Peptides 31 (2010) 1674–1677

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Novel family of antimicrobial peptides from the skin of *Rana shuchinae*

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ARTICLE INFO

Article history:
Received 30 April 2010
Received in revised form 17 May 2010
Accepted 17 May 2010
Available online 27 May 2010

Keywords:
Amphibian
Antimicrobial peptides
Skin
Rana shuchinae

ABSTRACT

So far numerous antimicrobial peptides have been characterized from amphibians. In this work, a new family of antimicrobial peptides, named shuchin, was purified and characterized from skin secretions of the frog, *Rana shuchinae* that lives in freezing mountains. Totally two members of shuchin (shuchin 1 and 2) were identified with the amino acid sequence of NALSMPRNKCNRALMCFG and NALSSPRNKCDRASSCFG, respectively. cDNAs encoding shuchins were cloned from the skin cDNA library of *R. shuchinae*. The precursors of shuchin are composed of 62 amino acid residues including the conserved signal peptides, acidic propieces, and mature antimicrobial peptides. Synthetic shuchins showed strong and broad antimicrobial activities against Gram-positive bacteria (*Staphylococcus aureus*, and *Bacillus cereus*; MICs < 12.5 μg/ml), Gram-negative bacteria (*Escherichia coli*, *Bacillus dysenteriae*, *Pseudomonas aeruginosa*; most MICs from 3.1 to 12.5 μg/ml), and yeast (*Candida albicans*; MICs of 6.25 μg/ml), but no hemolytic activity under the effective concentration, thereby provide more leading templates for designing novel anti-infection agents.

1. Introduction

Amphibian skin contains rich bioactive agents, such as peptides, proteins, and small organic molecules [2]. Bioactive peptides in amphibian skin have important functions, include (i) innate defensive functions, and (ii) regulatory or hormonal functions. Peptides that function in innate defense are considered as antimicrobial peptides, and they constitute an important part of the amphibian innate immune system. Hundreds of antimicrobial peptides with diverse structures and functions have been found in the skin secretions of amphibian families, such as Pipidae, Hylidae, Hyperoliidae, and Ranidae. Extensive studies have been conducted on antimicrobial peptides from the genus *Rana*, *Bombina*, *and Xenopus* [1,2,5–10,12,13,16–20]. In most of cases, more than one family of antimicrobial peptides can be found in a single species of amphibian. Recently, 107 antimicrobial peptides belonging to 30 families with variable lengths in amino acid sequences (from 9 to 47 residues) have been identified in skin secretions of the frog, *Odorrana grahami* [9], and most of these peptides belong to novel families, suggesting that many amphibian antimicrobial peptides have yet to be discovered. In addition, such an extreme diversity of antimicrobial peptides present in a single amphibian species indicates that amphibian skins are large resource of antimicrobial peptides. In this study, a novel family of antimicrobial peptide was discovered and identified from the skin secretions of *Rana shuchinae*.

2. Materials and methods

2.1. Collection of frog skin secretions

Specimens of adult *R. shuchinae* from both sexes ($n = 30$; weight range 30–35 g) were collected in Sichuan Province of China. Skin secretion was collected according to previous methods described by Li et al. [9]. The experimental animals were first rinsed with 0.1 M NaCl solution containing 0.01 M EDTA. Three hundred milliliters of skin secretion was collected (total absorbance at 280 nm was 400), quickly centrifuged, and the supernatant was lyophilized. The experimental animals were then cleaned with water, put into a clean wet container with free access to air, and allowed to recover. All animal experiments were approved by the Animal Ethics Committee of Nanjing Medical University.

2.2. Peptide purification

Lyophilized skin secretion sample of 1.5 g was reconstituted in 10 ml of 0.1 M phosphate buffer solution (PBS), containing 5 mM EDTA, pH 6.0. The sample was applied to a Sephadex G-50

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doi:10.1016/j.peptides.2010.05.014

