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Peptidomics and genomics analysis of novel antimicrobial peptides from the frog, *Rana nigrovittata*

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ABSTRACT

Much attention has been paid on amphibian peptides for their wide-ranging pharmacological properties, clinical potential, and gene-encoded origin. More than 300 antimicrobial peptides (AMPs) from amphibians have been studied. Peptidomics and genomics analysis combined with functional test including microorganism killing, histamine-releasing, and mast cell degranulation was used to investigate antimicrobial peptide diversity. Thirty-four novel AMPs from skin secretions of *Rana nigrovittata* were identified in current work, and they belong to 9 families, including 6 novel families. Other three families are classified into rugosin, gaegurin, and temporin family of amphibian AMP, respectively. These AMPs share highly conserved preproregions including signal peptides and spacer acidic peptides, while greatly diversified on mature peptides structures. In this work, peptidomics combined with genomics analysis was confirmed to be an effective way to identify amphibian AMPs, especially novel families. Some AMPs reported here will provide leading molecules for designing novel antimicrobial agents.

Introduction

Amphibian skins act as the first line to defend against noxious aggression by microorganisms, parasites, predators, and physical harm. Amphibians, being the first group of organisms forming a connecting link between land and water, are forced to adopt and survive in a variety of conditions laden with pathogens and predators. Amphibian skins play key roles in their everyday survival and their ability to exploit a wide range of habitats and ecological conditions [1]. Amphibians are endowed with an excellent chemical defense system composed of pharmacological and antimicrobial gene-encoded peptides [2,3]. These peptides have pharmacological effects including cardiotoxic, myotoxic, neurotoxic, and antimicrobial activities [1]. All the properties clearly adversely affect potential predators or pathogens.

A remarkably diverse array of antimicrobial peptides (AMPs), 10–50 residues in length, have been found in skins of anuran amphibians, particularly those belonging to the families of Pipidae, Hylidae, Hyperoliidae, and Ranidae [4-10]. They are released onto the outer layer of the skin to provide an effective and fast-acting defense against harmful microorganisms [4,5]. Most of amphibian AMPs have a common N-terminal preproregion, which is highly conserved both intra- and inter-specifically, followed by a markedly different C-terminal domain that corresponds to mature AMPs. They have been suggested to originate from a common ancestor [4,5,7].

In order to investigate the diversity of amphibian antimicrobial peptides, methods of peptidomics combined with genomics were used to identify novel AMPs from the skin of the frog, *Rana nigrovittata*.

Results

Purification of AMPs 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 our previous report [11]. Fractions IV and V containing antimicrobial activities were subjected to further purification by C_8 and C_{18} RP-HPLC, respectively. As indicated in Fig. 1A, more than 30 peaks were eluted from fraction IV by C_8 RP-HPLC, and peaks A1–A6 were found to contain antimicrobial activities. Fig. 1B showed that more than 40 peaks were eluted from fraction V by C_{18} RP-HPLC, and peaks B1–B10 were found to contain antimicrobial activities. These fractions were further

