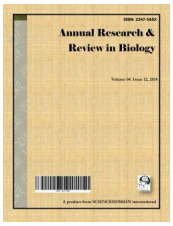

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Novel Cathelicidin Antimicrobial Peptides from *Paa robertingeri*

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Authors' contributions

This work was carried out in collaboration among all authors. Author JZ designed the study, performed the statistical analysis and wrote the protocol. Author CC wrote the first draft of the manuscript. Authors HD and MY managed the analyses of the study. Author QL managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

This study aimed to describe two cathelicidins (cathelicidin-PR1 and cathelicidin-PR2) from the skin of *Paa robertingeri* (*Anura*: *Ranidae*). The deduced mature peptides cathelicidin-PR1 and cathelicidin-PR2 were composed of 29 and 25 residues, respectively. Cathelicidin - PR1 has higher antimicrobial activity it could kill Gram-positive and Gram-negative bacteria and even some fungal species. Cathelicidin-PR1 exhibited more effective than AMP in antimicrobial activity against *Pseydomonas maltophilia* clinical strain. On the contrary, cathelicidin-PR2 had very weak antimicrobial activity. Furthermore, cathelicidin-PR1 and cathelicidin-PR2 exhibited very low hemolytic activity against human erythrocytes and little hemagglutinating activity. The results suggested that the cathelicidin-PR1 might serve as a template for developing novel antibiotics.

Keywords: Antimicrobial activity; antimicrobial peptide; hemagglutinating activity; hemolytic activity; Paa robertingeri.

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1. INTRODUCTION

Antimicrobial peptide (AMP) is a kind of small-molecule peptides characterized by strong and broad-spectrum bactericidal activity. In the last 30 years, the widespread distribution of AMPs has been discovered, providing insights into the innate defensive systems that permit multicellular organisms to live in harmony with microbes [1]. Cathelicidins and defensins are the two major AMP families in mammals [2,3]. They can defence against a variety of harmful microorganisms. The presence of cathelicidins in hagfish, the oldest jawless craniates, indicates that cathelicidin genes appeared early in phylogenesis [4-6], which illustrates their important role. Almost 100 kinds of cathelicidins were searched from the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php), and the data are constantly updated.

Since the discovery of the first cathelicidin (Bac5) from the cDNA of the bovine neutrophils [7], a variety of new cathelicidins have been found in most of the vertebrates, ranging from mammalians, birds, reptiles, amphibians to fishes [8,9]. Cathelicidins possess a conserved structure. Upon activation, most of the cathelicidin precursors are proteolytically cleaved to release the C-terminal mature peptide domain [5,6,10,11]. On the basis of the protein secondary structure, cathelicidins can be divided into four categories: α-helix cathelicidins, extension-spiral cathelicidins, cyclic cathelicidins, and β-sheet cathelicidins. Cathelicidins derived from mammals are mostly α-helical, and the primary structure generally contains 23–40 amino acids [12,13]. Most of the cathelicidins antibacterial peptides are the α-helical structure [14]. β-sheet cathelicidins generally contain 16–18 amino acids, which are folded into a hairpin structure stabilized by disulfide bonds [15].

A known mechanism of action of amphibian antimicrobial peptides is that the positively charged polypeptides interact with the microbial cell membrane and induce changes in the membrane structure, resulting in the cytoplasm outflow and eventually causing microbial death [16]. However, evidence shows that the cell membrane is not the only target of antimicrobial peptides. They also act on other parts of microorganisms, such as intracellular DNA and RNA, thereby interfering with microbial metabolic pathways [17].

The amphibians face the challenge of adapting to moist environments. Their skins secrete a large volume and variety of antimicrobial peptides. Also, amphibians lack lymphocytes. Hence, the secretion of mucous substances is particularly important in such an environment [18]. In this study, two cathelicidins were identified and characterized *by Paa robertingeri*.

2 MATERIALS AND METHODS

2.1 Tissue Preparation

An adult specimen of *Paa robertingeri* was captured from Fanjingshan in Guizhou province (108°45'55"–108°48'30" E; 27°49'50"–28°1'30" N). A 1- cm^2 piece of dorsal skin was removed from its back immediately and stored in liquid RNA protector (sample protector for RNA/DNA, TaKaRa, Japan) until use. After collection, this frog was sterilized with alcohol and then set free in its natural habitat.