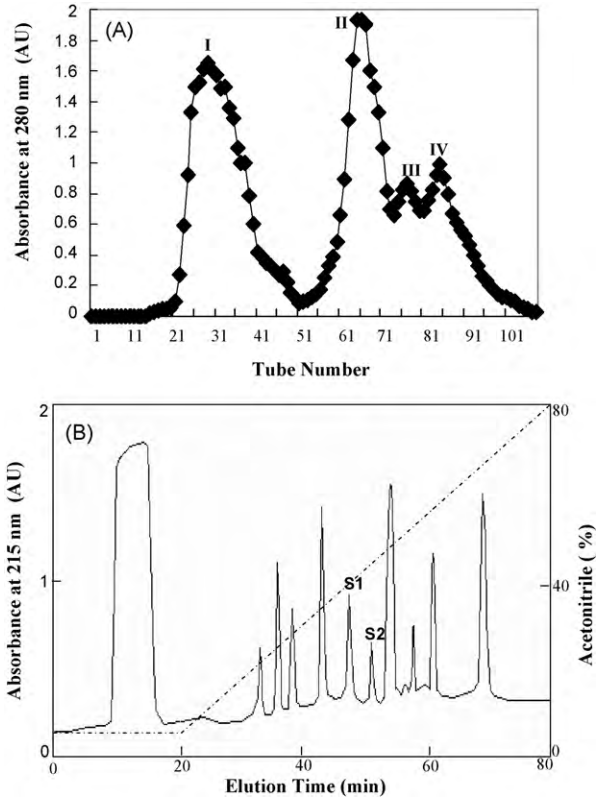

Fig. 1. Purification of shuchins from *Rana shuchinae* skin secretions. (A) shows Sephadex G-50 gel filtration of *R. shuchinae* skin secretions. *R. shuchinae* skin secretion was applied on a Sephadex G-50 column equilibrated with 0.1 M PBS. Elution was performed with the same buffer, collecting fractions of 3.0 ml (A). Fraction III from Sephadex G-50 exerted antimicrobial activities was further purified on a RP-HPLC column equilibrated with 0.1% (v/v) trifluoroacetic acid/water. The elution was performed with the indicated gradient of acetonitrile in (B) at a flow rate of 0.7 ml/min, and fractions were tested for antimicrobial activity. The purified peptides are indicated by S1 and S2, respectively (B).

(Superfine, Amersham Biosciences, 2.6 cm × 90 cm) gel filtration column previously equilibrated with 0.1 M PBS. The column was eluted with the same buffer, and 3-ml fractions were collected. The absorbance of the eluate was monitored at 280 nm. The antimicrobial activity of each fraction was measured as described below. Fractions having major antimicrobial activity were pooled, lyophilized, and re-suspended in 2 ml of 0.1 M PBS (pH 6.0) and further purified by reversed phase HPLC using a C_{18} column (RP-HPLC, Tigerkin C_{18}, 30 cm × 0.21 cm, Dalian Sipore Co. Ltd., Dalian, China) column.

2.3. Structural analysis

Complete peptide sequencing was determined by Edman degradation carried out with an Applied Biosystems pulsed liquid-phase sequencer, model 491. The actual molecular mass (MS) of the peptides were determined by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) performed on an AXIMA CFR (Krates Analytical) mass spectrometer in positive ion and liner mode with α-cyano-4-hydroxycinnamic acid (CHCA) matrix. The major parameters were as follows: ion acceleration voltage of 20 kV, accumulating time of single scanning of 50 s. Polypeptide mass standard (Kratos Analytical) was used as external standard for instrument calibration.

2.4. cDNA library construction

Total RNA was extracted from the skin of a single specimen of *R. shuchinae* using TRIzol reagent (Life Technologies, Ltd.). The cDNA was synthesized using a SMART™ PCR cDNA synthesis kit (Clontech, Palo Alto, CA). The primers used in the first strand synthesis were 3′ SMART CDS Primer II A, 5′-AAGCAGTGGTATCAACGCAGAGTACT (30) N-1N-3′ (N = A, C, G or T; N-1 = A, G or C), and 5′ SMART II A oligonucleotide, 5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′. The second strand was amplified by Advantage polymerase using 5′ PCR primer II A, 5′-AAGCAGTGGTATCAACGCAGAGT-3′.

2.5. Screening of cDNA library for sequences encoding antimicrobial peptides

The cDNA synthesized was used as template to screen the cDNAs encoding shuchins. Two oligonucleotide primers, S_1 5′-AA(T/C)GC(A/T/C/G)CT(A/T/G/C)AG(T/C)ATGCC(A/T/C/G)(A/C)G(A/T/C/G)-3′ in the sense direction, a specific primer designed according to the peptide sequences of shuchins and PCR primer II A in the antisense direction were used in PCR reactions. PCR was carried out with Advantage polymerase from Clontech (Palo Alto, CA) under the following conditions: 2 min at 94 °C, followed by 30 cycles of 10 s at 92 °C, 30 s at 50 °C, 40 s at 72 °C. The PCR products were cloned into pGEM®-T Easy vector (Promega, Madison, WI) and the identity of the DNA fragment was checked by DNA sequencing, performed on an Applied Biosystems DNA sequencer, model ABI PRISM 377.