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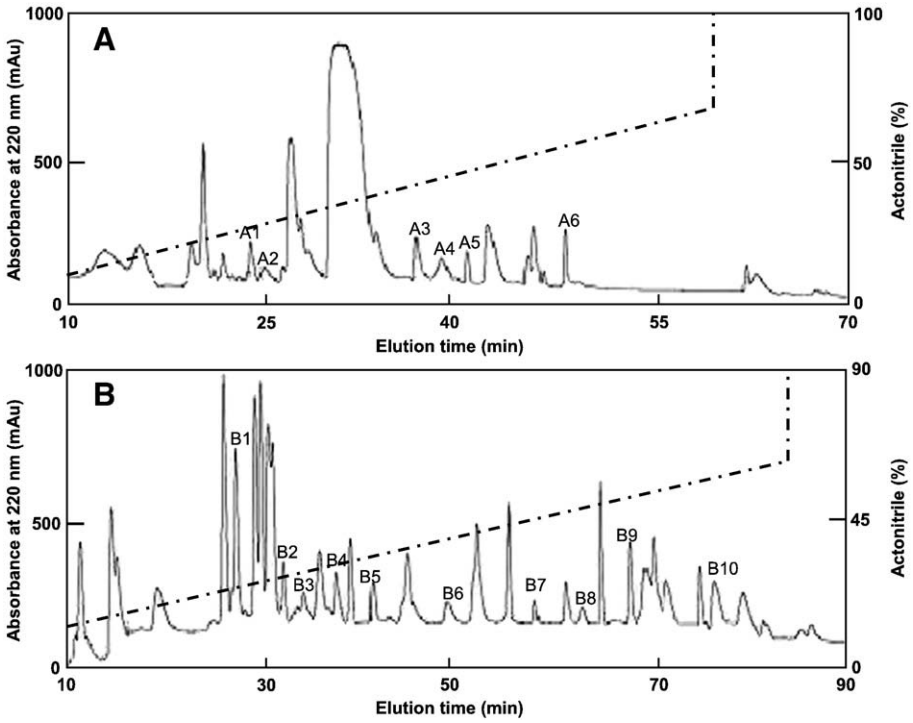

Fig. 1. RP-HPLC purification of antimicrobial peptides from *R. nigrovittata* skin secretions. Six fractions were eluted from Sephadex G-50 gel filtration as our previous report [11]. The fraction IV (A) and V (B) were subjected to further purification by C_8 and C_{18} RP-HPLC columns equilibrated with 0.1% (v/v) trifluoroacetic acid/water, respectively. The elutions were performed with the indicated gradient of acetonitrile with 0.1% (v/v) trifluoroacetic acid/acetonitrile at a flow rate of 0.7 ml/min, and fractions were tested for antimicrobial activity. The purified antimicrobial peptides were indicated in A (A1–A6) and B (B1–B10).

analyzed by MALDI-TOF mass spectrometry and subjected to amino acid sequencing.

Structure analysis

Peaks A1–A6 and B1–B10 were analyzed by MALDI-TOF mass spectrometry and amino acid sequencing. Peptides from the peaks A3, B6, and B7 have not been determined in current experiments. Thirteen purified AMPs with determined amino acid sequences were listed in Table 1. They belong to nine peptide families including nigroain-B to nigroain-E, nigroain-I, nigroain-K, gaegurin-RN, rugosin-RN, and temporin-RN.

These AMP families all contain one disulfide bridge except nigroain-C, nigroain-I, and temporin. Interestingly, there are three cysteines in the nigroain-B family while most of amphibian antimicrobial peptides contain two cysteines. After digestion by trypsin, nigroain-B3 was analyzed by MALDI-TOF mass spectrometry (data not shown). The result showed that two cysteines positioned at the C-terminus of nigroain-B3 formed a disulfide bridge and one cysteine at the N-terminus was free. The synthetic nigroain-B3 had the same bioactivity and mass spectrometry with the purified nigroain from the skin secretions, which further confirmed the structure of nigroain-B3. Most of the observed masses of these AMPs matched well with their theoretical masses deduced from primary structures, with an error less than 0.5 U (Table 1, Fig. S1). Only the mass error (+1.47) of rugosin-RN3 is larger than 0.5, and the reason is not clear yet. Another interesting antimicrobial family is nigroain-C, containing a single cysteine. Sequence and mass spectrometry analysis (mass error of −0.49) indicated that the single cysteine in nigroain-C2 was free.

cDNA screening of AMPs

One hundred and sixty-eight cDNA clones (Genbank accession nos. EU136401–EU136568) encoding AMP homologues were screened from the skin cDNA library of *R. nigrovittata*. These cDNA clones encode 34 different peptides including those purified from the skin secretions as described above, and belong to 9 peptide families (Table 1). BLAST search indicated that six families (nigroain-B to -E, nigroain-I, and nigroain-K) are novel peptide families because no similar sequences are found. The other three families designated as gaegurin-RN, rugosin-RN, and temporin-RN are similar to families of gaegurin [12], rugosin [13], and temporin [14], where RN is the abbreviation of *R. nigrovittata*.