2.2 cDNA Library Construction and Screening of the Skin cDNAs Encoding Cathelicidins

The stored skin was washed in water and then ground into powder in liquid nitrogen. The total RNA was extracted using TriZol reagent (Life Technologies, CA, USA). Then, the total RNA was used to construct the cDNA library using the Creator Smart cDNA Library Construction Kit (Clontech, CA, USA). First-strand cDNA synthesis was performed using SMARTScribe Reverse Transcriptase (Clontech) and SMARTer V Oligonucleotide and 3′ IF SMARTer CDS Primer. Second-strand cDNA synthesis was performed by a long-distance polymerase chain reaction (PCR) method using Advantage 2 Polymerase Mix (Clontech) in the presence of 5′ PCR Primer II A and 3′ IF SMARTer PCR Primer. The synthesized cDNA was used as a template for the following PCR to screen the cDNAs encoding the cathelicidin peptides [18].

On the basis of the conserved signal peptide domain of previously characterized host defence peptide (HDP) from ranid frogs, a sense oligonucleotide primer (5′-CCCCATGTTCACCTTGAAG-3′) was designed and coupled with 3′ antisense primer (5′-TACGCGACGCGATACGCGAAT-3′) according to the sequence of 3′ IF SMARTer CDS primer to screen the HDP encoded cDNAs. The PCR procedure was as follows: 5 min of denaturation at 94ºC; 30 cycles: denaturation at 94ºC for 30 s, primer annealing at ºC for 30 s, and extension at

72ºC for 1 min. The PCR product was purified by gel electrophoresis and cloned into pMD19-T vector (TaKaRa, Japan) for sequencing.

2.3 Alignment of Amphibian Cathelicidins

Sequencing results used the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool to remove the carrier and identify the fragment. Then the fragment sequences were translated into amino acids by ExPASy (http://www.expasy.org/). The sequences were input into NCBI database, the complete gene sequence encoding cathelicidins of *P. robertingeri* was identified, and the amino acid sequence of the mature peptide was predicted according to the characterized cathelicidins.

2.4 Peptide Synthesis

Cathelicidin-PR1 and -PR2 were synthesized by GL Biochem Ltd. (Shanghai, China) and analyzed by high-performance liquid chromatography and mass spectrometry to ensure purity of more than 95%.

2.5 Antimicrobial Assay

Seven strains of standard and clinically isolated microorganisms, including Gram-positive bacteria, Gram-negative bacteria, and fungi, were used in the antimicrobial assay. Minimal inhibitory concentrations (MICs) of the peptides were determined by a standard twofold microdilution method in a 96-well microtiter plate, as described previously [19]. Briefly, the microorganisms were incubated in Mueller–Hinton broth (MH) at 37°C to exponential phase and diluted with fresh MH broth to 10^6 colony-forming unit (CFU)/mL. Then, 50 µL of serial dilutions of peptides in MH broth were prepared in 96-well microtiter plates and mixed with 50 µL of diluted bacterial inoculum. The plates were incubated at 37°C for 18 h, and the minimal concentration at which no visible growth occurred was recorded. The traditional antibiotic ampicillin was used as a positive control, and the assay was conducted in triplicate.

2.6 Bacterial Killing Kinetics Assay

The bacterial killing kinetics of cathelicidin-PR1 against *Bacillus cereus* clinical strain was determined by measuring the changes in the viable bacterial counts after peptide treatment. *B. cereus* clinical strain was incubated in the Luria-Bertani (LB) liquid medium at 35ºC and 200 rpm for 10–16 h and diluted to 10^5 CFU/mL in the fresh LB liquid medium. Cathelicidin-PR1 was added to the bacterial suspension to a final concentration of 5× MIC, and the bacterial suspension was incubated at 37ºC for 0, 10, 20, 30, 45, 60, 90, and 120 min. At each time point, aliquots (10 µL) were removed and diluted with fresh LB broth 100 times. Next, 100 µL of the dilutions were coated on the LB solid medium and incubated for 10–16 h at 37ºC. The viable colonies were counted. Ampicillin was used as a positive control, and sterile deionized water was used as a negative control, the assay was conducted in triplicate at least and took the average.

