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Design and analysis of structure–activity relationship of novel antimicrobial peptides derived from the conserved sequence of cecropin

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Abstract: We have *de novo* designed four antimicrobial peptides AMP-A/B/C/D, the 51-residues peptides, which are based on the conserved sequence of cecropin. In the present study, the four peptides were chemically synthesized and their activities assayed. Their secondary structure, amphipathic property, electric field distribution and transmembrane domain were subsequently predicted by bioinformatics tools. Finally, the structure–activity relationship was analyzed from the results of activity experiments and prediction. The results of activity experiments indicated that AMP-B/C/D clearly possessed excellent broad-spectrum activity against bacteria, whereas AMP-A was almost inactive against most of the bacterial strains tested. AMP-B/C/D showed more potent activity against Gram-positive bacteria than against Gram-negative bacteria. By utilizing bioinformatics analysis tools, we found that the secondary structure of the four cation peptides was mainly α-helix, and the result of CD spectrum also displayed that all the peptides had considerable α-helix in the presence of either 50% TFE or SDS micelles. AMP-C showed much better activity than other peptides against most of the bacteria tested, owing to its remarkable cation property and the amphipathic character of its *N*-terminal. The study of structure–activity relationship of the designed peptides confirmed that amphipathic structure and high net positive charge were prerequisites for maintaining their activities. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antimicrobial peptide; structure–activity relationship; design; bioinformatics

INTRODUCTION

Antibiotics have become indispensable in the modern health care system, assisting and complementing the natural immune system. The emergence of microbes that are resistant to conventionally used antibiotics has triggered considerable interest in searching for more potent and efficient antibiotics [1]. Among the more potent antibiotics reported so far are small antimicrobial peptides (AMPs) such as cecropin, maganin, melittin, and defensin [2]. AMPs have broad-spectrum activity against bacteria, yeast, fungi, and virus, and play important roles in the defense system of many organisms, including plants, insects, amphibians, and mammals [3]. Despite the great diversity in the primary structure of these peptides, most natural AMPs are generally cationic peptides of 13–50 amino acids with approximately 50% hydrophobic residues [4], and show the amphipathic characteristic of adopting α-helix and β-sheet structures in hydrophobic environments [5]. As cationic AMPs are known to rapidly kill microorganisms by disrupting the cellular structure of the host cells, a mechanism different from other antibiotics which act by targeting the physiology of the pathogen, resistance is not likely to develop to them easily [6–8].

The mechanisms of action have been investigated for several AMPs, including defensin, cecropin, magainin, and melittin. The mechanism by which AMPs cause cell death does not involve binding to specific receptors on the cell membrane but rather a nonspecific interaction with membrane phospholipids [9]. The surface of bacterial cells is composed of negatively charged components, such as lipopolysaccharide and teichoic acids [10], and an electrostatic interaction between the cationic AMPs and the negatively charged bacterial cell surface plays an important role in their antibacterial activity [11–13]. The formation of transmembrane pores or ion channels on the cellular membrane causing leakage of essential metabolites and, finally, the disruption of microbial cell structures are the major killing mechanism for these cationic peptides [14]. So far, hundreds of AMPs have been isolated from various organisms and their great diversity in the primary structure results in the large difference in their antibacterial mechanisms [15].

In recent years, studies on AMPs were aimed at not only delineating the structural requirements for selective antimicrobial activity but also analyzing structure–activity relationship. In previous research [16,] on the basis of structure–activity relationship of cecropin we had discussed the novel design of AMPs through an approach of piecing together the conserved sequence of cecropin. Sixty-two types of

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cecropin AMPs were gathered from the Swiss-Prot database by utilizing the sequence retrieval system (SRS). They were divided into 11 groups according to their sources and, consequently, a series of conserved sequences were found by the method of pattern discovery via multiple sequences alignment in each group. On the basis of the criterion of 51-amino acid length, these conserved sequences were pieced together into 57 different combined peptides according to the location of the conserved sequences in the original sequences. Analysis was applied to each polypeptide on its chemicophysical characteristics, transmembrane conditions, electronic field distribution, and secondary structure. Finally, four 51-residue polypeptides (AMP-A/B/C/D) were designed as the polypeptides of interest based on the following rules: possession of certain amphipathy, positive electronic field distribution, at least two transmembrane sequences and an α-helix content exceeding 50%. In the present study, we will discuss structure–activity relationship of the four AMPs based on the results from activity experiments and structure prediction by bioinformatics tools.

