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Three novel antimicrobial peptides from the skin of the Indian bronzed frog *Hylarana temporalis* (Anura: Ranidae)‡

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Amphibian skin secretion is considered as a rich source of bioactive peptides. The present work describes the successful identification of three novel peptides named brevinin-1TEa, brevinin-2TEa and brevinin-2TEb present in the skin secretion of Indian bronzed frog *Hylarana temporalis*. The deduced open reading frame encoding the biosynthetic precursor of brevinin-1TEa consisted of 70 amino acid residues and brevinin-2TEa and brevinin-2TEb consisted of 71 and 72 amino acids, respectively. All the three peptides showed higher antimicrobial activity against Gram-negative than against Gram-positive bacteria. On the basis of the antibacterial and haemolytic activity, brevinin-2TEb is the most potent peptide reported in the present study. Further research on these peptides may provide potential clue towards newer drug development to combat various microbial diseases. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antimicrobial peptide; *Hylarana temporalis*; brevinin-1TEa; brevinin-2TEa; brevinin-2TEb

Introduction

The family *Ranidae* includes the most diverse and widely distributed group of anuran amphibians endowed with an excellent chemical defence system composed of pharmacologically active compounds. This holocrine skin secretion contains active peptides, cytosolic components and intact polyadenylated mRNAs encoding peptides and are stored in the granular skin glands and released on disturbances [1,2]. Membrane-disturbing mechanism of these peptides makes it difficult for the pathogen to develop drug resistance [3]. This make peptides from frog skin secretion an excellent leading biomolecule to design novel anti-infective therapeutic agents for the control of emerging microbial pathogens [4]. On the basis of extensive studies on the genus *Rana*, their active skin peptides are grouped into at least 13 distinct families of structurally related peptides including brevinin-1, brevinin-2, esculentin-1, esculentin-2, ranatuerin-1, ranatuerin-2, palustrin-1, palustrin-2, temporin, tigerinin, japonicin-1, japonicin-2 and nigrocin-2 [5]. Most of the frog skin antimicrobial peptides share a similar structural motif at their N-terminus, with phenylalanine or glycine as the first amino acid, while the C-terminus contains the Rana box, a cyclic heptapeptide domain [6].

Accumulated data from several studies have established that the organisation of amphibian skin peptide biosynthetic precursors is both highly ordered and conserved between different taxa [7]. In general, the hydrophobic amino acid residue-rich signal peptide is followed by a region rich in acidic amino acid residues and finally a single copy of bioactive peptide. From alignment of either nucleic acid sequences or open reading frames the amino acid sequences of cloned biosynthetic precursor transcripts from many species of amphibians showed highly conserved nucleic acid and amino acid sequences within the signal peptide as well as the 3′ and 5′ nontranslated regions. This permitted the design of general PCR primer sets that are class, family or genus specific within the amphibians [7]. Thus with a skin secretion-derived cDNA library from an unstudied species and a general primer set, one can amplify and clone peptide transcripts in the total absence of any specific peptide primary structural data [7]. Hence, the present work is designed to elucidate the natural peptides present in the skin secretion of Indian bronzed frog *Hylarana temporalis* using transcriptomic approaches.

Materials and Methods

Collection of Frog Skin Secretions

All the procedures involved in the present study were approved by the RGCB Institutional Animal Ethics Committee (IAEC/63/SG/2007/RGCB). Adult male and female ($n = 8$; 5–7 cm in length) frogs of *H. temporalis* were captured from different locations of Western Ghats, India. The skin secretions were obtained by mild transdermal electrical stimulation [8] and collected by washing the dorsal region with nuclease-free water and then the frogs were released immediately to the same habitat. The collected solutions were snap frozen with liquid nitrogen and lyophilized.

Construction of cDNA Library

Polyadenylated mRNA was isolated from stabilization buffer using magnetic oligo (dT) beads as directed by the manufacturer (Dy-

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Abbreviations used: *GRAVY, Grand average hydropathicity.*

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nal Biotech, UK). PCR-based cDNA library was constructed using SMART™ cDNA Amplification Kit (Clontech, UK) in strict accordance with the manufacturer's instruction. The first-strand cDNA was synthesised using MMLV RT by SMART II™ A Oligonucleotide Primer 5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′and 3′CDS Primer A 5′-AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$V N-3′ (N = A, C, G or T; V = A, G, C) supplied with the kit. The second strand was amplified using Advantage DNA Polymerase from Clontech-UK, by 3′CDS Primer A and 5′PCR primer 5′-AAGCAGTGGTATCAACGCAGAGT-3′.