2.7 Hemolytic Assay

Fresh human erythrocytes were collected, mixed in 5 mL of mixing Alsever's solution (8.0 g sodium citrate, 0.55 g citric acid, 20.5 g glucose, and 4.2 g NaCl in 1 L deionized H_2O, pH 6.1) at a volume ratio of 1:1, and centrifuged at 1000 rpm for 5 min. The supernatant was removed, washed with 0.9% saline three or four times, and resuspended to a final concentration of 2% (v/v). Serial dilutions of cathelicidin-PR1 and cathelicidin-PR2 were incubated with the erythrocyte solutions at 37ºC for 30 min, and then the cells were centrifuged at 1500 rpm for 10 min. The supernatant was collected, and the absorbance at 540 nm was measured. The assay was conducted in triplicate. 1% Triton X-100 (v/v) was used as a positive control, and 0.9% saline was used as a negative control. The assay was conducted in triplicate at least. Percentage of hemolysis (*I*%) was calculated according to the following formula:

$$I\% = (A_{sample} - A_{negative\ control})/(A_{positive\ control} - A_{negative\ control}) \times 100\%$$

2.8 Anti-oxidant Assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable aliphatic nitrogen-centered radical. It can be used to detect the anti-oxidant activity of antimicrobial peptides by radical scavenging assay. DPPH (Sigma, USA) was dissolved in methanol to a final concentration of 6×10^{-5}M. Next, 192 µL of DPPH solutions were mixed with 8 µL of serial concentrations of peptide solutions. The mixture was incubated in the dark at room temperature for 30 min, and the amount of reduced DPPH was quantified by measuring a decrease in absorbance at 517 nm. Deionized water was used as a negative control.

Inhibition of free radicals by DPPH in percentage (*I*%) was calculated according to the formula:

$$I\% = (A_{blank} - A_{sample})/A_{blank} \times 100\%$$

2.9 Erythrocyte Hemagglutination Assay

Lectins are glycan-binding proteins that can specifically recognize glycan structures and have been identified from a wide variety of organisms [20]. Fresh human erythrocytes were collected and stored in Alsever's solution to prevent coagulation. The assay was performed in U-well microtiter plates (96 wells) according to the method described by Li et al. [21]. The erythrocytes were washed twice with Tris buffered saline (TBS) buffer (6.06 g Tris base and 5.84 g NaCl in 1 L of H_2O, pH 7.5) and TBS + Ca^{2+} buffer (6.06 g Tris base, 5.84 g NaCl, and 1.12 g $CaCl_2$ in 1 L of H_2O, pH 7.5), centrifuged at 1000 rpm for 5 min, and resuspended in the same buffer to a final concentration of 2% (v/v). Then, 10 µL of peptide solutions (2 mg/mL) were mixed with 90 µL of erythrocyte solutions in a U-well microtiter plate. The plate was incubated at room temperature for 45 min, and the result was observed. Deionized water was used as negative control, and the assay was conducted in triplicate.

2.10 Bioinformatics Analysis and Structure Prediction

The physical and chemical parameters of cathelicidin-PR1 and cathelicidin-PR2 were determined by the Prot Param tool (http://web.expasy.org/protparam/) through ExPASy Bioinformatics Resource. The secondary structure was predicted using the PSIPRED protein structure prediction server provided by Bioinformatics Group of UCL Department of Computer Science (http://bioinf.cs.ucl.ac.uk/psipred/).

2.11 Circular Dichroism Analysis/ Spectroscopy

The samples were prepared by dissolving the peptide powder in 60mM sodium dodecyl sulfate (SDS)/H_2O solutions to a concentration of 0.5 mg/mL. The spectra were measured at 298 K (25ºC) between 192 and 250 nm using a 0.1-cm path length cell with 1-nm bandwidth, 1-s response time, and a scan speed of 100 nm/min. Three consecutive scans per sample were performed and averaged, followed by subtraction of the solvent signal.

3. RESULTS AND DISCUSSION

3.1 Identification and Characterization of *P. robertingeri* Cathelicidins

Total RNA was extracted from the skin of *P. robertingeri*, and cDNA library was constructed using a cDNA library construction kit. Two cDNAs encoding two different cathelicidins were obtained from the cDNA library by the PCR-based cDNA cloning method. The complete nucleotide sequences and translated amino acid sequences of the two cathelicidin precursors are shown in Fig. 1. The cDNAs encoding cathelicidin-PR1 and cathelicidin-PR2 precursors were composed of 587 bp and 607 bp, respectively. The translated protein precursors comprised of 147 and 145 amino acid residues, respectively. Consistent with other cathelicidins, precursors of cathelicidin-PR1 and cathelicidin-PR2 possessed a typical signal peptide sequence, a highly conserved cathelin domain, and a cationic C-terminal mature peptide sequence.

