Original Research

Synthesis and secretory expression of hybrid antimicrobial peptide CecA–mag and its mutants in *Pichia pastoris*

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Abstract

The hybrid peptide CA(1–7)–M(2–12) gene was designed according to the N-terminal 1–7 amino acid sequence of the antimicrobial peptide cecropin A (CA) and the N-terminal 2–12 amino acid sequence of maganin (M) and synthesized using *Pichia pastoris* preferred codons. The gene was cloned into pPICZαA and transformed into the *P. pastoris* recipient bacterium SMD1168, regulated by the alcohol oxidase (AOX). Expression of the cecA-mag hybrid antimicrobial peptide (MW, 1.9 kDa) revealed broad-spectrum antibiotic activity and to the ability to inhibit growth of most G^- and G^+ bacteria. Three mutants of cecA-mag were designed and synthesized by recombination polymerase chain reaction site-directed mutagenesis to investigate the relationship between the structure and function of this antimicrobial peptide. The inhibition titers of these mutants against *Staphylococcus aureus* were evaluated using the agar diffusion method. Under the conditions of the same concentration and volume, the bacteriostatic diameters of three cecA-mag mutants were 1.2, 1.2 and 1.5 times, respectively, compared with the diameters of wild-type cecA-mag.

Keywords: hybrid antimicrobial peptide CecA-mag, *Pichia pastoris* secretary expression, bacteriostatic activity

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Introduction

Antimicrobial peptides form an important component of insect immunity.[1,2] Cecropin A was the first antimicrobial peptide to be separated from *Hyalophora cecropia,* and exhibits a broad spectrum of bactericidal activity, high-temperature resistance and is not associated with toxicity or side-effects in eukaryotic cells.[3,4] The N-terminal region, which is critical for the activity of this peptide, is strongly alkaline and forms an amphipathic α-helical structure. Cecropin A is composed of 37 amino acid residues and is likely to be immunogenic. Therefore, this peptide is not an ideal candidate for antibacterial drug development.

Magainin is an alkaline antimicrobial peptide consisting of 21–27 amino acid residues without cysteines and has been identified from *Xenopus laevis*. These types of antimicrobial peptides have a broad spectrum of resistance and are hydrophilic in character with lower hemolytic activity than the lytic peptides melittin and bombinin. Magainin destroys Gram-positive and Gram-negative bacteria, fungi and protozoa, and even eukaryotic tumor cells at micromolar concentrations, without damaging somatocytes and red blood cells.[5–7]

The magainin antimicrobial peptides consist of fewer amino acids and therefore are more suitable for development as a treatment for bacterial, fungal or protozoon–protozoan infections in humans. Recombinant magainin is currently in phase-three clinical trials. Therefore, identification of short peptides with broad-spectrum antibacterial activity that are non-toxic in humans represents a critical focus of research. Bacterial resistance to antibiotics has increased dramatically with their wide application. Consequently, generation of synthetic antimicrobial peptides with high activity represents a new challenge in the development of novel antibiotics.

To address this issue, a hybrid peptide CA (1–8)–M (2–12) gene was designed and synthesized to encode the N-terminal 1–7 amino acid sequence of the antimicrobial peptide cecropinA (CA) and the N-terminal 2–12 amino acid sequence of maganin (M). The product, referred to as cecA-mag, is an 18-amino acid hybrid polypeptide. The recombinant gene was cloned into the *Pichia pastoris* expression vector, pPICZα-A, and expressed for the evaluation of peptide function and activity.

