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Development of novel antimicrobial peptides derived from anti-lipopolysaccharide factor of the swimming crab, *Portunus trituberculatus*

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ABSTRACT

Anti-lipopolysaccharide factors (ALFs) are a representative host defense protein in crustaceans. In this study, we successfully developed two novel antimicrobial peptides (AMPs), named crab-ALF2A and crab-ALF6A, which contain changes to the amino acid sequences of the lipopolysaccharide binding domain and signal peptide, respectively, of the ALF of the swimming crab *Portunus trituberculatus*. The crab-ALF2A peptide showed potent antimicrobial activity against the Gram-positive bacteria *Bacillus cereus*, *Staphylococcus aureus*, and *Streptococcus iniae* (minimal effective concentration [MEC] 1.51–1.93 μg/mL) and the Gram-negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli* (MEC 1.87–1.98 μg/mL), with maximal bactericidal activity at a peptide concentration of 5 μg/mL. The crab-ALF6A peptide also showed potent antimicrobial activity against *B. cereus*, *S. aureus*, and *S. iniae* (MEC 1.49–2.3 μg/mL) and *P. aeruginosa* and *E. coli* (MEC 1.72–1.19 μg/mL) at a peptide concentration of 5 μg/mL. Notably, the crab-ALF2A and crab-ALF6A peptides exhibited strong activity against *Candida albicans* (MECs of 2.11 and 1.95 μg/mL, respectively). These activities were stable following heat treatment. Moreover, the effect of crab-ALF2A and crab-ALF6A peptide treatment on microbe cell morphology was confirmed by scanning electron microscopy. Membrane disruption and damage, and the leakage of cytoplasmic content were clearly observed. A downsizing peptide approach illustrated that the hexapeptide ALF6A8 (RVLLRL) was the shortest peptide showing significant antimicrobial activity. Our approach allows for the generation of novel antimicrobial peptides in a cost effective manner as potential next-generation antibiotics.

1. Introduction

The inappropriate use of antibiotics in human and animal health care for the treatment and prevention of infections has caused a rapid increase in antibiotic-resistant pathogenic bacteria in recent decades. Therefore, there is an immediate need for the development of novel antimicrobial drugs with different mechanisms of action on target microorganisms than that of existing antibiotics [1]. Antimicrobial peptides (AMPs), which are ubiquitous in nature, play an important role as a first line of defense. All multicellular organisms, microorganisms, plants, and animals have an innate immune defense system that secretes AMPs. Endogenous AMPs are produced when infections occur and can also be stored in exposed tissues of animals and plants, constituting a rapid and efficient first line of defense. These peptides have broad-spectrum antimicrobial activity and are able to mount repair and adaptive immune responses in a concerted manner against multidrug-resistant bacteria [2].

A significant amount of research is currently focused on the development of novel AMPs that exhibit enhanced antimicrobial activity but decreased toxicity against eukaryotic cells. Various strategies in the design of novel synthetic analogs of AMPs have been applied, including modification of the typical features of AMPs such as chain length, net charge, hydrophobicity, and structure [3–6]. Among the various strategies used in the design of novel AMP analogs, the sequence-based approach, which correlates antimicrobial activity to the presence of specific amino acids or fragments at a specific position, is a direct and efficient method [7]. In our previous studies, we successfully developed novel and effective AMPs based on the amino acid sequence of several AMPs or proteins such as lipopolysaccharide (LPS) binding protein/bactericidal permeability-increasing protein of flounder [8] and LPS and β-1,3-glucan binding protein of abalone [9] by modification or substitution of particular amino acid residues. This approach is simple

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in design and does not usually require high-level computational techniques.

Anti-lipopolysaccharide factors (ALFs) belong to an important AMP family and are distributed exclusively in marine chelicerates and crustaceans. The first ALF was identified in the horseshoe crab (*Limulus polyphemus*) and numerous ALF homologs have subsequently been reported in many other crustacean species, including shrimp [10–13], lobster [14], crayfish [15,16], freshwater prawns [17,18], and crabs [19–27].

ALFs typically share a conserved three-dimensional structure, consisting of three α-helices packed against a four-stranded β-sheet, and contain a conserved LPS-binding domain (LBD), which is formed by a disulfide bond between two conserved cysteine residues where a cluster of hydrophilic and hydrophobic residues are present [28]. Typical ALF LBDs often display an alternating series of positively charged hydrophilic and hydrophobic residues with an amphipathic molecular structure [29]. Many synthetic peptides corresponding to these LBDs have been shown to possess antibacterial or antiviral activity and are used to mimic the functions of whole molecules, although some LBD peptides do not exhibit significant antibacterial activity [30]. Apart from amino acids in the LBD, some amino acids outside the LBD have also been predicted to participate in antibacterial activities [29]. Some synthetic cationic LBD peptides exhibit antimicrobial activity by disrupting the bacterial cell wall [31].

A signal peptide is a short (5–30 amino acids) peptide present at the N-terminus of the majority of newly synthesized proteins/peptides destined for the secretory pathway. These proteins/peptides include those that reside inside organelles, such as the endoplasmic reticulum, Golgi, or endosome, are secreted from the cell, or inserted into cellular membranes. Interestingly, there are several common features in amino acid composition or structure between AMPs and signal peptides. The common structure of signal peptides from various proteins/peptides is often described as a positively charged N-region, followed by a hydrophobic H-region and a neutral but acidic polar C-region. To be more detailed, almost all signal peptides begin with a short positively charged stretch of amino acids (N-region), which may help to enforce proper binding or topology of the polypeptide during translocation. The core of the signal peptide contains a long stretch of hydrophobic amino acids (5–16 residues) that has a tendency to form a single α-helix (H-region) for insertion into the membrane [32]. These features suggest that signal peptides can be used as a template for the design of novel membrane-targeted AMPs.

The aim of this study was to develop novel AMPs derived from a known protein sequence through structural or physiochemical modification. These data will provide useful information for the development of antimicrobial drugs with high activity.

2. Materials and methods

2.1. Structural and physiochemical prediction of the peptides

The secondary structure of the peptides was predicted using the Garnier-Osguthorpe-Robson (GOR) method (ExPASy). The molecular weight, theoretical isoelectric point (pI), net charge, and instability and aliphatic indices were calculated using the ProtParam tool (ExPASy) [33]. Helical wheel diagrams were produced using the EMBOSS Pepwheel program (European Bioinformatics Institute, Cambridge, UK) [33].

2.2. Peptide synthesis

The designed peptides were synthesized by Anygen Co., Ltd. (Gwangju, Korea) using solid-phase peptide synthesis methods. The peptides were dissolved in acidified distilled water (0.01% acetic acid) and stored at −20 °C until use.

