Skin bradykinin-related peptides (BRPs) and their biosynthetic precursors (kininogens): Comparisons between various taxa of Chinese and North American ranid frogs[☆]

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ABSTRACT

Bradykinins and related peptides (BRPs) occur in the defensive skin secretions of many amphibians. Here we report the structures of BRPs and their corresponding biosynthetic precursor cDNAs from the Chinese brown frog, *Rana chensinensis*, and the North American leopard frog, *Lithobates pipiens*. *R. chensinensis* skin contained four transcripts each encoding a different kininogen whose organizations and spectrum of encoded BRPs were similar to those reported for the pickerel frog, *Lithobates palustris*. In contrast, from *L. pipiens*, a single skin kininogen was cloned whose structural organization and spectrum of mature BRPs were similar to those reported for the Chinese piebald odorous frog, *Huia schmackeri*. These data also implied that the endogenous precursor processing proteases in each species pair have identical site-directed specificities, which in part may be dictated by the primary structures of encoded BRPs. Thus the spectra of skin BRPs and the organization of their biosynthetic precursors are not consistent with recent taxonomy. The natural selective pressures that mould the primary structures of amphibian skin secretion peptides are thought to be related to the spectrum of predators encountered within their habitats. Thus similarities and differences in skin bradykinins may be reflective of predator spectra rather than indicative of species relatedness.

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1. Introduction

Although most biomolecular research on amphibian defensive skin secretions has focused attention on the peptides with antimicrobial properties, there exists a plethora of additional bioactive peptides, many of which have structural/functional analogs in vertebrate neuroendocrine systems [10,13]. The bradykinins and related peptides (BRPs) are one of the major peptide families that have attracted much attention in the past 5 years, especially since cloning of the first skin kininogen (bombinakinin M/maximakinin from *Bombina maxima* [14,2]). A body of data now exists for the skin kininogen structures from

representative bombinid toads, ranid frogs and some hylids [1–8,14–19,21], and what has become apparent is the high degree of heterogeneity in both biosynthetic precursor organization and in the primary structures of mature, processed BRPs [1–8,14–19,21].

In the three species of bombinid toad studied, three different patterns of biosynthetic precursor organization have been reported. In the Oriental fire-bellied toad, *Bombina orientalis*, two different bradykinins are present in skin secretion – canonical bradykinin and its (Thr[6])-substituted variant – each encoded by a different precursor containing four copies and two copies of mature peptide, respectively [3]. In the European yellow-bellied toad, *Bombina variegata*, two BRPs are likewise present in skin secretion but these are the unique site-substituted variants, (Ala[3],Thr[6])-bradykinin and (Val[1],Thr[3],Thr[6])-bradykinin, each encoded as single copies on separate precursors [5]. In the Chinese large-webbed bell toad, *B. maxima*, bradykinin occurs with an N-terminal decapeptide extension and has been called bombinakinin M or maximakinin, and is encoded by several biosynthetic precursors containing different numbers of copies of this peptide [2,14]. Thus, even within a sample of closely related species of toad, there exists a high degree of heterogeneity in both mature BRP structures and in biosynthetic precursor organization.

In the ranids that have been examined to date, the situation is quite different with single, relatively large, tandem repeat-coding domain precursors and, in some species, smaller related isoforms probably arising from mRNA splicing events, encoding all or the majority of BRPs that have been identified within corresponding skin secretory peptidomes [15–17,21].

Phyllokinin precursors from the skins of phyllomedusine frogs possess only a single BRP coding sequence although a plethora of chemically discrete BRPs can be generated from this by multiple-site, post-translational modification [6].

The reason for the heterogeneity and diversity of BRPs in amphibian skin secretions is unclear but an emerging explanation, brought about through parallel bradykinin analog structural characterization in the blood of various vertebrate taxa, is that these may be targeted to endogenous receptors in the spectrum of vertebrate predators that each individual species encounters within different biotopes [7]. Recently, we have shown that the large number of BRPs in the skin secretion of the pickerel frog, *Lithobates palustris*, can be generated from a single biosynthetic precursor and several smaller precursors that probably arise from mRNA splicing events [17]. Here, we report that the spectrum of skin BRPs and the structural organization of their biosynthetic precursors in the Chinese brown frog, *Rana chensinensis*, are very similar to those of the North American pickerel frog, *L. palustris* [17]. Likewise, BRPs and their precursors from the North American leopard frog, *Lithobates pipiens*, closely resemble those of the Chinese piebald odorous frog, *Huia schmackeri* [15]. These data may indicate that the primary structural similarities observed in amphibian skin BRPs and the organization of the respective biosynthetic precursors between North American and Chinese ranid frogs, rather than reflecting phylogenetic relatedness, may arise from a fundamental survival requirement related to delivery of a defensive secretion that is molecularly tailored to specific predators.

2. Materials and methods

2.1. Acquisition of material

Chinese brown frogs (*R. chensinensis*, both sexes, $n = 6$, 4–6 cm snout-to-vent length) were captured in the vicinity of Xi'an, Shaanxi Province, People's Republic of China. Skins were dissected from pithed frogs and were rapidly sun-dried after which they were stored at $-20\,°C$ prior to analysis. Northern leopard frogs (*L. pipiens*, both sexes, $n = 10$, 5–8 cm snout-to-vent length) were obtained from a commercial supplier in the USA and were maintained in our purpose-designed amphibian facility for at least 2 years at 20 °C, under 12 h/12 h light/dark cycles, and fed multivitamin-loaded crickets three times per week. Skin secretion was obtained by gentle transdermal electrical stimulation, washed from the skin with deionized water, snap frozen in liquid nitrogen and lyophilized. Lyophilizate was stored at $-20\,°C$ prior to analysis.

