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Antibacterial Activity of Peptides Derived from the C-Terminal Region of a Hemolytic Lectin, CEL-III, from the Marine Invertebrate *Cucumaria echinata*

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Several synthetic peptides derived from the C-terminal domain sequence of a hemolytic lectin, CEL-III, were examined as to their action on bacteria and artificial lipid membranes. Peptide P332 (KGVIFAKASVSVKVTASLSK-NH_2), corresponding to the sequence from residue 332, exhibited strong antibacterial activity toward Gram-positive bacteria. Replacement of each Lys in P332 by Ala markedly decreased the activity. However, when all Lys were replaced by Arg, the antibacterial activity increased, indicating the importance of positively charged residues at these positions. Replacement of Val by Leu also led to higher antibacterial activity, especially toward Gram-negative bacteria. The antibacterial activity of these peptides was correlated with their membrane-permeabilizing activity toward the bacterial inner membrane and artificial lipid vesicles, indicating that the antibacterial action is due to perturbation of bacterial cell membranes, leading to enhancement of their permeability. These results also suggest that the hydrophobic region of CEL-III, from which P332 and its analogs were derived, may play some role in the interaction with target cell membranes to trigger hemolysis.

Key words: antibacterial peptide, hemolysin, lectin, lipid membrane, marine invertebrate.

Abbreviations: CD, circular dichroism; ONPG, *o*-nitrophenyl-β-D-galactoside; MIC, minimum inhibitory concentration; TBS, Tris-buffered saline; TSB, tryptic soy broth.

CEL-III is a Ca^{2+}-dependent, galactose/*N*-acetylgalactosamine-specific lectin (carbohydrate-binding protein) isolated from a sea cucumber, *Cucumaria echinata* (*1*). In addition to carbohydrate-binding activity, this lectin exhibits strong hemolytic activity (*2*) and cytotoxicity toward some cultured cell lines (*3*), by forming ion-permeable pores in target cell membranes after binding to carbohydrate chains on the cell surface (*4–6*). The activity of CEL-III varies depending on the cell type. For example, the hemolytic activity is strong for human and rabbit erythrocytes, but not for horse or chicken erythrocytes (*1*). The cytotoxicity also varies considerably for cultured cell lines; HeLa (LD_{50} = 82 ng/ml) and MDCK (LD_{50} = 53 ng/ml) cells are highly susceptible to CEL-III cytotoxicity, whereas CHO cells are very insensitive up to 10 μg/ml of CEL-III (*3*). These facts suggest that the action of CEL-III largely depends on the nature of the surface carbohydrates on the cells. We identified glycosphingolipids, such as lactosyl ceramide and globoside, as effective CEL-III receptors on human erythrocyte membranes (*7*). It is probable that complex carbohydrate chains containing galactose or *N*-acetylgalactosamine on cultured cells also determine the extent of the CEL-III cytotoxicity. After binding to the cell surface carbohydrate chains, CEL-III is assumed to undergo conformational changes, which lead to its oligomerization in the cell membrane. Similar protein toxins, which form ion-permeable pores in cell membranes, have been found in some bacteria. For example, α-hemolysin from *Staphylococcus aureus* (*8*, *9*) and aerolysin from *Aeromonas hydrophila* (*10*, *11*) are extensively studied bacterial pore-forming toxins. These proteins form membrane pores composed of their oligomers after activation on the surface of target cell membranes. The amino acid sequence of CEL-III was recently determined from the cDNA and partial amino acid sequences of the protein (*12*). Interestingly, the N-terminal two-thirds of CEL-III exhibits homology to the B-chains of ricin and abrin, plant lectins exhibiting high toxicity toward eukaryotic cells (*13*, *14*). Recently, the carbohydrate-binding activity of the N-terminal region of CEL-III was confirmed by examination of the N-terminal domains of CEL-III prepared by limited digestion as well as expression using *Escherichia coli* cells (*15*). On the other hand, the C-terminal one-third of CEL-III exhibits no homology with any other known proteins. This region contains a characteristic hydrophobic sequence with repetitive Val and Lys residues, and is therefore expected to be involved in the oligomerization of CEL-III in target cell membranes. In this study, we chemically synthesized peptides having partial sequences of this region, and

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examined their antibacterial activity. The results indicate that one of the peptides, P332, exhibits high antibacterial activity toward certain bacteria, suggesting a relationship with its potential role in the interaction with target cell membranes in the course of the hemolytic process. Also, examination of the effects of amino acid replacements in this peptide revealed the possibility for developing more effective antibacterial peptides.