Greater understanding of the structure, function and mechanism of antimicrobial peptides has led to attempts

to design novel antibiotics with improved and broader spectrum activity. Jaynes[8] designed and synthesized Shiva-1 using cecropin B as a blueprint and Fink *et al.*[9] chemically synthesized the hybrid antimicrobial peptide, AD, using a solid-phase method. The bactericidal activities of both peptides were higher than those of the natural peptides with broader antimicrobial spectra. The hybrid antimicrobial peptides designed and synthesized in this study, maintained the broad antimicrobial spectrum of cecropin A, and acquired the antibacterial activity of magainin while abrogating its cellular toxicity. Bacteriostatic analysis demonstrated that this peptide dramatically inhibited the growth and replication of *Staphylococcus aureus.* However, the bacteriostatic activity of this hybrid antimicrobial peptide was slightly lower in the bacteriostatic titration assay when compared with other antibiotics such as penbritin. This study aimed to identify improvements in the bacteriostatic activity of this hybrid antimicrobial peptide, by examining a number of mutants designed to improve the antibacterial activity of the hybrid cecA-mag polypeptide.

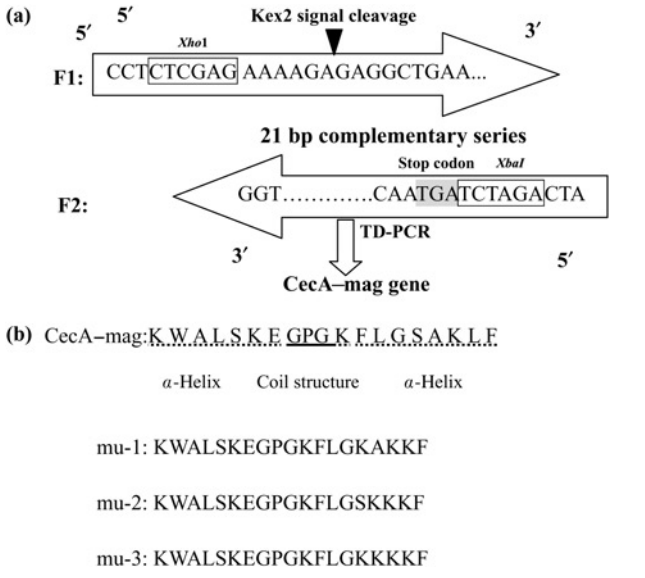

Figure 1 (a) Schematic representation of the strategy for the production of CecA-mag; (b) amino acid sequence and structure of CecA-mag and mutants

Methods and materials

Materials and reagents

Plasmids, the recipient bacterium indicator strain, *Pichia Saccharomyces pastorianus Pichia pastoris* recipient bacterium, and SMD1168 (His/Mut$^+$) were provided by Nanjing Agricultural University. The pPICZα-A expression vector was purchased from Invitrogen (Shanghai, China). Primers F1, F2, P1 and P2 were synthesized by Invitrogen. High-fidelity Pyrobest DNA polymerase, T4 DNA ligase, dNTPs and restriction endonucleases were purchased from Takara Biotechnology Co Ltd (Dalian, China). Tricine was purchased from Sangon Biotech Co Ltd (Shanghai, China). Zeocin was purchased from Invitrogen. Other reagents were all of analytical grade.

Design of the cecA-mag antimicrobial peptide

The strategy for the design of the cecA-mag antimicrobial peptide is illustrated in Figure 1a. The gene sequences of the cecA-mag hybrid peptides were designed based on the sequences of the cecropin A mature peptide (1–7) and magainin (2–12) in GenBank (accession number: Xo6672, J03193), using yeast-preferred codons and the signal peptide cleavage site. Kex2 was added at the N-terminal to ensure that the expressed peptides possessed the natural N-terminal. Primers F1 and F2 were designed to amplify the gene sequence of the recombinant cecA-mag hybrid peptides:

F1: 5′ CCTCTCGAGAAAAAGAAGTGGAAGCTGTTCA AGAAGATCGGTCCAGGTAAG 3′
F2: 5′ CTATCTAGAGAACTTCTTGGCACTGTGCAGGA ACTTACCTGGACCGATCTTCTT 3′

A 21-base complementary series was included in the 3′ ends of both primers. The *Xho*I and *Xba*I restriction endonuclease sites were designed in the 5′ and 3′ primers of the cecA-mag hybrid gene. Gene splicing by overlap extension (SOE) was used for gene synthesis.