2.2. Molecular cloning of skin bradykinin biosynthetic precursor (kininogen) cDNAs

Dried skin of *R. chensinensis* (approximately 50 mg) was chopped into small ($2\,mm^2$) pieces, placed in 1 ml of cell lysis/mRNA stabilization buffer (Dynal Biotech, UK) and extraction of polyadenylated mRNA was performed using Dynabeads® mRNA DIRECT™ Kit (Dynal Biotech, UK). Lyophilized skin secretion from *L. pipiens* (5 mg) was treated separately in the same manner as described above. The immobilized mRNA in each preparation was then reverse-transcribed. The resultant cDNA libraries from each species were subjected to 5′- and 3′-rapid amplification of cDNA ends (RACE) procedures to obtain full-length skin kininogen nucleic acid sequence data using a SMART-RACE kit essentially as described by the manufacturer (Clontech, UK). Briefly, the 3′-RACE reactions employed a nested universal primer (NUP) that was supplied with the kit and a sense primer (primer BK-S, 5′-CCRVCNGGGTTYASSCCWTTY-3′) for 3′-RACE was complementary to the amino acid sequences, -PPGFSPF and -PPGFTPF. 3′-RACE reactions were gel-purified and cloned using a pGEM-T vector system (Promega Corporation) and sequenced using an ABI 3100 automated DNA sequencer. The sequence data obtained from these 3′-RACE products were used to design a gene-specific antisense primer: BK-AS (5′-TCAATTCARGATTAAACGATTGCTGG-3′) to a region in the vicinity of the stop codon of the open-reading frame. 5′-RACE was carried out using this specific primer in conjunction with the NUP and the generated products were gel-purified, cloned and sequenced as described above. Following acquisition of these data, the RACE reactions were amplified using a second gene-specific sense primer: BK-S2 (5′-ATGTTCACCTTGAAGAAATCCCTG-TT-3′) designed to a site within the putative signal peptide domain and BK-AS. Products were likewise gel-purified, cloned and sequenced as described previously. All identified skin kininogen nucleotide sequences were represented in at least 10 individual sequenced clones.Identification of skin BRPs by reverse phase HPLC fractionation and mass spectrometry

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(A)      M   F   T   L   K   K   S   L   L   L   L   F   F   L   G   T   I
    1    ATGTTCACCT TGAAGAAATC CCTGTTACTC CTTTTCTTTC TTGGGACCAT
         S   L   S   L   C   E   Q   E   R   D   A   D   E   D   E   Y
   51    CTCCTTATCT CTCTGTGAAC AAGAGAGAGA TGCTGACGAA GACGAATATG
         A   G   D   A   K   A   E   D   V   K   R   A   G   Y   S   R   V
  101    CAGGGGACGC TAAAGCCGAA GACGTTAAAA GAGCAGGATA CTCGAGAGTG
         I   S   L   P   A   G   L   S   P   L   R   I   A   P   A   S   S
  151    ATCAGTCTCC CAGCTGGGCT GAGCCCACTT CGTATTGCAC CAGCGTCTTC
         R   M   I   R   R   P   P   G   F   S   P   F   R   I   A   P
  201    GAGAATGATA AGACGCCCAC CTGGGTTTAG CCCATTTCGT_ATTGCACCAG
         A   S   T   L   K   R   D   A   D   E   D   E   Y   A   G   E   A
  251    CATCTACTCT CAAGAGAGAT GCCGATGAAG ACGAATATGC AGGGGAAGCT
         K   A   E   D   V   K   R   A   G   I   R   R   P   P   G   F   S
  301    AAAGCCGAAG ACGTTAAAAG AGCAGGGATC AGACGCCCAC CTGGGTTTAG
         P   L   R   I   A   P   A   S   S   R   M   I   R   R   P   P
  351    CCCACTTCGT ATTGCACCAG CGTCTTCGAG AATGATCAGA CGCCCACCTG
         G   F   S   P   F   R   I   A   P   A   I   V   *
  401    GGTTTAGCCC ATTTCGTATT GCCCCAGCAA TCGTTTAATC CTGAATTGAA
  451    AATCATCTGA TGTTAAATGT AATTTAGCTA AATGCACAAC AGATGTCTAA
  501    TAAAAAATAA AATGGTCACA TAAAAAAAAA AAAAAAAA


(B)      M   F   T   L   K   K   S   L   L   L   L   F   F   L   G   T   I
    1    ATGTTCACCT TGAAGAAATC CCTGTTACTC CTTTTCTTTC TTGGGACCAT
         S   L   S   L   C   E   Q   E   R   D   A   D   E   D   E   Y
   51    CTCCTTATCT CTCTGTGAAC AAGAGAGAGA TGCTGACGAA GACGAATATG
         A   G   D   A   K   A   E   D   V   K   R   A   G   Y   S   R   V
  101    CAGGGGACGC TAAAGCCGAA GACGTTAAAA GAGCAGGATA CTCGAGAGTG
         I   S   L   P   A   G   L   S   P   L   R   I   A   P   A   S   S
  151    ATCAGTCTCC CAGCTGGGTT GAGCCCACTT CGTATTGCAC CAGCGTCTTC
         R   M   I   R   R   P   P   G   F   S   P   F   R   I   A   P
  201    GAGAATGATA AGACGCCCAC CTGGGTTTAG CCCATTTCGT ATTGCCCCAG
         A   I   V   *
  251    CAATCGTTTA ATCCTGAATT GAAAATCATC TGATGTTAAA TGTAATTTAG
  301    CTAAATGCAC AACAGATGTC TAATAAAAAA TAAAATGGTC ACATAAAAAA
  351    AAAAAAAAAA A


(C)      M   F   T   L   K   K   S   L   L   L   L   F   F   L   G   T   I
    1    ATGTTCACCT TGAAGAAATC CCTGTTACTC CTTTTTTTTC TTGGGACCAT
         S   L   S   L   C   E   Q   E   R   D   A   D   E   D   E   Y
   51    CTCCTTATCT CTCTGTGAAC AAGAGAGAGA TGCTGACGAA GACGAATATG
         A   G   D   A   K   A   E   D   V   K   R   A   G   Y   S   R   V
  101    CAGGGGACGC TAAAGCCGAA GACGTTAAAA GAGCAGGATA CTCGAGAGTG
         I   S   L   P   A   G   F   S   P   F   R   I   A   P   A   I   V
  151    ATCAGTCTCC CAGCTGGGTT TAGTCCATTT CGTATTGCCC CAGCAATCGT
         *
  201    TTAATCTTGA ATTGAAAATC ATCTGATGTT AAATGTAATT TAGCTAAATG
  251    CACAACAGAT GTCTAATAAA AAATAAAATG GTCACATAAA AAAAAAAAAA
  301    AAAA