2.3. Antimicrobial assays

The antimicrobial activity of each peptide was tested by ultrasensitive radial diffusion assay (URDA) as described previously [34]. The antimicrobial activities of the synthetic peptides were tested against the Gram-positive bacteria, *Bacillus cereus* ATCC21772, *Staphylococcus aureus* ATCC6538, *Streptococcus iniae* FP5229, and *Streptococcus mutans* KCTC3065; the Gram-negative bacteria, *Escherichia coli* KCTC1116 and *Pseudomonas aeruginosa* KCTC2004; and the yeast, *Candida albicans* KCTC7965. The bacterial strains were grown in brain-heart infusion medium (BHI; BD Biosciences, San Jose, CA, USA) at the appropriate temperature (25 °C for *P. aeruginosa* and *S. iniae*, and 37 °C for the other strains). The yeast strain *C. albicans* was grown in yeast medium (YM) at 25 °C. After 16–18 h of incubation, the bacterial and *C. albicans* suspensions were diluted to a McFarland turbidity standard of 0.5 (Vitek Colorimeter #52–1210; Hach, Loveland, CO, USA) corresponding to approximately 10^8 colony forming units (CFU)/mL for bacteria and 10^6 CFU/mL for *C. albicans*. A 500-mL aliquot of the diluted bacterial or *C. albicans* suspension was added to 9.5 mL of underlay gel containing 5×10^6 or 5×10^4 CFU/mL in 10 mM phosphate-buffered saline (PBS; pH 6.6) with 0.03% tryptic soy broth or 0.03% Sabouraud dextrose broth and 1% type I low-electroendosmosis agarose. The purified peptide was serially diluted two-fold in 5 μL of acidified water (0.01% HAc), and each dilution was added to 2.5-mm diameter wells made in the 1-mm thick underlay gel. After a 3-h incubation at 25 °C (*P. aeruginosa*, *S. iniae*, and *C. albicans*) or 37 °C (the remaining strains), the bacterial or yeast suspension was overlaid with 10 mL of double-strength overlay gel containing 6% BHI or 6% YM prepared in 10 mM PBS (pH 6.6) and 1% agarose. The plates were incubated for an additional 18–24 h, after which the clearing zone diameters were measured. After subtracting the diameter of the well, the clearing zone diameter was expressed in units (0.1 mm = 1 U).

2.4. Minimum effective concentrations (MECs)

All tested bacteria and yeast were prepared as described above. The minimum effective concentration (MEC; μg/mL) of the synthetic peptides was calculated as the *x*-intercept of a plot of the above-described units against the $\log_{10}$ of the peptide concentration [34]. The antimicrobial assay was performed in triplicate, and the results were averaged.

2.5. Bactericidal test

Bactericidal tests of the analogs were performed with *E. coli* ML35p using the broth microdilution method and plate assay [35]. The bacterial strain was grown overnight to mid-logarithmic phase as described above and diluted to an appropriate concentration. The diluted bacterial suspension (90 μL of 10^6 CFU/mL) was added to each well of the 96-well polypropylene microtiter plates (Costar; Corning Incorporated, Corning, NY, USA) and 10 μL serially two-fold diluted analog solution in 0.01% acetic acid was loaded into the 96-well polypropylene microtiter plates for a final concentration ranging from 100 to 3.13 μg/mL. The plate was incubated at 37 °C for 16–18 h. After 16–18 h, 10 μL aliquots were withdrawn from each well, plated on TSA plates, and incubated overnight at 37 °C for 16–18 h. The bactericidal activity was determined by the concentration of analog without bacterial growth.

2.6. Effects of pH, temperature, and salt on antimicrobial activity

The influence of pH (5.0, 6.0, and 7.0), temperature (heating at 80 °C for 6 h), and supplementation with NaCl (0.5, 1.0, 1.5, and 2.0%) on the activities of crab-ALF2A and crab-ALF6A were evaluated with the URDA. To explore thermal stability at different pH levels, the peptide was dissolved in 50 mM sodium acetate buffer (pH 5.0 or 6.0) or Tris-HCl buffer (pH 7.0), incubated at 80 °C for 6 h, cooled, and used

MARVSLLLIVLSIALVAPSQGFLKDLLFGEAKKALLEDGTTEILDRGCNFRVMPRLRSWELYFRGDVWCP
ccccchhhhhhhhhccccccchhhhhhhhhhhhhhhhhccceeeecccccceeeccceeeeeeeccccccc

GWTVIKGESLTRSRTRVVNKAVADFAQKALAQGLITQEDAQPLLE
cceeeccccccccchhhhhhhhhhhhhhhhhhhhhhhhhhcceec

Fig. 1. Prediction of the secondary structure of anti-lipopolysaccharide factor (ALF) from the swimming crab, *Portunus trituberculatus* (ADU25042). The signal peptide and the lipopolysaccharide-binding domain are underlined and double underlined, respectively. The parental sequences used for the design of the antimicrobial peptide analogs are highlighted in gray. h, c, and e represent the helix, random coil, and extended strand secondary structures, respectively.

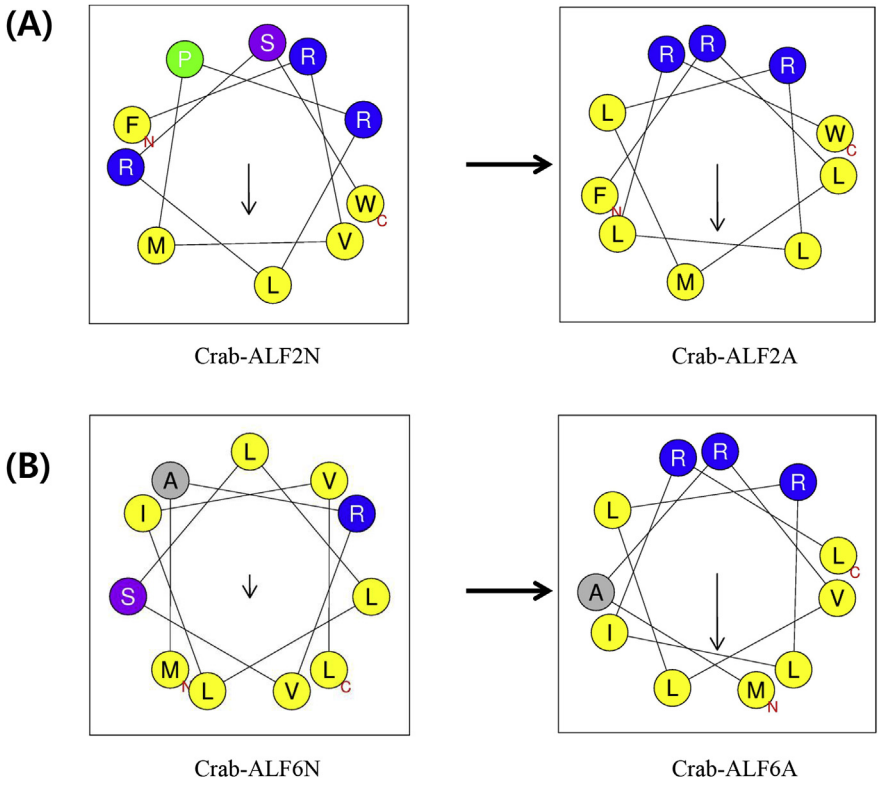

Fig. 2. Schiffer-Edmundson helical wheels projection of crab-ALF2 (A) and crab-ALF6 series (B). The hydrophobic residues are yellow, positively charged hydrophilic residues are blue, small polar residues are purple, and small neutral residues are gray. The hydrophobic face is indicated by an arrow.

in the URDA against *B. cereus*, *E. coli*, and *C. albicans*. To explore the effect of salinity, the underlay medium was supplemented with NaCl at the desired concentrations (0.5, 1.0, 1.5, and 2.0%) and overlaid with medium containing each microbe. The plates were incubated for 24 h, after which zones of inhibition were measured.

2.7. Inner membrane permeabilization assay

To assess the bacterial inner membrane permeabilizing activity of crab-ALF analogs, *o*-nitrophenyl-β-D-galactoside (ONPG) and total nucleotide leakage were determined with *E. coli*. The inner membrane permeabilization was determined by measuring the β-galactosidase activity of *E. coli* ML35p, a lactose permease-deficient strain, using ONPG as a substrate as previously described [36]. In brief, the overnight culture of *E. coli* ML35p was inoculated in fresh Luria-Bertani (LB) medium containing 1 mM isopropyl β-D-1-thiogalactopyranoside to induce the expression of β-galactosidase. At each time point (0, 10, 20, 30, 40, 50, and 60 min), the supernatant was collected and ONPG was added to a final concentration of 0.4 mM and incubated at 37 °C for 10 min.