MATERIALS AND METHODS

Peptide Synthesis

AMP-A/B/C/D, listed in Table 1, were synthesized by the standard Fmoc chemistry under solid phase peptide synthesis according to the literature procedure [17]. Deprotection and cleavage was achieved by treatment with a mixture of TFA/water/thioanisole/ethendithiol (8.5/0.5/0.5/0.5, v/v/v/v) at room temperature for 3–4 h. After cleavage of the product from the resin, the peptides were purified by preparative HPLC using a water (0.1% TFA)–acetonitrile (0.1% TFA) gradient (10–50% acetonitrile during 40 min). The success of the synthesis of the peptides was confirmed by analysis using an ESI-mass spectrometer (Platform II, Micromass, UK) and a MALDI TOF mass spectrometer (Voyager-DESTR, Applied Biosystem). The homogeneity (>95%) of the compound was confirmed by analytical HPLC with a C_{18} column and HPLC–mass spectrometer (PlatformII, Manchester, Micromass, UK).

Circular Dichroism (CD) Studies

CD spectra were recorded on a Jasco J-715 spectropolarimeter (Tokyo, Japan) using a quartz cell of 1-mm path length between 190 and 245 nm at room temperature. The concentration of peptides was 10^{-4} M in 10 mM sodium phosphate buffer (pH 7.4) containing 50% trifluoroethanol (TFE, v/v) or 25 mM sodium dodecyl sulfate (SDS). Two scans with a scan speed of 10 nm/min were averaged for each peptide. CD spectral results were expressed as the mean residue ellipticity and the α-helix content was calculated from the mean residue ellipticity [θ] at 222 nm [18].

Antimicrobial Assays

Antimicrobial activity was expressed as the minimal inhibitory concentration (MIC), which is defined as the lowest concentration of peptides that completely inhibits microbial growth. MIC of peptides against microbes was determined by a standard microdilution method using sterile 96-well microtiter cell-culture plates [19].

Single colonies of bacteria were inoculated in Lauria–Bertani broth and cultured overnight at 37 °C (fungal strains in potato dextrose broth (PDB), 28 °C). An aliquot of each was transferred to 10 ml of fresh culture medium and incubated for additional 3–5 h at 37 °C to obtain mid-logarithmic-phase organisms. Then, 50 µl of a set of two-fold serial dilutions of peptides in 1% bactopeptone (fungal strains in PDB media) was added to 100 µl of bacterial suspension (1.0×10^6 colony-forming units per milliliter) in 96-well microtiter plates and the plates were incubated at 37 °C (fungal strains at 28 °C) for 12 h. After incubation, microbial growth was determined by the increase in the turbidity of each well measured at 630 nm using a microtiter ELISA reader. The lowest concentration that resulted in complete inhibition of growth was recorded as the 100% MIC. Each MIC was determined from two independent experiments performed in triplicate.

Analysis Tools

The following strategy was adopted:

Protein database:
Swiss-Prot http://us.expasy.org
Sequences search:
SRS v 5.1.0 http://us.expasy.org/ srs/
Sequence comparison:
1. IBM Pattern Discovery Tools: Multiple Sequence Alignment http://cbcsrv.watson.ibm.com/Tmsa.html
2. NPS@(Network Protein Sequence @nalysis in Hierarchical Neural Network)http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_n n.html

Protein chemicophysical characteristics analysis:
ProtParam: http://us.expasy.org/tools/protparam.html
Transmembrane-prediction analysis:
PredictProtein: http://cubic.bioc.columbia.edu/predictprotein/

Table 1 The sequences of AMP-A/B/C/D

AMP-A	KYFVVLVV**LAL**I**L**AIGIKKIGKKLEGVGKRVAVIS**AAP**A**VA**LVGQAAALAN
AMP-B	KYFVVLVV**AGK**I**A**AIGIKKIGKKLEGVGKRVAVIS**DSH**A**KR**LVGQAAALAN
AMP-C	KYFVVLVV**AGK**I**A**AIGIKKIGKKLEGVGKRVAVIS**AAP**A**VA**LVGQAAALAN
AMP-D	KYFVVLVV**LAL**I**L**AIGIKKIGKKLEGVGKRVAVIS**DSH**A**KR**LVGQAAALAN

The bold and underlined indicate different substitutions of amino acid residues.

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Analysis software for protein structures and chemicophysical characteristics:

Structure and electric field simulation software: ArgusLab4.0.1.

Protein analysis software: Anthewin4.3: Peptool 2.0(Demo version).