Compared with other members, some of the peptides from families like nigroain-B4, -B5, -K1, and gaegurin-RN6 obviously are size and structure different. Extreme diversity of skin AMPs could be observed in a single individual. In the case of gaegurin-RN6, the stop codon mutation results in reading frame extension, creating a peptide of 47 amino acids, compared with the original length of 24 residues. In contrast to gaegaurin-RN6, nigroain-B4, -B5, and -K1 have a length of 12, 8, and 22 amino acid residues, respectively, shortened from the original length (19 and 31 amino acid residues), due to the creation of a premature termination codon. In addition, in these cases, these truncated peptides have lost the carboxyterminal conserved disulfide-bridged heptapeptide segments (CKLTGNC, CRVLGRC, CKVAGGC). A single point mutation in nigroain-D destroyed the disulfide bridge by changing the second cysteine into an arginine while maintaining the original length of the peptide.

The diversity of AMPs in *R. nigrovittata* also resides in disulfide motif in their sequences. Nigroain-B and -K, gaegurin-RN, and rugosin-RN have a seven-residue loop disulfided by two cysteines positioned at the C-terminus as found in most of amphibian skin AMP families. Nigroain-D has a 13-residue loop disulfided by two cysteines positioned at the N-terminus, while nigroain-E has the same loop in the middle of its sequence. To our knowledge, disulfide bridges composed of 11–13 residues only have been described in the frog of *Odorrana grahami* [7].

In addition, nigroain-D and -E are interesting templates or leading structures to design peptide antibiotics. The whole sequences of

Table 1
Antimicrobial peptides from the skin of the frog, *R. nigrovittata*.

Peptides	Sequences	Elution position in Fig. 1	OM	TM	MD
Nigroain-B1	CVISAGWNHKIRCKLTGNC				
Nigroain-B2	CVISAGWDHKVRCKLTGNC				
Nigroain-B3	CKI-ALPYH-MRCRVLGRC	B5	2017.48	2017.54	−0.06
Nigroain-B4	CVISAGWNHKIR				
Nigroain-B5	CKIALPYT				
Nigroain-C1	FKTWKRPPFQTSCSGIIKE				
Nigroain-C2	FKTWKRPPFQTSCWGIIKE	B9	2352.29	2352.78	−0.49
Nigroain-D1	CVHWQTNPARTSCIGP				
Nigroain-D2	CVHWQTNPARTSRIGP				
Nigroain-D3	CVHWQTNTARTSCIGP	B2	1772.49	1772.00	+0.49
Nigroain-E1	DCTRWIIGINGRICRD	B3	1889.91	1889.20	+0.71
Nigroain-E2	GCTQWINNIHGRICVRN				
Nigroain-I	SFLSKFKDIALDVPRMRARVY	B8	2513.30	2513.00	+0.30
Nigroain-K1	SLWETIKNAGKGFIQNILDKIR				
Nigroain-K2	SLWETIKNAGKGFILNILDKIRCKVAGGCKT	A4	3376.58	3376.05	+0.53
Gaegurin-RN1	FIGPVLKIAAGILPTAICKIFKKC	A1	2542.94	2543.27	−0.33
Gaegurin-RN2	FIGPVLKIATSILPTAICKIFKKC				
Gaegurin-RN3	FLGPIIKIATGILPTAICKILKKC				
Gaegurin-RN4	FVGPVLKIAAGILPTAICKIYKKC	B10	2545.14	2545.24	−0.10
Gaegurin-RN5	FLGPIIKIATGILPTAICKFLKKC	A2	2587.55	2587.32	+0.23
Gaegurin-RN6	FLGPIIKIATGILPTAICKILKKMLKLWKWKSS DVEYHLAKCTSDVL				
Gaegurin-RN7	FLGPIIKIATGILPTAICKILKNVETLEMEII				
Rugosin-RN1	SIRDKIKTIAIDLAKSAGTGVLKTLICKLDKSC				
Rugosin-RN2	SIRDKIKTIAIDLAKGAGTGVLKTLICKLDKSC				
Rugosin-RN3	SIRDKIKTIAIDLAKSAGMGILKTLICKLDKSC	A6	3547.83	3546.36	+1.47
Rugosin-RN4	SIRDKIKTIAIDLAKSAGTGVLKTSICKLDKSC				
Rugosin-RN5	SIRDKIKTIAIDLAKSAGTGVLKTLICKLNKSC	A5	3501.08	3501.26	−0.18
Rugosin-RN6	SFLSKFKDIALDVAKNAGKGVLTTLACKIDGSC				
Rugosin-RN7	RFLSKFKDIALDVAKNAGKGVLTTLACKIDGSC				
Rugosin-RN8	SFLSKFKDIALDVAKNAGKGVLTTLARKIDGSC				
Rugosin-RN9	SFLSKIKDIALDVAKNAGKGVLTTLACKIDGSC				
Temporin-RN1	FLPLVLGALSGILPKIL-NH2	B1	1762.95	1763.27	−0.32
Temporin-RN2	FFPLLFGALSSLLPKLF-NH2				
Temporin-RN3	FFPLLFGALSSHLPKLF-NH2	B4	1933.45	1933.35	+0.10