Design of CecA-mag antimicrobial peptide mutants

It is important to ensure that the amino acid composition within the structural domain is retained by the design strategy adopted for reconstruction of the antimicrobial peptide in order to maintain their steric configuration. The antimicrobial peptide gene and mutants are illustrated in Figure 1b. The physicochemical properties of hybrid peptides and their mutants were analyzed with DNAStar 5.0 Software (Lasergene System; DNAStar, Madison, WI, USA). In comparison with the original hybrid peptide, the mutants exhibited changes in the degree of α-helix structure, amphipathic characteristics, the flexible structure of the central connecting region and isoelectric points (pI). The pI values for mutants 1–3 were predicted as 10.778, 10.85 and 10.90, respectively. It can be speculated that these factors are responsible for the observed changes in the antibiotic activities of the mutant antimicrobial peptides.

Synthesis of hybrid antimicrobial peptide gene

A pair of primers was designed for identification of the recombinant vector and yeast. The upstream primer targeted the 5′ AOX gene region of *P. pastoris,* and the downstream primer targeted the insertion region of CecA-mag. The amplification length was 496 bp. The primer sequences were as follows:

P1: 5′ GTCTCCACATTGTATGCTTC 3′
P2: 5′ CTGTGCAGGAACTTGAT 3′

F1 and F2 fragments were used as templates and primers for polymerase chain reaction (PCR) amplification using the SOE method and Touchdown PCR (TD-PCR)[10] was used to optimize the PCR conditions to ensure specificity. The TD-PCR reaction mixture (50 μL) contained: 5 μL 10$\times$ PCR buffer (Mg^{2+} free), 3 μL $MgCl_2$ (25 mmol/L), 1 μL dNTPs (10 mmol/L), 2 μL F1 and F2 (40 pmol/L), 0.5 μL Takara ExTaq^{TM} and 38.5 μL double-distilled water. The reaction

mixture was vortexed and centrifuged briefly prior to the TD-PCR procedure according to the following conditions: pre-denatured at 94°C for two minutes followed by 30 cycles of: 94°C 30 s, annealing temperature decreasing from 65 to 50°C with a gradient of 0.5°C/min, and extension at 72°C for one minute. Subsequently, the temperature was reduced to 50°C and a further 15 cycles performed at the optimal annealing temperature of 52°C with a final extended incubation at 72°C for six minutes. TD-PCR products (1.0 μL) were separated to 1.5% agarose for electrophoresis and the results were observed and recorded under the gel documentation system.

Construction of recombinant yeast expression vectors (*P. pastoris* expression system)

PCR products and pPICZαA were digested with *Xho*1 and *Xba*1 prior to ligation with T4 DNA ligase. Constructs were transformed into *Escherichia coli* DH5α. Recombinant expression vectors were identified by PCR analysis and restricted endonuclease digestion.[11] Positive plasmids were sequenced to confirm the identity of pPICZα-CA (Invitrogen).

Synthesis of CecA-mag peptide mutants

Mutants of the CecA-mag peptide were synthesized from the pPICZα-CA template by PCR using the following primers:

Universal primer P: 5′ CTAGTCTAGAACAAAAACTC 3′
Mutant primer P1: 5′ AACTTCTTGGCCTTGTGCAGG AA 3′
Mutant primer P2: 5′ AACTTCTTCTTACTGTGCAGG AA 3′
Mutant primer P3: 5′ AACTTCTTCTTCTTGTGCAGG AA 3′