RESULTS

Antimicrobial Activities

The antimicrobial activities of the designed peptides against bacteria and fungi were determined by measuring their MIC (Table 2). AMP-C exhibited good antibacterial activity against both Gram-negative and Gram-positive bacteria tested with MIC ranging between 6.2 and 25 μg/ml. AMP-B also exhibited activity against all the bacteria tested, but AMP-C was slightly more active compared to AMP-B with the exception of *Lactic acid galactococcus*. AMP-D also had a moderate activity against the bacterial strains tested, except for *S. typhymurium*, against which it did not show any activity. Interestingly, they clearly possessed as excellent and broad spectrum of activities against bacteria as the naturally occurring peptides. Among all the bacteria tested, AMP-B/C/D showed much more potent activities against *Staphylococcus aureus* compared to other bacteria. AMP-A was almost inactive against most of the bacteria tested; only a very weak activity was exhibited against *B. subtilis* and *S. aureus*. Comparing the activities of AMP-B/C/D against Gram-negative and Gram-positive bacteria tested, we could find that all the three antibacterial peptides displayed outstanding activities against Gram-positive bacteria as against Gram-negative bacteria. Obviously, all the four AMPs did not possess antifungal activity against *Cerevisiae fermentum, Blue mold* and *Aspergillus niger*.

Chemcophysical Characteristics Analysis of the Designed Peptides

Chemicophysical parameters of the designed peptides were analyzed by ProtParam and the parameters, *viz.*

Table 2 Minimal inhibitory concentration (MIC) for four AMPs against different microorganisms

Microorganisms	G^+/G^-	MIC(μg/ml)[a]			
		AMP-A	AMP-B	AMP-C	AMP-D
Escherichia coli	G^-	>200	25	25	25–50
Salmonella typhymurium	G^-	>200	25–50	25	>100
Bacillus subtilis	G^+	100–200	12.5–25	12.5	25
Staphylococcus aureus	G^+	100–200	12.5	6.2	12.5
Bacillus thuringiensis	G^+	>200	12.5	6.2–12.5	25–50
Bacillus acidi lactici	G^+	>200	12.5–25	12.5	12.5
Lactic acid galactococcus	G^+	>200	12.5	12.5–25	12.5–25
Streptococcus riridans	G^+	>200	12.5	12.5	25
Cerevisiae fermentum	—	>200	>200	>200	>200
Blue mold	—	>200	>200	>200	>200
Aspergillus niger	—	>200	>200	>200	>200

[a] Each MIC was determined from two independent experiments performed in triplicate with a standard deviation of 17%.

Table 3 Results of the chemicophysical characteristics analysis

Peptides	Molecular mass (Da)	pI	pI in the functional domain	GRAVY[a]	Net charge[b]	Aliphatic index
AMP-A	5126.3	10.39	9.70	1.282	+6	164.51
AMP-B	5257.3	10.53	10.0	0.541	+8	131.96
AMP-C	5043.2	10.47	10.0	1.010	+9	143.53
AMP-D	5340.5	10.46	10.0	0.814	+7	152.94

[a] Peptide GRAVY index.
[b] Estimated at physiological pH.

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molecular mass, theoretical isoelectric point, isoelectric point in the functional domain, GRAVY, net positive charge and aliphatic index, are shown in Table 3. According to aliphatic index, mostly based on the content of the four aliphatic amino acids (Ala, Val, Leu and Ile) in the polypeptide, we can conjecture the heat stabilities of the AMPs. GRAVY index represents the hydrophilicity of the polypeptides. Analytical results of the chemicophysical parameters indicated that they were all cation polypeptides at physiological pH, and possessed good heat stabilities.

Analysis of Secondary Structure of the Designed Peptides

Bioinformatics has sped up the progress of protein structure prediction and provided a new platform for protein studies. Secondary structure compositions of the designed peptides had already been analyzed by hierarchical neural network (HNN). The results indicated that secondary structure of four polypeptides was mostly α-helix, 64.71, 56.86, 52.94, and 66.67% of α-helix for AMP-A/B/C/D, respectively. Statistical results (data not shown) of the secondary structural content of different cecropin AMPs and those in the literature [20–22] suggest that a very important characteristic in many AMPs is the content of α-helix exceeding 50%.