MATERIALS AND METHODS

Synthesis of Peptides—Peptides were synthesized by the solid-phase method using Fmoc-amino acids and 4-(2′,4′-dimethoxyphenylaminomethyl)phenoxy resin (Rink Amide resin) (*16*). The protecting groups and the resin were removed with TFA in the presence of m-cresol (2%) and thioanisole (12%) at room temperature for 60 min. For the Arg-containing peptide (P332-R), the filtered solution was mixed with trimethylbromosilane at a final concentration of 2.0 M (*17*). After incubation at 0°C for 1 h, the crude peptides were precipitated with diethylether, and then purified by gel filtration on a Sephadex G-10 column and reverse-phase HPLC on a Wakosil 5C4 column. The amino acid sequences of the resulting peptides were confirmed with a protein sequencer, PPSQ-21 (Shimadzu).

Measurement of Antibacterial Activity—Antibacterial activity was measured by the serial solution dilution method as previously described (*18*), using two Gram-positive bacteria (*Staphylococcus aureus* IFO 12732 and *Bacillus subtilis* IFO 3134) and two Gram-negative bacteria (*Escherichia coli* ATCC 43827 and *Pseudomonas aeruginosa* ATCC 27853). Each cell suspension was diluted to 10^4 cells/ml with tryptic soy broth (TSB) medium (pH 7.4). Several concentrations of each peptide solution (10 μl) were placed in the wells of a 96-well microplate, and 90 μl aliquots of the cell suspension in TSB were added. After incubation for 6 h at 37°C, the growth of the bacteria was expressed as the turbidity, *i.e.* as measured as the absorbance at 620 nm, using a microplate reader. Comparison of the antibacterial activity among various analog peptides was performed using the ratios of the peptide concentrations giving 50% inhibition of bacterial growth, as measured as the absorbance at 620 nm.

Measurement of Inner Membrane Permeabilization by the Peptides—The inner membrane permeability of the bacterial cells was determined using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (*19*). Bacteria grown to the logarithmic phase were adjusted to A_{600} = 0.5 with TSB, and then mixed with two volumes of 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl. To this solution (0.8 ml), 0.1 ml of peptides (0.5 mM) and 0.1 ml of ONPG (25 mM) in PBS were added, followed by incubation at 37°C. The inner membrane permeability was monitored as the production of *p*-nitrophenol, as measured as the absorbance at 420 nm.

Preparation of Liposomes—Liposomes were prepared as small unilamellar vesicles. Egg phosphatidylcholine (5 μmol) was dissolved in $CHCl_3/CH_3OH$ [2:1 (v/v), 0.4 ml], and then dried under a stream of N_2 gas. The dried lipid was hydrated in TBS using a Branson bath-type sonicator. The suspension was sonicated for 10 min at 50°C using a Titech Ultrasonic Processor Model VP-5T at an intensity of 10 W. The liposomes were allowed to stand for 30 min at 25°C before measurements were made. The lipid concentration was 1 mM. Vesicles containing carboxyfluorescein were similarly prepared by hydrating the dried lipid in TBS containing 0.1 M carboxyfluorescein. The vesicles containing carboxyfluorescein were separated from the free dye by gel filtration using Sephadex G-75 (1 × 22 cm) in TBS.

Measurement of Carboxyfluorescein-Leakage from Liposomes—The liposome solution was diluted to 990 μl with TBS, and then placed in a quartz cuvette kept at 25°C. A peptide solution (10 μl) in the same buffer was then added to this solution and, after mixing, the fluorescence intensity at 518 nm, after excitation at 490 nm, was immediately recorded for an appropriate period using a Hitachi F-3010 Fluorescence Spectrophotometer. For 100% leakage of the carboxyfluorescein, Triton X-100 was finally added to a final concentration of 0.1% (v/v), and then the fluorescence intensity was measured.

Circular Dichroism (CD) Spectroscopy—Far-UV CD spectra of the peptides were recorded in TBS using a JASCO J-720 spectropolarimeter. The spectra were measured using a quartz cell with a 1-mm path length at 20°C. The peptide concentration was 0.1 mM.