2.8. DNA binding assay

DNA binding assays were performed to examine the inhibition of the rate of migration of DNA bands through agarose gels. A commercial molecular weight marker, λ-*Hin*dIII-digested DNA (50 ng) (Roche, Basel, Switzerland), was mixed with varying amounts of peptide (0, 0.156, 0.313, 0.625, and 1.25 μg) in 0.01% acetic acid, incubated at room temperature for 5 min, and electrophoresed in a 1.0% agarose gel containing 0.5 μg/mL ethidium bromide (EtBr).

2.9. DNA polymerase inhibition assay

The crab-ALF2A and crab-ALF6A were dissolved in 0.01% acetic acid at a concentration of 1 mg/mL. To assess polymerase chain reaction (PCR) inhibition, various concentrations of crab-ALF2A or crab-ALF6A (2.5, 1.25, 0.625, or 0.313 μg/mL) were added to the respective reaction mixtures. Genomic DNA of *E. coli* (100 ng) was used as a template for PCR with the primers 16S—F1 (5′-CTCCTACGGGGGCAG CAG-3′) and 16S-R3 (5′—CCAGGGTATCTAATCCTG-3′) and a predicted amplicon of approximately 1.5 kilobases. The PCR was performed as follows: 30 cycles each of 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C. After the PCR, all amplicons were electrophoresed in 1.0% agarose gels containing EtBr.

2.10. Scanning electron microscopy (SEM)

The morphology of *E.coli*, *S. aureus* or *C. albicans* following treatment with the crab-ALF analogs were observed by scanning electron microscopy (SEM). Mid-logarithmic phase cultures of *E.coli*, *S. aureus*, or *C. albicans* were prepared as described above and resuspended in 495 μL of fresh PBS buffer. Each microbe mixed with 5 μg of crab-

Table 1
Sequences and physiochemical properties of the peptides used in this study.

Peptide name	Sequence	Length	MW	pI	Net charge	Hydro phobicity (kcal/mol)	Boman index (kcal/mol)	Instability index
Crab-ALF2N	FRVMPRLRSW-NH_2	10	1347.65	12.3	+3	−0.41	3.15	108.6
Crab-ALF2A	FRLMLRLLRW-NH_2	10	1403.80	12.3	+3	−3.02	1.74	104.8
Crab-ALF6N	MARVSLLLIVL-NH_2	11	1227.61	9.5	+1	−1.53	−1.68	12.59
Crab-ALF6A	MARVLLRLIRL-NH_2	11	1353.78	12.3	+3	−0.11	1.08	37.82

MW, molecular weight; pI, isoelectric point.

Table 2
Antimicrobial activities of the peptide analogs crab-ALF2A and crab-ALF6A.

Microbe	Gram	Minimal effective concentration (μg/mL)	
		Crab-ALF2A	Crab-ALF6A
B. cereus	+	1.51	1.88
S. aureus RM4220	+	1.87	1.79
S. iniae FP5229	+	1.93	2.3
S. mutans	+	1.73	1.49
P. aeruginosa KCTC2004	–	1.98	1.72
E. coli	–	1.87	1.79
C. albicans KCTC7965	Yeast	2.11	1.95

ALF2A or crab-ALF6A for 2 h and collected, individually. The untreated cells were used as a control. The collected cells were subsequently fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) overnight, washed three times with 0.1 M phosphate buffer (pH 7.4), and dehydrated with a graded ethanol series (30, 50, 70, 90, 95, and 100%). After critical point drying, the samples were mounted on 1-cm stubs and platinum-coated using a sputter coater (Q150T, Quorum Technologies, Laughton, East Sussex). The specimens were then observed under SE2 mode (ETH = 5 kV) with SEM (SUPRA-55VP, Carl Zeiss, German).

3. Results

3.1. Design and synthesis of peptide analogs

To develop a novel AMP, we designed peptide analogs of the crab ALF (NCBI accession no. ADU25042) based on the amino acid sequences of its LBD and signal peptide. Two parent peptides were selected using the GOR algorithm (ver. 4) and named crab-ALF2N and

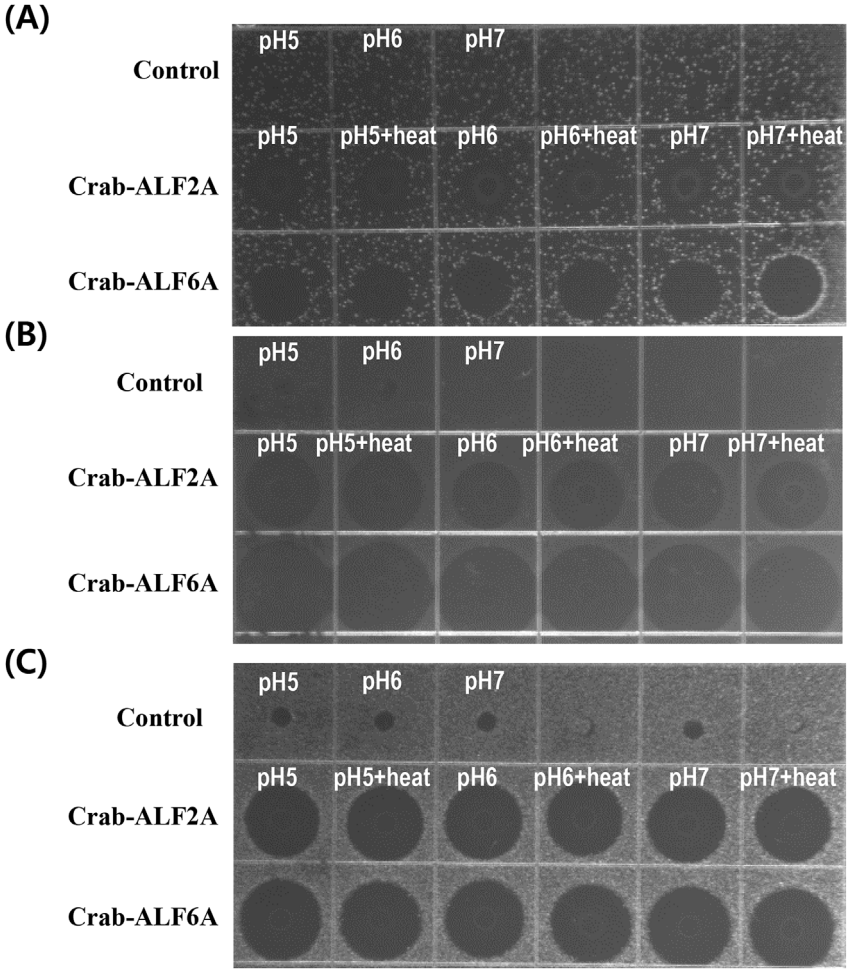

Fig. 3. pH and thermal stability of crab-ALF analogs with activity against *Bacillus cereus* (A), *Escherichia coli* (B), and *Candida albicans* (C). The ultrasensitive radial diffusion assay (URDA) was performed by incubating the crab-ALF2A or crab-ALF6A peptides in the various pH conditions (pH 5, 6, and 7) with or without heating at 80 °C. The antimicrobial activities of peptides were not altered by pH or heat treatment.