(D)      M   F   T   L   K   K   S   L   L   L   L   F   F   L   G   T   I
    1    ATGTTCACCT TGAAGAAATC CCTGTTACTC CTTTTCTTTC TTGGGACCAT
         S   L   S   L   C   E   Q   E   R   D   A   D   E   D   E   Y
   51    CTCCTTATCT CTCTGTGAAC AAGAGAGAGA TGCTGACGAA GACGAATATG
         A   G   E   A   K   A   E   D   V   K   R   A   G   Y   S   R   M
  101    CAGGGGAAGC TAAAGCCGAA GACGTTAAAA GAGCAGGATA CTCGAGAATG
         I   R   R   P   P   G   F   S   P   F   R   I   A   P   A   I   V
  151    ATCAGACGCC CACCTGGGTT TAGCCCATTT CGTATTGCCC CAGCAATCGT
         *
  201    TTAATCCTGA ATTGAAAATC ATCTGATGTT AAATGTAATT TAGCTAAATG
  251    CACAACAGAT GTCTAATAAA AAATAAAATG GTCACATAAA AAAAAAAAAA
  301    AAAA
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Fig. 1 – Nucleic acid sequences and translated open-reading frames of (A) *Rana chensinensis* skin kininogen-1 (RCSK-1), (B) RCSK-2, (C) RCSK-3 and (D) RCSK-4. Putative signal peptide residues are double-underlined, mature bradykinin residues are single-underlined and the stop codons are indicated by asterisks. Both cDNA strands were sequenced but complementary strand omitted for brevity.

Samples from the dried skins of two *R. chensinensis* (dry weight 90 mg) were chopped into small (2 mm^2) pieces and placed directly into 10 ml of boiling deionized water for 10 min. Following this, the extract was removed from the heat source and permitted to cool to room temperature. Microparticulates were removed by centrifugation at 1500 × *g* for 10 min after which the resultant clear supernatant was decanted. Five milligrams of lyophilized skin secretion from *L. pipiens* was dissolved in 1 ml of 0.05% (v/v) aqueous trifluoroacetic acid (TFA) and clarified of microparticulates as above. The resultant clear supernatant was decanted. Both samples were subjected separately to fractionation by being pumped directly onto a reverse phase HPLC column (Phenomenex C-18, 25 cm × 0.45 cm) and peptides were eluted using a gradient formed from 0.05/99.5 (v/v) TFA/water to 0.05/19.95/80.0 (v/v/v) TFA/water/acetonitrile in 240 min at a flow rate of 1 ml/min. A Cecil CE4200 Adept (Cambridge, UK) gradient reverse phase HPLC system was employed and fractions were collected automatically at 1 min intervals. The molecular masses of polypeptides in each chromatographic fraction were analyzed off-line using matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI–TOF MS) on an Applied Biosystems 4700 Proteomics Analyzer in MS mode. Peptides with masses coincident with deduced sequences from cloned cDNAs were further analyzed using the same instrument in MS/MS mode.

3. Results

Following cloning and DNA sequencing, four different kininogen cDNAs were consistently identified in the cDNA library of *R. chensinensis* skin, each being represented in at least 10 different sequenced clones (Fig. 1). *R. chensinensis* skin kininogen-1 (RCSK-1) possessed an open-reading frame of 145 amino acid residues that encoded four BRPs —a single copy of R-13-R, a single copy of A-15-A and two copies of canonical bradykinin. RCSK-2 possessed an open-reading frame of 86 amino acid residues that encoded two BRPs—a single copy of R-13-R and a single copy of canonical bradykinin. RCSK-3 possessed an open-reading frame of 67 amino acid residues and encoded a single copy of the novel BRP, (Phe^9, Phe^{12}) R-13-R. RCSK-4 also possessed an open-reading frame of 67 amino acid residues but encoded a single copy of canonical bradykinin (Fig. 2). All of the predicted precursors had identical signal peptides and first acidic spacer peptides. A BLAST search in the EMBL Protein Sequence Database revealed that these skin kininogens also exhibited high degrees of structural similarity to the skin BRP precursors from *L. palustris* [17]. MS

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Rana chensinensis skin kininogen-1

SP     MFTLKKSLLLLFFLGTISLSLCEQE
1.     R DADEDEYAGDAKAEDV KR AGYS RVISLPAGLSPLR IAPASS
2.                              RMIR RPPGFSPFR IAPASTL
3.  KR DADEDEYAGEAKAEDV KR        AGIRRPPGFSPLR IAPASS
4.                              RMIR RPPGFSPFR IAPAIV.


Rana chensinensis skin kininogen-2

SP     MFTLKKSLLLLFFLGTISLSLCEQE
1.     R DADEDEYAGDAKAEDV KR AGYS RVISLPAGLSPLR IAPASS
2.                              RMIR RPPGFSPFR IAPAIV.


Rana chensinensis skin kininogen-3

SP     MFTLKKSLLLLFFLGTISLSLCEQE
1.     R DADEDEYAGDAKAEDV KR AGYS RVISLPAGFSPFR IAPAIV.


Rana chensinensis skin kininogen-4

SP     MFTLKKSLLLLFFLGTISLSLCEQE
1.     R DADEDEYAGDAKAEDV KR AGYS RMIR RPPGFSPFR IAPAIV.
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Fig. 2 – Domain architecture of *R. chensinensis* skin kininogens 1–4 (RCSK 1–4). Mature BRP sequences are in bold typeface. SP, signal peptide (putative).

and MS/MS spectra of R-13-R and the novel variant, (Phe[9], Phe[12]) R-13-R are shown in Figs. 3 and 4, respectively.