Screening of cDNA for Encoding Antimicrobial Peptides

Two sense primers, including a degenerate (S1; 5′-GAWYYAYY HRAGCCYAAADATG 3′) and a specific primer were designed from a highly conserved domain of the signal peptide and 5′-untranslated regions of antimicrobial peptide-encoding cDNAs from ranid frogs. 3′CDS primer A was used in PCR reactions as the antisense primer. The PCR was done with Advantage DNA Polymerase (Clontech, UK) under the following conditions: 94 °C for 2 min; followed by 30 cycles of 92 °C for 10 s, 50 °C for 30 s, 72 °C for 40 s; and again followed by a final extension at 72 °C for 10 min. PCR products were gel purified and finally cloned into pGEM-T easy vector system (Promega Corp.). Plasmids were isolated, purified and sequenced using an ABI 3730 automated sequencer.

Bioinformatic Analysis and Physicochemical Properties of the Antimicrobial Peptides

The primary structure of deduced peptides was subjected to homology searches using BLAST (NCBI). Theoretical molecular weight, theoretical pI, net charge and grand average of hydropathicity (GRAVY) [9] were computed using ProtParam (http://expasy.org/tools/protparam.html).

Peptide Synthesis

Mature peptides were prepared by solid-phase synthesis using amino acids protected at the Nα position with Fmoc. In short, Tentagel resin was used as the support to obtain a carboxylated C-terminal peptide. Synthesis was carried on at a scale of 0.1 mmol with 1-h coupling time. O-Benzotriazole-N,N,N′,N′-tetramethyluronium-hexafluorophosphate (HBTU)/N-hydroxybenzotriazole (HOBt) were the coupling reagents and the deprotection reaction was for 30 min. The Kaiser test was performed after each amino acid coupling step and the coupling was repeated if necessary. The peptides were cleaved from the resin by treatment with the cocktail (94.5% TFA, 2.5% water, 2.5% EDT, 1% TIS) for 3 h. The synthetic peptides were then purified by RP-HPLC and their identity was confirmed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry, MALDI-TOF-MS (Voyager DE Pro, Applied Biosystems).

Circular Dichroism (CD) Spectroscopy

CD spectroscopy was performed using a Jasco J-715 spectrophotometer to analyse the secondary structure of the peptides. The spectra were recorded from 190 to 250 nm with a bandwidth of 2 nm and step of 1 nm, at a scanning rate of 200 nm/min. Samples with a peptide concentration of 0.5 mg/ml were prepared in the solvents: water and trifluroethanol (TFE)/water (50% (v/v), and added in a quartz optical cell with a pathlength of 1 nm at 25 °C. The spectra were averaged over three consecutive scans, followed by subtraction of the CD signal of the solvent. The mean residue molar ellipticity was calculated using the formula $(\Theta)_{222\ nm} = (\Theta_{obs} \times m.r.w)/(10 \times d \times c)$, where Θ_{obs} is the observed ellipticity in degrees [10].

Antimicrobial Assays

Bacterial strains used for antimicrobial assays included Gram-positive bacteria (*Staphylococcus aureus*: ATCC 25923; *Bacillus cereus*: NCLM 2156) and Gram-negative bacteria (*Pseudomonas aeruginosa*: ATCC 10145; *E. coli*: NCLM 2064; *Vibrio cholerae*: PL 91). Microorganisms were grown in Luria–Bertani (LB) broth to an absorbance of 0.8 OD at 600 nm. The minimum inhibitory concentrations (MICs) were determined in 96-well microtiter plates by growing the bacteria (4×10^5 CFUs/ml) in the presence of different concentrations of peptide. MICs were determined as the lowest peptide concentration at which no bacterial growth occurred. Serial dilutions of ampicillin and kanamycin were used as Gram-positive and Gram-negative bacterial control antibiotics, respectively.

