Structure-Activity Relationship of Model Peptides Based on Pleurocidin, an Antibacterial Peptide

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Pleurocidin (Ple) isolated from the skin of the winter flounder is a cationic amphiphilic peptide with antibacterial activity. To search for a simple peptide with strong antibacterial activity but no hemolytic activity, we designed three model peptides based on Ple, Ple I, Ple II, and Ple III. In the model peptides, Leu or Ala, and Lys were used instead of some hydrophobic amino acids and hydrophilic ones in Ple, respectively, to simplify the amino acid composition. The order of their α-helicity and hydrophobicity is Ple I > Ple II > Ple III. Ple I and Ple II showed antibacterial activity similar to Ple amide (Ple-NH_2). However, Ple I had very strong hemolytic activity. Ple III exhibited negligible biological activity. Ple II was found to be a desirable candidate to promote the study of the structure-activity relationship.

Most organisms have a common innate protective mechanism using antibacterial peptides against inversion of pathogens. Hundreds of antibacterial peptides have been isolated from various kinds of organisms, including human, animals, amphibians, fish, and microorganisms, and their action mechanisms and structure-activity relationships have been investigated.[1] Many antibacterial peptides have 15–40 amino acid residues including several Lys and/or Arg residues. These peptides are often in epithelial cell and phagocytes, and exhibit activities against Gram-positive bacteria, Gram-negative bacteria, fungi, and protozoa. Cationic amphiphilic peptides usually act on bacterial cell membranes, and suppress generation of tolerant bacteria because of their induction of rapid death of bacteria.[2] Therefore, these peptides are expected to be useful candidates for new drugs.

Pleurocidin (Ple) is a cationic antibacterial peptide isolated from the skin secretions of the winter flounder (*Pleuronectes americanus*). Ple consists of 25 amino acid residues, and becomes amphiphilic when it takes on an α-helical structure (Fig. 1).[3] We previously reported that Ple showed properties similar to those of magainin 2 (M2), a well-known antibacterial peptide,[4] and Ple might form ion channel-like pores in phospholipid membranes.[5] Ple had strong antibacterial activity against Gram-positive and Gram-negative bacteria, whereas it had weak hemolytic activity, indicating that Ple is one of the desirable antibacterial peptides. In this connection, replacement of two Gly residues by Ala at the middle region (positions 13 and 17) in Ple resulted in remarkable changes in its properties including biological activities. However, replacement of two Gly residues by Ala at the N-terminal region (positions 1 and 3) gave little effect on the activtity.[5] Furthermore, we examined the biological activities of other two analogs, [Ala^{13}]Ple-NH_2 and [Ala^{17}]Ple-NH_2, and found that the $Gly^{13} \rightarrow$ Ala substitution influenced the peptide-membrane interaction more than the $Gly^{17} \rightarrow$ Ala substitution.[6] To search for a peptide with high antibacterial activity but no hemolytic activity, we designed three simple model peptides based on Ple, Ple I, Ple II, and Ple III (Fig. 1). In Ple I and Ple II, some hydrophobic amino acids and hydrophilic ones in the N-terminal 17 residues in Ple were replaced with Leu or Ala, and Lys, respectively. In Ple III, all the hydrophobic amino acids except Gly^{13} and hydrophilic ones were substituted with Ala and Lys, respectively, to simplify amino acid composition. Then we examined their biological activities. Ple II was found to show biological activities superior to Ple I and Ple III.

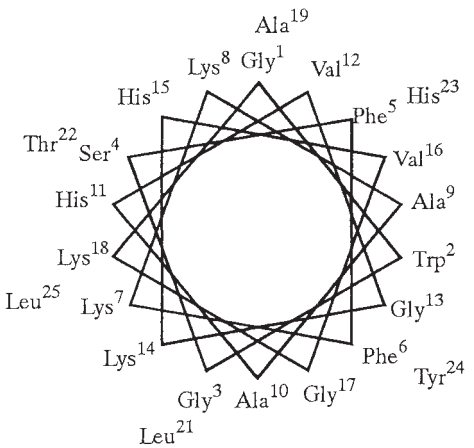

Fig. 1. α-Helical wheel of Ple (a) and amino acid sequences of the synthetic peptides (b). Common amino acid residues in Ple-NH_2 and M2 are underlined.