In contrast, a single multi-domain BRP precursor (LPSK-1) was consistently cloned from the skin secretion-derived cDNA library of *L. pipiens*. The open-reading frame consisted of 287 amino acid residues that contained 6 tandem repeat BRP-encoding domains (Fig. 5). These encoded one copy of (Val[1], Thr[6]) bradykinin, three copies of bradykinyl-IAPAS (that could generate canonical bradykinin), one copy of (Thr[6])-bradykinyl-IAPAS (that could generate (Thr[6]) brady-kinin) and a terminal domain that encoded canonical bradykinin.

The structural organization of skin kininogens from representative Chinese and North American ranid frogs are compared in Fig. 6. In species that contain a number of skin kininogens that are probable splice variants, comparisons are made with largest forms. The skin kininogens from *R. chensinensis* and *L. palustris* [17] exhibited a high degree of similarity in both coding domain structure and primary structures of mature BRPs (Fig. 6A). Likewise, skin kininogens from *L. pipiens* and *H. schmackeri* [14] exhibited a high degree of similarity in both attributes (Fig. 6B). However, skin kininogen B from the Chinese ranid, *Hylarana guentheri* [20], although exhibiting a similar tandem repeat BRP coding domain
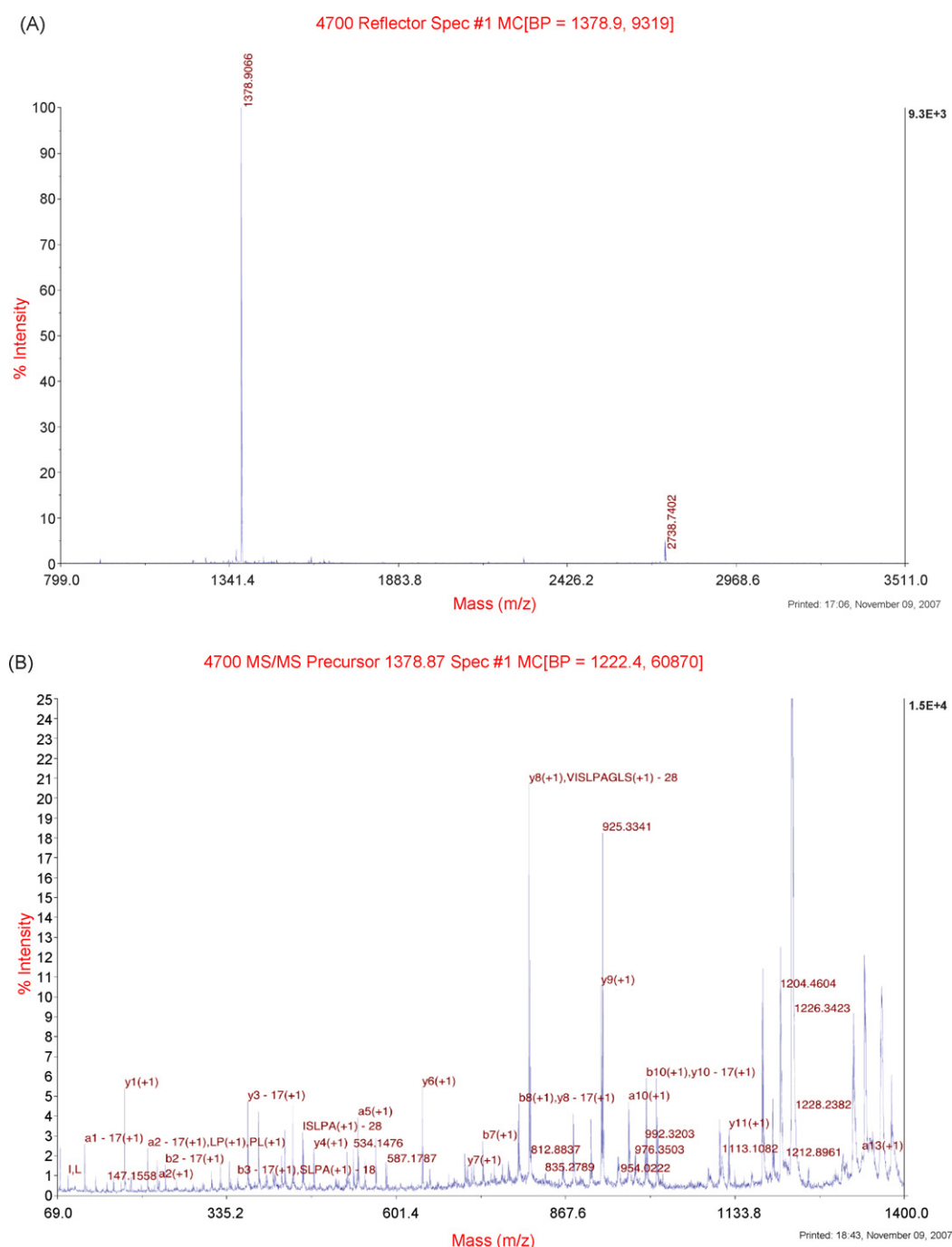

