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Two novel antimicrobial peptides from skin secretions of the frog, *Rana nigrovittata*

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Two novel antimicrobial peptides with similarity to brevinin-2 family are purified and characterized from the skin secretions of the frog, *Rana nigrovittata*. Their amino acid sequences were determined as GAFGNFLKGVAKKAGLKILSIAQCKLSGTC (*brevinin-2-RN1*) and GAFGNFLKGVAKKAGLKILSIAQCKLFGTC (*brevinin-2-RN2*), respectively, by Edman degradation. Different from brevinin-2, which is composed of 33 amino acid residues (aa), both *brevinin-2-RN1 and -RN2* contain 30 aa. Five cDNA sequences (Genbank accession numbers, EU136465-9) encoding precursors of *brevinin-2-RN1 and -RN2* were screened from the skin cDNA library of *R. nigrovittata*. These precursors are composed of 72 aa including a predicted signal peptide, an acidic spacer peptide, and a mature *brevinin-2-RN*. Both *brevinin-2-RN1 and -RN2* showed strong antimicrobial activities against gram-positive and gram-negative bacteria and fungi. The current work identified and characterized two novel antimicrobial peptides with unique primary structure. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antimicrobial peptides; amphibian; skin; brevinin-2; *Rana nigrovittata*

Introduction

Amphibians are a pool to isolate and identify bioactive compounds with versatile functions. Much attention has been paid on amphibian peptides for their wide-ranging pharmacological properties, clinical potential, and gene-encoded origin [1]. Especially, amphibian skins are the first line to interact with environments. A large amount of bioactive peptides or proteins have been found in amphibian skins [1]. These peptides exhibit microbe-killing, defense against either predators or parasites, wound healing, and oxidant scavenging activities [2–9].

Amphibian antimicrobial peptides have been intensively studied from the families of Pipidae, Hylidae, Hyperoliidae, and Ranidae [3,4,9,10]. More than 50 families of antimicrobial peptides have been identified from amphibian skins. Most of them are 10–50 residues in length with remarkably diverse structures. They are released onto the outer layer of the skin to provide an effective and fast-acting defense against harmful microorganisms [3,4,9–12]. Our previous work has identified nine families of antimicrobial peptides from the frog, *Rana nigrovittata*. They are nigroain B–E, nigroain I, nigroain K, rugosin, gaegurin, and temporin [10]. No brevinin-2-like antimicrobial peptides have been identified. In order to investigate the diversity of antimicrobial peptides in the skin of *R. nigrovittata*, we continue our attempt to purify and characterize new antimicrobial peptides.

Materials and Methods

Collection of Frog Skin Secretions

As described in our previous work, adult specimens of *R. nigrovittata* of both sexes ($n = 30$; weight range 30–40 g) were collected from the Yunnan Province of China and skin secretions were collected [10]. The collection of skin secretions is according to our reported method [10]. Frogs were anaesthetized by a piece of absorbent cotton immersed with anhydrous ether in a cylinder container. After 1–2 min, frog skin surface was seen exuding copious secretions. Skin secretions were collected by washing the dorsal region of each frog with 0.1 M NaCl solution (containing protease inhibitor cocktail). The collected solutions (500 ml of total volume) were quickly centrifuged at 5000 × *g* for 20 min and the supernatants were lyophilized.

Peptide Purification

As reported in our previous work [10], lyophilized skin secretion sample of *R. nigrovittata* (1.2 g, total OD280 nm of 300) was dissolved in 10 ml 0.1 M phosphate buffer, pH 6.0 (PB), containing 5 mM EDTA. The sample was applied to a Sephadex G-50 (Superfine, Amersham Biosciences, USA, 2.6 × 100 cm) gel filtration column equilibrated with 0.1 M PB. Elution was performed with the same buffer, collecting fractions of 3.0 ml. The absorbance of the elute was monitored at 280 nm. The antimicrobial activity of fractions was determined as described below. The fractions containing

