Structural Characterization and Cytolytic Activity of a Potent Antimicrobial Motif in Longicin, a Defensin-Like Peptide in the Tick *Haemaphysalis longicornis*

Md. Morshedur RAHMAN¹⁾, Naotoshi TSUJI²⁾, Damdinsuren BOLDBAATAR¹⁾, Banzragch BATTUR¹⁾, Min LIAO¹⁾, Rika UMEMIYA-SHIRAFUJI¹⁾, Myungjo YOU³⁾, Tetsuya TANAKA¹⁾ and Kozo FUJISAKI^{1)*}

¹⁾Department of Frontier Veterinary Medicine, Kagoshima University, Korimoto, Kagoshima 890-0065, ²⁾National Institute of Animal Health, National Agricultural Research Organization, Ibaraki 305-0856, Japan and ³⁾Department of Veterinary Parasitology, College of Veterinary Medicine, Chonbuk National University, Duckjin-dong, Jeonju 561-756, Korea

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ABSTRACT. Longicin, a defensin-like peptide, was recently identified in the hard tick *Haemaphysalis longicornis*. Longicin and one of its synthetic partial analogs (P4) displayed antimicrobial/fungicidal/parasitocidal activity. In the present study, we compared longicin-derived synthetic analogs in order to characterize the antimicrobial motif (P4) by analyzing some structural features using various bioinformatic tools and/or CD spectroscopy. According to the chemico-physical characteristics, P4 is suggested to be a cationic peptide with hydrophobic and amphipathic character. The predicted secondary structure indicated the existence of a β -sheet, which was also observed in the modeled tertiary structure. CD spectroscopic results also showed the existence of a β -sheet and transition to a helical conformation in the presence of membrane-mimicking conditions. These structural observations on P4 suggested that the antimicrobial activity could be due to the β -sheet as well as the α -helix. In addition, a sequence homology search showed that molecules identified in other ticks and organisms also have the P4 analogous domain at their C-terminal, which indicates P4 as a conserved domain. The peptide P4 also showed low cytolytic activity. Based on the present result and previously reported studies, the peptide P4 could be suggested as a novel antimicrobial domain indicating future therapeutic agent against bacteria.

KEY WORDS: antimicrobial peptides, longicin, P4, structure.

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Antimicrobial peptides represent an essential alternative first line of defense. Searching for more potent and effective antibiotics to combat pathogens having resistance to conventionally used antibiotics is of great interest [32]. Naturally occurring molecules involved with the innate immune system in disease-bearing vectors, such as mosquito and the tick, could be the target due to their well developed and diverse protective immune system. Disease-transmitting arthropod vectors have an extensive spectrum of innate immunity mechanisms comprising different types of useful and important molecules [2, 5, 6, 8, 11]. Therefore, these molecules could be the effective agents against pathogens. Small antimicrobial peptides, such as cecropin, defensin, maganin, and melittin, have been reported as the most potent naturally occurring antibiotics [20].

Ticks encounter various microbes through sucking blood from an infected host or a microbe-friendly environment, and they play as important vectors of a wide variety of disease-causing bacteria, viruses, protozoa, and other pathogenic microorganisms. Defensin-like proteins in several ticks have already been reported [3, 7, 13, 17, 19, 23, 27, 40]. We also recently explored the expression of a defensin-like peptide, longicin, from the midgut epithelium of the hard tick *Haemaphysalis longicornis* [33]. Longicin consists of 74 amino acids, of which 22 residues were detected

as a signal peptide. We also evaluated the antimicrobial activity of longicin and four of its synthetic analogs, namely, P1 (residues 23–37), P2 (33–45), P3 (42–57), and P4 (53–73). Longicin and one of its synthetic analog (P4) displayed antimicrobial/fungicidal/parasitocidal activity and they were, therefore, proposed to be therapeutic agents against tick-borne pathogens. Interestingly, three other synthetic analogs of longicin (P1, P2, and P3) were inactive against microorganisms [33]. Thus, the peptide P4 is indicated as a potent antimicrobial motif in longicin. These findings led us to analyze the structural differences among the synthetic analogs derived from longicin.