RESULTS AND DISCUSSION

CEL-III is composed of three portions based on its amino acid sequence. The N-terminal two-thirds exhibit relatively low, but definite, homology with the B-chains of ricin and abrin, galactose-specific plant lectins. On the other hand, the C-terminal domain exhibits no homology with any other proteins. Since CEL-III exerts hemolytic activity by forming ion-permeable pores in target cell membranes after binding to the cell-surface carbohydrate chains, the C-terminal domain possibly plays an important role in the interaction with the target cell membranes. Since there are two hydrophobic segments in the C-terminal domain of CEL-III (Phe314–Val326 and Val334–Val345) (*12*), it seems likely that these regions may be primarily responsible for the initial interaction with the lipid membranes. Table 1 shows the sequences of the peptides used in this study. Among them, P303 (residues 303–322), P313 (residues 313–332) and P332 (residues 332–351) were derived from the hydrophobic region of the C-terminal domain of CEL-III.

Table 1. **Amino acid sequences of the peptides derived from the hydrophobic region in the C-terminal domain of CEL-III.** The C-terminal residues of the peptides were amidated. Substituted residues in P332-derived peptides are boldfaced.

Peptide	Amino acid sequence
P303	KVSQQISNTISFSSTVTAGV-NH_2
P313	SFSSTVTAGVAVEVSSTIEK-NH_2
P332	KGVIFAKASVSVKVTASLSK-NH_2
P332-1A	**A**GVIFAKASVSVKVTASLSK-NH_2
P332-7A	KGVIFA**A**ASVSVKVTASLSK-NH_2
P332-13A	KGVIFAKASVSV**A**VTASLSK-NH_2
P332-20A	KGVIFAKASVSVKVTASLS**A**-NH_2
P332-R	**R**GVIFA**R**ASVSV**R**VTASLS**R**-NH_2
P332-L	KG**L**IFAKAS**L**S**L**K**L**TASLSK-NH_2

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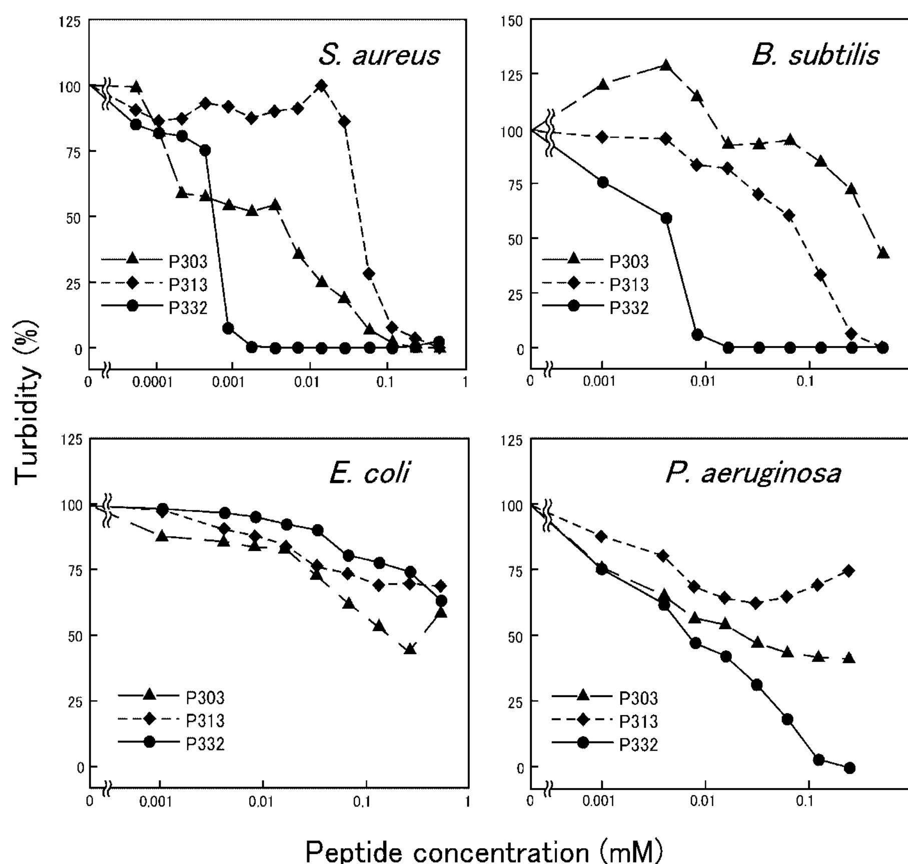

Fig. 1. **Antibacterial activities of P303, P313, and P332.** The antibacterial activities toward four bacteria were measured as the turbidity at 620 nm after incubation for 6 h with the indicated concentrations of the peptides.