The reaction mixture was composed of: 5 μL 5× Prime STARTMHS DNA Polymerase (Takara Biotechnology), 1 μL dNTPs, 10 mmol/L, 1 μL template, 2 μL each of universal primer P and specific primer P1 to amplify pPICZα-CA-Mu-1, 40 pmol/L, 0.5 μL PrimeSTAR™ DNA Polymerase (Takara Biotechnology) and 38.5 μL double-distilled water. The following PCR conditions were used: pre-denatured at 94°C for two minutes followed by 30 cycles of: 94°C for one minute, annealed at 65°C for one minute, 72°C for two minutes, with a final extension incubation at 72°C for 10 min. Fragments of approximately 3700 bp were amplified and recovered from agarose gel with a kit (Promega Wizard SV Gel and PCR Clean-up System; Promega Biotechnology Co Ltd, Shanghai, China) according to the instructions provided by the manufacturer. The fragments were cloned into pPICZαA, transformed into *E. coli* DH5α and identity confirmed by sequencing. Three recombinant expression plasmids were selected corresponding to the CecA-mag hybrid peptide mutants, pPICZα-CA-Mu-1, pPICZα-CA-Mu-2 and pPICZα-CA-Mu-3.

Transformation of *P. pastoris* with pPICZα-CA and mutants

Competent *P. pastoris*, SMD1168 (His/Mut$^+$) (80 μL) was transformed by electroporation (1.5 kV, 25 μF, 200 Ω for 5 ms) with *Sac*I linearized pPICZαA-CA and mutants (5 μg) using a Pichia Expression Kit (Invitrogen) according to the instructions provided by the manufacturer. Precooled sorbierite (1 mL, 1 mol/L) was added to the electroporation mix and 200 μL cultured onto YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1 mol L^{-1} sorbitol, 2% agar) plates at 30°C until the appearance of single colonies.

Positive *P. pastoris* transformants were identified by PCR production of a 460-bp fragment. Templates were prepared by the alternating freeze–thaw method[12] and the expected fragment amplified with primers P1 and P2 using the previously described reaction mixture. The PCR conditions were as follows: 94°C for five minutes; 25 cycles of 94°C 45 s; 48°C, 45 s; 72°C 45 s, followed by 72°C for six minutes.

Induced expression of *P. pastoris*

The selected positive colony was inoculated into 5 mL BMGY medium (1% yeast extract, 2% peptone, 100 mmol/L potassium phosphate, pH 6.0, 1.34% YNB [1.34% yeast nitrogen base with ammonium sulfate without amino acids], 4×10^{-5}% biotin, 1% glycerol) and cultured in a shaking incubator at 30°C (speed 230 rpm) for 22 h (A_{600} 3–6). Cultures were centrifuged at room temperature at a speed of 3000 rpm for two minutes and the cells were re-suspended in 25 mL BMMY medium (1% yeast extract, 2% peptone, 100 mmol/L potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol) to induce peptide expression. Cells were cultured in a shaking incubator at 28°C (speed at 250 rpm) for 60 h, with the addition of methanol every 24 h to a final concentration of 1% (v/v). After 72 h, the cells were centrifuged at 5000 rpm for 10 min and the supernatant collected for analysis of bacteriostatic activity.[13]

Purification of secreted cecA-mag

Culture medium (100 mL) was collected after 60 h by centrifugation at 12,000 *g* for 10 min. The supernatant was filtered using the Amicon ultrafiltration device (Millipore Biotechnology Co Ltd, Shanghai, China). The filtrate, which contained proteins ranging from 3 to 10 kDa in size, was dialyzed overnight in 0.1 mol/L sodium acetate and then applied to a CM-Sepharose CL-6B column (Pharmacia Biosciences, Madison, WI, USA) pre-equilibrated with 0.1 mol/L sodium acetate (pH 4.5). The column was washed with 0.1 mol/L acetate buffer and the proteins were eluted using a linear gradient of 0.1–1.0 mol/L sodium acetate (pH 4.5). The purified proteins were analyzed by Tricine-sodium dodecyl sulfate-polyacrylamide electrophoresis (Tricine-SDS-PAGE).[14]

Detection of antibiotic activity of cecA-mag peptide and mutants

Antibiotic activity was analyzed by standard agar diffusion using *S. aureus Cowan I* and *E. coli K88* as test bacteria. Test bacteria (200 μL, $D_{600} = 0.2–0.3$) were mixed with 25 mL Luria–Bertani solid culture medium at 55°C and plated out for future use. Once solidified, 20 μL expression

culture supernatant was added to holes (5 mm in diameter) punched in the agar and cultured at 37°C (8–12 h). Equal volumes of supernatant from empty vector pPICZα-A-transformed yeast and ampicillin (25 mg/mL) served as negative and positive controls, respectively. The bacteriostatic diameter was measured after two days.