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Results and Discussion

Peptide Design and Synthesis. Our aim of this study is to find a simple peptide with high antibacterial activity but no hemolytic activity, because such a peptide would be valuable from the viewpoint of practical use.[7] α-Helicity and hydrophobicity ($\langle H \rangle$) of cationic α-helical peptides are known to be important factors for their biological activities. Indeed, the replacement of the $Gly^{13,17}$ residues by Ala in Ple resulted in the increase in both antibacterial and hemolytic activities.[5] This result suggested that increase in the α-helicity and/or hydrophobicity of Ple may strengthen undesirable hemolytic activity. In this study, we designed three model peptides, Ple Ⅰ, Ple Ⅱ, and Ple Ⅲ, in which Ala, Leu, and Lys were mainly used for clear estimation of important factors for antibacterial activity. M2 is a natural antibiotic peptide,[4] and shows similar biological activities to those of Ple.[5] Ple and M2 have six common amino acid residues at the same positions; these are underlined in Fig. 1. These residues are maintained in Ple Ⅰ, and Ple Ⅱ contains Ala instead of Phe[5] in Ple Ⅰ. Ple Ⅲ exclusively consists of Ala and Lys. The amino acid sequences of the synthesized peptides are shown in Fig. 1, and their $\langle H \rangle$ values are given in Table 1. Although natural Ple possesses a free C-terminus, the peptides were obtained as peptide amides because of easy preparation. All the peptides were synthesized by the solid phase method using Fmoc-amino acids and Rink Amide resin in the usual manner. After purification by gel chromatography followed by RP-HPLC,[8] the final products were identified by MALDI-TOF-MS (Table 1).

Circular Dichroism. 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) SUVs to examine the secondary structures of the peptides. The α-helical contents calculated according to the method of Scholtz et al.[9] are listed in Table 1. All the peptides were random in the buffer (pH 7.4). Amphiphilic short peptides are often found to be random in an aqueous environment.[5,10] In TFE, which is an α-helix-inducing solvent, the peptides showed α-helical patterns with moderate α-helical contents of 35–45%. This result indicates that the peptides may have the identical tendency to take an α-helical structure. On the other hand, different α-helical contents of the peptides were observed in the presence of egg PC. The α-helical contents of Ple Ⅰ and Ple Ⅱ were lower than the α-helical content of Ple-NH_2, whereas Ple Ⅲ was still random. Low or no α-helicity of the model peptides may be due to their insufficient interaction with neutral egg PC SUVs. The result is reasonable if one considers their low hydrophobicity. However, another tendency was found in the presence of egg PC/egg PG (3:1) SUVs. Ple Ⅰ and Ple Ⅱ had almost the same α-helicities of ca. 50% to Ple-NH_2. Charge interaction of the basic peptides with acidic SUVs may participate in α-helix formation, in addition to hydrophobic interaction between the peptides and the lipids.

Antibacterial and Hemolytic Activities. There are many cationic amphiphilic peptides that have antibacterial activity.[1,2,11] Antibacterial activity was examined by the serial solution dilution method using two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and two Gram-negative bacteria (*Eschelichia coli* and *Pseudomonas aureginosa*). The MICs of the peptides are listed in Table 2. Ple-NH_2, Ple Ⅰ, and Ple Ⅱ were active against both Gram-positive and Gram-negative bacteria. In this connection, we previously reported antibacterial activity of Ple.[5] The activity of Ple-NH_2 was about 4 times stronger than that of Ple. The basicity of Ple-NH_2 is stronger than that of Ple. This difference may increase the charge interaction between Ple-NH_2 and anionic bacterial cell membrane. The activities of Ple Ⅰ and

Table 1. Analytical Data of the Peptides

Peptide	MALDI-TOF-MS		$\langle H \rangle$[a]	α-Helical Content/%[b]			
	Found	Calcd ([M + H]$^+$)		Buffer[c]	TFE[d]	PC[e]	PC/PG (3:1)[e]
Ple-NH_2	2710.8	2711.2 ($C_{129}H_{193}N_{37}O_{28}$)	−0.026	R[f]	41	37	50
Ple Ⅰ	2626.4	2626.3 ($C_{124}H_{213}N_{35}O_{27}$)	−0.121	R[f]	38	26	51
Ple Ⅱ	2550.5	2551.2 ($C_{118}H_{209}N_{35}O_{27}$)	−0.135	R[f]	38	23	47
Ple Ⅲ	2452.7	2453.0 ($C_{111}H_{195}N_{35}O_{27}$)	−0.162	R[f]	35	R[f]	20

a) Hydrophobicity/residue. Hydrophobicity was calculated using the consensus value of hydrophobicity value for each amino acid residue.[18] b) [Peptide] = 20 μM; 25 °C. c) 20 mM Tris HCl buffer (pH 7.4). d) 98% TFE. e) [egg PC], [egg PC/egg PG (3:1)] = 1 mM. f) Random.