The structural characterization of antimicrobial peptides is very important to understand the peptide activity. So far, hundreds of antimicrobial peptides have been isolated from various organisms, and their great diversity in the primary structure results in the large difference in their antibacterial mechanisms [35]. In addition, harmful side effects, such as the lysis of red blood cells [1, 16, 29] or cytotoxicity towards mammalian host cells [9, 37, 38], which are commonly associated with antimicrobial peptides as potential therapeutic agents, should also be elucidated. In this study, we analyzed some structural features of longicin and longicin-derived synthetic peptides in relation with their antimicrobial potential using various bioinformatic tools and/or CD spectroscopy. We also determined the cytolytic activity of P4 peptide for evaluating the safety/toxicity of the peptide.

* CORRESPONDENCE TO: FUJISAKI, K., Department of Frontier Veterinary Medicine, Kagoshima University, Korimoto, Kagoshima 890-0065, Japan.
e-mail: tick@ms.kagoshima-u.ac.jp

Table 1. Predicted chemicophysical properties of longicin and its partial peptides

Peptides ^{a)}	Sequence ^{b)}	Predicted chemicophysical characteristics						
		Length	Molecular mass (Da) ^{c)}	Mean amphipathicity ^{d)}		GRAVY index ^{c)}	Aliphatic index ^{c)}	Net charge ^{c)}
Longicin	QDDSDVPHVRVRGFGCPLNQGACHNHCRSIGRRGGYAGIIKQTCTCYRK	52	5820.6	0.76	0.54	-0.783	50.58	9.15 + 5
P-1	QDDSDVPHVRVRRG	15	1764.8	0.39	0.53	-1.627	58.00	5.43 - 1
P-2	RVRGFGCPLNQG	13	1459.6	1.16	1.55	-0.769	55.78	11.7 + 3
P-3	LNQGACHNHCRSIGRR	16	1822.0	0.93	0.28	-1.056	55.00	10.41 + 3
P-4	SIGRRGGYAGIIKQTCTCYR	21	2306.7	1.11	0.25	-0.214	60.48	9.50 + 4

a) Longicin and one of its analog, P4, were reported to be active against bacteria, parasites, and fungi, as described by Tsuji *et al.* [33].

b) The longicin sequence represents only the putative mature protein; sequences were taken from Tsuji *et al.* [33]; the cysteine residues are underlined.

c) Predicted by ProtParam, a primary structure analysis tool located on the ExPASy server.

d) Calculated using the software WHAT [39] assuming that peptides adopt either an α -helical (100° angle) or a β -sheet (180° angle) conformation.

MATERIALS AND METHODS

Synthetic peptides: The synthetic peptides used in this study were prepared by Sigma Aldrich, Japan with a purity of >95%. The sequences and chemicophysical characteristics of them are listed in Table 1.

Structure prediction: Various bioinformatic tools and software were used to predict the peptides' structure. The tool ProtParam (<http://br.expasy.org/tools/protparam.html>), located on the ExPASy Server, was used to analyze the peptides' chemicophysical characteristics. The mean amphipathicity was calculated using the program WHAT [39]. The software Arguslab 4.0.1 was used to generate a broken-heaven pdb file, which was then loaded on the software CCP4MG [25] to compute the electrostatic potential. The Hierarchical Neural Network (HNN) (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_nn.html), also located on the ExPASy Server, was used to predict the secondary structure of peptides. The disulphide bond was predicted by Dipro (<http://scratch.proteomics.ics.uci.edu/>). The modeled tertiary structure was generated through the Swiss-Model Protein Modeling Server (<http://swiss-model.expasy.org/SWISS-MODEL.html>). A helical wheel was projected using the software Antheprot 2000 V 6.0. A sequence similarity search against protein databases was performed using the FASTA and SSEARCH programs (<http://www.ebi.ac.uk/Tools/fasta33/index.html>).

Circular dichroism (CD) spectroscopy: To determine the secondary structure of the peptides P1 (as negative control) and P4, CD spectroscopy was performed with a Jasco J-820 spectropolarimeter (JASCO, Tokyo, Japan) using a 1-cm path length cell over the 190–250 nm range. The spectra were acquired at 25°C every 0.5 nm with a 1 s averaging time per point and a 1 nm band pass. Four scans with a scan speed of 20 nm/min were also averaged for each peptide. Peptides were dissolved in Mili-Q water containing 50% trifluoroethanol (TFE) or 25 mM sodium dodecyl sulfate (SDS) or in Mili-Q water only at a concentration of 0.2 mg/ml. CD spectral results were expressed as the mean residue ellipticity. The contents of four types of the secondary

structural elements, namely, α -helix, β -sheet, β -turns, and random coils, were determined.