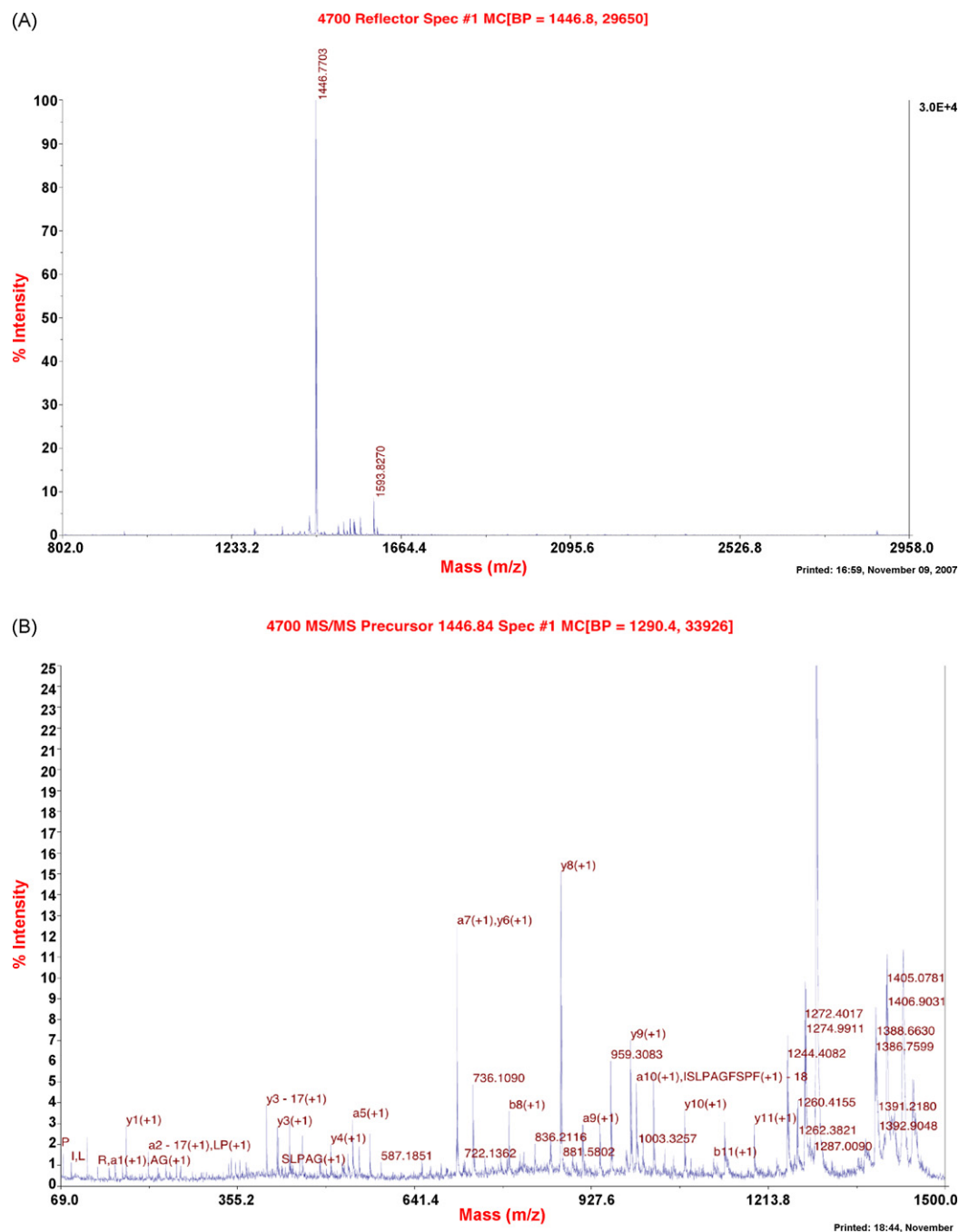Fig. 3 – (A) MS spectrum of R-13-R and (B) annotated MS/MS spectrum of R-13-R.

(A)

(B)

Fig. 4 – (A) MS spectrum of (Phe⁹, Phe¹²) R-13-R and (B) MS/MS spectrum of (Phe⁹, Phe¹²) R-13-R.

structure to the others, has an attenuated acidic spacer peptide of quite different primary structure and hence is obviously different (Fig. 6C). The only other known ranid frog skin kininogen that has been reported to date is that of the Chinese rufous-spotted torrent frog, *Amolops loloensis* (Fig. 6D). This contains a single BRP encoding domain. The mature peptide arising from this, amolopkinin, is an N-terminally extended (Val^1, Thr^6)-bradykinin [16].

BRPs predicted from cloned skin kininogen cDNAs were located by MALDI–TOF MS in HPLC fractions of skin secretions (Fig. 7) from both *R. chensinensis* and *L. pipiens* and their primary

structures were confirmed by MS/MS fragmentation. Those detected in each species are shown in Table 1.

4. Discussion

The Chinese Brown frog, *R. chensinensis*, is relatively common and widely distributed in Northern and Central China and its dried skin has been used in traditional chinese medicine (TCM) for many hundreds of years as a general tonic or a topical wound dressing [22]. Recently, using dried skins from another