The mature peptides of cathelicidin-PRs were predicted in this study. Cathelicidin-PR1 was composed of 29 amino acid residues, and the amino acid sequence was RKCNLFCKAKQKLKSLSSVIGTVVHPPRG. In contrast, cathelicidin-PR2 was composed of 25 amino acid residues, and the amino acid sequence was KECKDYLCKLLMKLG SSSHIESIDP.

3.2 Antimicrobial Activity of cathelicidin-PRs

Cathelicidin-PR1 and cassthelicidin-PR2 were chemically synthesized and their purity was confirmed to be 95%. The minimal inhibitory concentrations (MICs) of the two peptides against seven microorganisms, including Gram-positive bacteria, Gram-negative bacteria, and fungi, were determined. As listed in Table 1, except for *Acinetobacter baumannii,* cathelicidin-PR1 exhibited potent and broad-spectrum antimicrobial activity against most in the tested clinical strain. Cathelicidin-PR1 is more effective than AMP in antimicrobial activity against *Pseydomonas maltophilia* clinical strain. Unlike cathelicidin-PR1, cathelicidin-PR2 exhibited very weak antimicrobial activity.

3.3 Bacterial Killing Kinetics of cathelicidin-PR1

Using ampicillin as a positive control, the killing kinetics of cathelicidin - PR1 against

Table 1. Antimicrobial activity of cathelicidin-PR1 and cathelicidin-PR2

Microorganisms	Minimal inhibitory concentrations (μg/ml)		
	Cathelicidin-PR1	Cathelicidin-PR2	Amp
Acinetobacter baumannii clinical strain	＞100	＞100	4.69
Pseydomonas maltophilia clinical strain	75	＞100	＞100
Staphylococcus aureus clinical strain	37.5	＞100	＜0.10
Bacillus cereus clinical strain	37.5	＞100	4.69
Bacillus subtilis clinical strain	37.5	＞100	4.69
Candida albicans clinical strain	37.5	＞100	4.69
Candida glabrata clinical strain	37.5	＞100	4.69

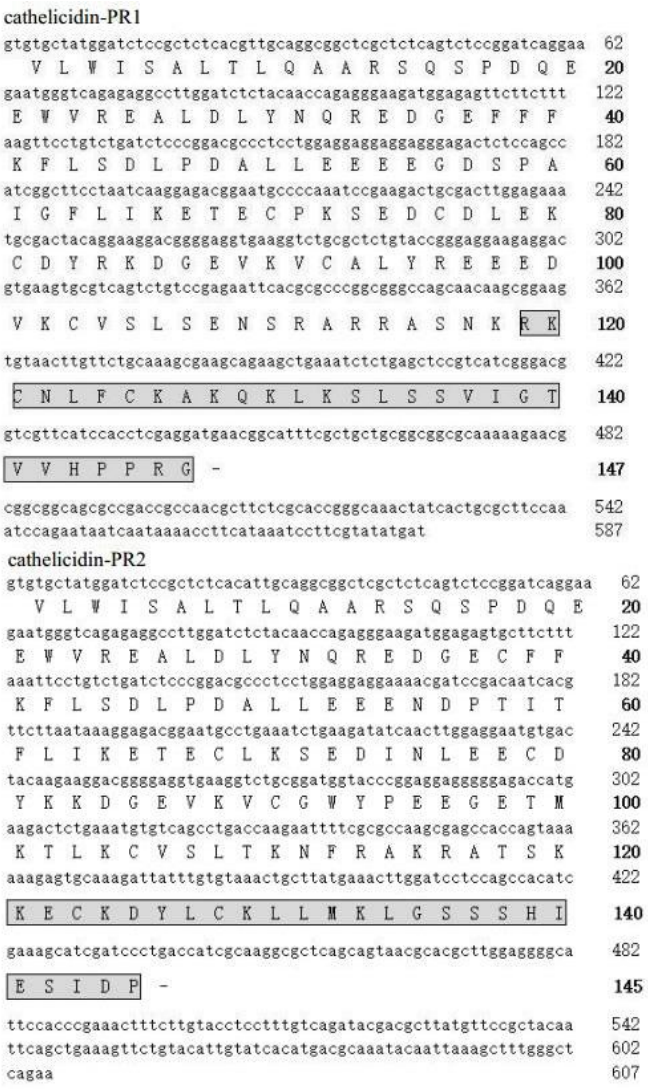

Fig. 1. cDNA sequences encoding cathelicidin-PR1 and the predicted prepropeptide sequences. The putative mature peptides of cathelicidin-PRs are boxed and shaded

Bacillus cereus clinical strain was investigated by a colony counting method. As illustrated in Table 2, at a concentration of 5× MIC, cathelicidin-PR1 rapidly exerted its antimicrobial function. It just took 45 min for cathelicidin-PR1 to kill all the *B. cereus* clinical strain cells. More importantly, the

colony forming units (CFUs) remained zero when the incubation time was extended to 120 min, implying that the antimicrobial property of cathelicidin-PR1 was lethal. In contrast, at the same concentration of 5× MIC, it took at least 90 min for the positive control ampicillin to completely kill the *B. cereus* clinical strain cells.