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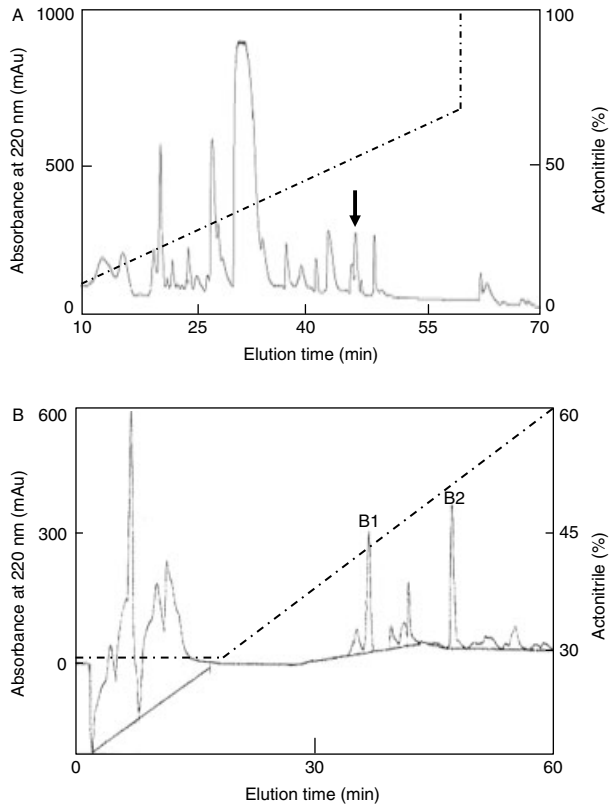

Figure 1. RP-HPLC purification of antimicrobial peptides from *R. nigrovittata* skin secretions. Six fractions were eluted from Sephadex G-50 gel filtration as in our previous report [10]. (A) Fraction IV was subjected to C18 RP-HPLC purification. The elution was performed with the indicated gradient of acetonitrile with 0.1% (v/v) trifluoroacetic acid/acetonitrile at a flow rate of 0.7 ml/min. (B) The fraction indicated by an arrow in Figure 1(A) was purified further by the same C18 RP-HPLC with indicated gradient of acetonitrile with 0.1% (v/v) trifluoroacetic acid/acetonitrile at a flow rate of 0.7 ml/min. The purified antimicrobial peptides were indicated as B1 and B2.

antimicrobial activities were pooled, lyophilized, and resuspended in 0.1 M PB, and purified further by C18 reverse-phase high-performance liquid chromatography (RP-HPLC, Hypersil BDS C18, 30 × 0.46 cm, Dalian Elite Analytical Instruments Co. Ltd., DEAIC) as illustrated in Figure 1.

Structural Analysis

The eluted fractions containing antimicrobial activity from RP-HPLC were analyzed by a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF-MS) cFR plus (Shimadzu Corporation, Kyoto, Japan and Kratos Analytical, Manchester, UK) in positive ion and linear mode. The operation parameters were as follows: the ion acceleration voltage was 20 kV, the accumulating time of single scanning was 50 s, polypeptide mass standard (Kratos Analytical) serving as external standard. The accuracy of mass determinations was within 0.1%. Complete amino acid sequences of antimicrobial peptides were determined by Edman degradation on an model 491 (Applied Biosystems, Foster City, CA, USA).

SMART cDNA Synthesis

As in our previous report, SMART™ techniques (Clontech) were used to synthesize the cDNA using total RNA extracted from the skin of a single frog by TRIzol (Life Technologies Ltd, Gaithersburg, MD, USA) [10]. For the cDNA synthesis, a SMART™ PCR cDNA synthesis kit (Clontech, Palo Alto, CA, USA) was used. The first strand was synthesized using cDNA 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 SMART II an oligonucleotide, 5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′. 5′ PCR primer II A, 5′-AAGCAGTGGTATCAACGCAGAGT-3′ provided by the kit was used to synthesize using Advantage polymerase (Clontech).

cDNA Cloning

Antimicrobial peptide precursors from Ranidae amphibians have been found to share conserved preproregion. The preproregion comprises a hydrophobic signal peptide of 22 residues followed by a 16–25 residue acidic propiece which terminates by a typical prohormone processing signal Lys-Arg. Most of the signal peptides of Ranidae AMP precursor have a sequence of MF(T/P)(L/M)KKS(L/M/F/P)LL(L/V)L [10]. According to the conserved sequence, a primer (RS1, 5′-CCAAA(G/C)ATGTTCACC(T/A)TGAAGAAA(T/C)-3′) was designed to screen antimicrobial peptides. RS1 in the sense direction and primer II A as mentioned in 'SMART cDNA synthesis' in the antisense direction were used in PCR reactions. The cDNA synthesized by SMART™ techniques was used as a template for PCR. The DNA polymerase was Advantage polymerase from Clontech. The PCR conditions were as follows: 2 min at 94 °C, followed by 30 cycles of 10 s at 92 °C, 30 s at 50 °C, and 40 s at 72 °C. Finally, the PCR products were cloned into pGEM® -T Easy vector (Promega, Madison, WI, USA). DNA sequencing was performed on an (Applied Biosystems, Foster City, CA, USA).