Table 2. Antibacterial and Hemolytic Activities of the Peptides

Peptide	MIC/μM[a]				Hemolysis/%[b]
	S. aureus IFO 12732	*B. subtilis* IFO 3134	*E. coli* IFO 12734	*S. aeruginosa* IFO 12582	
Ple-NH_2	2	1	1	4	9
Ple Ⅰ	2	2	1	2	47
Ple Ⅱ	4	2	2	2	6
Ple Ⅲ	64	64	>64	>64	1
Ple	16[c]	4[c]	4[c]	16[c]	6[c]

a) Method, serial solution dilution method; medium, TSB medium (pH 7.4); inoculum size, 10^4 cells mL^{-1}. b) Percentage of hemolysis at a peptide concentration of 100 μM. c) Data from Ref. 5.

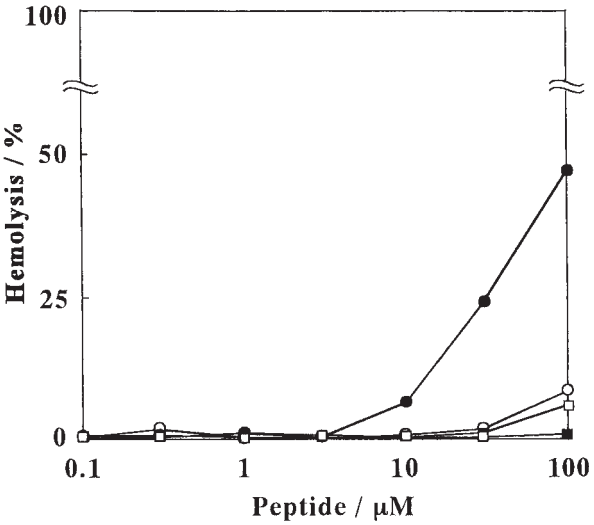

Fig. 2. Profiles of hemolysis as a function of the peptide concentration for rabbit blood cells. Ple-NH_2 (○), Ple I (●), Ple II (□), and Ple III (■).

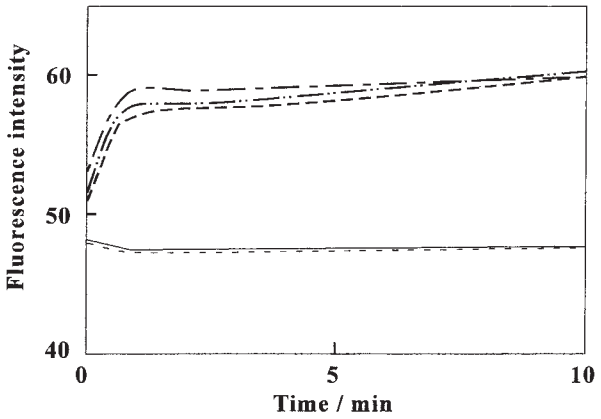

Fig. 3. Effect of the peptides on the permeabilization of outer membrane of *E. coli*. [Peptide] = 1 μM, [NPN] = 10 μM, $\lambda_{ex} = 350$ nm and $\lambda_{em} = 420$ nm. Ple-NH_2 (— ·· —), Ple I (— — —), Ple II (– – –), Ple III (——), and control (- - - - - -).

Ple II were slightly stronger than or comparable to those of Ple-NH_2. Ple III had very weak activity against Gram-positive bacteria and no activity against Gram-negative bacteria up to a peptide concentration of 64 μM. The order of antibacterial activities of the peptides is accord with that of α-helical contents in the presence of aionic SUVs, indicating that α-helicity may be closely related to antibacterial activity.[5] It is noteworthy that Ple I and Ple II had strong antibacterial activity, although their $\langle H \rangle$ values are considerably different from the $\langle H \rangle$ value of Ple-NH_2 (Table 1). The $\langle H \rangle$ of α-helical antibacterial peptides is important factors for their activity.[12] Natural α-helical antibacterial peptides have a variety of the $\langle H \rangle$ values: −0.026 for Ple, −0.036 for M2, and 0.198 for maculatin 1.1.[13] Therefore, it is likely that the $\langle H \rangle$ values of Ple I and Ple II may be incidentally suited to antibacterial activity. The very weak activity of Ple III seems to be due to too low $\langle H \rangle$ value of −0.162. The low α-helicity of Ple III in the presence of egg PC/egg PG (3:1) SUVs is compatible with its weak antibacterial activity.