It indicated that cathelicidin-PR1 could rapidly and efficiently kill *B. cereus* clinical strain cells. Therefore, cathelicidin-PR1 might be used as a potential antibiotic.

3.4 Hemolytic and Anti-oxidant Activity

Cathelicidin-PR1 did not show any hemolytic activity. At a concentration of 200 μg/mL, the rate of hemolysis of cathelicidin-PR1 and cathelicidin-PR2 was 1.78% and 2.01%, respectively. However, at a concentration of 100 μg/mL, the rate of hemolysis of cathelicidin - PRs was 3.87% and 1.12%, respectively (Table 3).

At a concentration of 80 μg/mL, cathelicidin-PR1 and cathelicidin-PR2 showed slight DPPH radical scavenging activity, with *I*% values of 2.92% and 2.30%, respectively.

3.5 Erythrocyte Hemagglutinating Activity

In this study, cathelicidin-PR1 did not show any hemagglutinating activity irrespective of the presence of Ca^{2+}. However, cathelicidin-PR2 showed a weak hemagglutinating activity in the presence of Ca^{2+}, but it did not show any hemagglutinating activity in the absence of Ca^{2+} (Fig. 2).

3.6 Physical Properties Analysis and Secondary Structure Prediction

The physical and chemical parameters of the two cathelicidin-PRs were computed by ProtParam (http://web.expasy.org/protparam/); they are listed in Table 4. Besides, the secondary structures of the two cathelicidin-PRs were also predicted by the online prediction software from the University College London (UCL) Department of Computer Science (http://bioinf.cs.ucl.ac.uk/psipred/). Cathelicidin-PR1 was mainly composed of a helix and random coil (Fig. 3). Cathelicidin-PR2 was similar to cathelicidin-PR1.

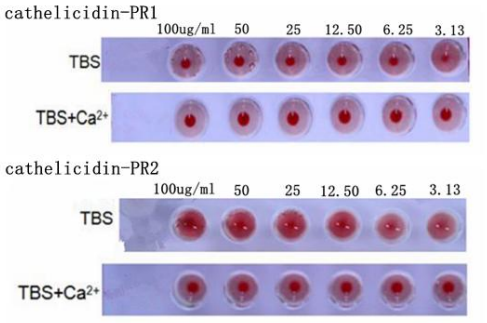

Fig. 2. Erythrocyte hemagglutinating activity of different concentrations of cathelicidin-PRs

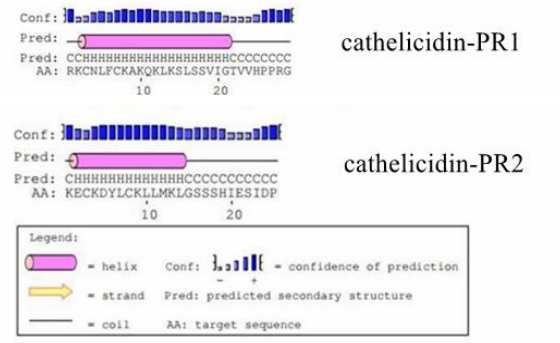

Fig. 3. Secondary structure prediction of cathelicidin-PRs

Table 2. Bacterial killing kinetics of cathelicidin PR1 against *Bacillus cereus* clinical strain

Time	Colony Forming Units (CFUs)							
	0min	**10min**	**20min**	**30min**	**45min**	**1h**	**1.5h**	**2h**
cathelicidin-PR1(5xMIC)	49.33±12.06	51±8.19	41±3.00	14.33±7.02	0±0.00	0±0.00	0±0.00	0±0.00
Ampicillin(5xMIC)	37.67±6.11	31.33±11.15	26.67±11.02	15.67±2.52	7.00±2.65	1.33±2.31	0.33±0.58	0±0.00
Blank control (sterile water)	39.67±10.07	32.33±7.57	26.33±3.06	55±9.64	95.67±20.21	98.33±14.57	132.33±15.37	219.67±11.15