Hemolysis assay: The peptide P4 was challenged against the erythrocytes of different species, and the hemolytic activity was determined as described previously [31] with some modifications. Briefly, fresh anticoagulated blood was collected either from cattle, dogs, or rabbits and washed twice with sterilized physiological saline (0.9% NaCl) by centrifugation at $1,000 \times g$ for 10 min. The resulting erythrocytes were then resuspended in saline to 4% (v/v). The peptide P4 was dissolved in saline, and 100 μ l of a twofold serially diluted peptide solution and 100 μ l of an erythrocyte suspension were dispensed into 96-well plates in triplicate and incubated at 37°C for 1 hr. Following centrifugation at $1,000 \times g$ for 5 min, the supernatants were transferred to new 96-well plates and monitored by measuring the absorbance (*A*) at 540 nm for released hemoglobin. Controls for 0 and 100% hemolysis consisted of cells suspended in saline and in 0.1% Triton X-100, respectively. Percentage hemolysis (%) was calculated as follows:

$$\text{Percentage hemolysis (\%)} = \frac{(A_{540 \text{ nm, peptide}} - A_{540 \text{ nm; 0.9\%NaCl}}) / (A_{540 \text{ nm; 0.1\%TritonX-100}} - A_{540 \text{ nm; 0.9\%NaCl}})}{1} \times 100.$$

Cytotoxicity assay: The CellTiter 96® Non-Radioactive Cell Proliferation Assay System (Promega) was applied to examine the toxicity of the P4 peptide against Vero cells. After being grown in an Eagle's medium, Vero cells were harvested and resuspended in a fresh medium at a final concentration of 1×10^5 cells/ml. Fifty microliters of the cell suspension was dispensed into each well of a 96-well microtiter plate containing an equal volume of either the two-fold serially diluted P4 peptide or only the medium. After an additional 72 hr of incubation, color was developed by adding respective dye solution followed by 4 hr of additional incubation. The reaction was then stopped by adding a solubilization solution, and absorbance was recorded at 550 nm in a microplate reader (BIO-RAD). Cytotoxicity was expressed as the percentage inhibition of cell growth (%) and was calculated as follows:

$$\text{Percentage cell growth inhibition (\%)} = 1 - B/A, \text{ where } A \text{ and } B \text{ represent the absorbance value in the presence or}$$

absence of the peptide.

RESULTS

Chemicophysical characteristics: Antimicrobial peptides are typically relatively short (12 to 100 amino acids), positively charged (net charge of +2 to +9), amphiphilic, and hydrophobic, as reviewed by many researchers [15, 18, 26, 36]. Table 1 shows the predicted chemicophysical properties of the longicin sequence (without signal peptide) and its four synthetic analogs. The size of the peptides ranged from 13 to 52 amino acids. All the synthetic analogs (except P1) and longicin were positively charged, ranging from +3 to +5, and had a pI value higher than 9. It has been reported that a peptide net charge from +3 to +5 results in increasing antibacterial activity, whereas a net charge from +6 to +7 leads to increased hemolytic propensity and loss of antimicrobial activity [4].

Hydrophobicity is an essential feature for antimicrobial peptide membrane interaction. However, increase of the hydrophobicity levels is strongly correlated with mammalian cell toxicity and loss of antimicrobial specificity. The GRAVY index, which indicates peptide hydrophobicity, was higher (but at moderate level) in P4 than those of longicin and other partial peptides. The aliphatic indices were found to be almost the same in all peptides, suggesting no differences in heat stability.

Pathak *et al.* [24] suggested that amphipathicity is more important than hydrophobicity and α -helical content in governing antimicrobial peptide activity. When the average amphipathicity of each peptide was estimated using the software WHAT, the peptide was assumed to adopt either an α -helical or a β -sheet conformation, as shown in Table 1.