Haemolysis Assays

Haemolytic assays were carried out in liquid medium using human red blood cells (20% suspension (v/v)) from a healthy donor. Different concentrations of the peptide were incubated with human blood cells at 37 °C for 30 min. After centrifugation (3000 × g for 5 min), the absorbance of the supernatant was measured at 540 nm. Samples treated with deionised water were treated as the control for 100% haemolysis.

Results

cDNA Cloning

Three different cDNA sequences, one encoding brevinin-1 and two encoding brevinin-2 precursor proteins, were obtained from the skin cDNA library of *H. temporalis*. The nucleic acid sequences of each cDNA were confirmed in at least five replicate clones. The nucleotide and deduced amino acid sequences of the three antimicrobial peptides are illustrated in Figure 1. By BLAST search, the first peptide was predicted with a sequence identity of 85% to gaegurin (brevinin-1 family) from *Hylarana nigrovittata* [11] and the remaining two peptides had a sequence identity of 84% (Figure 2) to brevinin-2LTa from *Hylarana latouchii* [12]. The new peptides were named brevinin-1TEa, brevinin-2TEa and brevinin-2TEb, respectively (TE = temporalis), in accordance with nomenclature rules recently suggested for frog skin peptides [5].

The deduced open reading frame encoding the biosynthetic precursors of brevinin-1TEa consisted of 70 amino acid residues, while those of brevinin-2TEa and brevinin-2TEb consisted of 71 and 72 amino acids, respectively. The mature peptide of brevinin-1TEa comprises 24 residues, brevenin-2TEa 29 and brevinin-2TEb 30. The domain topology of the peptide precursors are shown in Figure 3. The conserved prepro-regions of each precursor open reading frame include a putative 22 amino acid residue signal peptide followed by an N-terminal acidic spacer domain. Release of different mature C-terminal antimicrobial peptides is effected by the termination of N-terminal acidic spacer domain in a dibasic cutting site Lys-Arg (K-R) for trypsin-like proteases cleavage [13].

Brevinin-1TEa

```
     M  F  T  L  K  K  S  M  L  L  L  F  F  L  G  T  I  N  L  S
  1 ATGTTCACCTTGAAGAAATCCATGTTACTCCTTTTCTTCCTTGGGACCATAAACCTATCT

     L  C  E  E  E  R  N  A  E  E  E  R  R  D  D  D  D  K  R  N
 61 CTCTGTGAGGAAGAGAGAAATGCAGAAGAAGAAAGAAGAGATGATGATGATAAAAGGAAT

     T  E  V  Q  K  R  F  F  G  P  L  I  K  I  A  T  G  V  L  P
121 ACTGAAGTGCAAAAACGATTTTTTGGACCCTTGATAAAGATTGCTACTGGTGTTTTGCCT

     N  L  I  C  K  A  L  G  K  C  *
181 AATTTAATATGTAAAGCATTAGGGAAATGTTGAAACTTGAATTGGAAATCATCTGATGCT
241 GAATAACATTTAGCTAAACACATATCAGATGTCTTACGAAAAAAAAAAAAAAAAAAAAA
301 AA
```

Brevinin-2TEa

```
     M  F  T  M  K  K  P  M  L  L  L  F  F  L  G  T  I  S  L  S
  1 ATGTTCACCATGAAGAAACCCATGTTACTCCTTTTCTTCCTTGGGACCATCTCCTTATCT

     L  C  Q  E  E  R  G  A  D  E  D  D  G  G  E  M  T  E  E  E
 61 CTCTGTCAGGAAGAGAGAGGTGCCGATGAAGACGATGGAGGGGAAATGACAGAAGAAGAA

     K  R  G  I  G  S  M  L  L  G  L  A  K  N  V  G  M  S  L  L
121 AAAAGAGGTATTGGGAGTATGCTCTTGGGGCTCGCCAAGAATGTGGGCATGTCGTTGTTG

     N  K  A  Q  C  K  I  S  G  K  C  *
181 AATAAGGCTCAATGTAAAATTTCTGGAAAATGTTAAAACATGAATTGGAAGTCATCCAAT
241 GTGGAATATCATTTATTAAATGCTAAATGTCTGGTAAAAAAAAAAAAAAAAAAAAAAAA
301 AAAAA
```