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       M   F   T   L   K   K   S   L   L   L   L   F   F   L   G   T   I
  1  ATGTTCACCT TGAAGAAATC CCTGTTACTC CTTTTCTTTC TTGGGACCAT
       N   L   S   L   C   E   Q   E   R   D   A   D   E   D   E   N   E
 51  CAACTTATCT CTCTGTGAAC AAGAGAGAGA TGCTGATGAA GATGAAAATG
       R   E   A   K   V   E   D   V   K   R   A   G   Y   S   R   M
101  AACGGGAAGC TAAAGTGGAA GATGTTAAAA GAGCGGGATA CTCGAGAATG
       I   R   R   P   P   G   F   S   P   F   R   I   A   P   A   S   S
151  ATCAGACGCC CACCTGGGTT TAGCCCATTT CGCATTGCAC CAGCGTCTTC
       L   K   R   D   A   D   E   D   E   D   A   G   E   A   K   T   E
201  TCTCAAGAGA GATGCAGATG AAGATGAAGA TGCAGGGGAA GCTAAAACTG
       D   V   K   R   A   G   Y   S   R   M   I   R   R   P   P   G
251  AAGACGTTAA AAGAGCGGGA TACTCAAGAA TGATCAGACG CCCACCTGGG
       F   T   P   F   R   I   A   P   A   S   T   L   K   R   D   R   D
301  TTTACCCCAT TTCGTATTGC ACCAGCGTCT ACTCTCAAGA GAGATCGAGA
       E   D   E   Y   A   G   Q   A   K   A   E   D   V   K   R   A   G
351  CGAAGACGAA TATGCAGGGC AAGCTAAAGC CGAAGACGTT AAAAGAGCAG
       Y   S   R   M   I   R   V   P   P   G   F   T   P   F   R   I
401  GATACTCAAG AATGATCAGA GTCCCACCTG GGTTTACCCC ATTTCGTATT
       A   P   A   S   T   L   K   R   D   A   D   E   D   E   Y   A   G
451  GCACCAGCGT CTACTCTCAA GAGAGATGCT GATGAAGACG AATATGCAGG
       E   A   K   A   E   D   V   K   R   A   G   Y   S   R   M   I   R
501  GGAAGCTAAA GCTGAAGACG TTAAAAGAGC AGGATACTCG AGAATGATCA
       R   P   P   G   F   S   P   F   R   I   A   P   A   S   T   L
551  GACGCCCACC TGGGTTTAGC CCATTTCGTA TTGCACCAGC GTCTACTCTC
       K   R   D   A   D   E   D   E   Y   A   G   E   A   K   V   E   D
601  AAGAGAGATG CCGATGAAGA CGAATATGCA GGGGAAGCTA AAGTCGAAGA
       V   K   R   A   G   Y   S   R   M   I   R   R   P   P   G   F   S
651  CGTTAAAAGA GCAGGATACT CGAGAATGAT CAGACGCCCA CCTGGGTTTA
       P   F   R   I   A   P   A   S   T   L   K   R   D   S   D   E
701  GCCCATTTCG TATTGCACCA GCGTCTACTC TCAAGAGAGA TTCCGATGAA
       D   E   Y   A   G   E   A   K   A   E   D   V   K   R   A   G   Y
751  GACGAATATG CAGGGGAAGC TAAAGCCGAA GACGTTAAAA GAGCAGGATA
       S   R   M   I   R   R   P   P   G   F   S   P   F   R   I   A   P
801  CTCGAGAATG ATCAGACGCC CACCTGGGTT TAGCCCATTT CGTATTGCAC
       A   I   V   *
851  CAGCAATCGT TTAATCTTAA ATTGAAAATT ATCTGATGTA AAATGTAATT
901  TAGCTAAATG CACAACAGAT GTCTAATAAA AAATAAGATG GTCACTTAAA
951  AAAAAAAAAA AAAAAAAAAA
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Fig. 5 – Nucleic acid sequence and translated open-reading frame of *Lithobates pipiens* skin kininogen-1. Putative signal peptide residues are double-underlined, mature bradykinin residues are single-underlined and the stop codons are indicated by asterisks. Both cDNA strands were sequenced but complementary strand omitted for brevity.

species used in TCM, the Helongjiang brown frog, *Rana amurensis*, the presence of antimicrobial peptides and their mRNAs was demonstrated—a fact that might explain their use in topical wound dressings [22]. Since BRPs are common components of the skins of ranid frogs, it was decided to investigate the skin of *R. chensinensis* for the presence of BRPs and to attempt to clone their precursor cDNAs using our validated molecular cloning technique [4], through design of PCR primers to highly conserved domains identified through alignment of multiple ranid frog skin peptide cDNAs. Subsequent to successful molecular cloning of precursor (skin kininogen) cDNAs and translation of open-reading frames, the molecular masses of putative mature BRPs were calculated and these were sought by interrogation of mass spectra generated from reverse phase HPLC fractions of skin extracts. Using this approach, canonical bradykinin and three additional BRPs were confirmed as fully mature peptides within the skin secretion. While two of the BRPs (RVISLPAGLSPLR, R-

13-R; IRRPPGFSPLR, I-11-R) have been identified before as components of the skin peptidome of the North American pickerel frog, *L. palustris* [17], the third was a novel variant – RVISLPAGFSPFR – (Phe[9], Phe[12]) R-13-R. While three bradykinin-encoding cDNAs were cloned from *L. palustris* skin secretion, four were consistently cloned from *R. chensinensis*. All open-reading frames of *L. palustris*-derived clones contained multiple BRP-encoding domains in contrast to two of those identified in *R. chensinensis* that contained single BRP-encoding domains. *R. chensinensis* skin kininogen cDNA open-reading frames were generally smaller than those of *L. palustris* with RCSK(1–4) containing 4, 2, 1, and 1 BRP-encoding domains, respectively, compared to RPSK1–3, that contained 8, 6 and 5 BRP-encoding domains respectively [17]. RCSK-3 encoded a single copy of the novel BRP, (Phe[9], Phe[12]) R-13-R and RCSK-4 encoded a single copy of canonical bradykinin. This latter kininogen open-reading frame consisted of 67 amino acid residues and with removal of the putative signal peptide that

(A)
Rana chensinensis skin kininogen-1

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SP    MFTLKKSLLLLFFLGTISLSLCEQE
1.    R  DADEDEYAGDAKAEDV  KR  AGYS  RVISLPAGLSPLR  IAPASS
2.                              RMIR  RPPGFSPFR  IAPASTL
3.    KR  DADEDEYAGEAKAEDV  KR        AGIRRPPGFSPLR  IAPASS
4.                              RMIR  RPPGFSPFR  IAPAIV.
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Lithobates palustris skin kininogen-1

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SP    MFTLKKSLLLLFFLGTISLSLCEQE
1.    R  DADEDEYAGDAKAEDV  KR  AGYS  RVISLPAGLSPLR  IAPASS
2.                              RMIR  RPPGFSPFR  IAPASTL
3.    KR  DADEDEYAGEAKAEDV  KR  AGYS  RVISLPAGLSPLR  IAPASTL
4.    KR  DADEDEYAGEAKAEDV  KR  AGYA  RVISLPAGLSPLR  IAPASTL
5.    KR  DADEDEYAGEAKAEDV  KR  AGYA  RVISLPAGLSPLR  IAPASTL
6.    KR  DADEDEYAGEAKAEDV  KR  ARYS  RENSLPAGLSPLR  IAPASTL
7.                        KR        AGIRRPPGFSPLR  IAPASS
8.                              RMIR  RPPGFSPFR  IAPAIV.
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(B)
Lithobates pipiens skin kininogen-1