Regarding the α -helix, P2 and P4 were highly amphipathic, whereas P3 and longicin were moderately amphipathic, and P1 was less so (Table 1). Similar results were found when the peptides were analyzed by the software Antheprot 2000 V 6.0; they are presented as a helical wheel in Fig. 1. On the other hand, regarding the β -sheet, P2 was also found to be highly amphipathic, although P3 and P4 were slightly amphipathic, and P1 and longicin were moderately so (Table 1). It is also noteworthy that longicin had four negatively charged acidic residues, and all of them were located at the N-terminal representing the P1 peptide (Table 1). The only active partial peptide (P4) was located at the C-terminal of longicin.

Secondary structure prediction: It is generally agreed that the structure of a protein is more highly conserved than its sequence. Therefore, we attempted to predict the secondary structure using the Hierarchical Neural Network (HNN), as shown in Fig. 2. The figure shows that longicin is characterized by a β -strand and a short α -helix. The P4 peptide represents the well-developed β -strand part, and P3 represents the helix part. The P1 is composed of a short β -strand with a random coil, whereas P2 is fully composed of a random coil. As shown in Table 1, longicin is rich in cysteine residues (6 cysteines), three of which were distributed in P4, two in P3, and one in P2. It is believed that disulphide bonds are involved in stabilizing the peptide structure and may play a role in the antimicrobial activity [36]. With the bioinformatics tool Dipro, the disulphide bonds were predicted in the peptides. Dipro classified only longicin having 3 disulphide bonds between cysteine positions, 18–39, 25–47 and 29–49; however, the probability of forming a single disulphide bond in P3 (between Cys5–10) and P4 (between Cys9–17) was also suggested.

Fig. 1. Helical wheel projection of longicin and its four synthetic analogs. The red circles are hydrophilic amino acids, and the blue circles, hydrophobic ones. The green circles indicate cysteine residues.

Fig. 2. The secondary structures of longicin (without signal peptide) and its four synthetic analogues were predicted using a Hierarchical Neural Network (HNN).

Fig. 3 Modeled tertiary structure of longicin (A) and its two analogs, P3 (B) and P4 (C), based on the template 1fjn.pdb (an arthropod defensin). The position of the cysteine residues is indicated in blue.

Modeled tertiary structure: The longicin sequence (without the signal peptide) was submitted to the Swiss-Model Protein Modeling Server (<http://swissmodel.expasy.org/SWISS-MODEL.html>) for automated modeling via the “first approach mode”. The server generated a three-dimensional model of longicin based on the template from the Mediterranean muscle defensin (1fjnA.pdb) [34]. The modeled longicin was composed of residues ranging from 15 to 51 and had a 63.2% sequence identity with the template molecule. The partial peptides P3 and P4 were also separately modeled based on the same template using a Swiss-Model alignment mode. The modeled molecules were further processed by RasMol and are shown in Fig. 3. However, P1 and P2 could not be modeled.

Based on the structure, antimicrobial peptides are classified into four major classes: β -sheet, α -helical, loop and extended [15, 26]. The predicted secondary structure (Fig. 2) and modeled tertiary structure (Fig. 3) suggested longicin as a mixed-structure defensin class peptide (Fig. 3A), whereas P3 is suggested to be a helical peptide (Fig. 3B) and P4, a β -sheet peptide (Fig. 3C). These predicted structures indicated that the antimicrobial activity of P4 could be due to the well-developed β -sheet. The modeled structure also showed that longicin has 3 disulphide bonds (blue line) cor-

responding to the predicted results by DIpro, whereas P3 and P4 have no disulphide bond.

Electrostatic potential: According to the molecular electroporation theory, the electrostatic potential on the molecular surface plays an important role for the electrostatic bonding between the antimicrobial peptides and the negatively charged bacterial cell membrane [10]. With the software Arguslab 4.01, we generated a brokenheaven pdb file that was then loaded onto software CCP4MG to compute the electrostatic potential, as shown in Fig. 4. Red color is used to indicate 0.5 below the normal range, whereas blue color indicates 0.5 above the normal range. The electrostatic potential of the P4 peptide was obviously superior to that of P1 and also slightly higher than that of longicin because of the acidic amino acid residues located on the P1 peptide, which produced an electrostatic potential below the normal range, as indicated by red color. The peptides P2 and P3 also showed an electrostatic potential almost identical to that of P4. Although longicin had a greater net positive charge (+5) than P4 (+4), the presence of a negatively charged P1 (−1) domain was probably due to the lower electrostatic potential as well as slightly lower activity than that of P4, as reported by Tsuji *et al.* [33].