From the viewpoint of practical use as drugs, many attempts have been made to find antibacterial peptides without hemolytic activity.[12,14] Hemolytic activity was assayed using fresh rabbit blood cells (Fig. 2 and Table 2). Hemolytic activity of Ple-NH_2 was 9% and was found to be slightly higher than that of Ple. Ple I unexpectedly showed strong hemolytic activity of 47% at 100 μM peptide concentration, whereas the hemolytic activity of Ple II was only 6%. The difference between Ple I and Ple II is that they have Phe and Ala, respectively, at position 5. This result suggests that the Phe residue has substantial influence on peptide–membrane interaction or there is a critical $\langle H \rangle$ value between Ple I and Ple II referring to hemolytic activity. Ple-NH_2 showed a slightly stronger activity than Ple II, and the activity of Ple III was negligible, as was presumed.

Outer and Inner Membrane Permeability. The outer membranes of Gram-negative bacteria contain lipopolysaccharide as a main component, which plays an important role in membrane translocation.[15] Bacteria are often resistant for a variety of antibiotics by the use of lipopolysaccharide as a defense wall. *N*-phenyl-1-naphthylamine (NPN), a hydrophobic fluorescent probe, can not penetrate into intact bacterial outer membrane, but it is taken up into the membrane interior when the membrane is disturbed.[16] Since the fluorescence intensity of NPN increases in hydrophobic environment, it is easily applicable to the permeability measurement. Outer membrane permeabilities of the peptides are shown in Fig. 3. All the peptides except for Ple III had permeabilities. The order of their permeabilities is Ple I > Ple-NH_2, Ple II ≫ Ple III, although the difference between permeabilities of the peptides except for Ple III was small. Ple III showed negligible outer membrane permeability. It is interesting that the fluorescence intensity increased rapidly and then very slowly, suggesting that the main change of the outer membrane structure occurred in the early stage.

The inner membrane permeability was evaluated by measuring β-galactosidase activity using *o*-nitrophenyl β-galactopyranoside (ONPG) as a substrate.[17] When the permeability of the inner membrane is increased by the peptide, ONPG moves into the cytoplasm, and is hydrolized by β-galactosidase to produce *o*-nitrophenol, which is colored under weakly alkaline conditions. The result is shown in Fig. 4. Absorbance at 420 nm gradually increased with the passage of time, and the order of permeability of the peptides was Ple I ≥ Ple-NH_2, Ple II ≫ Ple III. Permeability of Ple I was slightly greater than that of Ple-NH_2 and Ple II in the early time, but no difference between their permeabilities was observed after 40 min. The result in membrane permeabilities of the peptides is consistent with that in their antibacterial activities, and indicated that the peptides except for Ple III were able to permeabilize the outer and inner membranes of *E. coli* rapidly.

In conclusion, Ple I and Ple II exhibited antibacterial activity slightly higher than or compatible to Ple-NH_2. However, only Ple I had unexpected strong hemolytic activity. This observation suggests that the remarkable increase in hemolytic activity of Ple I may be due to subtle difference between $\langle H \rangle$ values of Ple I and Ple II or the presence of the aromatic side chain of Phe residues in Ple I. It is interesting that Ple II has antibacterial activity comparable with that of Ple-NH_2 and weaker he-

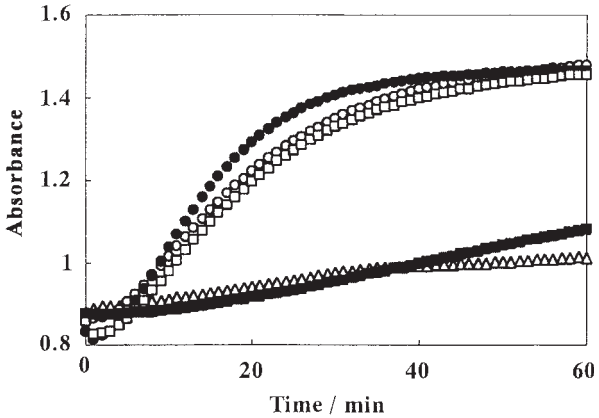

Fig. 4. Effect of the peptides on the permeabilization of inner membrane of *E. coli*. [Peptide] = 1 μM, [ONPG] = 50 μM. Ple-NH_2 (○), Ple Ⅰ (●), Ple Ⅱ (□), Ple Ⅲ (■), and control (△).