During investigation of the interaction of these peptides with lipid membranes, we found that they, especially P332, show antibacterial activity. Figure 1 shows the effects of P303, P313 and P332 on the growth of four bacteria, Gram-positive *S. aureus* and *B. subtilis*, and Gram-negative *E. coli* and *P. aeruginosa*. The growth of the bacteria was expressed as the turbidity, as measured as the absorbance at 620 nm, after incubation for 6 h with the peptides at 37°C. As can be seen in this figure, P332 exhibited strong inhibition of the growth of Gram-positive bacteria, especially *S. aureus*; the minimal concentration of P332 required to inhibit bacterial growth (MIC) was about 1 μM for *S. aureus*, and 10 μM for *B. subtilis*. These values are comparable to those for naturally occurring antibacterial peptides, such as magainin (*20*, *21*) and pleurocidin (*18*). However, the growth inhibition of Gram-negative *E. coli* and *P. aeruginosa* by this peptide was very weak; the MIC for *P. aeruginosa* was about 100 μM, and growth inhibition of *E. coli* was not observed up to 250 μM. Many known antibacterial peptides have an amphiphilic nature, cationic and hydrophobic residues being located so as to form amphiphilic α-helices on binding to the target bacterial membranes (*22*, *23*). To determine the secondary structures of P303, P313, and P332, their far UV-CD spectra in TBS were measured. As shown in Fig. 2, P303 and P313 exhibited negative peaks around 200 nm, indicating they have no regular structure, while P332 showed a shallow negative peak around 210–220 nm, which suggests a β-sheet like structure (*24*). It is not known whether or not such a difference in the secondary structure of the peptides in aqueous solution is associated with their antibacterial activities, since a structural change would occur on binding to bacterial membranes, as is the case of many antibacterial peptides (*23*). Therefore, we also attempted to measure far UV-CD spectra of the peptides in the presence of liposomes composed of neutral (dipalmitoyl phosphatidyl choline) and anionic (dipalmitoyl phosphatidyl choline:dioleoyl phosphatidyl glycerol = 3:1) lipids. However, we were not successful because of the turbidity due to aggregation of the vesicles induced by the peptides; marked turbidity was especially observed with acidic liposomes. Although the actual conformation of the membrane-bound forms of

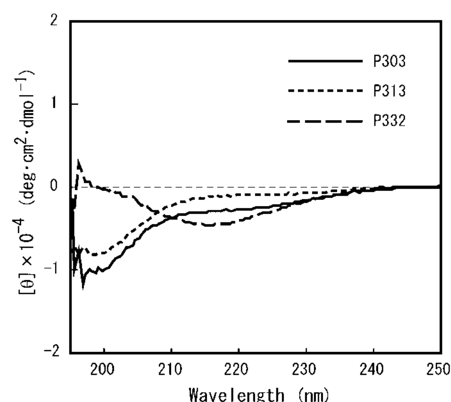

Fig. 2. **Far UV-CD spectra of P303, P313, and P332.** Measurement was performed with 0.1 mM peptides in TBS at 20°C. The values ([θ]) are expressed as the mean residue molar ellipticity.

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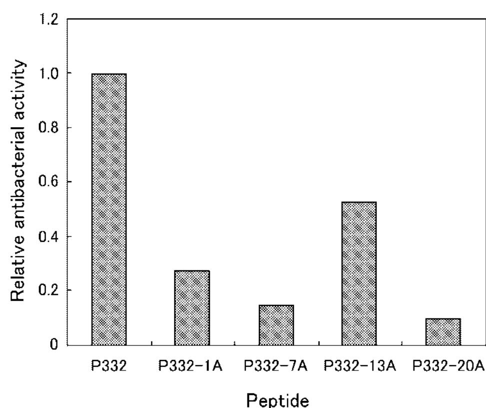

Fig. 3. **Comparison of the antibacterial activities of the P332-analog peptides, in which one Lys residue was replaced by an Ala residue.** The relative antibacterial activities toward *Staphylococcus aureus* were compared with that of P332 using the IC50 values for the peptides.

these peptides is not clear, these results suggest that the peptides could interact with bacterial cell membranes.