Antimicrobial Testing

Microorganisms including gram-positive bacterium *Staphylococcus aureus* (ATCC2592), *S. aureus* (ATCC43300, methicillin resistance), *Bacillus subtilis*, gram-negative bacteria *Escherichia coli* (ML-35P) (penicillin resistance), *Pseudomonas aeruginosa* PA01 (streptomycin resisitance), *P. aeruginosa* (ATCC27853), and fungus *Candida albicans* (ATCC2002) were obtained from the Kunming Medical College. They were first grown in LB (Luria–Bertani) broth or yeast extract–peptone–dextrose broth as in our previous methods [10]. Minimal inhibitory concentrations (MICs) of tested samples against these microorganisms were determined as in previous reports [10]. The MIC was defined as the lowest concentration of test peptides inhibiting microorganism growth.

Hemolytic Assays

In order to test hemolytic ability, antimicrobial peptides were incubated with rabbit red blood cells in Alsever's solution (in g/L: NaCl, 4.2; citric acid· 3Na· $2H_2O$, 8.0; citric acid·H_2O, 0.55; *d*-glucose, 20.5) according to the methods reported by Bignami [13]. Antimicrobial peptides were serially diluted and incubated with rabbit red blood cells at 37 °C for 30 min. After centrifugation, the absorbance in the supernatant was measured at 540 nm. Maximum hemolysis was determined by adding 1% Triton X-100 to the red cells.

Synthetic Peptides

All peptides used for bioassays were synthesized by GL Biochem (Shanghai Ltd, Shanghai, China) and analyzed by HPLC and mass spectrometry to confirm purity higher than 98%.