The secondary structure of linear antibacterial peptides in lipid membranes must correlate well with the activity. To investigate the secondary structure of the peptide in lipid membranes, the CD spectrum was measured in a membrane-mimic condition – in the presence of TFE/H_2O (50 : 50, v/v) or SDS micelles. As shown in Figure 1(A), the CD spectrum of AMP-C exhibited double minimum bands at 207 and 221 nm, which indicated that AMP-C adopted a well-defined α-helical structure in the presence of 50% TFE. The CD spectrum of AMP-B showed a slight shift in its minima to lower wavelengths. It is likely that AMP-B adopted a coil and α-helical structure simultaneously. According to the α-helix calculated from the molar ellipticity at 222 nm, all the peptides had considerable α-helix in the presence of 50% TFE. In the presence of SDS micelles, all the peptides had slightly different secondary structures, as shown in Figure 1(B). The CD spectrum of AMP-C measured in this condition was similar to that of AMP-C measured in the presence of TFE; AMP-C adopted α-helical structure in the presence of either TFE or SDS micelles. The band minimum at 230 nm indicated that AMP-A must have considerable β-sheet structures in the presence of SDS micelles compared to the secondary structures in the presence of 50% TFE. The band minima at 208 and 230 nm indicated that AMP-D had β-sheet and α-helical structures. AMP-B adopted coil and α-helical structures simultaneously in the presence of SDS micelles.

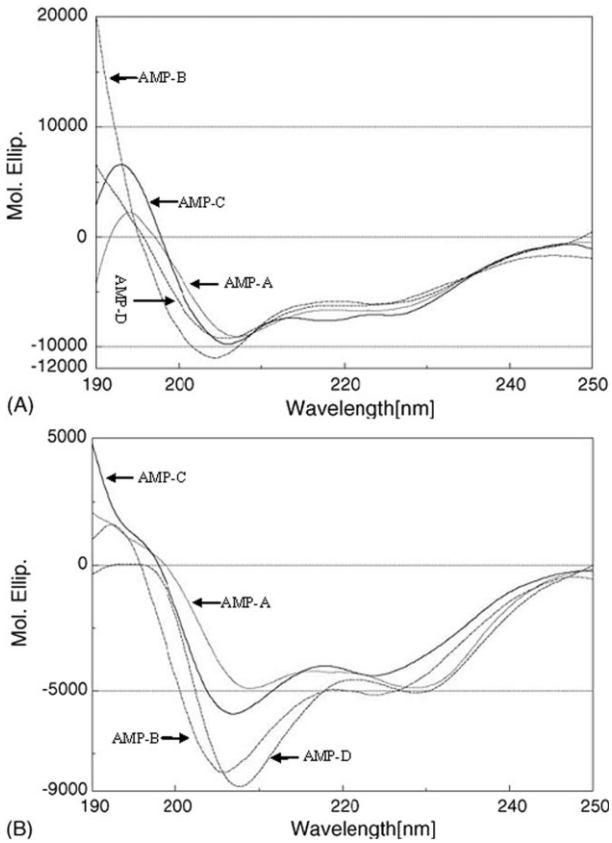

Figure 1 Circular dichroism (CD) of peptides in the presence of TFE or SDS micelles. CD spectra were measured at the concentration of sample in 10 mM sodium phosphate buffer (pH 7.4) including (A) 50% TFE (v/v) or (B) 25 mM SDS.

The exact distribution of secondary structure in polypeptide chains had also been further predicted by NPS@. The secondary structure of the designed polypeptides could be mimicked by the biology software ArgusLab 4.0 (as shown in Figure 2). The analytical results of two software indicated that the region of residues$^{8-21}$ and residues$^{37-48}$ were two primary α-helix domains in the polypeptide chain. In the AMP-A chain, Pro^{38} is located at the initial site of α-helix domain, which resulted in a distinct reverse-turn region on the *C*-terminal; hence the configuration of AMP-A evidently differed from the other three designed peptides.

The peptides of interest had been submitted to alignment tool (MAXHOM alignment) for multiple sequence alignment with position-specific iterated BLAST (PSI-BLAST), as presented in Table 4. The results of alignment indicated that the *C*-terminal sequences of the designed peptides and cec2-manse (WNPFKELERAGQRVRDAVISAAPAVATVGQAAAIARG) from the cecropin family, an antimicrobial peptide in tobacco hornworm with powerful antibacterial activity against many Gram-negative and Gram-positive bacteria [23], had very high homology, 70.0, 55.2, 70.0, and 55.2% of homology rate for AMP-A/B/C/D, respectively.