Brevinin-2TEb

```
     M  F  T  L  K  K  S  K  L  L  L  F  F  L  G  T  I  S  L  S
  1 ATGTTCACCTTGAAGAAATCCAAGTTACTCCTTTTCTTTCTTGGGACCATCTCCTTATCT

     L  C  E  E  E  R  G  A  D  E  E  D  G  G  E  M  T  E  E  V
 61 CTCTGTGAGGAAGAGAGAGGTGCTGATGAAGAAGATGGAGGGGAAATGACAGAAGAAGTA

     K  R  G  F  M  G  D  T  L  K  G  I  A  R  N  A  A  L  A  L
121 AAAAGAGGTTTCATGGGGGATACACTCAAGGGTATAGCCAGGAATGCGGCCTTGGCATTG

     M  N  A  A  Q  C  K  L  S  G  K  C  *
181 ATGAACGCGGCTCAATGTAAACTTTCTGGAAAATGTTAAAACAGGAATAATACATCATCT
241 GATGTGGAATATCATTTAGCTAAATGCAAAATGTCTGGTAAAAAAAAAAAAAAAAAAAA
301 AAAAAAAAA
```

Figure 1. Nucleic acid sequences of cDNAs encoding the novel peptides brevinin-1TEa, brevinin-2TEa and brevinin-2TEb cloned from the skin secretion library of *Hylarana temporalis*. Putative signal peptides are single underlined, mature peptides are double underlined and stop codons are indicated by asterisks.

Chemical Synthesis and MALDI–TOF Mass Spectrometry

The solid-phase chemical synthesis of brevinin-1TEa, brevinin -2TEa, brevinin-2TEb was found to be highly successful. The major products obtained in each case represented approximately 95% of the synthetic mixture. By MALDI-TOF-MS analysis, the observed molecular weights of the three peptides matched well with the theoretical molecular weights (Table 1).

Circular Dichroism (CD) Spectroscopy

The CD spectra (Figure 4) of all the peptides were solvent-dependent. In a membrane-mimetic solvent such as 50% TFE/water, the CD spectra were supportive of α-helical structure. In contrast, in water all the peptides exhibited mainly random coil conformations. Secondary structural analysis of the mature peptides using CD spectra showed that 2TEa has the lowest molar ellipticity at 222 nm, while 2TEb showed the highest ellipticity value in TFE/water (Figure 4). The grand average hydropathicity (calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence) of the mature peptide was found to be positive indicating hydrophobic nature of the deduced peptides which may enable them to interact easily and efficiently with microbial membrane. Theoretical pI, net charge and hydropathicity of all three peptides are shown in Table 1.

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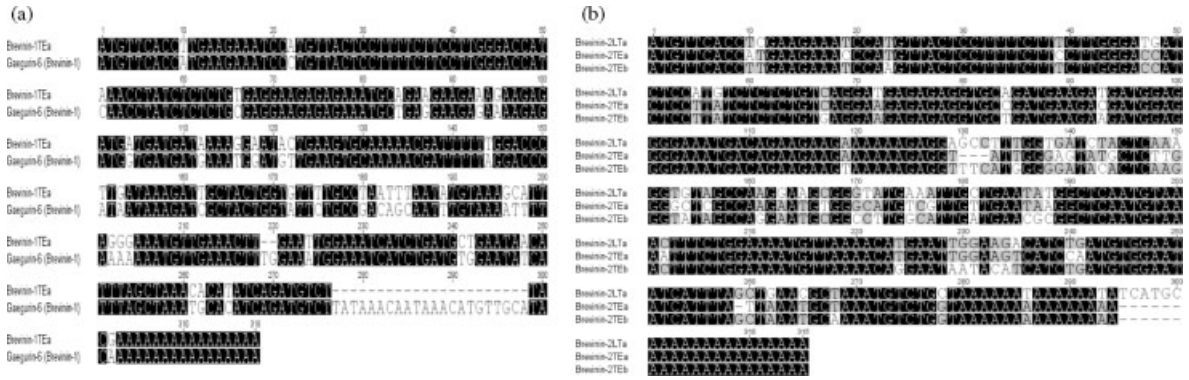

Figure 2. (a) Alignment of nucleotide sequences of cDNA clones encoding brevinin-1TEa compared with brevinin-1(gaegurin) of *Hylarana nigrovittata*. (b) Alignment of nucleotide sequences of cDNA clones encoding brevinin-2TEa, brevinin-2TEb compared with brevinin-2LTa of *H. latouchii*. Conserved nucleotides shown white on black and consensus nucleotides shown black on grey, gaps have been included to maximise alignment.