molytic activity than that of Ple-NH_2, in spite of its simple structure and inspite of having a different $\langle H \rangle$ value from that of Phe-NH_2. Ple Ⅱ is a candidate to promote the study on the structure-activity relationship. Work along this line using other model peptides is in progress.

Experimental

Peptide Synthesis. Peptides were synthesized by the solid-phase method using Fmoc-amino acids and 4-(2,4-dimethoxyphenylaminomethyl)phenoxy resin (Rink Amide resin). The products were purified by gel chromatography on a Sephadex G-10 column, followed by RP-HPLC on a YMC-Pack ODS column. The purity of the final products was evaluated by analytical RP-HPLC and MALDI-TOF-MS, and was found to exceed 95%.

Preparation of Phospholipid Vesicles. SUVs of egg PC and egg PC/PG (3:1) were prepared for the CD measurement. Phospholipid (5 μmol) was dissolved in $CHCl_3$/MeOH (2:1 v/v, 2 mL), then dried under 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 Titech Ultrasonic Processer VP-5T at an intensity of 10 W. The vesicles were allowed to stand for 30 min before the measurements were made. The lipid concentration was 1 mM.

Circular Dichroism Measurement. CD spectra were recorded on a JASCO J-720 W 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 containing 1 mM phospholipid vesicles. The concentration of the peptides was 20 μM. Measurements were done at 25 °C. The mean residue ellipticity is given in deg cm^2 $dmol^{-1}$, and the helical contents were calculated according to the method of Scholtz et al.[9]

Antibacterial and Hemolytic Assays. The serial solution dilution method was used to determine the MIC values as described by Yoshida et al.[5] The cell suspension was dissolved with tryptic soy broth (TSB) medium (pH 7.4) to 10^4 cells mL^{-1}. Several concentrations of the peptide solution were placed in the test tubes, made up to 20 μL with the medium, and cell suspension (180 μL) was added. After incubation for 24 h at 37 °C, absorbance at 620 nm was measured. Hemolytic activity was assayed according to the procedure of Yoshida et al.[5] Phosphate buffered saline (PBS) (pH 7.5, 3 mL) was added to fresh rabbit blood (1 mL). The resulting mixture was centrifuged at 2000 rpm for 3 min, and the precipitates were collected. After being washed with PBS three times, the obtained precipitates were suspended in PBS (2 mL). PBS (1 mL) was added to the rabbit erythrocyte solution (5 μL) followed by the peptides. The resulting suspension was incubated for 20 min at 37 °C, and then centrifuged. The supernatant was monitored at 413 nm using a Hitachi U-2000 spectrophotometer. To measure the absorbances 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 Permeability. Outer membrane permeability of the peptides was measured according to the procedure by Wu and Hancock.[16] 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 permeability was performed by measuring the fluorescence intensity of NPN using a Hitachi F-3010 fluorescence spectrophotometer. A hundred microliters of 10 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 permeability 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 Permeability. Determination of the inner membrane permeability was performed by measuring the β-galactosidase activity using ONPG as a substrate according to the procedure by Pellegrini et al.[17] A suspension of *E. coli* (10^4 cells mL^{-1}, 880 μL) was added to a solution of 2.5 mM ONPG in TSB medium (pH 7.4, 20 μL). After 15 min of incubation, 10 μM peptide solution (100 μL) was added. The inner membrane permeability was monitored by measuring the rate of *o*-nitrophenol production at 420 nm every 1 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 dichroism; MALDI-TOF-MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; MIC, minimum inhibitory concentration; NPN, *N*-phenyl-1-naphthylamine; PBS, phosphate buffered saline; PC, 3-phosphatidylcholine; PG, 3-phosphatidylglycerol; RP-HPLC, reversed phase high-performance liquid chromatography; SUV, small unilamellar vesicle; TFE, 2,2,2-trifluoroethanol; TSB, tryptic soy broth.

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