Results

Identification of recombinant yeast expression plasmids of cecA-mag

Identification of positive colonies by restriction enzyme digestion was made difficult by the absence of restriction sites and the small size of the inserted cecA-mag gene fragment. However, incorporation of *Xho*I and *Xba*I sites into the cecA-mag gene fragment results in loss of all restriction endonuclease sites (*Eco*RI, *Pml*I, *Kpn*I, *Not*I, *Sac*I, *Sal*I) in the recombinant between the *Xba*I and *Xho*I sites when the fragment is inserted in the correct orientation. Therefore, digestion with these enzymes was used to identify positive colonies in which the recombinant plasmids were not linearized due to the loss of these sites.

Synthesis of cecA-mag peptide mutants

The synthesized and correctly sequenced cecA-mag gene was used as a template for PCR amplification of the mutant peptides using the described universal and mutant primers. Amplification of a 3700-bp fragment was visualized by 1% agarose gel electrophoresis. The major product was slightly larger due to the use of a slightly larger template and multiple minor PCR products were also observed although the major product dominated. The correct structures of the three CecA-mag hybrid peptide mutant genes were confirmed by sequencing.

Transformation and identification of recombinant yeast

The recombinant plasmid pPICZα-A-CA and mutants were linearized with *Sac*I according to the instructions of the manufacturer (Invitrogen) for *P. pastoris*. The linearized recombinant plasmids were electro-transformed into *P. pastoris* SMD1168 competent cells to obtain Zeocin^{TM} resistant colonies. Positive colonies were identified by PCR using primers P1 and P2. Empty plasmid pPICZα-transformed yeast genomic DNA was used as a control.

Tricine-SDS-PAGE of CecA-mag antimicrobial peptide mutants

The recombinant hybrid peptides and their mutants were confirmed by optimal resolution and banding pattern visualized using a modified cine electrophoresis system (Figure 2).

Bactericidal activity of recombinant antimicrobial peptides

The hybrid peptide CecA-mag exhibited bacteriostatic and bactericidal activity against *E. coli* and *S. aureus* (Figures 3a–c).

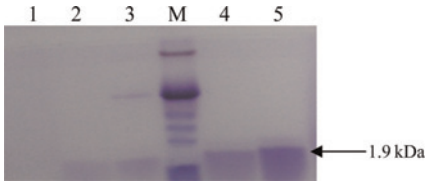

Figure 2 Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the antibacterial peptide, CecA-mag, and mutant (M). Ultra-low molecular-weight markers were used for proteins (26.6, 17.0, 14.4, 6.5, 3.5 and 1.06 kDa, respectively). Lane 1: SMD1168/pPICZαA; lane 2: SMD1168/pPICZαA-CA; lane 3: SMD1168/pPICZαA-CA-mu-1; lane 4: SMD1168/pPICZαA-CA-mu-2; and lane 5: SMD1168/pPICZαA-CA-mu-3. (A color version of this figure is available in the online journal)

Discussion

Antibiotic resistance and the development of broad-spectrum resistant bacteria represent a significant threat to human health and the ability to control resistant strains is a major scientific challenge. Antimicrobial peptides are promising biomolecules suitable for the development of substitutes for traditional antibiotics.