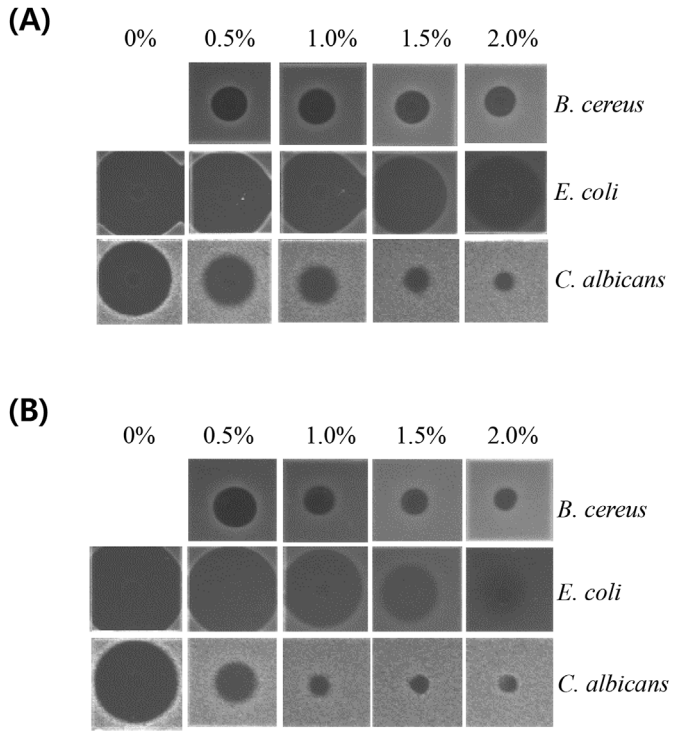

Fig. 4. Salinity stability of the activities of crab-ALF2A (A) and crab-ALF6A (B) against *B. cereus*, *E. coli*, and *C. albicans*. The ultrasensitive radial diffusion assay (URDA) showed that NaCl affected the antimicrobial activities of the analogs in a concentration-dependent manner. In particular, the antimicrobial activities of crab-ALF2A and crab-ALF6A against *C. albicans* were abolished with medium containing 1.5% and 1.0% NaCl, respectively.

crab-ALF6N. The secondary structure of the LBD was predicted to be a random coil and extended strand and the signal peptide exhibited a random coil and α-helical structure at residues 51–60 (crab-ALF2N: FRVMPRLRSW) and 1–11 (crab-ALF6N: MARVSLLLIVL), respectively (Fig. 1). Schiffer-Edmundson helical wheel projections were also used to predict an amphipathic structure in which hydrophobic and hydrophilic residues containing basic residues are positioned on opposite sides in the secondary structure of the synthetic peptides crab-ALF2A (FRLMLRLLRW) and crab-ALF6A (MARVLLRLIRL) (Fig. 2).

The predicted pI, net charge, hydrophobicity, and Boman and instability indices are listed in Table 1. The crab-ALF-2A peptide had the same pI value and net charge as the native parental crab-ALF-2N peptide, but the hydrophobicity and Boman index were lower than those of crab-ALF-2N. The crab-ALF-6A peptide had a higher pI value and net charge than the parental crab-ALF-6A peptide.

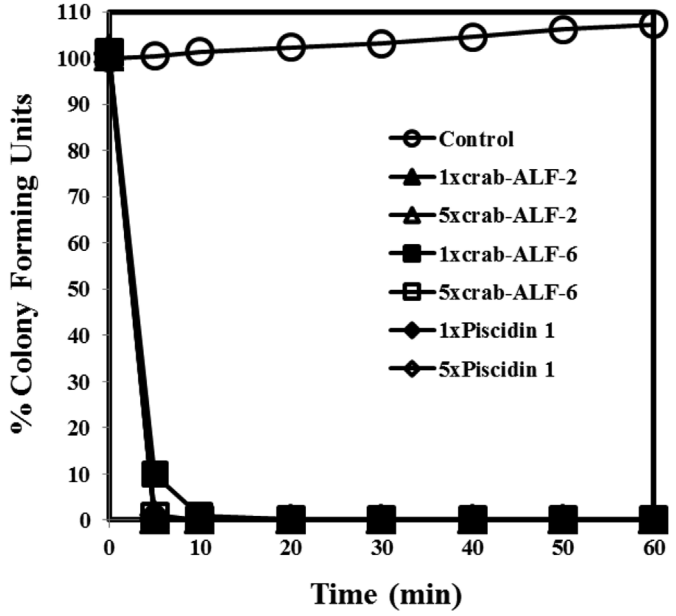

Fig. 6. Bactericidal kinetics of the crab-ALF analogs and piscidin-1 against *E. coli* ML35p. Bacteria were incubated in the presence of 1 × or 5 × minimal effective concentration of the crab-ALF analog peptides or piscidin-1. Control sample did not contain a peptide.

3.2. Antimicrobial activity of synthetic crab-ALF analogs

3.2.1. Minimum effective concentration (MEC)

We determined the antimicrobial activity of the synthetic peptides by measuring the MECs against several bacterial species and one fungus (*C. albicans*) by URDA. While the parent peptides (ALF2N and ALF6N) did not exhibit any antimicrobial activity against the microbes tested (data not shown), the crab-ALF-2A peptide showed potent antimicrobial activity against the Gram-positive bacteria *B. cereus*, *S. aureus*, and *S. iniae* (MEC 1.51–1.93 μg/mL) and the Gram-negative bacteria *P. aeruginosa* and *E. coli* (MEC 1.87–1.98), with maximal bactericidal activity at a peptide concentration of 5 μg/mL. The crab-ALF-6A peptide also showed potent antimicrobial activity against *B. cereus*, *S. aureus*, and *S. iniae* (MEC 1.49–2.3 μg/mL) and *P. aeruginosa* and *E. coli* (MEC 1.72–1.19 μg/mL) at a peptide concentration of 5 μg/mL. Notably, crab-ALF2A and crab-ALF6A exhibited strong activity against *C. albicans* (MECs of 2.11 and 1.95 μg/mL, respectively) (Table 2).

3.2.2. Effects of pH, temperature, and salinity

To investigate the effects of pH, heat, and salinity on the antimicrobial activities of the two analogs, 5 μg/mL of the synthetic crab-ALF2A or crab-ALF6A peptide was incubated at 80 °C for 6 h at pH 5, 6, and 7 and cooled before testing in the URDA against *E. coli* (Gram-negative), *B. cereus* (Gram-positive), and *C. albicans* (fungus). The

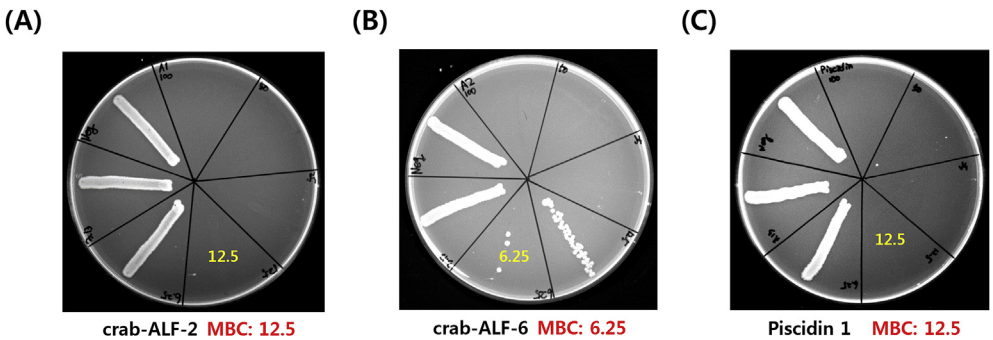

Fig. 5. Bactericidal activity of the analogs and piscidin-1 against *E. coli* ML35p. Bactericidal tests of crab-ALF2A (A), crab-ALF6A(B), and piscidin-1(C) were performed with the broth microdilution method and plate assay at concentrations of 3.13–100 μg/mL against *E. coli* ML35p. The bactericidal concentration was determined by the concentration of peptide without bacterial growth.