2.6. Antimicrobial assays

The microorganisms used in the antimicrobial assays included the Gram-positive bacteria *Staphylococcus aureus* (ATCC2592), *Bacillus cereus*, and *Bacillus dysenteriae*, the Gram-negative bacteria *Escherichia coli* (ATCC25922), *Psecdomonas aeruginosa*, and fungus *Candida albicans* (ATCC2002) were obtained from Nanjing Medical University. Antimicrobial assays were carried out as described by Li et al. [9]. In brief, bacteria were first grown in Luria-Bertani (LB) broth to an $OD_{600\,nm}$ of 0.8. Next a 10 μl aliquot of the bacteria was then taken and mixed with 8 ml of fresh LB broth containing 0.7% agar, and then poured over a 90 mm Petri dish containing 25 ml of 1.5% agar in LB broth. After the agar had hardened, a 20 μl aliquot of the test sample filtered through a 0.22 μm Millipore filter paper was dropped onto a paper disc that was placed on the surface of the bacterial-agar and allowed to dry completely before the plate was incubated at 37 °C for overnight. Samples that showed positive antimicrobial activity would have a clear zone around the filter paper, representing inhibition of bacterial growth.

Minimal inhibitory concentration (MIC) was measured by standard micro-dilution broth method using 96-well microtiter plate. The peptides were subjected to serial dilutions in LB, and then 50 μl of the diluted samples were dispensed into a 96-well microtiter plate and mixed with 50 μl of bacteria or yeast inoculums in LB (1×10^6 cfu/ml). The microtiter plate was incubated at 37 °C for 18 h for bacteria or 48 h for yeast before being measured of absorbance at 595 nm using a microtiter plate spectrophotometer. MIC was defined as the lowest concentration of peptide that completely inhibits growth of the microbe determined by visual inspection or spectrophotometrically growth percentage was less than 5% compared to that of negative control.

2.7. Hemolysis assays

Hemolysis assays were performed using rabbit red blood cells as described by Bignami [3]. Serially diluted peptides were added to a suspension of cells, and after incubating at 37 °C for 30 min, the cells were centrifuged and the absorbance of the supernatant

Fig. 2. The nucleotide sequences encoding shuchin from *R. shuchinae* and the deduced amino acid sequences of the precursor polypeptide, and their sequence comparison with other antimicrobial peptide precursors. (A) The nucleotide sequence encoding shuchin 1. (B) The nucleotide sequence encoding shuchin 2. (C) The sequence comparison of antimicrobial peptide precursors from *R. shuchinae and Amolops loloensis* [11]. The bar (-) indicates stop condon. The mature peptides are boxed.

was measured at 540 nm. Maximum hemolysis was determined by adding 1% Triton X-100 to a sample of cells.

2.8. Synthesis of peptides

The peptides shuchin 1 and 2 (NALSMPRNKCNRALMCFG and NALSSPRNKCDRASSCFG) were synthesized by GL Biochem (Shanghai Ltd.) and analyzed by HPLC and MALDI-TOF-MS. The purity of the synthesized peptides was determined to be higher than 98%. All peptides were dissolved in double distilled water.

3. Results

3.1. Purification of antimicrobial peptides

Four major fractions were obtained when the lyophilized sample of *R. shuchinae* skin secretion was resolved by gel filtration (Fig. 1A). Among these eluted fractions, fraction III was found to have antimicrobial activity. Thus it was further purified by RP-HPLC. More than 10 peaks were resolved by RP-HPLC (Fig. 1B), but only two of these S1 and S2 had antimicrobial activity. S1 and S2 were therefore designated as shuchin 1 and shuchin 2, respectively.

3.2. Structural characterization

The purified antimicrobial peptides were subjected to amino acid sequence analysis by automated Edman degradation. The amino acid sequences of shuchin 1 and 2 were NALSMPRNKCNRALMCFG and NALSSPRNKCDRASSCFG, respectively. The MWs of shuchin 1 and 2 were determined to be 2024.2 and 1911.0, respectively, both of which matched their predicted MWs (2024.4, 1911.1), considering shuchin 1 and 2 contain an intra-molecular di-sulfide bridge. Synthesized shuchin 1 and 2 containing an intra-molecular di-sulfide bridge showed the same RP-HPLC elution patterns and the same MW as native shuchin 1 and 2. These results confirmed the presence of di-sulfide bridge in native shuchin 1 and 2.