Direct expression of antimicrobial peptides in prokaryotic systems is not possible due to bactericidal activity. Such peptides have generally been expressed in yeast or as an inactive fusion protein for post-synthetic cleavage and activation.[15] As an eukaryotic expression system, *P. pastoris* possesses many advantages over others in the expression of exogenous genes. Yeast is an eukaryote and therefore has lower susceptibility to many anbibacterial agents. Its rapid rate of reproduction and low nutrient requirements mean that culture medium is inexpensive and suitable for

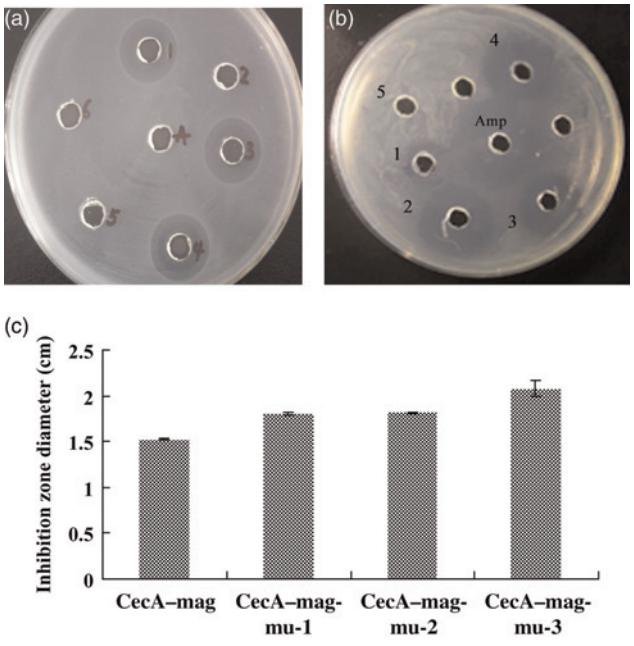

Figure 3 Antibacterial activity of CecA-mag against *Escherichia coli* and *Staphylococcus aureus*: (a) A: Amp control (25 μg/mL); 1, 3 and 4: antibacterial peptides CecA-mag; 2, 5 and 6: SMD1168/pPICZαA; (b) Amp: Amp control (25 μg/mL); 1: antibacterial peptides CecA-mag; 2: antibacterial peptides CecA-mag-mu-1; 3: antibacterial peptides CecA-mag-mu-2; 4: antibacterial peptides CecA-mag-mu-3; 5: SMD1168/pPICZαA; (c) antibacterial peptides CecA-mag and its mutant against *S. aureus*. (A color version of this figure is available in the online journal)

industrialized production. Vectors derived from this system integrate with the nuclear genome with high stability, resulting in maintenance of the exogenous gene during the production process. Furthermore, processing and modification of the expressed protein in an eukaryotic system ensure correct folding and post-translational processing that may be critical for its biological activity. *P. pastoris* allows intracellular and secreted expression of recombinant proteins, which are simple to purify due to the absence of endogenously expressed secretory proteins. The recipient yeast used in this study, *P. pastoris* SMD 1168, is a protease-deficient strain that reduces degradation of exogenous proteins and increases the half-life of secretory proteins.

Despite numerous investigations, the mechanism of antimicrobial peptide activity remains to be elucidated. The current model of antimicrobial peptide function is based on a number of reports that postulate the formation of ion channels in the cell membrane, increasing member permeability and subsequent cell death. Scott *et al*.[16] suggested that antimicrobial peptides are attracted to the surface of the cell membrane by electrostatic interactions where the hydrophobic tails of the peptides become inserted into the hydrophobic regions on the cell membrane, resulting in altered membrane configuration and ion channel formation. Fink *et al*.[9] hypothesized that only the C-terminal hydrophobic helix is inserted into the membrane, while the N-terminal amphiphilic helices only bind to the surface of the membrane. Lehrer *et al.*[17–19] speculated that the first step of the antimicrobial function occurred due to the interaction of the positively-charged peptides and the negatively-charged phospholipid groups in the membrane, followed by insertion and polymerization of the hydrophobic helices into the membrane to form the pore canal. In order to further investigate these hypotheses, three mutants of the cecA-mag hybrid peptide were designed and shown to possess marked bacteriostatic and bactericidal functions against some routinely resistant pathogenic bacterial strains.