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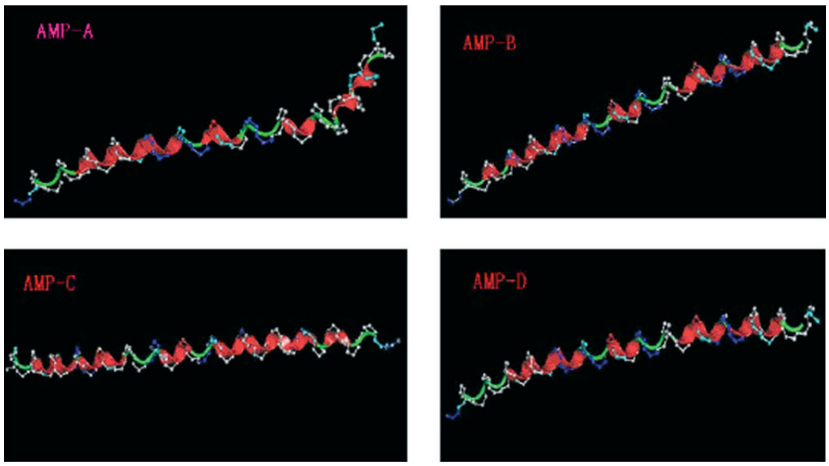

Figure 2 ArgusLab4.0 mimics of the secondary structure of AMP-A/B/C/D. AMP-A had a distinct reverse-turn region on the *C*-terminal.

Table 4 Alignment of sequence homology of designed peptides and cec2-manse

AMP-A	1 predict_h4970 100.0% KYFVVLVVLALILAIGIKKIG**K**K**LE**GV**G**K**RVAVISAAPAVA**LV**GQ**AAA**L**A**N
	2 cec2-manse 70.0%—**K**E**LE**RA**G**Q**RV AVISAAPAVA**TV**GQ**AAA**I**A**R
AMP-B	1 predict_h4270 100.0% KYFVVLVVAGKIAAIGIKKIG**K**K**LE**GV**G**K**RVAVIS**DSH**A**KRL**VGQ**AAA**L**A**N
	2 cec2-manse 55.2%—**K**E**LE**RA**G**Q**RV AVIS**AAP**A**VAT**VGQ**AAA**I**A**R
AMP-C	1 predict_h4440 100.0% KYFVVLVVAGKIAAIGIKKIG**K**K**LE**GV**G**K**RVAVISAAPAVA**LV**GQ**AAA**L**A**N
	2 cec2-manse 70.0%—**K**E**LE**RA**G**Q**RV AVISAAPAVA**TV**GQ**AAA**I**A**R
AMP-D	1 predict_h4500 100.0% KYFVVLVVLALILAIGIKKIG**K**K**LE**GV**G**K**RVAVIS**DSH**A**KRL**VGQ**AAA**L**A**N
	2 cec2-manse 55.2%—**K**E**LE**RA**G**Q**RV AVIS**AAP**A**VAT**VGQ**AAA**I**A**R

The bold and underlined indicate homologous sequences of the *C*-terminals between AMP-A/B/C/D and cec2-manse.

The results of homology comparison and secondary structure prediction suggested that the designed peptides clearly possessed the characteristics of a membrane-bound protein. Consequently, we inferred that the designed peptides should belong to membrane-bound AMPs and further analyzed the transmembrane domains of the polypeptides.

Transmembrane Predictions of the Designed Peptides

The transmembrane domains of the designed peptides had been predicted by PredictProtein, the protein structure prediction software located at bioinformatics website ExPASy (http://www.expasy.ch/), the results of which are shown in Table 5. The results of transmembrane analysis revealed that variant amino acid residues in all the four polypeptides consisted of transmembrane domains, which were the likely reason for the difference in the activity among these peptides.

Analysis of the Relationship between Antibacterial Activity and Amphipathy, Hydrophobicity of the Designed Peptides

The antimicrobial effect of many of the membrane-active AMPs is exerted via permeabilization of the target membrane, due to the formation of pore-like

Table 5 The results of transmembrane domain predictions of the designed peptides

AMP-A	**KYFVVLVVLALILAIGIKKIGKKLEGVGKRVAVISAAPAVALVGQAAALAN**
AMP-B	**KYFVVLVVAGKIAAIGIKKIGKKLEGVGKRVAVISDSHAKRLVGQAAALAN**
AMP-C	**KYFVVLVVAGKIAAIGIKKIGKKLEGVGKRVAVISAAPAVALVGQAAALAN**
AMP-D	**KYFVVLVVLALILAIGIKKIGKKLEGVGKRVAVISDSHAKRLVGQAAALAN**

The letters in yellow represent transmembrane domains and the gray the fold domain.

