C, the MIC values were measured as the lowest final concentration at which no growth was observed.

Hemolytic activity was assayed according to the procedure

of Yoshida et al.[3] Phosphate buffered saline (PBS) (pH 7.5, 0.5 mL) was added to fresh rabbit blood (0.5 mL). The mixture was centrifuged at 2000 rpm for 3 min, and the precipitates were collected. After being washed with PBS three times, the precipitates were suspended in PBS (1 mL). PBS (1 mL) was added to the rabbit erythrocyte solution (5 μL) followed by the peptides. The mixture was incubated for 20 min at 37 °C, and then centrifuged. The supernatant was monitored at 576 nm using a Hitachi U-200 spectrophotometer. To measure the absorbance of 100% and 0% activities, 10% Triton X-100 (10 μL) and water (10 μL) instead of peptide were respectively added to the erythrocyte solution.

Outer Membrane Permeabilization. Outer membrane permeabilization of the peptides was measured according to the procedure by Wu and Hancock.[14] Single colonies of *E. coli* grown on trypticase soy agar plates were inoculated in TSB medium (5 mL) and grown overnight at 37 °C. The suspension was diluted with TSB medium to the absorbance of 0.5–0.6 at 600 nm. Determination of the outer membrane permeabilization was performed by measuring the fluorescence intensity of NPN using a Hitachi F-3010 fluorescence spectrophotometer. A hundred microliters of 160 μM peptide solution were added to a mixture of the bacterial suspension (880 μL) and 0.5 mM NPN in acetone (20 μL). The outer membrane permeabilization was monitored by measuring the intensity of NPN at 420 nm (excited at 350 nm) for 10 min at 25 °C. An equivalent volume of water was used instead of the peptide solution in the control assay.

Inner Membrane Permeabilization. Determination of the inner membrane permeabilization was performed by measuring the β-galactosidase activity using ONPG as a substrate according to the procedure by Pellegrini et al.[14] A suspension of *E. coli* (10^4 cells mL^{-1}, 800 μL) was added to a solution of 2.5 mM ONPG in water (100 μL). And then, a 320 μM peptide solution (100 μL) was added. The inner membrane permeabilization was monitored by measuring the rate *o*-nitrophenol production at 420 nm every 1 min between 30 min. An equivalent volume of water was used instead of the peptide solution in the control assay.

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8 Abbreviations used are as follows: CD, circular dichromism; egg PC, egg yolk phosphatidylcholine; egg PG, egg yolk phosphatidylglycerol; MALDI-TOF-MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; MIC, minimum inhibitory concentration; NPN, *N*-phenyl-1-naphthylamine; ONPG, *o*-nitrophenyl β-galactopyranoside; PBS, phosphate bufferd saline; RP-HPLC, reversed phase high-performance liquid chromatography; TFE, 2,2,2-trifluoroethanol; TSB, tryptic soy broth.

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