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SP    MFTLKKSLLLLFFLGTINLSLCEQE
1.    R  DADEDENEREAKVEDV  KR  AGYSRMIR  RPPGFSPFR  IAPAS  SL
2.    KR  DADEDEDAGEAKTEDV  KR  AGYSRMIR  RPPGFTPFR  IAPAS  TL
3.    KR  DADEDEYAGQAKAEDV  KR  AGYSRMIR  VPPGFTPFR  IAPAS  TL
4.    KR  DADEDEYAGEAKAEDV  KR  AGYSRMIR  RPPGFSPFR  IAPAS  TL
5.    KR  DADEDEYAGEAKVEDV  KR  AGYSRMIR  RPPGFSPFR  IAPAS  TL
6.    KR  DSDEDEYAGEAKAEDV  KR  AGYSRMIR  RPPGFSPFR  IAPAIV.
```

Huia schmackeri skin kininogen-1

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SP    MFTLKKSLLLLFFLGTINLSLCKEQE
1.    R  DADEDENEREAKVEDV  KR  AGYSRMIR  RPPGFSPFR  VAPASSL
2.    KR  DADEDENEREAKVEDV  KR  AGYARMIR  RPPGFSPFR  VAPASSL
3.    KR  DADEDENEREAKVEDV  KR  AGYSRMIR  RPPGFSPFR  VAPASSL
4.    KR  GADEDENEREAKVEDV  KR  AGYSRMIR  RPPGFSPFR  VAPASSL
5.    KR  DADEDENEREAKVEDV  KR  AGYARMIR  RPPGFSPFR  VAPASSL
6.    KR  DADEDENEREAKVEDV  KR  AGYSRMIR  RPPGFSPFR  VAPASSL
7.    KR  GADEDENEREAKVEDV  KR  AGYARMIR  RPPGFSPFR  IAPAIV.
```

(C)
Hylarana guentheri skin kininogen-B

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SP    MLTLKKSLLLLFFFGTISLSLCEQE
1.    R  DADEDENESRTV  R  VPPGFTPFR  IAPASSL
2.    KR  NADADETERRIS  K  RPPGFSPFR  IAPASSL
3.    KR  NADADETERRIS  K  RPPGFSPFR  IAPASSL
4.    KR  NADADETERRIS  K  RPPGFSPFR  IAPASSL
5.    KR  NADADETERRIS  K  RPPGFSPFR  IAPASSL
6.    KR  NADEDENESRMV  R  VPPGFSPFR  IAPEIV.
```

(D)
Amolops loloensis skin kininogen

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SP    MFTLKESLLLLFFLGAISLSLCEQE
1.    R  DADEEETEGGAKVETV  K  RAPVPPGFTPFR  VAPEIV.
```

Fig. 6 – Comparisons of structural organizations and derived bradykinins of largest skin kininogens deduced from cDNA cloning in Chinese and North American ranid frogs. (A) Skin kininogen-1 from *Rana chensinensis* and *Lithobates palustris* exhibit similar organization and spectrum of encoded bradykinins. Tandem repeat domain length is identical. (B) Skin kininogen-1 from *Lithobates pipiens* and *Huia schmackeri* exhibit similar organization and spectrum of encoded bradykinins. Tandem repeat domain length is identical. (C) Skin kininogen-B from *Hylarana guentheri* exhibits an organization and spectrum of encoded bradykinins that differentiates this species from those in either group (A) or (B) above. (D) Skin kininogen from Amolops loloensis exhibiting classical organization and unique single copy BRP.
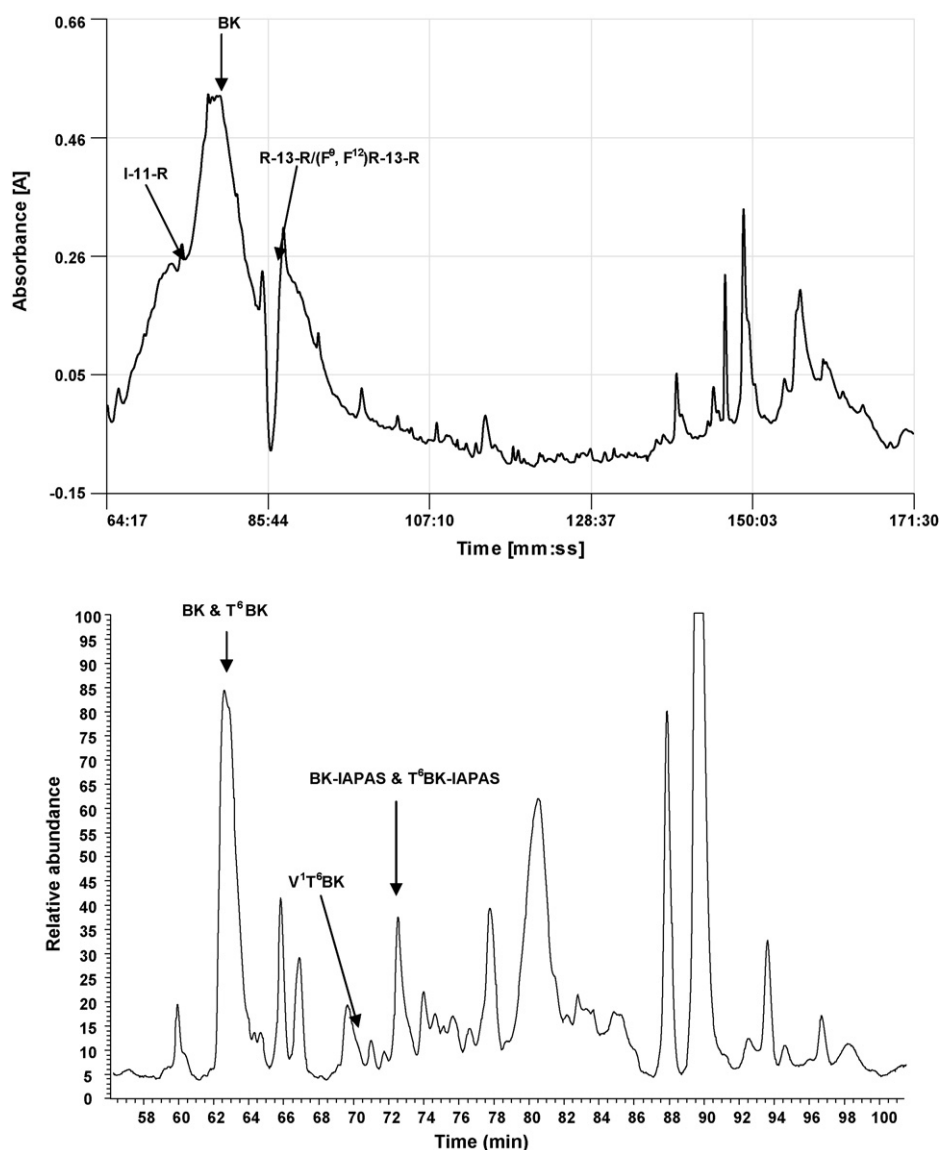
Fig. 7 – Regions of reverse phase HPLC chromatograms from _R. chensinensis_ skin extract (upper panel) and _L. pipiens_ skin secretion (lower panel). Retention times of respective BRPs in each chromatogram are indicated.