P332 has four Lys residues at intervals of 6 or 7 residues in its sequence, which is a conspicuous feature of this peptide. To assess the implication of these Lys residues in its antibacterial activity, we synthesized four analogs derived from P332 (P332-1A, P332-7A, P332-13A, and P332-20A), in which each Lys residue was replaced by an Ala residue (Table 1). As shown in Fig. 3, the antibacterial activities of these analogs toward *S. aureus* were reduced in comparison with that of P332. Although the largest decrease in activity was observed for P332-20A in this assay, there were no significant differences in activity among these analogs, except in the case of P332-13A. These results suggest that Lys residues are important for the antibacterial activity, but critical responsibility for the activity could not be attributed to a particular Lys residue. On the other hand, we examined another analog, P332-R, in which all four Lys residues had been replaced by Arg residues (Table 1). This peptide exhibited about two-fold higher antibacterial activity toward *S. aureus*, compared to the original P332 (Fig. 4). This result suggested the importance of positively charged residues at these positions, while Lys residues are not necessarily required. Interestingly, another peptide, P332-L, in which Val residues had been replaced by Leu exhibited markedly increased antibacterial activity toward all the bacteria used in this study; this peptide exhibited a definite increase in activity toward Gram-negative *E. coli* and *P. aeruginosa*, while P332 showed little activity toward these two bacteria. The reason for such enhancement of the activity is not obvious. Since enhancement of antibacterial activity is well correlated with permeabilization of the bacterial inner cell membrane by these peptides, as described below, substitution of Leu for Val may have enhanced the interaction of the peptide with the bacterial cell membrane. Our previous study (*25*) indicated that analogs of an amphiphilic antibacterial peptide, 4_3, whose Ala residues had been replaced by Val residues, showed a tendency to form a β-sheet structure, and had decreased antibacterial activities. Due to the bulky β-branched structure, Val residues may reduce the flexibility of the peptide. The enhancement of the antibacterial as well as the membrane permeabilizing activity might be due to an increase in the flexibility of P332-L on the change from Val to Leu, which facilitated the structural changes on binding to the cell membrane.

To examine the interaction of the peptides with bacterial cell membranes, permeabilization assays were performed. *S. aureus* cells were incubated with the peptides together with ONPG, a substrate for β-galactosidase in the cytosol of the bacterial cells, to monitor the permeabilization of the bacterial inner membrane. As shown in Fig. 5, P332 and its analogs, P332-R and P332-L, caused a considerable increase in the permeability of the inner membrane when incubated with *S. aureus* cells, whereas the other peptides, P303 and P313, had little effect. This

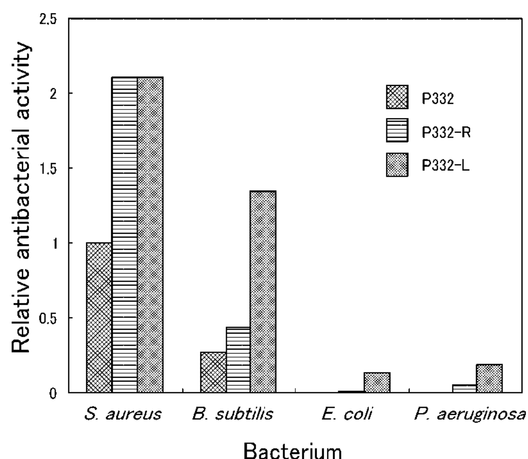

Fig. 4. **Antibacterial activities of P332, P332-R, and P332-L.** The relative antibacterial activities toward four bacteria were determined in comparison with that of P332 toward *Staphylococcus aureus* using the IC50.