OM, observed mass; TM, theoretical mass; MD, mass difference.

nigroain-D and -E are nearly fallen into the circles connected by disulfide bonds.

We have purified and sequenced only 13 of the 34 peptides represented by the cDNAs we have characterized (Table 1). Other 21 peptides are just deduced from the cDNAs. However, to our knowledge, mRNA sequences isolated from frog skin universally are found to be translated into peptides. We suggest, but have not yet proven, that expressed mRNAs for which no peptide has as yet been isolated are actually translated into stable peptides.

Peptide precursor sequences

The precursors encoding nine families of AMP from *R. nigrovittata* were aligned in Fig. 2. These AMPs are from the precursors that have a common N-terminal preproregion, which is highly conserved, followed by a markedly different C-terminal domain that corresponds to the mature AMPs. They also share the conserved enzymatic processing sites (-Lys-Lys-, Lys-Arg-, or -Arg-Arg-) to release the mature peptides. The remarkable similarity of preproregions of precursors that give rise to very different AMPs suggests that the corresponding genes form a multigene family originating from a common ancestor. Many gene diversifications forming multigene family have been found. For example, 180 cDNA sequences of four-loop conotoxins have been reported from two species of the venomous gastropod *Conus* [15]. It was suggested that gene duplication and diversifying selection result in the formation of functionally variable conotoxins that are linked to ecological diversification and evolutionary success of this genus. Their skeletons of the gene products are not changed although they have experienced extensive diversification. All of the *Conus* toxins encoded by these diversified genes contain a "-C————C————CC—C——C-" cysteine "backbone" [15]. In contrast to the *Conus* toxins, the "backbones" in translated products of the diversified genes reported in this paper are extensively changed. Most of AMP families have lost the loop disulfided by cysteines. A similar type of structural diversity is seen in the vertebrate cathelicidin family precursor, where the C-terminal can include linear proline/arginine rich segments, linear alpha-helical segments, and segments with disulfide bonds, sometimes even from a single species (i.e., the pig) [16].

Antimicrobial activities

The diversification on primary structures of the AMPs from *R. nigrovittata* skin may underlie their diverse activities. Peptides that were representative of different families in the current population of skin peptides exhibited diverse activities (Table 2). However, peptides belonging to the same family also exhibited diverse activities. Nigroain-C3 and temporin-RN3 exhibit antimicrobial activities against all of the tested microbes including Gram-positive and Gram-negative bacteria and fungi. Nigroain-D3 and -E1 appear to target narrow antimicrobial spectrum, with low potency. Gaegurin-RN, rugosin-RN, and temporin-RN showed comparably antimicrobial abilities. The functional significance of structure diversification among these different families of an AMP has been compared as mentioned above. Considering that the sequence diversification not only occurred in different families but also occurred within the same ones, we checked the functional significance of structure diversification that occurred within families of nigroain-K, gaegurin-RN, rugosin-RN, and temporin-RN. As listed in Table 2, different AMP