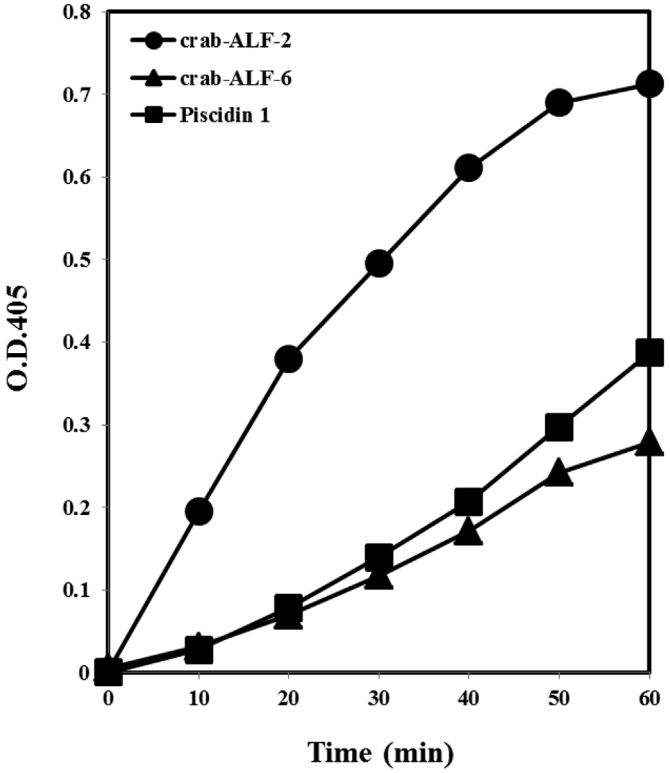

Fig. 7. Inner membrane permeabilization of *E. coli* ML35p by crab-ALF analog peptides and piscidin-1. Cytoplasmic membrane permeabilization was monitored as an increase in fluorescence intensity by the hydrolysis of the impermeable, chromogenic substrate *o*-nitrophenyl-β-D-galactoside in the presence of each peptide.

antimicrobial activities of the peptides were not greatly altered by pH or heat treatment (Fig. 3), as evidenced by their strong antimicrobial activities against the tested strains. However, the antimicrobial activities of the peptides were affected by sodium chloride in a concentration-dependent manner (Fig. 4). In particular, the antimicrobial activities of crab-ALF2A and crab-ALF6A against *C. albicans* were abolished with medium containing 1.5% and 1.0% sodium chloride, respectively.

3.2.3. Bactericidal test

To determine whether the antimicrobial function of crab-ALF2A and crab-ALF6F was a result of a bactericidal or bacteriostatic activity, a bactericidal test was performed using *E. coli* ML35p. The ALF2A and ALF6A showed bactericidal activity at 12.5 μg/L and 6.25 μg/L, respectively (Fig. 5). These results suggest that the antimicrobial function of crab-ALF2A and crab-ALF6A is bactericidal same as piscidin-1 used for control. To evaluate the inhibition mode of the analogs, we performed analysis of bactericidal kinetics against *E. coli* ML35p at 1 × and 5 × MEC of the analogs (Fig. 6). Crab-ALF2A, crab-ALF6A, and the control piscidin-1 rapidly killed *E. coli* ML35p within 5 min at 5 × MEC and within 10 min at 1 × MEC. These results indicate that the inhibition mode of crab-ALF2A and crab-ALF6A is a bactericidal process.

3.3. Inner membrane permeabilizing assay

Hydrophobicity is also required for bacterial membrane permeabilization. Hydrophobic residues facilitate interactions with the fatty acyl chains of the bacterial membrane. The relatively low hydrophobicity prevents binding to the zwitterionic membranes found in mammalian cells, resulting in low toxicity [37,38]. It is well known that the eukaryotic cell membrane, in contrast to the prokaryotic membrane, is generally characterized by the presence of zwitterionic phospholipids, a relatively large amount of cholesterol and sphinogomyelin, and the absence of the high, intracellular negative transmembrane potential present in prokaryotic membranes. Some studies have demonstrated that above an optimum level or threshold of hydrophobicity [39], a further increase leads to a loss of antimicrobial activity and increased toxicity [40].

Inner membrane permeabilization was investigated using the *E. coli* ML35 isolate, which lacks lactose permease, an enzyme that functions in the uptake of lactose from the extracellular environment into the bacterial cell, using ONPG as a chromogenic substrate for the β-galactosidase activity assay (Fig. 7). The *o*-nitrophenol produced upon cleavage of ONPG by β-galactosidase was detected spectrophotometrically by measuring the absorbance at 405 nm (A405). If the crab-ALF analog permeabilized the inner membrane of *E. coli*, β-galactosidase activity would be detected outside the bacterial cell. Following exposure to the crab analogs, a rapid increase in the A405 value (β-galactosidase activity) in the supernatant was observed from 0 to 60 min. The β-galactosidase activity of crab-ALF2 was stronger than that of crab-ALF6.

3.4. DNA-binding and polymerase inhibition assay

To investigate the binding ability of the peptides to DNA, we performed an electrophoretic mobility shift assay with crab-ALF2A and crab-ALF6A. The electrophoretic mobility of the DNA was almost completely inhibited following exposure to 1.25 μg crab-ALF2A or crab-ALF6A compared with non-complexed DNA (Fig. 8A). To determine

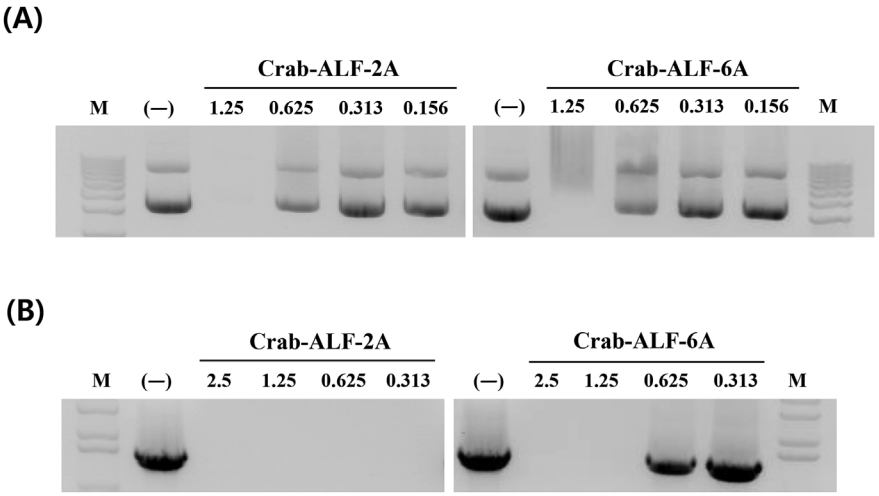

Fig. 8. DNA binding and DNA polymerase inhibition assay of crab-ALF analog peptides. (A) Binding of peptides to DNA was assessed by measuring the retardation of pEGFP C2 vector DNA (500 ng) migration through an agarose gel. The peptide concentration is indicated above each lane and represents 1.25, 0.625, 0.313, and 0.156 μg. The control (−) reaction was performed without peptide. (B) The effect of the peptides on DNA polymerase was tested by polymerase chain reaction amplification of *E. coli* 16S ribosomal DNA. The peptide concentration is indicated above each lane and represents 2.5, 1.25, 0.25, and 0.313 μg. The control (−) reaction was performed without peptide.