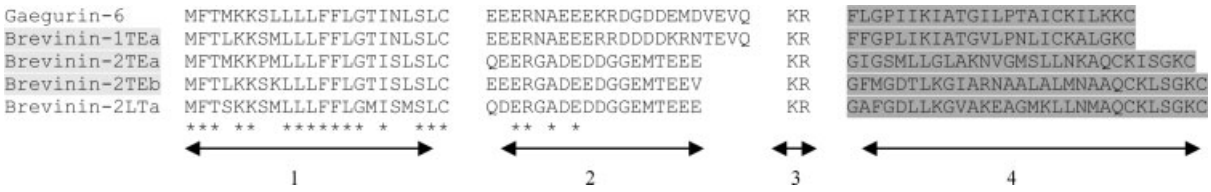

Figure 3. The comparison of the domain topology of peptide precursors, deduced from cDNAs cloned from the skin of *Hylarana temporalis*, with other Rana antimicrobial peptide precursors. Gaegurin-6 and Brevinin-2LTa from Genbank [accession nos: EU136546 and FJ647173]. (1) The putative signal peptide domain; (2) Variable length acidic 'spacer' peptide domain; (3) dibasic residue propeptide convertase cleavage site; (4) variable mature peptides encoding domain. Mature peptide sequences are shaded in grey. Conserved residues are indicated by asterisks.

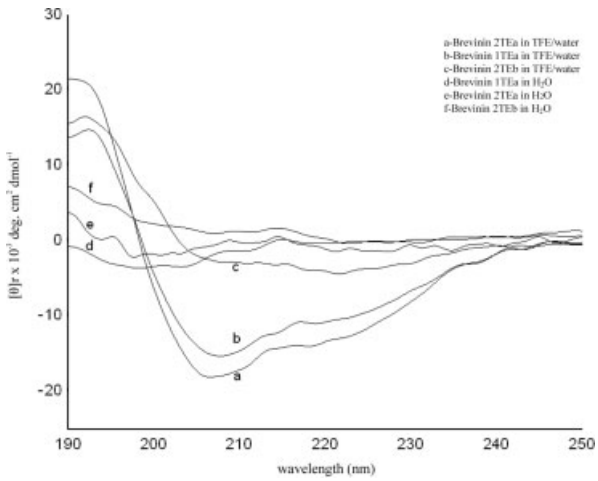

Figure 4. Circular dichroism spectra of antimicrobial peptides from *Hylarana temporalis*.

Antimicrobial activity

Brevinin-1TEa, brevinin-2TEa and brevinin-2TEb exhibited different antimicrobial activities against the tested bacteria. Table 2 shows the MIC value of each peptide against tested Gram-negative bacteria (*E. coli, P. aeruginosa* and *V. cholerae)* and Gram-positive bacteria (*B. cereus* and *S. aureus)*. The three peptides were more active against Gram-negative than Gram-positive bacteria.

Haemolytic Activity

In this study, we used human red blood cells to examine the haemolytic activity. It was observed that brevinin-2TEb revealed little haemolytic activity even at peptide concentrations up to 200 µg/ml, whereas brevinin-1TEa and brevinin-2TEa exhibited haemolytic activity (Table 2).

Discussion

Genus *Hylarana* is mainly distributed in tropical and subtropical regions of Asia as well as in the savannas of Africa. In this study, we purified and sequenced three novel antimicrobial peptides from the skin secretion of *H. temporalis*. Three different mature antimicrobial peptides belonging to two families were deduced from the cDNA sequences encoding peptide precursors. Primary structural characterization of the *H. temporalis* antimicrobial peptides revealed that they were members of the brevinin-1 and brevinin-2 families. Thus, according to a recent nomenclature recommendation by Conlon [5], the peptides from *H. temporalis* isolated in this study were named brevinin-1TEa, brevinin-2TEa and brevinin-2TEb.