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structure or other membrane-disrupting modes of organization of the peptides [24–29]. The cecropins and most analogs can all induce the formation of big transmembrane pores in artificial lipid-bilayer-mimicking bacterial membranes at physiological pH. Their amphipathic α-helix structure and hydrophobic terminal are prerequisites for maintaining activity. Both amphipathy of transmembrane domain (*N*-terminal) and hydrophobicity of *C*-terminal of the designed peptides had already been analyzed by the protein analysis software Antherwin4.3. The results indicated that the *N*-terminals of the four peptides had certain amphipathy, whereas the *N*-terminal of AMP-B/C had more remarkable amphipathic character than that of AMP-A/D, with the replacement of hydrophobic residue Leu^{11} by hydrophilic residue Lys^{11} on the hydrophilic face, as described in Figure 3. The *N*-terminal of AMP-A/D had considerable hydrophobicity because of the presence of more hydrophobic residues. The substitution of hydrophilic residues Asn, Ser, His, Lys, and Arg on the *C*-terminal of AMP-B/D by hydrophobic amino acids Ala, Ala, Pro, Val and Ala at position 36, 37, 38, 40, and 41 resulted in the increase of hydrophobicity and the decrease of amphipathic character of the *C*-terminal of AMP-A/C, as shown in Figure 4. AMP-C showed more powerful activity against most of the bacteria tested, owing to the more prominent amphipathic character of its *N*-terminal and hydrophobicity of the *C*-terminal than the other designed peptides. Though the amphipathic character of the *N*-terminal and hydrophobicity of the *C*-terminal of AMP-D were weaker than those of other peptides, amphipathic character of the *C*-terminal and hydrophobicity of the *N*-terminal were outstanding. This result demonstrated that antibacterial peptides possessing an amphipathic *C*-terminal and a hydrophobic *N*-terminal, as in the case of AMP-D, likewise had an antibacterial activity. Consequently, we could further infer that the amphipathic helical structure and the hydrophobic terminal of AMPs were pivotal factors to maintain activity.

Analysis of the Relationship Between Electric Field Distribution and Activity of the Designed Peptides

High net positive charge is generally regarded as a prerequisite for antibacterial activity because lipid membranes of bacteria are negatively charged and

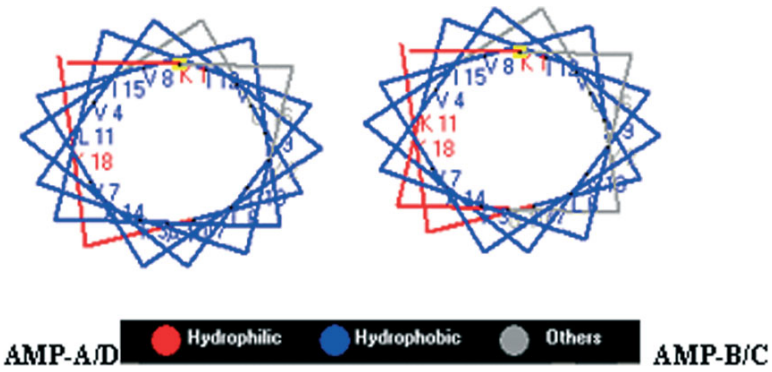

Figure 3 Helical wheel diagram of the *N*-terminals of the four peptides. AMP-A and AMP-D possess same *N*-terminal. AMP-B and AMP-C have the same *N*-terminal. The red lines are hydrophilic faces and the blue lines hydrophobic faces. The substitution of Leu with Lys at position 11 resulted in the increase of amphipathic character of *N*-terminal of AMP-B/C.

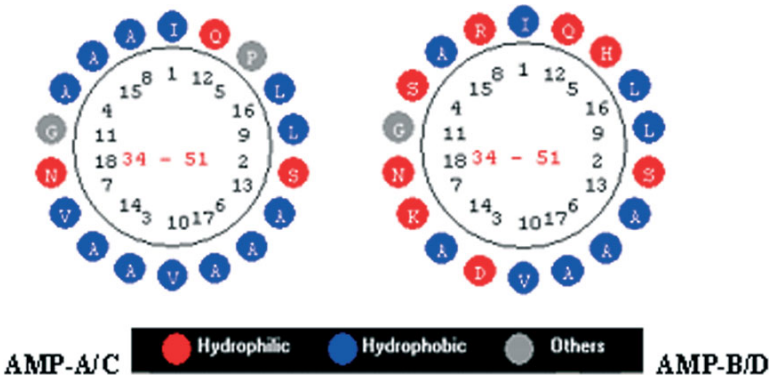

Figure 4 Helical wheel diagram of the *C*-terminals (amino acid residues$^{34-51}$) of the four peptides. AMP-A and AMP-C possess the same *C*-terminal. AMP-B and AMP-D have the same *C*-terminal. The red filled circles are hydrophilic amino acids and the blue filled circles are the hydrophobic amino acids.