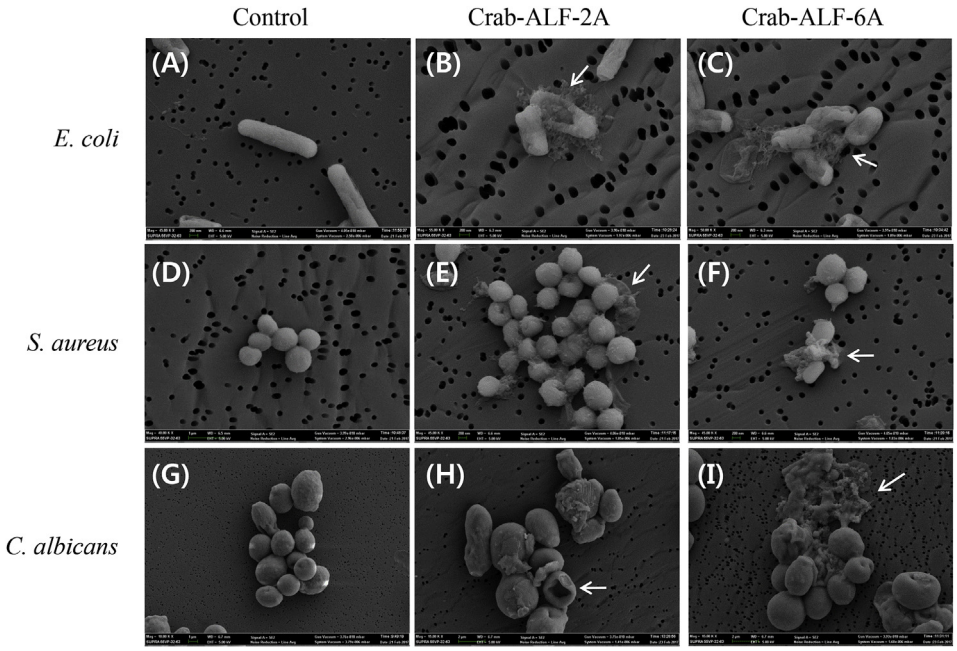

Fig. 9. Morphological observations of microbes by scanning electron microscopy. *E. coli* (A–C), *S. aureus* (D–F), and *C. albicans* (G–I) were treated individually with 5 μg of crab-ALF2A (B, E, H) or crab-ALF6A (C, F, I) for 2 h. White arrows indicate coarse surfaces, cellular debris and cell lysis.

Table 3
Physicochemical properties and antimicrobial activities of serial downsized crab-ALF6A variants.

No.	Peptide Name	Sequence	Length	M.W.	p.I.	Net Charge	Hydro-phobicity (kcal/mol)	Instability index	Boman Index (kcal/mol)	*E. coli*	*B. cereus*	*C. albicans*
1	ALF6A	MARVLLRLIRL(-NH_2)	11	1353.79	12.3	+3	-0.11	37.82	1.08			
2	ALF6A1	-ARVLLRLIRL(-NH_2)	10	1222.59	12.3	+3	0.12	28.26	1.43			
3	ALF6A2	MARVLLRLIR-(-NH_2)	10	1240.62	12.3	+3	0.45	40.60	1.68			
4	ALF6A3	-ARVLLRLIR-(-NH_2)	9	1109.43	12.3	+3	0.68	30.29	2.13			
5	ALF6A4	--RVLLRLIR-(-NH_2)	8	1038.35	12.3	+3	0.51	32.83	2.63			
6	ALF6A5	-ARVLLRLI--(-NH_2)	8	953.24	12.0	+2	-0.13	32.83	0.53			
7	ALF6A6	--RVLLRLI--(-NH_2)	7	882.16	12.0	+2	-0.3	36.09	0.87			
8	ALF6A7	---VLLRLI--(-NH_2)	6	725.97	9.72	+1	-1.11	40.43	-1.46			
9	ALF6A8	--RVLLRL---(-NH_2)	6	769.004	12.0	+2	0.01	40.43	1.84			
10	ALF6A9	---VLLRL---(-NH_2)	5	612.816	9.72	+1	-0.8	46.52	-0.77			
11	ALF6A10	--RVLLR----(-NH_2)	5	655.844	12.0	+2	0.57	46.52	3.19			

whether the peptides inhibited DNA polymerase activity, we performed DNA polymerase inhibition assays using crab-ALF2A and crab-ALF6A. Crab-ALF2A exhibited complete inhibitory activity at all concentrations tested (0.313–2.5 μg), but crab-ALF6A showed less inhibitory activity at 1.25 μg compared with crab-ALF2A (Fig. 8B).

3.5. Lysis of microbes observed by electron microscopy

We investigated the effect of peptide analog treatment on the morphology of microbes (*C. albicans*, *E. coli*, and *S. aureus*) by SEM. Changes in cell morphology were observed in all the microbes treated with 5 μg/mL crab-ALF2A or crab-ALF6A after 2 h. Control microbes had intact and smooth surface without cell lysis or debris. However, after treatment with peptide, the morphology of microbes changed significantly. Compared with the control, microbes treated with crab-ALF2A or crab-ALF6A exhibited shrinkage, which could be a result of leakage of cytoplasmic content (Fig. 9).

3.6. Downsizing of AMP

Crab-ALF2A and crab-ALF6A exhibited similar antimicrobial activity in the MEC test. However, the instability index of crab-ALF2A was greater than that of crab-ALF6A (104.80 and 12.59, respectively; Table 2). The instability index is a measure used to determine whether a protein will be stable in a test tube. AMPs, with index values less than 40, tend to be considered stable, while a value above 40 predicts that a protein may be unstable [41]. Therefore, we performed further analysis with crab-ALF6A to downsize the synthetic peptide while maintaining antimicrobial activity.

We produced a synthetic peptide series (ALF6A1–ALF6A10) by serial removal of N− and/or C-terminal ends of the template peptide of ALF6A (Table 3). The antimicrobial activity was observed with a URDA against *E. coli*, *B. cereus*, and *C. albicans*. The hexa-peptide ALF6A8 (RVLLRL) maintained high antimicrobial activity against *E. coli* and *C. albicans*, but the hexa-peptide ALF6A7 (VLLRLI) did not exhibit antimicrobial activity. The penta-peptides ALF6A9 and ALF6A10 also did not exhibit any antimicrobial activity against the tested microbes (Table 3).

4. Discussion

ALFs have been regarded as important effectors of the innate immune system in crustaceans [42] and have been shown to exhibit active antimicrobial activity *in vitro* against a broad range of Gram-positive and Gram-negative bacteria, certain fungi, and viruses [31]. These antimicrobial proteins, including ALFs, are considered good templates for the development of new antimicrobials. We have previously shown that synthetic peptides derived from the C-terminus of the olive flounder LBP/BPI precursor protein (ofLBPs) [8] and derived from the LPS and β-1,3-glucan binding protein (LGBP) of abalone (HDH-LGBPs) [9] exhibited high inhibitory activity against a broad range of microbes. Additionally, HDH-LGBP analogs showed antitumor activity in human cancer cell lines [9].

In this study, we used the amino acid sequences of the LBD of ALF as a parent sequence for designing novel AMPs. Several previous studies have reported that LBD-derived peptides could rapidly kill both Gram-negative *E. coli* and Gram-positive *Staphylococcus epidermidis* by binding the bacteria directly with high affinity to LPS and lipoteichoic acid (LA) [43], thus resulting in the successful design of novel AMPs [31,44]. Additionally, we developed novel AMPs by modifying the signal peptide sequence of ALF as a template. There are several common features between AMPs and signal peptides, as signal peptides are also short peptides consisting of 5–30 amino acids at the N-terminus of secreted proteins. Most signal peptides contain a positively charged N-region, followed by a hydrophobic H-region and a neutral but polar C-region. Therefore, we expected that a signal peptide would be a good template sequence for the design of novel AMPs.

Antimicrobial activity also depends on various physicochemical parameters including pI, cationicity (net charge), hydrophobicity, and amphipathicity. Therefore, many studies have concluded that the natural biological activities of these peptides are coordinated by a sophisticated modulation of the hydrophobicity, amphipathicity, positive charge, and a reduction in the hydrophobic moment [2,45,46].

The pI is directly correlated with solubility, and proteins are soluble, and do not aggregate, at pH values greater than 1 or 2 on either side of the pI. Because AMPs need to access lipid membranes from an aqueous phase, they need to be soluble in both environments. If AMPs aggregate in solution, they will lose their ability to interact with the cell membrane. The AMPs used in this study, with the exception of ALF6N, have a calculated pI greater than 12 (Table 1), which is considered to be sufficient to avoid self-association driven by a loss of side chain charge at physiological pH.