The first seven amino acids in cecropin antimicrobial peptides are conserved and form the first α-helix. These residues were therefore not selected for mutation. The central coil forms the flexible structure of cecropin antimicrobial peptides and is associated with the activity of these peptides. It has been reported that the size of this region directly affects peptide activity and reconstruction in these areas has been performed in several laboratories. Mutation of the flexible GPG sequence to IP resulted in increased activity and was therefore selected as the target for the mutations in this study. In C-terminal reconstructions, the emphasis was placed on generation of increased positive charges without affecting the structure of the α-helix, thus facilitating secure adsorption to the surface of the bacterial membrane. The natural cecropin antimicrobial peptides have an amphipathic α-helix in the N-terminal. The positively-charged hydrophilic groups are located on one side of the vertical axis of the columniform molecule and the opposite side forms a hydrophobic region. The collapsible hinged region formed by glycine and proline is located between the N- and C-terminals. The N-terminal is positively charged at physiological concentrations, although the C-terminal is amidated.[20–23] The second amino acid, Trp, is highly conserved in all cecropin antimicrobial peptides. Cecropins exhibit structural homology and cecropin B and D possess 65% and 62% molecular homology, respectively, with cecropin A. These features are critical to the ability of antimicrobial peptides to destroy the membrane of the bacteria.

Natural cecropins exhibit broad-spectrum antimicrobial activities in *Antheraea yamamai*. Alterations in the amino acid residues in the peptide chain are associated with narrowing of the antimicrobial spectrum, while the amidate of the C-terminal results in broadening of the antimicrobial spectrum. Therefore, this investigation of the effects of modifications of the hybrid peptide was targeted to the more flexible GPG amino acids in the hinge region while maintaining the N-terminal structure for preservation of the natural α-helical structure of cecropin.

There is a direct correlation between the strength of the bactericidal activity and the positively-charged amino acid content of cecropin. The peptide domain close to the C-terminal end contains three arginine residues and one lysine through which the antimicrobial peptide binds to the surface of bacterial membrane, mainly through electrostatic adsorption. The free N-terminal of the antimicrobial peptides is inserted into the membrane, thus destroying the integrity of the membrane resulting in irreversible cell damage.[24]

In this study, three mutant forms of the CecA-mag hybrid peptides were rationally designed to increase the positively-charged nature of the peptides as well as amphiphilic activities, with the aim of increasing adsorption to the surface of bacterial membrane for increased antimicrobial activity. Furthermore, mutations were introduced at sites selected to minimize conformational changes and to avoid the main functional regions. There are four known positively-charged amino acids (arginine, lysine, proline and histidine) among the 20 naturally occurring amino acids. Cecropin is a cationic peptide rich in arginine. Three mutants of the cecA-mag hybrid peptide gene were deigned and generated by PCR site-directed mutagenesis for the analysis of the relationship between structure and function of this antimicrobial peptide. It has been reported that positively-charged oligomeric lysine groups are important in targeting drug binding to the cell membrane and have been used in broad applications.[16] Although the bacteriostatic titer of the mutants increased only marginally, the hybrid peptides were associated with higher antibacterial activities. It is clear that antimicrobial peptides represent a suitable platform for the development of drugs for use in a wide variety of applications.

Author contributions: XW, MZ and AZ carried out the experiments. FY wrote the manuscript. PC conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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REFERENCES

1 Hultmark D, Steiner H, Rasmusson T, Boman HG. Insect immunity purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of Hyalophora cecropia. *Eur J Biochem* 1980;**106**:7–16

2 Boman HG, Hultmark D. Cell-free immunity in insects. *Annu Rev Microbiol* 1987;**41**:103–26

3 Steiner H, Hultmark D, Engstrom A, Bennich H, Boman HG. Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* 1981;**292**:246–8

4 Zhang Y, Wu J, Gu L. Isolation of antifungal peptides from hemolymph of housefly larvae by gel filtration. *Chin J Vector Biol Control* 2007;**18**:200–4