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charge–charge interaction between cationic antibacterial peptides and bacterial membranes is critical for peptide binding to target lipid membranes [30,31]. We searched for partial AMPs of artificial design reported in the literature [3,20,32] and found that they had a net positive charge, ranging from +2 to +8. There was no comparability among the amino acid sequences of these designed peptides, but they all exhibited powerful antibacterial activities and their structures were known to share two common features: a net positive charge and an amphipathic secondary structure, as most natural AMPs do.

According to molecular electroporation theory, the electrostatic potential on the molecular surface plays an important role during the electrostatic bonding between the AMPs and the negatively charged bacterial cell membrane. With the software Arguslab3.0 and the quantum mechanical QM-ZINDO method, we computed the electrostatic potential and electron density, respectively, of the *N*-terminals of the designed peptides (Figure 5). The results of the comparison revealed that the cation property of the *N*-terminal of AMP-B/C was obviously much superior to that of AMP-A/D, because of the alkaline amino acid residue Lys^{11} on AMP-B/C chain compared to the nonpolar amino acid residue Leu^{11} on the AMP-A/D chain. The electric field of AMP-B/C formed an arc, which made the scope of action of their positive electric field much broader than that of AMP-A/D, augmenting the interaction area and bonding force between the AMPs and bacterial cell membranes. That AMP-C had more net positive charge (+9) than AMP-B (+8) was probably one reason causing the activity of AMP-C better than that of AMP-B.

DISCUSSION

In present study, we determined the antimicrobial activities and predicted secondary structure, amphipathic property, electric field distribution, and transmembrane domain of four designed peptides. All the four designed peptides were inactive against the fungus tested. The composition of the bacterial cytoplasmic cell membrane is rich in acidic phospholipids, whereas the plasma membrane of fungal cells contains a much higher proportion of zwitterionic phosphatidylcholine and sphingomyelin phospholipids [10]. Consequently, an increase in peptide cationicity should promote interaction with the more negatively charged bacterial cell membrane and increase antimicrobial potency. The positive charge on the peptide is also believed to facilitate interaction with, and passage across, the bacterial cell wall, both in the case of Gram-negative bacteria that contain negatively charged lipopolysaccharide and of Gram-positive bacteria that contain negatively charged teichoic and teichuronic acids [33]. All the active peptides showed more potent activity against Gram-positive than Gram-negative bacteria. This result may be explained by the differences in the composition of bacterial lipid membranes. For example, the lipid membranes of *S. aureus* among Gram-positive bacteria mainly consisted of negatively charged lipids such as phosphatidyl glycerol and cardiolipin, whereas the outer lipid membranes of *E. coli* among Gram-negative bacteria contained an uncharged lipid, phosphatidyl ethanolamine [34]. According to the composition of lipid membranes, Gram-positive bacteria have more negatively charged lipid membranes than those of Gram-negative bacteria. Thus, the peptides showed a more potent activity against the Gram-positive bacteria by increasing charge–charge interaction between the peptide and the lipid membranes with a more negatively charged surface.

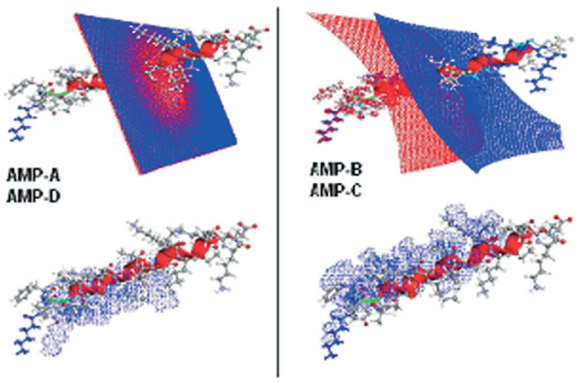

Figure 5 Electrostatic potential and electron density of *N*-terminals of polypeptides. AMP-A and AMP-D possess same *N*-terminal. AMP-B and AMP-C have the same *N*-terminal. Blue indicates positive charge and red indicates negative charge.

It is generally agreed that the structure of a protein is more highly conserved than its sequence. The statistical results of the secondary structure content of 62 cecropin AMPs searched revealed that the secondary structure of cecropins mainly existed with the α-helix conformation, approximately 61.83, 30.33% for random coil and 7.79% for β-sheet. With different structure prediction methods, we found that the secondary structure of four polypeptides was mostly α-helix, and the result of CD spectrum also showed that all peptides had considerable α-helix in the presence of either 50% TFE or SDS micelles. It is reported that the secondary structure of a majority of membrane-bound antibacterial peptides is based on the amphiphilic α-helix. The importance of amphiphilic helices has been implicated in a wide variety of biologically active polypeptides whose function is related to the binding of lipids and cell membranes [8].