CD Spectroscopy analysis: Many peptides are reported to

Fig. 4. Electrostatic potential of longicin and its four synthetic analogs. Red and blue indicate 0.5 below and 0.5 above the normal range of the electrostatic potential, respectively.

Fig. 5. CD Spectra of P1 (A) and P4 (B) in the presence or absence of membrane-mimic conditions. The content of secondary structural elements is shown in tabular form (C).

exist in relatively unstructured or extended conformations prior to interaction with bacterial membranes [15]. Therefore, the secondary structure of P1 and P4 peptides in aqueous solution or in the presence of membrane-mimicking conditions were observed by performing CD spectroscopy. The membrane-mimicking condition was provided using structure-promoting agents, such as TFE or SDS. Peptides were dissolved in Mili-Q water containing 50% TFE or 25 mM SDS or in Mili-Q water only at a concentration of 0.2

mg/ml. As shown in Fig. 5, both peptides had almost an identical β -sheet content with a very low helical content and a high random coil content in the aqueous solution. However, no significant changes in the secondary structure of the peptide P1 were observed in the presence of structure-promoting agents. Conversely, P4 was largely unstructured in the aqueous solution, and no α -helix content was observed (Figs. 5B and 5C); however, the peptide underwent a significant transition to a typical α -helical conformation follow-

Table 2. Searching for sequence similarity of the peptide P4 against ticks and other organisms

Organism Source	Sequence	Identity (%)	Similarity (%)
Synthetic peptide (P-4)	SIGRRGGYCAGIIKQTCTCYR 21		
Ticks			
<i>Haemaphysalis longicornis</i>	QDDESDVPHVRVRGFGCPFNQGACHNHCRSIGRRGGYCAGIIKQTCTCYRK 74	100	100
<i>Ixodes scapularis</i> (SG)	QEEENQVAHVVRVRGFGCPFDQGACHRHCSIGRRGGYCAGFIKQTCTCYHN 74	95	100
<i>I. scapularis</i>	QEEENQVAHVVRVRGFGCPFDQGACHRHCSIGRRGGYCAGFIKQTCTCYHN 74	95	100
<i>Rhipicephalus (Boophilus) microplus</i>	VPAESEMAHLRVRGFGCPFNQGACHRHCSIRRRGGYCAGLIKQTCTCYRN 74	90.48	95.24
<i>Dermacentor variabilis</i>	APAEEVAHLRVRGFGCPFNQGACHNHCRSIRRRGGYCAGIIKQTCTCYRN 74	90.48	95.24
<i>D. andersoni</i>	APAEEVAHLRVRGFGCPFNQGACHNHCRSIRRRGGYCAGIIKQTCTCYRN 74	90.48	95.24
<i>Argas monolakensis</i>	DDGDDDAALTRVRGFGCPFNQGACHRHCSIGRRGGYCAGLIKQTCTCYRH74	80.95	100
Scorpion			
Sahara scorpion	GFGCPFNQGACHRHCSIRRRGGYCAGLIKQTCTCYR 37	85.71	95.24
Yellow scorpion	GFGCPFNQGACHRHCSIRRRGGYCAGFIKQTCTCYRN 38	85.71	95.24
Mussel			
Eastern oyster	GFGCPWNRQCHSHCRSIGRLGGYCAGSLRLTCTCYRS 38	76.19	85.71

ing the addition of SDS (39.7%), and a moderate transition was observed by the addition of TFE (26.6%). With the increase of the helical content, a concomitant reduction of the random coiled structure was observed in the presence of TFE or SDS. However, a drastic reduction of the random coil was found in the presence of SDS, whereas the presence of TFE showed a moderate influence (Fig. 5C). The β -sheet structure was constant in the presence of TFE but increased slightly with the addition of SDS, as shown in Fig. 5C. In the presence of SDS, P4 also showed a low β -turn content (Fig. 5C). The β -sheet peptide tachyplesin I was also reported to have a type I turn that connects an antiparallel β -sheet [26].

Sequence homology of P4: We searched the sequence similarity of the P4 peptide from protein databases using the program FASTA and SSEARCH. The alignment result presented in Table 2 showed that the molecules identified in other ticks and organisms have the P4 analogous domain at the C-terminal.