5 Zasloff M. Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc Natl Acad Sci USA* 1987;**84**:5449–53

6 Zasloff M, Martin B, Chen H. Antimicrobial activity of synthetic magainin peptides and several analogues. *Proc Natl Acad Sci USA* 1988;**85**:910–3

7 Jacob L, Zasloff M. Potential therapeutic applications of magainins and other antimicrobial agents of animal origin. In: Marsh J, Goode JA, eds. *Ciba Foundation Symposium 186-Antimicrobial Peptides*. Chichester, UK: John Wiley & Sons, 1997. DOI: 10.1002/9780470514658.ch12

8 Jaynes JM. Use of genes encoding novel lytic peptides and proteins that enhance microbial disease resistance in plants. *ISHS Acta Hort* 1993;**336**:33–9

9 Fink J, Boman A, Boman HG, Merrifield RB. Design, synthesis and antibacterial activity of cecropin-like model peptides. *Int J Pept Protein Res* 1989;**33**:412–21

10 Wang T, Zhang G, Xue L. A simple and efficient modified touchdown PCR. *Prog Biotechnol* 2003;**23**:80–2

11 Sambrook J, Russell DW. *Molecular Cloning: A Laboratory Manual*. 3rd edn. NY, USA: Cold Spring Harbor Laboratory Press, 2001

12 Ju H, Liang D, Guo G, Zhang J. Comparison of four methods to prepare pichia genomic DNA for PCR. *Tianjing Med J* 2003;**31**:270–2

13 Wang X, Zhu M, Yang G, Zhang A, Yang F, Chen P. Expression of Cecropin B in *Pichia pastoris* and its bioactivity *in vitro*. *Exp Ther Med* 2011;**2**:655–60

14 Schagger H, Von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100KD. *Anal Biochem* 1987;**166**:368–79

15 Koliaraki V, Marinou M, Samiotaki M. Iron regulatory and bactericidal properties of human recombinant hepcidin expressed in *Pichia pastoris*. *Biochimie* 2008;**90**:726–35

16 Scott MG, Gold Michael R, Hancock Robert EW. Interaction of cationic peptides with lipoteichoic acid and gram-positive bacteria. *Infect Immun* 1999;**67**:6445–53

17 Lehrer RL, Barton A, Daher KA, Harwig SS, Ganz T, Selsted ME. Interaction of human defensins with *Escherichia coli*: mechanism of bactericidal activity. *J Clin Invest* 1989;**84**:553–61

18 Michaut L, Fehlbaum P, Moniatte M, Van Dorsselaer A, Reichhart J-M, Bulet P. Determination of the disulfide array of the first inducible antifungal peptide from insects: drosomycin from *Drosophila melanogaster*. *FEBS Lett* 1996;**395**:6–10

19 Saberwalg G, Nagaraj R. Cell-lytic and antibacterial peptides that act by perturbing the barrier function of membranes. *Biochim Biophys Acta* 1994;**1197**:109–31

20 Yechiel S. Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by α-helical antimicrobial and cell non-selective membrane-lytic peptides. *Biochim Biophys Acta* 1999;**1462**:55–70

21 Park Y, Lee DG, Jang S-H, Woo E-R, Jeong HG, Choi C-H, Hahm K-S. A Leu-Lys-rich antimicrobial peptide: activity and mechanism. *Biochim Biophys Acta* 2003;**1645**:172–82

22 Ibrahim HR, Sugimoto Y, Aoki T. Ovotransferrin antimicrobial peptide (OTAP-92) kills bacteria through a membrane damage mechanism. *Biochim Biophys Acta* 2000;**1523**:196–205

23 Bechinger B. Structure and functions of channel-forming peptides: magainins, cecropins, melitin and alamethicin. *Mem Biol* 1997;**156**:197–211

24 Yang D, Chertov O, Oppenheim JJ. The role of mammalian antimicrobial peptides and proteins in awakening of innate host defenses and adaptive immunity. *Cell Mol Life Sci* 2001;**58**:978–89

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