3.3. cDNA cloning

Two cDNA clones encoding the precursors of shuchin were isolated and sequenced from the skin cDNA library of *R. shuchinae*. Their cDNA sequences and deduced amino acid sequence are shown in Fig. 2. Each of the peptides was derived from a precursor of 62 amino acid residues in length. The structural organization of the two precursors is quite similar, comprising a signal peptide sequence, an N-terminal spacer peptide region containing several aspartic and glutamic acid residues, and the mature shuchins at the C-terminus. There is a di-basic site for trypsin-like enzymes processing between the spacer peptide region and the mature shuchin as is found in the precursors of other antimicrobial peptides (Fig. 2C). All of the precursors have signal sequences that are highly conserved, although the sequences of the mature peptides are significantly different from each other (Fig. 2C). The amino acid sequences deduced from the cDNA sequences were identical to the sequences determined by Edman degradation. BLAST search revealed no sequence similarity between the two

Table 1
Antimicrobial activity of Shuchins.

Microoganism	MIC (μg/ml)[a]	
	Shuchin 1	Shuchin 2
Gram-positive bacteria		
Staphylococcus aureus ATCC2952	12.5	6.25
Bacillus cereus	12.5	12.5
Gram-negative bacteria		
Escherichia coli ATCC25922	50	50
Bacillus dysenteriae	6.25	3.1
Pseudomonas aeruginosa	12.5	6.26
Yeast		
Candida albicans ATCC2002	6.25	6.25

[a] MIC: the lowest concentration of peptide that completely inhibits growth of the microbe determined by visual inspection or spectrophotometrically growth percentage was less than 5% compared to that of negative control.

shuchins and other antimicrobial peptides. Thus, these shuchins were considered to represent a novel class of antimicrobial peptides.

3.4. Antimicrobial activity

Shuchins showed antimicrobial activities against all the tested microorganisms including Gram-positive bacteria, Gram-negative bacteria and fungi (Table 1). For *S. aureus*, *B. dysenteriae* and *P. aeruginosa*, the antimicrobial activities of shuchin 2 were stronger than that of shuchin 1. As for the other microorganisms, *B. cereus*, *E. coli*, and *C. albicans*, the antimicrobial activities exerted by shuchin 1 and 2 were similar. The antimicrobial activity was proven to be lethal for the sensitive strain. The sensitive strains were not capable of resuming growth on agar plates after a 6-h treatment with peptide concentrations above the corresponding MICs.

3.5. Hemolytic activity

Some antimicrobial peptides exhibit hemolytic activities [8,12]. Rabbit red blood cells were used to check for hemolytic capability in our experiments. Shuchins had little hemolytic activity, and could only lyse 2% of the rabbit red blood cells at a concentration up to 200 μg/ml.

4. Discussion

Antimicrobial peptides are recognized as an important component of the innate defense system [4,14–16]. Their syntheses are either constitutive or inducible. Many antimicrobial peptides have been identified from animals and plants. Most of the amphibians require special living environments and they were the first group of organisms that formed a link between land and water. These kinds of environments may be laden with pathogenic microbes. In addition, the skins of most amphibians are fragile and easy to be destroyed, and therefore, they are endowed with an excellent chemical defense system composed of pharmacological and antimicrobial peptides [4]. A considerable variety of antimicrobial peptides have been characterized from amphibians. Frogs of the genus *Rana*, a widely distributed group with 48 species, synthesize and secrete a remarkably diverse array of peptides with antimicrobial activity that is believed to have arisen as a result of multiple gene duplication events [6]. Antimicrobial peptides from ranid frogs represent a potential source of new therapeutic agents, and they have been considered for use as taxonomic and phylogenetic markers [6].

As a species of *Rana*, *R. shuchinae* is mainly distributed in the south-west of China. They live in high altitude (altitude 3000–3600 m) with extremely cold weather. In this report, we identified a novel family of antimicrobial peptides present in the skin of *R. shuchinae*, and named it shuchin. The similarity in overall structures between pro-shuchins and antimicrobial peptide precursors from Ranidae amphibians (Fig. 2C) suggested that they may have a common ancestral gene. Despite such similarity of overall structures, the structure of mature shuchins was rather unique. All these results suggested that *R. shuchinae* possesses a different family of antimicrobial peptide compared to other *Rana* amphibians, despite all belonging to the same genus. The high altitude and extreme cold condition that constitute the living environment of *R. shuchinae* may have a contributing factor to such specialty. The shuchin family of antimicrobial peptide may also serve as taxonomic and phylogenetic markers. Its discovery would also increase the diversity of antimicrobial peptides and provides more templates for designing the anti-infection agents.

Acknowledgements

We thank Dr. Alan K. Chang for his help with the revision of the manuscript. This work was supported by the grants from National Natural Science Foundation (30900240).

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