There is no universal agreement regarding the precise mechanism by which bacterial cell death occurs, and it is clear that no single mechanism is applicable to all peptides and bacteria. Generally, cationic peptides exert their activities in a first step process by parallel binding to the microbe membrane's surface with

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electrostatic interaction between the positively charged domain and the negatively charged components of the bacterial membrane. Then the peptides oligomerize and reorient so that the hydrophobic domain inserts into the hydrophobic core of the lipid bilayer, leading to membrane permeabilization and eventual disintegration into peptide-coated vesicles. Membrane collapse may be facilitated by the formation of transient transmembrane holes or pores that will allow passage of low molecular mass molecules, generating osmotic imbalance. In addition, peptides may exercise a growth-inhibitory action, independent of membrane destruction, by interacting with intracellular targets to inhibit macromolecular synthesis after penetration across the cell membrane [27,35]. According to these mechanisms of action, the amphipathic helix structure in transmembrane domain of the designed peptides has played a very important role for maintaining activity. Commonly, the length of the transmembrane domain required is at least 15 amino acid residues; otherwise they cannot make up transmembrane sequences. The results of transmembrane predictions indicated that the designed peptides possessed two transmembrane sequences consisting of 18 residues, which matched the structural character of cecropins.

We had found that the remarkable hydrophobicity and the weak amphipathy on two terminals of AMP-A might be one reason causing almost inactivity. In addition, the net positive charge of AMP-A was +6, but the distribution of positively charged residues on the whole peptide chain appeared relatively dispersed, easy to be effected by the lateral chain of the surrounding residues and hard to form effective positive electric field acting with bacterial cell membrane. The helix structure on two terminals of AMP-B had prominent amphipathy, analogous to the structure of the artificial designed peptide CE-MA (KWKLFKKIGIGKFLHSAKKF-NH_2), cecropin A (1–8)-magainin2 (1–12), a hybrid peptide bonding eight residues on the *N*-terminal of cecropin A with 12 residues on the *C*-terminal of magainin 2 [36]. This hybrid peptide had put up a very potent antibacterial activity without hemolytic activity. The present research revealed that the action mechanism of CE-MA was different from the pore-like model of cecropin, CE-MA permeabilizing the outer membrane of the bacterial cell and bonding lipopolysaccharide with high affinity. The secondary structure of AMP-B and CE-MA were similar, though with lower sequence homology. Here, we inferred that the action mechanism of AMP-B might be similar to that of CE-MA owing to structure conservation rather than sequence conservation of AMPs. Also, the structural property of the polypeptide was influenced by too many redundant amino acids in the peptide chain, which probably resulted in the moderate activity of AMP-B. With the remarkable amphipathic α-helix of the *N*-terminal and hydrophobic α-helix of the *C*-terminal, just like in cecropon's structure, and augmentation of positive charge after the substitution of the nonpolar residue Leu by the alkaline residue Lys at position 11 on *N*-terminal, AMP-C possessed a much higher activity than other designed peptides. The structure of AMP-D was a little special with weaker amphipathic α-helix on the *N*-terminal, mainly showing hydrophobicity, and good amphipathic character on the *C*-terminal owing to more alkaline residues. This kind of structure was quite contrary to cecropin's structure, but AMP-D likewise exhibited certain activity. Therefore, we deduced that the action of membrane-bound AMPs to bacteria was independent of the distribution of functional group whether on the *N*-terminal or the *C*-terminal.

In conclusion, we synthesized four analogs designed to study the structure–activity relationship of cecropins. The results of our investigations suggest a new strategy for the rational design of AMPs. It consists of a first fictive decomposition, to single conserved sequences of homologous polypeptides with well-known bactericidal properties, followed by peptide piecing together and combination of different conserved sequences. Some simple considerations based on amphipathy and net positive charge of the linear polypeptides may be sufficient to design AMPs with considerable bactericidal activity. In addition, the present peptide chain was so long that the redundant residues appended on the *N*-terminals might increase the flexibility and wobble of the *N*-terminals, and too long sequences would make the positive charge on the amphipathic face more dispersive, which work against the action on bacterial cell membrane. Consequently, it is necessary to consider further reduction in chain length of the peptides.

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