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Nigroain-B1    MFTMKKSLFLLFFLGTISLSLCEEERDADEEDGGEATEQEERDVQRRCVISAGWNHKIRCKLTGNC
Nigroain-C1    MFPMKKSLLLLFFLGVISLSLCKQKRHADEEGNEVSGGEAKVEEVKRFKTWKRPPFQTSCSGIIKE
Nigroain-D1    MFTMKKSLLLIFFLGTISLSLCEQERNADEEDNEENGGEAKVEDVKRCVHWQTNPARTSCIGP
Nigroain-E     MFTMKKSLLLLFFLGTISLSLCEQERDADEEENEENGEETNLEVVKRDCTRWIIGINGRICRD
Nigroain-I     MFTMKKSLLLLFFLGTISLSLCEVERGADEDDGVEMTEEEVKRSFLSKFKDIALDVPRMRARVY
Nigroain-K2    MFTMKKSLLLLFFLGFVSLSLCEQERGADEDEGEDIEEVKRSLWETIKNAGKGFILNILDKIRCKVAGGCKT
Rugosin-RN1    MFTMKKSLLFLFFLGTISLSFCEEERSADEDDEGEMTEEEKRSIRDKIKTIAIDLAKSAGTGVLKTLICKLDKSC
Gaegurin-RN3   MFTMKKSLLLLFFLGTINLSLCEEERNAEEEKRDGDDEMDVEVQKRFLGPIIKIATGILPTAICKILKKC
Temporin-RN1   MFTMKKSLLLLFFLGTINLSLCEEERNTEEEKRDGDDEGSVEMQKRFLPLVLGALSGILPKILGK
Odorranain-A1  MFTMKKSLLLLFFLGTISLSLCEQERDADEEEGSENGAEDIKLNRVVKCSYRLGSPDSRCN
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Fig. 2. The sequence alignment of the precursors of antimicrobial peptides. The predicted signal peptides are italic; the mature peptides are underlined; Odorranain-A1 is from Li et al. [7].

belonging to the same family displayed extraordinarily different specificity and potential.

Hemolytic activity

Some AMPs were found to exert hemolytic activities [6,7]. Rabbit red blood cells were used to check for hemolytic capability in our experiments. Most AMPs exhibited weak hemolytic activity at the concentration of 100 μg/ml (Table 3). In contrast, nigroain-K2, gaegurin-RN1, temporin-RN1, and temporin-RN3 had strong hemolytic activity against rabbit red cells. Nigroain-K2 and temporin-RN1 could destroy 85–95% red cells even at the concentration of 50 μg/ml. Compared with nigroain-K2, nigroain-K1 lost the C-terminal disulfide bridge, and had a much weaker hemolytic activity. The current result implies that the C-terminal disulfide bridge may contribute to the hemolytic activity.

Mast cell degranulation assays (MCD)

Mast cells are secretory cells central to specific and innate immunity, allergy, and inflammation. Mast cells are present in most body tissues, but are particularly numerous in connective tissue, such as the dermis (innermost layer) of skin. In an allergic response, an allergen stimulates the release of antibodies, which attach themselves to mast cells. Following subsequent allergen exposure, the mast cells release substances such as histamine (a chemical responsible for allergic symptoms) into the tissue [17-22]. As listed in Table 4, most of AMPs (50 μg/ml) from the skin of *R. nigrovittata* could induce mast cell degranulation, with the control of 0.1% (v/v) Triton X-100, suggesting that these AMPs can regulate or mediate antimicrobial response by stimulating mast cell degranulation. Among them, the families of gaegurin-RN and temporin-RN showed much stronger ability to induce mast cell degranulation than other families. Half of the tested peptide could induce histamine release (Table 4). Nigroain-B1 had weak ability to induce mast cell degranulation, but showed a strong histamine-releasing activity. On the contrary, nigroain-E1 could induce mast cell degranulation but inhibited histamine release. These results together suggest that mast cell degranulation and histamine release induced by amphibian AMPs are not consistent although histamine is a kind of chemical mediator released by mast cell.