Brevinin-1 was first identified from the skin of *Rana brevipoda porosa* (renamed as *Pelophylax porosus*) [14,15]. The antimicrobial peptides belonging to the brevinin-1 and -2 families have the common Rana-box motif (i.e. a C-terminal cyclic heptapeptide domain stabilized by a single disulfide bridge). The synthetic peptides in the present study were oxidised using 10% DMSO in water to form the Rana-box motif which was confirmed by analytical HPLC and MALDI-TOFF MS analyses. Nearly 80% amino acid sequence of brevinin-1TEa was similar to brevinin-1 from the skin of *H. nigrovittata*. It was found that the Phe^{1}, Ala^{9}, Val^{12} and Pro^{14} residues were strongly conserved in the brevinin-1 family which might be important functional determinants of antimicrobial activity. It has been suggested that Pro^{14} residue produces a stable kink in the molecule [16] and this feature

Table 1. Structural parameters of the mature peptides from the skin cDNA library of *Hylarana temporalis*

Peptide	GRAVY	Number of amino acids	Net charge	Theoretical pI	Theoretical mass	Observed mass
Brevinin-1TEa	1.117	24	+3	9.39	2517.16	2515.43
Brevinin-2TEa	0.486	29	+4	9.70	2935.6	2933.98
Brevinin-2TEb	0.293	30	+3	9.39	3054.6	3053.05

Table 2. Action of antimicrobial peptides of *Hylarana temporalis* against microorganisms and haemolytic activity against human red blood cells

	MIC (μg/ml)				
	Control				
Microorganism	Amp	Kan	Brevinin-1TEa	Brevinin-2TEa	Brevinin-2TEb
Gram-negative bacteria					
E. coli (NCLM 2064)	–	13	30	40	20
P. aeruginosa (ATCC 10145)	–	33	150	100	40
V. cholerae (PL 91)	–	20	150	150	60
Gram-positive bacteria					
B. cereus (NCLM 2156)	76	–	100	>150	150
S. aureus (ATCC25923)	43	–	>150	>150	40
Peptide concentration in μg/ml				Percentage haemolysis	
50	–	–	95	49.7	9.42
100	–	–	96	87.2	15.8
150	–	–	97.5	92.5	20.53
200	–	–	97.8	100	23.79

might be important in producing transmembrane pores that lead to bacterial cell lysis [17]. Brevinin-2TEa and brevinin-2TEb consist of 29 and 30 amino acids, respectively. Brevinin-2TEa contains just one amino acid deletion in the position two when compared to the previously identified brevinin-2LT a peptide from *Hylarana latouchii*. The peptide sequences obtained in the present study were deposited in GeneBank (accession nos. GU233779, GU233777, GU233778).

The actual antimicrobial mechanism of antimicrobial peptides is still unclear, although many hypotheses have been presented [18]. In our study, brevinin-1TEa, brevinin-2TEa and brevinin-2TEb exhibited different antimicrobial activity against tested bacteria (Table 2). The CD spectral measurements in TFE/water showed high ellipticity and lowest α-helicity for brevinin 2TEb than brevinin 1TEa and 2TEa (Figure 4). The reduced haemolytic activity of brevinin-2TEb (net charge +3) may be due to such a reduced α-helicity as observed by other authors [19]. The primary structure of brevinin-2 is very poorly conserved among the frog species studied as well as among individual members of the family within a single species with only three amino acid residues invariant in the peptide chain while no variant residue was noticed in brevinin1 [20]. Each antimicrobial peptide from a particular species has a unique amino acid sequence and is rarely identical in another species even when they are closely related phylogenetically. Further studies on this genus may enable the identification of a large number of antimicrobial peptides.