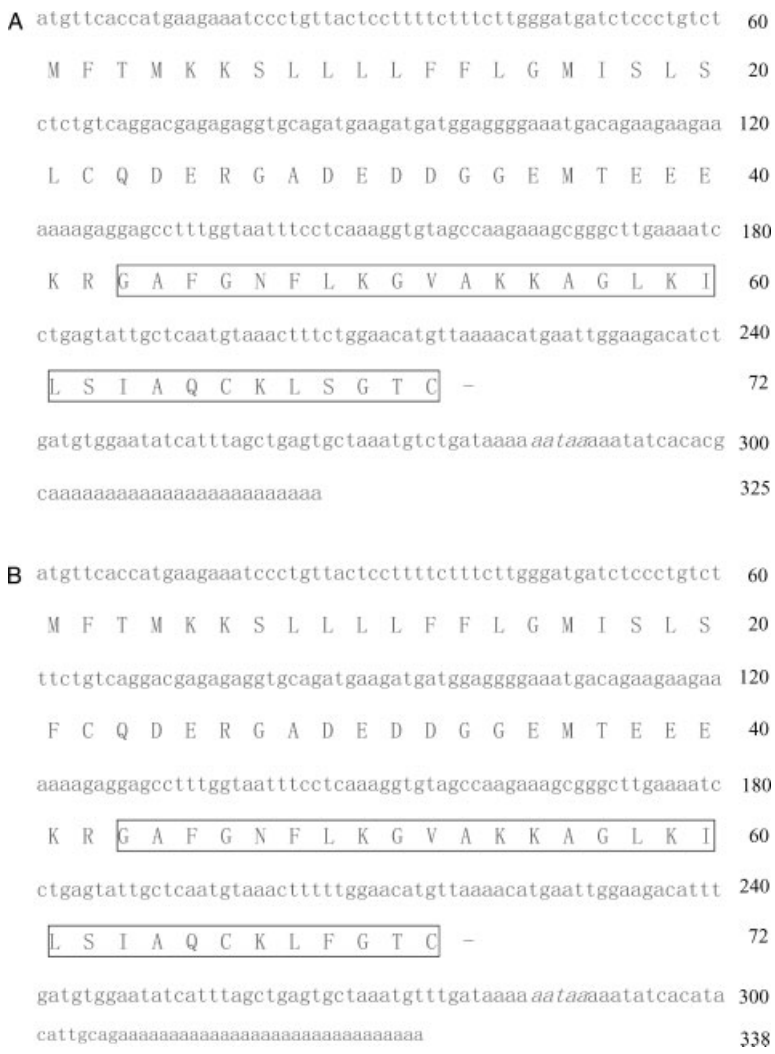

Figure 2. cDNAs encoding brevinin-2-RN1 (A) and -RN2 (B) and the deduced amino acid sequences. Mature antimicrobial peptides are boxed. Stop codons are indicated by a bar (—). Polyadenylation signals are in italic.

Results

Purification of Antimicrobial Peptides from Skin Secretions of the Frog, *R. nigrovittata*

The skin secretions of *R. nigrovittata* were fractionated into six fractions by Sephadex G-50 gel filtration as in our previous report [10]. Antimicrobial activities were found to be concentrated on fractions IV and V as in our previous report [10]. As illustrated in Figure 1(A), more than 30 peaks were eluted from fraction IV by C18 RP-HPLC. The eluted fraction indicated by an arrow in Figure 1 was found to contain antimicrobial activity; it was subjected to further purification by the C18 RP-HPLC using indicated acetonitrile gradient in Figure 1(B). Two eluted peaks (B1 and B2) were found to contain antimicrobial activities. These peaks were further analyzed by MALDI-TOF mass spectrometry and subjected to amino acid sequencing.

Structure Analysis

Peaks B1 and B2 were analyzed by MALDI-TOF mass spectrometry. It gave an observed mass of 3022.5 and 3082.5, respectively. Edman degradation gave an amino sequence of GAFGNFLKGVAKKAGLKILSIAQCKLSGTC and GAFGNFLKGVAKKAGLKILSIAQCKLFGTC, respectively. They are named *brevinin-2-RN1* and *-RN2*, respectively. By BLAST search, they showed sequence similarity to the amphibian antimicrobial family of brevinin-2 [11]. Brevinin-2 contain an intra-molecular disulfide bridge. Both *brevinin-2-RN1* and *-RN2* contain two half-Cys, which form a Rana-box as found in many amphibian antimicrobial peptide [10,11]. The observed masses (3022.5 and 3082.5) are matched with the theoretical masses (3022.7 and 3082.8) of *brevinin-2-RN1* and *-RN2* with an intra-molecular disulfide bridge. The error is −0.2 and −0.3 U, respectively.

cDNA Cloning

Five different cDNA seqeunces encoding precursors of *brevinin-2-RN1* and *-RN2* were screened and sequenced from the skin cDNA library of *R. nigrovittata* (GenBank accession numbers, EU136 465-9). cDNA sequences of EU136 465-8 encode *brevinin-2-RN1*; the cDNA sequence of EU136 469 encodes *brevinin-2-RN2*. The mature peptides deduced from the cDNA sequences are identical to the amino acid sequences determined by Edman degradation (Figure 2). These precursors share conserved signal peptide sequences composed of 22 aa as illustrated in Figure 2. The structural organization of these precursors is the same, comprising a signal peptide

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Table 1. Antimicrobial activity of antimicrobial peptides from the skin of *R ana nigrovittata*

Microorganisms	MIC[a] (μg/ml) RN1	RN2
S. aureus ATCC25923	3.10	6.25
S. aureus ATCC43300	3.10	6.25
B. subtilis	12.50	12.50
C. albicans ATCC2002	6.25	3.10
E. coli ML-35P	25.00	50.00
P. aeruginosa PA01	12.50	12.50
P. aeruginosa ATCC27853	25.00	12.50

[a] Minimal peptide concentration required for total inhibition of cell growth in liquid medium. These concentrations represent mean values (±20%) of three replicates.
RN, Brevinin-2-RN.

sequence, an *N*-terminal spacer peptide region containing several Asp and Glu residues, and the mature peptides at the *C*-terminus. They are composed of 72 aa including predicted signal peptides (22 aa), acidic propieces (18 aa), and mature antimicrobial peptides (30 aa). All these precursors share the same di-basic site (-Lys-Arg-) for enzymatic processing. The bi-basic site is located between spacer peptide and mature antimicrobial peptide (Figure 2).