Discussion

The growing problem of resistance to conventional antibiotics has stimulated the need for developing new human therapeutics. The gene-encoded AMPs play an important role in innate immunity against noxious microorganisms [1]. Since they cause much less drug resistance of microbes than conventional antibiotics, AMPs nowadays attract considerable attention for the development of new antibiotics [1]. Amphibian skin is a multifunctional organ acting as defence, respiration, and water regulation although it seems susceptible. Amphibian skins are easy to be harmed by biological or non-biological attacks such as microorganism infection. Among vertebrates, skins of amphibian are exposed to more dangers of microorganism injury than others. Amphibian skins are potential reservoir to explore antimicrobial agents. Recently, we identified 107 AMPs belonging to 30 different families from *O. grahami* skin, 24 out of which are of novel families. They have similar precursors and theoretically originate from the common ancestor [7]. So many AMPs from a single species suggest that the possible amount of amphibian AMPs might be far more than those already known. Traditional biochemical methods, such as peptide purification and sequencing, encounter difficulties in identifying peptides expressed in trace. It is necessary to develop new strategy to identify defensive agents from amphibian skins and to avoid killing many amphibians. Precursors of Ranidae amphibian antimicrobial peptides have been proved to share conserved signal peptide sequence. Based on conserved signal peptide sequence in precursors of AMPs, peptidomics combined with cDNA trapping is an effective way to investigate novel amphibian AMPs [4,5,7]. The current results further confirmed the efficiency. Nine different families AMPs including six novel families were isolated from the skin secretions of *R. nigrovittata*. Another three families are belonging to

Table 2
Antimicrobial activity of antimicrobial peptides from skin of *R. nigrovittata*.

Microorganisms	MIC[a] (μg/ml)													
	B1	C2	D3	E1	K1	K2	GN1	GN4	GN5	RN1	RN3	RN5	TN1	TN3
S. aureus ATCC25923	50.00	27.50	ND	18.75	9.38	11.25	2.34	1.17	3.75	4.69	16.51	15.00	4.69	3.75
S. aureus ATCC43300	50.00	55.00	ND	ND	ND	9.38	9.38	9.38	62.50	ND	ND	12.50	ND	7.50
B. subtilis	ND	110.00	55.00	ND	21.88	9.38	4.69	2.34	4.69	9.38	18.75	18.75	9.38	7.50
C. albicans ATCC2002	ND	13.75	110.00	75.00	32.74	15.00	4.69	2.34	4.69	5.63	15.00	18.75	8.26	3.75
E. coli ML-35P	ND	110.00	ND	ND	37.50	ND	18.75	ND	37.50	25.00	25.00	25.00	ND	15.00
P. aeruginosa PA01	25.00	55.00	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	4.69	3.75
P. aeruginosa ATCC27853	25.00	55.00	ND	ND	ND	ND	ND	ND	ND	>100[b]	>100[b]	ND	4.69	3.75

ND, no detectable antimicrobial activity in a dose up to 100 μg/ml; B-E, nigroain-B to -E; K, nigroain-K; GN, gaegurin-RN; RN, rugosin RN; TN, temporin-RN.
[a] Minimal peptide concentration required for total inhibition of cell growth in liquid medium.
[b] Means have detectable antimicrobial activity by the method of filter paper but not total inhibition of cell growth in liquid medium in a dose up to 100 μg/ml.

Table 3
Red cell hemolysis activity of antimicrobial peptides from skin of *R. nigrovittata*.

Samples	Hemolysis (%)[a]	Samples	Hemolysis (%)[a]
B1	ND	GN4	18.64 ± 0.25
C2	ND	GN5	16.16 ± 3.22
D3	ND	RN1	6.69 ± 1.51
E1	7.62 ± 1.60	RN3	ND
K1	22.49 ± 4.80	RN5	ND
K2[b]	85.52 ± 3.26	TN1[b]	95.7 ± 4.12
GN1	46.13 ± 1.49	TN3	87.2 ± 3.67

The percentages represent values ± SD (standard deviations) of three independent experiments performed in duplicate. ND, no detectable hemolysis in a dose up to 100 μg/ml; B-E, nigroain-B to -E; K, nigroain-K; GN, gaegurin-RN; RN, rugosin RN; TN, temporin-RN. Triton X-100 (0.1%, v/v) was used as 100% control.
[a] The sample concentration was 100 μg/ml.
[b] The sample concentration is 50 μg/ml.

amphibian skin AMP families of rugosin, gaegurin, and temporin, respectively.