Note: 5xMIC is 5 times of the minimum inhibitory concentration; cathelicidin-PR1 concentration is 187.5ug/ml; ampicillin concentration is 23.45ug/ml; the results are the average value of three independent repeated experiments (M±SD)

Table 3. Physical and chemical parameters of cathelicind-PR1 and cathelicind-PR2

Peptide	Number of amino acids	Molecular weight (Da)	Net charge	Theoretical pI	Grand Average of Hydropathicity (GRAVY)
Cathelicidin-PR1	29	3195.88	+7	10.59	-0.226
Cathelicidin-PR2	25	2838.34	0	6.74	-0.328

Table 4. The hemolysis ratios of cathelicidin-PR1 and cathelicidin-PR2

	cathelicidin-PR1	cathelicidin-PR2
100ug/ml	3.87%	1.12%
200ug/ml	1.78%	2.01%

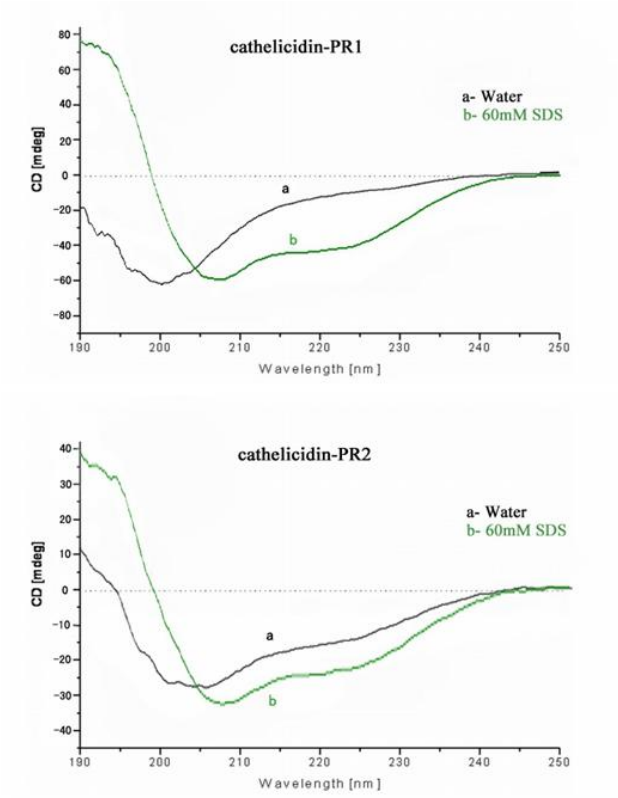

Fig. 4. Circular dichroism analysis of cathelicidin-PRs in ultrapure water (a) and SDS (60mM, b) solvent

Circular dichroism analysis is shown in Fig. 4. Both cathelicidin-PR1 and cathelicidin-PR2 had random coil configuration in sterile deionized water, while in 60mM sodium dodecyl sulfate (SDS) solvent, they had helix configuration, as predicted.

4. CONCLUSIONS

Recent studies have shown that cathelicidins act by interacting with the cell membrane of pathogenic microorganisms, leading to the formation of holes in the cell membrane, leakage of cell contents, and hence killing of pathogens [22]. Not every cathelicidin has antimicrobial activity [15,23]. This study showed that the charge of cathelicidin-PR1 was +7, while the cathelicidin-PR2 net charge was 0, and the cathelicidin-PR2 no antibacterial activity. It suggested that the antibacterial activity of cathelicidins was related to not only it's α- helix structure, but also its charge number.

Cathelicidins have potential clinical and agricultural value. At present, Cathelicidin PR1 and cathelicidin PR2 genes were tandem ligated and successfully expressed in *E. coli* BL21 by prokaryotic expression [24].

Bacillus cereus can cause human food poisoning, causing symptoms such as nausea, vomiting and abdominal pain. Cathelicidin PR1 has higher antimicrobial activity than ampicillin on the kill *B. cereus,* and also cathelicidin-PR1 has broad-spectrum antimicrobial activity. This indicates that Cathelicidin-PR1 is an important resource for the development of new anti-infection drugs, especially some strains that are resistant to traditional antibiotics. Cathelicidin-PR1 and cathelicidin-PR2 exhibited very low hemolytic activity against human erythrocytes and little hemagglutinating activity. The results suggested that the cathelicidin-PR1 might serve as a template for developing novel antibiotics.

ETHICAL APPROVAL

All animal experimental protocols were approved by the Animal Care and Use Ethics Committee of Guizhou Normal University.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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