occurs during translocation across the Golgi membranes, the proprotein/probradykinin contains but 45 residues making this the smallest canonical bradykinin biosynthetic precursor found thus far in amphibian skin and also in nature. These

Table 1 – Bradykinins identified in stimulated defensive skin secretions of _Rana chensinensis_ and _Lithobates pipiens_

Rana chensinensis	Lithobates pipiens
RPPGFSPFR	RPPGFSPFR
RVISLPAGLSPLR	RPPGFTPFR
RVISLPAGFSPFR	RPPGFSPFRIAPAS
IRRPPGFSPLR[a]	RPPGFTPFRIAPAS
	VPPGFTPFR

Leu and Ile differentiated on basis of unambiguous codons in cloned cDNA sequences.
[a] Major mature product of the (Leu⁸)-bradykinin-encoding domain.

data further substantiate the fact that prediction of BRP primary structures, structural heterogeneity, biosynthetic precursor structural organization and post-translational processing/modification is not possible for even a single species within a rather well-defined amphibian taxon and that further in-depth study will undoubtedly provide researchers with even greater numbers of permutations with perhaps a few surprises.

Recent taxonomic revision of the Amphibia [9,11,12], including the family Ranidae, maintained _R. chensinensis_ within the genus _Rana_, changed the generic assignation of both _Rana palustris_ and _Rana pipiens_ to _Lithobates_, changed _Odorrana schmackeri_ to the genus _Huia_, but maintained the generic assignations of _H. guentheri_ and _A. loloensis_. These are to date (including the present study), all of the ranid species from which both skin BRP and skin kininogen structural data are available [14–19,21]. The spectrum of skin bradykinins and the domain architecture of their corresponding biosynthetic

precursors are shown in Fig. 3. The putative signal peptides are highly conserved across the species examined, in fact, these are subject to a high degree of primary structural conservation across the skin peptide precursors of many different anuran taxa [20]. The acidic amino acid residue-rich spacer peptides are likewise highly conserved in both primary structure and length across ranid species being hexadecapeptides in *R. chensinensis*, *L. palustris* and *pipiens* and in *H. schmackeri*. However, in *H. guentheri*, these domains differ in primary structure from type and are attenuated by four residues, consistent with its separate generic status. It is in the region distal to this domain that some of the most striking differences in precursor architecture are observed and these features probably dictate to a large degree, the cleavage patterns of convertases that ultimately produce diversity of BRPs that are encoded downstream. In the multiple tandem repeat domains that are found in *L. pipiens* and *H. schmackeri* skin kininogens, classical double basic residue propeptide convertase cleavage sites (-KR-) are located immediately after the acidic spacer peptide domain. These are flanked by a highly conserved octapeptide (AGYS/ARMIR) whose terminal arginyl (R) residue constitutes the P1 position of the convertase whose action generates the N-terminus of the mature bradykinins (either R or V). The bradykinin nonapeptide domains are flanked by a highly conserved heptapeptide (I/VAPASS/TL) and the open-reading frame terminates in a highly conserved hexapeptide (IAPAIV). In the skin secretion itself, isolation of mature BRPs would suggest that cleavage after the C-terminal arginyl residue of nonapeptide bradykinin is only partially achieved as C-terminally extended forms such as bradykinyl-I/VAPAS are often major products. Thus the mature BRPs, propeptide convertase cleavages and structural organization of skin kininogens are all very similar in *L. pipiens* and *H. schmackeri*. This was a rather counter intuitive finding as *L. pipiens* and *L. palustris* display very similar phenotypes and are often sympatric in nature. Indeed, many other features have suggested their congeneric classification in a recent taxonomic revision [11]. *L. palustris* skin BRPs and respective kininogens, however, were found to be most similar to those of *R. chensinensis* in terms of primary structure, propeptide convertase cleavages and domain architecture although the kininogens of *R. chensinensis* skin were smaller than those of *L. palustris*. Thus the skin BRPs and the domain architecture of their respective kininogens are quite different in the congeneric *Lithobates* species, each displaying highest structural similarities with species from two other ranid genera. The corresponding data from *Hylarana* and *Amolops* would be consistent with their different taxonomic status. The primary structures of BRPs and the organization of their biosynthetic precursors in ranid frogs, albeit from a small sample of species, do not appear to reflect current taxonomic status. This may be due to sample size, choice of species or may indicate that these parameters are not good taxonomic clues. On the other hand, this phenomenon may result from other external environmental selection pressures such as dictated by specific predator spectra (colubrid snakes, monitor lizards, birds and mammals) or by aggressive sympatrics (the gopher frog (*Lithobates capito*) is so named as it co-habits gopher tortoise (*Gopherus polypnemus*) burrows). The noxious properties of amphibian skin secretions have evolved for just this purpose

so it would be expected from a molecular evolutionary point of view, that these external factors play a major role in bioactive peptide selection based upon effectiveness and hence survival.

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