Some AMPs found in current work have unique structures, such as nigroain-B with three cysteines, nigroain-C with single cysteine, and nigroain-D and -E of which the whole sequences are nearly fallen into the circles connected by disulfide bonds. Such special structure might grant the peptides increased stability and strengthened resistance against protease. Therefore, these AMPs provide novel templates or leading structures to design antimicrobial agents.

Materials and methods

Collection of frog skin secretions

Adult specimens of *R. nigrovittata* of both sexes ($n = 30$; weight range 30–40 g) were collected in Yunnan Province of China and skin secretions were collected as follows. Frogs were put into a cylinder container, a piece of absorbent cotton immersed with anhydrous ether was put on the top of the container, and the container was covered with a lid and permeated with volatilized anhydrous ether. After 1–2 min, frog skin surface was seen to exude 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

Lyophilized skin secretion sample of *R. nigrovittata* (1.2 g, total $OD_{280\ nm}$ of 300) was dissolved in 10 ml 0.1 M phosphate buffer, pH 6.0, containing 5 mM EDTA. The sample was applied to a Sephadex G-50 (Superfine, Amersham Biosciences, 2.6 × 100 cm) gel filtration column equilibrated with 0.1 M phosphate buffer, pH 6.0. 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 indicated below. The fractions containing antimicrobial activities were pooled, lyophilized, and re-suspended in 0.1 M phosphate buffer, pH 6.0, and purified further by C_{18} or C_8 reverse-phase high-performance liquid chromatography (RP-HPLC, Hypersil BDS C_{18} or C_8, 30 × 0.46 cm) as illustrated in Fig. 1.

Structural analysis

Complete peptide sequencing was undertaken by Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer, model 491. The fractions with antimicrobial activity from RP-HPLC were analyzed by MALDI (Kratos Analytical). Mass fingerprints (MFPs) were obtained using a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF-MS) AXIMA CFR (Kratos Analytical) in positive ion and linear mode. The specific 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%.

SMART cDNA synthesis

Total RNA was extracted using TRIzol (Life Technologies Ltd.) from the skin of a single frog. cDNA was synthesized by SMART™ techniques by using a SMART™ PCR cDNA synthesis kit (Clontech, Palo Alto, CA). The first strand was synthesized by 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′. The second strand was amplified using Advantage polymerase by 5′ PCR primer II A, 5′-AAGCAGTGGTATCAACGCAGAGT-3′.

cDNA cloning

AMPs from Ranidae amphibians have been intensively studied. The conserved preproregion in Ranidae AMP precursor 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 [7]. The cDNA synthesized by SMART™ techniques was used as template for PCR to screen the cDNAs encoding AMPs. Two oligonucleotide primers, RS_1 (5′-CCAAA(G/C)ATGTTCACC(T/A)TGAAGAAA(T/C)-3′), in the sense direction, a specific primer designed according to the signal peptide sequences of AMPs from ranid frogs and primer II A as mentioned in "SMART cDNA synthesis" in the antisense direction were used in PCR reactions. The DNA polymerase was Advantage polymerase from Clontech (Palo Alto, CA) 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, 40 s at 72 °C. Finally, the PCR products were cloned into pGEM®-T Easy vector (Promega, Madison, WI). DNA sequencing was performed on an Applied Biosystems DNA sequencer, model ABI PRISM 377.

Antimicrobial testing

Microorganisms including Gram-positive bacterium *Staphylococcus aureus* (ATCC2592), *S. aureus* (ATCC43300, methicillin resistance), Gram-negative bacteria *Escherichia coli* (ML-35P) (penicillin resistance), *Bacillus dysenteriae*, *Pseudomonas aeruginosa* PA01, *P. aeruginosa* (ATCC27853), and fungus *Candida albicans*

Table 4
Mast cell degranulation (MCD) and histamine-releasing (HR) activity of antimicrobial peptides from skin of *R. nigrovittata*.