Being cationic is a key feature of AMPs with a net charge. Biophysical studies have provided models for the mechanism underlying membrane damage; the main proposed modes of action are the carpet model, barrel stave model, and toroidal-pore model [47]. In all of the proposed models, the initial interaction between the peptide and the bacterial membrane is electrostatic and involves the positively charged residues of the peptide and the negatively charged moieties on the surface of the bacterial membrane. Among the parent AMPs, the net charge of ALF6N was +1, so we substituted two leucine residues with charged arginine residues to increase the cationicity (Table 1, Fig. 2).

Through SEM observation of the surface of the bacteria, we found that the ALF2A and ALF6A peptides could disrupt not only the bacterial membrane but also the fungal membrane and cause cytoplasmic leakage (Fig. 8).

The variant synthetic peptide series, ALF6A1–ALF6A10, was generated through peptide downsizing of the N− and/or C-terminal ends of the ALF6A template peptide (Fig. 9). Antimicrobial activity was observed until the hexa-peptide of ALF6A8 (RVLLRL), but not with ALF6A7 (VLLRLI). The physicochemical parameters of ALF6A7, such as pI, net charge, and hydrophobicity, were altered due to the removal of an arginine residue from the N-terminal end. The ALF6A8 peptide, which contained a terminal arginine residue, maintained its activity (Fig. 9). However, the penta-peptides, ALF6A9 and ALF6A10, showed reduced antimicrobial activity despite maintaining the same pI and net charge as ALF6A8. It is thought that some proteins, including peptides, elicit their biological function through a single short α-helical segment (usually 4–15 amino acids in 1–4 helical turns) that interacts with nucleic acids or proteins [48,49]. Taken together, the antimicrobial activity of crab ALF-derived peptide analogs depends not only on physicochemical parameters such as pI, cationicity, and hydrophobicity, but also sequence length. Our study will be useful for the development of novel antimicrobial agents.

Conflicts of interest

The authors declare that they have no competing interests exist.

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References

[1] M.P. Kieny, The Evolving Threat of Antimicrobial Resistance: Options for Action,