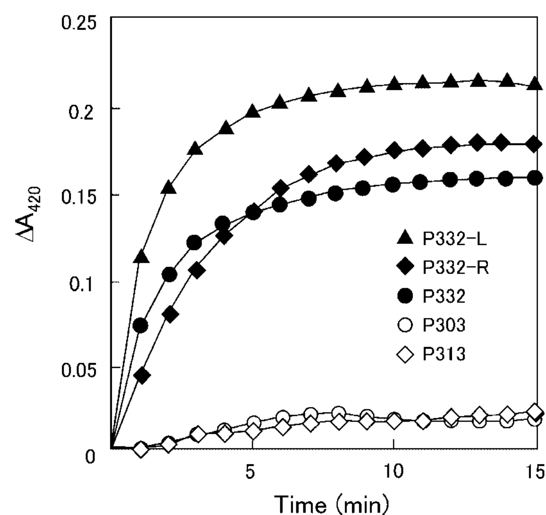

Fig. 5. **Permeabilization of the inner membrane of *S. aureus* induced by the peptides.** Bacterial cells were incubated with the peptides (0.05 mM) and ONPG (2.5 mM) at 37°C, and the production of *p*-nitrophenol due to the increasing permeability of the inner cell membrane was monitored as the absorbance at 420 nm.

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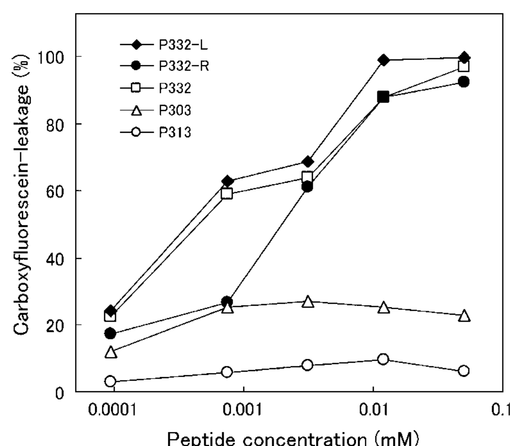

Fig. 6. **Carboxyfluorescein-leakage from liposomes induced by the peptides.** The indicated concentrations of the peptides were added to small unilamellar liposomes containing 0.1 M carboxyfluorescein, and the induced leakage of carboxyfluorescein was measured as the fluorescence intensity at 518 nm, after excitation at 490 nm. The fluorescence intensity after the addition of 0.1% Triton X-100 to the liposomes was taken as 100%.

indicates a correlation between the antibacterial activity and the membrane-perturbing ability of P332 and its analogs. To further examine the effects of the peptides on lipid membranes, carboxyfluorescein-leakage assays were performed. Figure 6 shows the effects of the peptides on egg phosphatidylcholine-liposomes containing carboxyfluorescein. When P332, P332-R, and P332-L were added to the liposome solution, remarkable increases in the fluorescence intensity at 518 nm were observed due to the leakage of carboxyfluorescein. This leakage was very rapid and reached a plateau within 1 min (data not shown). The leakage was nearly 100% with concentrations of P332 above 10 μM, compared to the value after treatment with 0.1% Triton X-100 taken as 100%. In contrast, P303 and P313 induced much less leakage. These results show the close correlation between the carboxyfluorescein-leakage and the inner membrane permeabilizing activities of these peptides, and also confirm that P332 and its analogs exert antibacterial activity through permeabilization of the cell membrane.

Although the intact CEL-III molecule has no antibacterial activity, the antibacterial activity of P332 based on its membrane-perturbing ability suggests that the region around residues 332–351 may be involved in the interaction with target cell membranes during the hemolytic action of CEL-III. The lack of antibacterial activity of intact CEL-III might be due to the difference in the carbohydrate chains as CEL-III receptors present on the surfaces of mammalian and bacterial cells. We recently found that C-terminal domain fragments of CEL-III (residues 281–432), produced on tryptic digestion, tend to oligomerize spontaneously, and also that its oligomerized form can agglutinate rabbit erythrocytes without carbohydrate-binding activity (*15*). These results indicate that the C-terminal domain has the ability to interact with the lipid membranes of erythrocytes. The data we obtained in this study suggest that the cationic and hydrophobic region corresponding to the P332 sequence may play an important role in such an interaction with the target cell membrane in the course of the hemolytic process.

The enhancement of the antibacterial activity on substitution of the amino acid residues in P332 also demonstrates the potential of P332-derived peptides as new antibacterial agents, which could be further developed to have higher antibacterial activities by modifying their sequences. For use of these peptides as antibacterial agents, it also appears advantageous that these peptides do not induce hemolysis, at least up to 0.5 mM (data not shown), since attempts to increase antibacterial activity by changing the sequences of peptides is often accompanied by an increase in their hemolytic action (*25*).

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