Samples	MCD (%)[a]	HR (%)[a]	Samples	MCD (%)[a]	HR (%)[a]
B1	15.04 ± 2.29	32.52 ± 7.63	GN4	45.99 ± 0.36	38.68 ± 2.46
C2	7.31 ± 1.63	ND	GN5	56.12 ± 7.98	54.71 ± 1.02
D3	ND	ND	RN1	14.33 ± 4.86	ND
E1	8.87 ± 1.63	−8.42 ± 0.16	RN3	4.43 ± 0.35	ND
K1	10.94 ± 1.49	ND	RN5	ND	ND
K2	29.78 ± 4.24	24.03 ± 0.60	TN1	77.57 ± 4.32	25.67 ± 2.76
GN1	63.86 ± 10.17	24.84 ± 3.48	TN3	80.43 ± 0.97	24.52 ± 6.98

The percentages represent values ± SD (standard deviations) of three independent experiments performed in duplicate. ND, no detectable activity in a dose up to 50 μg/ml; B-E, nigroain-B to -E; K, nigroain-K; GN, gaegurin-RN; RN, rugosin RN; TN, temporin-RN. Triton X-100 (0.1%, v/v) was used as 100% control.
[a] The percent of activity in a dose up to 50 μg/ml.

(ATCC2002) were obtained from Kunming Medical College and were first grown in LB (Luria–Bertani) broth or yeast extract–peptone–dextrose broth as our previous report [7]. MICs (minimal inhibitory concentration) of AMP against tested microorganisms were determined as previous reports [7]. The MIC was defined as the lowest concentration of test peptides inhibiting microorganism growth.

Hemolytic assays

Hemolytic assays were undertaken using rabbit red blood cells in liquid medium as reported [23]. Serial dilutions of the peptide were used, and after incubation at 37 °C for 30 min, the cells were centrifuged and the absorbance in the supernatant was measured at 540 nm. Maximum hemolysis was determined by adding 1% Triton X-100 to a sample of cells.

Mast cell degranulation assays

Mast cell degranulation was determined by measuring the release of β-D-glucosaminidase from lysed mast cells. Wistar rats were killed by cervical dislocation and mast cells were obtained by peritoneal washing of adult Wistar rats with Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.36 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 11.9 mM NaH_2CO_3, and 5.04 mM D-glucose). Ten microliter samples were added to 90 μl mast cells suspensions and incubated at 37 °C for 15 min. After centrifugation, 50 μl supernatants were added to 50 μl of the substrate (3 mg of *ρ*-nitrophenyl-*N*-acetyl-β-D-glucosaminidine dissolved in 10 ml of 200 mM sodium acetate, pH 4.5 solution) for β-D-glucosaminidase assay and the mixtures were incubated at 37 °C for 6 h. The reaction was terminated by addition of 100 μl 0.1 M Na_2CO_3 (pH 10.0). The absorbance of the colored product was assessed at 405 nm and the values were expressed as the percentage of total β-D-glucosaminidase activity from rat mast cell suspensions in the presence of 0.1% (v/v) Triton X-100 (used as 100% control).

Histamine release assay

Mast cells were obtained by peritoneal washing of adult Wistar rats with Tyrode's solution. Ten microliter samples were added to 90 μl mast cells suspensions and incubated at 37 °C for 15 min. The reaction was terminated by adding the ice-cold Tyrode's solution (2.9 ml). The cell suspensions were then centrifuged and both the supernatants and cell pellets were tested for histamine concentration as described[24]. Histamine-releasing activity was expressed as the percentage of the total cellular content and the values were corrected for spontaneous release without 5% exceeding in the absence of peptides.

Synthetic peptides

All peptides used for bioassays were synthesized by GL Biochem (Shanghai) Ltd. (Shanghai, China) and analyzed by HPLC and mass spectrometry to confirmed purity higher than 95%. All peptides were solved in water.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2009.09.004.

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