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References

1 Chen T, Farragher S, Bjourson AJ, Orr DF, Rao P, Shaw C. Granular gland transcriptomes in stimulated amphibian skin secretions. *Biochem. J.* 2003; **371**: 125–130.
2 Simmaco M, Mignogna G, Barra D. Antimicrobial peptides from amphibian skin: what do they tell us? *Biopolymers* 1998; **47**: 435–450.
3 Mangoni ML. Temporins, anti-infective peptides with expanding properties. *Cell. Mol. Life Sci.* 2006; **63**: 1060–1069.
4 Lu Y, Li J, Yu H. Two families of antimicrobial peptides with multiple functions from skin of rufous-spotted torrent frog *Amolops loloensis*. *Peptides* 2006; **27**: 3085–3091.
5 Conlon JM. Reflections on a systematic nomenclature for antimicrobial peptides from the skin of frogs of the family Ranidae. *Peptides* 2008; **29**: 1815–1819.
6 Park JM, Jung JE, Lee BJ. Antimicrobial peptides from the skin of a Korean frog, *Rana rugosa*,. *Biochem. Biophys. Res. Commun.* 1994; **205**: 948–954.
7 Shaw C, Chen T. Analysis of the peptidomes of amphibian skin granular gland secretions – an integrated functional genomic strategy. In *Peptidomics-Methods and Applications*, Soloview M, Shaw C, Andren P (eds). Wiley-Interscience: UK, 2007; 3–23.
8 Tyler MJ, Stone DJ, Bowie JH. A novel method for the release and collection of dermal, glandular secretions from the skin of frogs. *J. Pharmacol. Toxicol. Methods* 1992; **28**: 199–200.
9 Kyte J, Doolittle RF. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 1982; **157**: 105–132.
10 McCubbib WD, Kay CM, Sorasakn N, Kisilevsky R. Circular-dichroism studies on two murine serum amyloid A proteins. *Biochem. J.* 1988; **256**: 775–783.
11 Ma Y, Liu C, Liu X, Wu J, Yang H, Wang Y, Li J, Yu H, Lai R. Peptidomics and genomics analysis of novel antimicrobial peptides from the frog, *Rana nigrovittata*. *Genomics* 2010; **95**: 66–71.

12 Wang H, Lu H, Zhang X, Hu Y, Yu H, Liu J, Sun J. The novel antimicrobial peptides from skin of Chinese broad folded frog *Hylarana latouchii* (Anura: Ranidae). *Peptides* 2009; **30**: 273–282.
13 Li J, Xu X, Xu C, Zhou W, Zhang K, Yu H, Zhang Y, Zheng Y, Rees HH, Lai R, Yang D, Wu J. Anti-infection peptidomics of amphibian skin. *Mol. Cell. Proteomics* 2007; **6**: 882–894.
14 Frost DR, Amphibian species of the world: an online reference version 5.2, American Museum of Natural History, New York, USA, 2008; Electronic Database accessible at, http://research.amnh.org/herpetology/amphibia/index.php. [Accessed July 2008].
15 Morikawa N, Hagiwara K, Nakajima T. Brevinin-1 and 2, unique antimicrobial peptides from the skin of the frog, Rana brevipoda porsa. *Biochem. Biophys. Res. Commun.* 1992; **189**: 184–190.
16 Suh JY, Lee KH, Chi SW, Hong SY, Choi BW, Moon HM, Choi BS. Unusually stable helical kink in the antimicrobial peptide – a derivative of gaegurin. *FEBS Lett.* 1996; **392**: 309–312.
17 Isaacson T, Soto A, Iwamuro S, Knoop FC, Conlon JM. Antimicrobial peptides with atypical structural features from the skin of the Japanese brown frog *Rana japonica*. *Peptides* 2002; **23**: 419–425.
18 Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol. Rev.* 2003; **55**: 27–55.
19 Subasinghage AP, Conlon JM, Hewage CM. Development of potent anti-infective agents from Silurana tropicalis: conformational analysis of the amphipathic, alpha-helical antimicrobial peptide XT-7 and its non-haemolytic analogue [G4K]XT-7. *Biochim. Biophys. Acta* 2010; **1804**: 1020–1028.
20 Conlon JM, Al-Ghaferi N, Abraham B, Jiansheng H, Cosette P, Leprince J, Jouenne T, Vaudry H. Antimicrobial peptides from diverse families isolated from the skin of Asian frog, Rana graham. *Peptides* 2006; **27**: 2111–2117.