Cytolytic activity of the P4 peptide: The peptide P4 was challenged against red blood cells of different species for hemolytic activity and also against Vero cells for cytotoxicity in order to evaluate the safety/toxicity of the peptide. The result presented in Fig. 6A showed that the P4 peptide had a very low hemolytic activity against erythrocytes of all three species even at high concentrations. The highest hemolysis percentage (less than 15%) was estimated against bovine erythrocytes at a concentration of 500 μ M. On the other hand, although the maximum inhibition rate of Vero cell growth was estimated to be less than 50% (Fig. 6B), significant growth inhibition started to occur at a concentration of 16 μ M.

DISCUSSION

Structural comparison of longicin and its synthetic analogs: Structural parameters, such as the conformation, charge, hydrophobicity, hydrophobic moment, amphipathicity, and polar angle of peptides, are considered to be rele-

Fig. 6. Hemolytic activity of P4 against erythrocytes of cattle, dog, or rabbit (A) and cytotoxicity of P4 against Vero cells (B). Values are representative of two separate experiments in triplicate. The standard error mean was less than 10%.

vant to antimicrobial activity [36]. Therefore, some of these molecular determinants of longicin and its synthetic analogs was predicted using various bioinformatic tools and were compared to find out the characteristics which are responsible for P4 as an active antimicrobial domain in the present examination. The positive charge on the peptide is believed to be important for the initial electrostatic attraction of antimicrobial peptides to negatively charged phospholipid membranes of bacteria and other microorganisms [15]. Although the predicted secondary structure of P1 (Fig. 2) indicated a short β -sheet region with a random coil, according to the chemico-physical characteristics, P1 was found to be negatively charged, less hydrophobic, and less amphipathic (Table 1). A low electrostatic potential (Fig. 4) and a poor relationship between hydrophobicity and amphipathicity, as projected in the helical wheel diagram (Fig. 1), were also observed. These features could be responsible for the inactivity of P1. As shown in Table 1, P2 was positively charged with a high pI value and an electrostatic potential almost identical to that of P4 (Fig. 4). In addition, P2 had a GRAVY index almost identical to that of longicin as well as high amphipathicity either as an α -helix or a β -sheet (Table 1). The peptide also had a good relationship between amphipathicity and hydrophobicity, as projected in the helical wheel (Fig. 1). Despite these structural features, the reasons for the inactivity of P2 could be attributed to the lack of a secondary structure. As shown in Fig. 2, the P2 contained a random coil and only one cysteine residue. On the other hand, the predicted secondary structure (Fig. 2) and modeled three-dimensional structure (Fig. 3) indicated that the peptide P3 had a short helical region and the possibility of forming a disulphide bond due to the presence of two cysteine residues. The α -helix is an important conformation of antimicrobial peptides. Magainins and cecropins are examples of an α -helical peptide with an amphiphilic region [21]. However, although P3 had a positive charge with a high pI value, good amphipathicity as an α -helix and a good electrostatic potential, the peptide was less hydrophobic (Table 1), as was also projected in the respective helical wheel diagram (Fig. 1).

Structural analysis of the P4 peptide: From the findings described above, it appears that the peptide P4 has the following characteristics: optimum charge, moderate hydrophobicity, amphipathicity, good electrostatic potential, the possibility of forming a disulphide bond, and a well-developed beta sheet. Therefore, we further analyzed the peptide P4 by CD spectroscopy. In the aqueous environment, CD spectroscopy results corresponded to the predicted secondary structure, as shown in Fig. 2. However, the helical transition of P4 under membrane-mimetic conditions suggested that both the β -sheet and α -helix could be involved with the antimicrobial mechanism of P4. Therefore, it could be assumed that like the larger β -sheet family peptides, P4 also contains minor helical segments. Until now, many molecules have been identified in different ticks. In our previous study [33], P4 is indicated as an active motif of longicin. The sequence homology search displayed in Table 2, sug-

gesting P4 as a conserved antimicrobial domain.

The hemolytic activity and cytotoxicity of antimicrobial peptides are very important factors for evaluating their safety/toxicity. Various parameters, such as the hydrophobicity [14, 22, 28], cationicity [4, 30], number of disulphide bridges, and length of the peptides [12], are related with the cytolytic activity of antimicrobial peptides. Our previous study showed that P4 can kill bacteria at a concentration ranging from 2–10 μ M [33]. Therefore, the P4 peptide could be developed as a future therapeutic agent against bacteria.

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