World Health Organization, 2012.
[2] M.R. Yeaman, N.Y. Yount, Mechanisms of antimicrobial peptide action and resistance, Pharmacol. Rev. 55 (2003) 27–55.
[3] P. Lazarovici, N. Primor, L.M. Loew, Purification and pore-forming activity of two hydrophobic polypeptides from the secretion of the Red Sea Moses sole (*Pardachirus marmoratus*), J. Biol. Chem. 261 (1986) 16704–16713.
[4] S.Y. Shin, M.K. Lee, K.L. Kim, K.S. Hahm, Structure-antitumor and hemolytic activity relationships of synthetic peptides derived from cecropin A-magainin 2 and cecropin A-melittin hybrid peptide, J. Pept. Sci. 50 (1997) 279–285.
[5] C.-Y. Maeng, M.S. Oh, I.H. Park, H.J. Hong, Purification and structural analysis of the hepatitis B virus preS1 expressed from *Escherichia coli*, Biochem. Biophys. Res. Commun. 282 (2001) 787–792.
[6] M. Dathe, H. Nicolenko, J. Meyer, M. Beyermann, M. Bienert, Optimization of the antimicrobial activity of magainin peptides by modification of charge, FEBS Lett. 501 (2001) 146–150.
[7] C.-F. Le, M.Y.M. Yusof, H. Hassan, S.D. Sekaran, *In vitro* properties of designed antimicrobial peptides that exhibit potent antipneumococcal activity and produces synergism in combination with penicillin, Sci. Rep. 5 (2015) 9761.
[8] B.H. Nam, J.Y. Moon, E.H. Park, Y.O. Kim, D.G. Kim, H.J. Kong, W.J. Kim, Y.J. Jee, C.M. An, N.G. Park, J.K. Seo, Antimicrobial activity of peptides derived from olive flounder lipopolysaccharide binding protein/bactericidal permeability-increasing protein (LBP/BPI), Mar. Drugs 12 (2014) 5240–5257.
[9] B.H. Nam, J.Y. Moon, E.H. Park, H.J. Kong, Y.O. Kim, D.G. Kim, W.J. Kim, C.M. An, J.K. Seo, Antimicrobial and antitumor activities of novel peptides derived from the lipopolysaccharide- and β-1,3-glucan binding protein of the pacific abalone *Haliotis discus hannai*, Mar. Drugs 14 (2016) 227.
[10] P.S. Gross, T.C. Bartlett, C.L. Browdy, R.W. Chapman, G.W. Warr, Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas n th Pacific White Shrimp, *Litopenaes vannamei*, and the Atlantic White Shrimp *L. setiferus*, Dev. Comp. Immunol. 25 (2001) 565–577.
[11] P. Supungul, S. Klinbunga, R. Pichyangkura, I. Hirono, T. Aoki, A. Tassanakajon, Antimicrobial peptides discovered in the black tiger shrimp *Penaeus monodon* using the EST approach, Dis. Aquat. Org. 61 (2004) 123–135.
[12] H.S. Jiang, Q. Zhang, Y.R. Zhao, W.M. Jia, X.F. Zhao, J.X. Wang, A new group of anti-lipopolysaccharide factors from *Marsupenaeus japonicus* functions in antibacterial response, Dev. Comp. Immunol. 48 (2015) 33–42.
[13] H. Yang, S. Li, F. Li, J. Xiang, Structure and Bioactivity of a Modified peptide derived from the LPS-Binding domain of an anti-lipopolysaccharide factor (ALF) of Shrimp, Mar. Drugs 14 (2016) 96.
[14] K.M. Beale, D.W. Towle, N. Jayasundara, C.M. Smith, J.D. Shields, H.J. Small, et al., Anti-lipopolysaccharide factors in the American lobster *Homarus americanus*: molecular characterization and transcriptional response to *Vibrio fluvialis* challenge, Comp. Biochem. Physiol. Genom. Proteonomics 3 (2008) 263–269.
[15] H. Liu, P. Jiravanichpaisal, I. Soderhall, L. Cerenius, K. Soderhall, Anti- lipopolysaccharide factor interferes with white spot syndrome virus replication in vitro and in vivo in the crayfish *Pacifastacus leniusulus*, J. Viol. 80 (2006) 10365–10371.
[16] C. Sun, W.T. Xu, H.W. Zhang, L.P. Dong, T. Zhang, X.F. Zhao, et al., An anti-lipopolysaccharide factor from red swamp crayfish, *Procambarus clarkia*, exhibited antimicrobial activities *in vitro* and *in vivo*, Fish Shellfish Immunol. 30 (2011) 295–303.
[17] K.Y. Lu, H.J. Sung, C.L. Liu, H.H. Sung, Differentially enhanced gene expression in hemocytes from *macrobachium rosenbergii* challenged *in vivo* with lipopolysaccharide, J. Invertebr. Pathol. 100 (2009) 9–15.
[18] Q. Ren, Z. Zhang, X.C. Li, J. Du, K.M. Hui, C.Y. Zhang, W. Wang, Three differnet anti-lipopolysaccharide factors identified from giant freshwater prawn, *Macrobrachium rosebergii*, Fish Shellfish Immunol. 33 (2012) 766–774.
[19] R. Yedery, V.V.R. Reddy, Identification, cloning, characterization and recombinant expression of an anti-lipopolysaccharide factor from the hemocytes of Indian mud crab, *Scylla serrate*, Fish Shellfish Immunol. 27 (2009) 275–284.
[20] Y. Zhang, L. Wang, L. Wang, J. Yang, Y. Gai, L. Qiu, L. Song, The second anti-lipopolysaccharide factor (EsALF-2) with antimicrobial activity from *Eriocheir sinensis*, Dev. Comp. Immunol. 34 (2010) 954-952.
[21] Y. Liu, Z. Cui, X. Li, C. Song, G. Shi, A newly identified anti-lipopolysaccharide factor from the swimming crab *Portunus trituberculatus* with broad spectrum antimicrobial activity, Fish Shellfish Immunol. 32 (2013) 463–470.
[22] V.V. Afsal, S.P. Antony, V.N. Sanjeevan, P.R.A. Kumar, I.S.B. Singh, R. Philip, A new isoform of anti-lipopolysaccharide factor identified from the blue swimmer crab, *Portunus pelagicus*: molecular characteristics and phylogeny, Aquaculture 356–357 (2012) 119–122.
[23] C. Imjongjirak, P. Amparyup, A. Tassanakajon, S. Sittipraneed, Anti-lipopolysaccharide factor (ALF) of mud crab *Scylla paramamosain*: molecular cloning, genomic organization and the antimicrobial activity of its synthetic LPS binding domain, Mol. Immunol. 44 (2007) 3195–3203.
[24] C. Imjongjirak, P. Amparyup, A. Tassankajon, Molecular cloning, genomic organization and antibacterial activity of a second isoform of antilipopolysaccharide factor (ALF) from the mud crab, *Scylla paramamosain*, Fish Shellfish Immunol. 30 (2011) 58–66.
[25] Y. Liu, Z.X. Cui, X.H. Li, C.W. Song, Q.Q. Li, S.Y. Wang, Molecular cloning, expression pattern and antimicrobial activity of a new isoform of anti-lipopolysaccharide factor from the swimming crab *Portunus trituberculatus*, Fish Shellfish Immunol. 33 (2012) 85–91.
[26] L. Zhu, J.F. Lan, Y.Q. Huang, C. Zhang, J.F. Zhou, W.H. Fang, X.J. Yao, H. Wang, X.C. Li, SpALF4: a newly identified anti-lipopolysaccharide factor from the mud crab *Scylla paramamosain* with broad spectrum antimicrobial activity, Fish Shellfish Immunol. 36 (2014) 172–180.
[27] W. Sun, W. Wan, S. Zhu, S. Wang, S. Wang, X. Wen, H. Zheng, Y. Zhang, S. Li, Characterization of a novel anti-lipopolysaccharide factor isoform (SpALF5) in mud crab, *Scylla paramamosain*, Mol. Immunol. 64 (2015) 262–275.
[28] A. Hoess, S. Watson, G.R. Siber, R. Liddington, Crystal structure of an endotoxin-neutralizing protein from the horseshoe crab, Limulus anti-LPS factor at 1.5 A resolution, EMBO J. 12 (1993) 3351–3356.
[29] Y. Yang, H. Boze, P. Chemardin, A. Padilla, G. Moulin, A. Tassanakajon, NMR structure of rALF-Pm3, an anti-lipopolysaccharide factor from shrimp: model of the possible lipid A-binding site, Biopolymers 91 (2009) 207–220.
[30] R.D. Rosa, A. Vergnes, J. de Lorgeril, P. Goncalves, L.M. Perazzolo, L. Saune, Functional divergence in shrimp anti-lipopolysaccharide factors (ALFs): from recognition of cell wall components to antimicrobial activity, PloS One 8 (2013) e67937.
[31] P. Jaree, A. Tassanakajon, K. Somboonwiwat, Effect of the anti-lipopolysaccharide factor isoform 3 (ALFPm3) from *Penaeus monodon* on *Vibrio harveyi* cells, Dev. Comp. Immunol. 38 (2012) 554–560.
[32] L. Käll, A. Krogh, E.L.L. Sonnhammer, A combined transmembrane topolygy and signal peptide prediction method, J. Mol. Biol. 338 (2004) 1027–1036.
[33] J.K. Seo, M.J. Lee, B.H. Nam, N.G. Park, cgMolluscidin, a novel dibasic residue repeat rich antimicrobial peptide, purified from the gill of the Pacific oyster, *Crassostrea gigas*, Fish Shellfish Immunol. 35 (2013) 480–488.
[34] J.K. Seo, J.M. Crawford, K.L. Stone, E.J. Noga, Purification of a novel arthropod defensin from the American oyster, *Crassostrea virginica*, Biochem. Biophys. Res. Commun. 338 (2005) 1998–2004.
[35] A. Patrzykat, J.W. Gallant, J.K. Seo, J. Pytyck, S.E. Douglas, Novel antimicrobial peptides related from flatfish genes, Antimicrob. Agents Chemother. 47 (2003) 2464–2470.
[36] J.K. Seo, M.J. Lee, H.G. Jung, H.J. Go, Y.J. Kim, N.G. Park, Antimicrobial function of SHβAP, a novel hemoglobin β chain-related antimicrobial peptide, isolated from the liver of skipjack tuna, *Katsuwonus pelamis*, Fish Shellfish Immunol. 37 (2014) 173–183.
[37] B. Mojsoska, H. Jenssen, Peptides and peptidomimetics for antimicrobial drug design, Phamaceuticals 13 (2015) 366–415.
[38] L.M. Yin, M.A. Edwards, J. Li, C.M. Yip, C.M. Deber, Roles of hydrophobicity and charge distribution of cationic antimicrobial peptides in peptide-membrane interactions, J. Biol. Chem. 287 (2012) 7738–7745.
[39] E. Glukhov, L.L. Burrows, C.M. Deber, Membrane interactions of designed cationic antimicrobial peptides: the two thresholds, Biopolymers 89 (2008) 360–371.
[40] Y. Chen, M.T. Guarnieri, A.I. Vasil, M.L. Vasil, C.T. Mant, R.S. Hodges, Role of peptide hydrophobicity in the mechanism of action of α-helical antimicrobial peptides, Antimicrob. Agents Chemother. 51 (2007) 1398–1406.
[41] K. Guruprasad, B.V. Reddy, M.W. Pandit, Correlation between stability of a protein and its dipeptide composition: a novel approach for predicting in vivo stability of a protein from its primary sequence, Protein Eng. 4 (1990) 155–161.
[42] S. Tanaka, T. Nakamura, T. Morita, S. Iwanaga, Limulus anti-LPS factor: an anticoagulant which inhibits the endotoxin mediated activation of Limulus coagulation system, Biochem. Biophys. Res. Commun. 105 (1982) 717–723.
[43] K. Somboonwiwat, E. Bachere, V. Rimphanitchayakit, A. Tassanakajon, Localization of anti-lipopolysaccharide factor (ALFPm3) in tissues of the black tiger shrimp, penaeus monodon, and characterization of its biding properties, Dev. Comp. Immunol. 32 (2008) 1170–1176.
[44] H. Yang, S. Li, F. Li, X. Lv, J. Xiang, Recombinant expression and functional analysis of an isoform of anti-lipopolysaccharide factors (FcALF5) from Chinese shrimp *Fenneropenaeus chinensis*, Dev. Comp. Immunol. 53 (2015) 47–54.
[45] D. Fjell, J. Hiss, R.E.W. Hancook, G. Schneider, Designing antimicrobial peptides: form follows function, Nat. Rev. Drug Discov. 11 (2012) 37–51.
[46] K. Matsuzaki, Control of cell selectivity of antimicrobial peptides, Biochim. Biophys. Acta 1788 (2009) 1687–1692.
[47] Y. Shai, Mode of action of membrane active antimicrobial peptides, Biopolymers 6 (2002) 236–248.
[48] L. Pal, P. Chakrabarti, G. Basu, Sequence and structure patterns in proteins from an analysis of the shortest helices: implications for helix nucleation, J. Mol. Biol. 326 (2003) 273–291.
[49] J. Wang, J.A. Feng, Exploring the sequence patterns in the α-helices of proteins, Protein Eng. 16 (2003) 799–807.