Antimicrobial Activities

Both *brevinin-2-RN1* and *-RN2* were synthesized and their antimicrobial abilities were tested. They exerted potent antimicrobial activities against the tested microorganisms including gram-positive and gram-negative bacteria and fungi as listed in Table 1. In addition, their antimicrobial abilities against antibiotics-resistant strains (*S. aureus* ATCC43300 and *P. aeruginosa* PA01) are no weaker than those of common strains (*S. aureus* ATCC2592 and *P. aeruginosa* ATCC27853). The antibiotic activity was proven to be lethal for the sensitive strain (*S. aureus* ATCC43300, *P. aeruginosa* PA01, and *E. coli* ML-35P). The sensitive strains were not capable of resuming growth on agar plates after a 6-h treatment with concentrations above the corresponding MICs. Among the tested microorganisms, they showed the strongest antimicrobial activities against *S. aureus* and *C. albicans*. The MICs against *S. aureus* and *C. albicans* are $<$6.25 μg/ml.

Hemolytic Activity

Some antimicrobial peptides can act on eukaryotic membrane and exhibit hemolytic activities because of their hydrophobic structures [3]. In our experiments, rabbit red blood cells were used to check for hemolytic capabilities of *brevinin-2-RN1* and *-RN2*. They showed moderate hemolytic activity. At the concentration of 100 μg/ml, *brevinin-2-RN1* and *-RN2* could induce 17 and 21% rabbit red blood cell hemolysis, respectively.

Discussion

The growing problem of resistance to conventional antibiotics has stimulated the need for developing new anti-infective medicines. They are expressed on the primary barriers of the organism such as skin and mucosal epithelia, preventing the colonization of

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Brevinin-2-RN1 GAFGNFLKGVAKKAG---L-KILSIAQCKLSGTC 30
Brevinin-2-RN2 GAFGNFLKGVAKKAG---L-KILSIAQCKLFGTC 30
Brevinin-2     GLLDS-LKGFAATAGKGVLQSLLSTASCKLAKTC 33
               *     *** * **   *   **** ***  **
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Figure 3. Sequence comparison of brevinin-2-RNs with brevinin-2. Gaps (–) have been introduced to optimize the sequence homology. Stars indicate identical amino acid residues. The sequence of brevinin-2 is from Ref. 11.

host tissues by pathogens [14]. The gene-encoded antimicrobial peptides play key roles in innate immunity against noxious microorganisms [15]. As they cause much less drug resistance of microbes than conventional antibiotics, antimicrobial peptides nowadays attract considerable attention for the development of new antibiotics [15]. Recently, novel biological effects of antimicrobial peptides have been documented such as endotoxin neutralization, chemotactic and immunomodulating activities, induction of angiogenesis, and wound repair [16].

Amphibian skins are potential reservoir to explore antimicrobial agents. Amphibian naked skins are morphologically, biochemically, and physiologically complex organs, which act as defence, respiration, and water regulation, although it seems susceptible as amphibians are exposed to more dangers of microorganism injury than others [1]. Amphibian skins synthesize and secrete a remarkably diverse array of antimicrobial peptides to play key roles in their innate defense system [17–21].

R. nigrovittata is distributed in tropical regions, such as the Yunan province of China, Vietnam, Myanmar, and Thailand. In our previous work, nine families of antimicrobial peptides have been identified and characterized from *R. nigrovittata* by the method of peptidomics combined with cDNA trapping [10]. The current work identified and characterized another family of antimicrobial peptides, brevinin-2, which was found in some amphibian species belonging to the genus of *Rana*. Most of the antimicrobial peptides of brevinin-2 have 33 aa, but both *brevinin-2-RN1* and *-RN2* found in this work have only 30 aa (Figure 3). Compared with brevinin-2 containing three net positive charges, both *brevinin-2-RN1* and *-RN2* have five net positive charges. In addition, they have two phenylalanines in their N-terminus (Figure 3).

Antibacterial peptide chain formed polymer in solution by hydrophobic interaction, the higher hydrophobicity can increase its antibacterial activity and hemolytic activity [22]. *Brevinin-2-RN1* and *-RN2* having 15 and 16 hydrophobic amino acid residues, respectively, maybe the main reason to cause moderate hemolytic activity.

Because of the special structure and relatively low hemolytic activities, *brevinin-2-RN1* and *-RN2* might provide novel templates or leading structures to design antimicrobial agents.

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