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SHORT REPORT

Molecular Cloning of a Novel Bradykinin-Related Peptide from the Skin of Indian Bronzed Frog *Hylarana Temporalis*

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Abstract: Bradykinin-related peptides (BRPs) constitute one of the most studied groups of bioactive peptides in amphibian skin secretions. The present study describes the successful isolation of a novel BRP (hylaranakinin TE) from the skin secretion of the Indian bronzed frog *Hylarana temporalis*. The deduced open reading frame consisted of 115 amino acid residues with a putative signal peptide of 22 amino acid residues, followed by a spacer region and mature peptide regions that encode for two BRPs: a canonical bradykinin R-9-R with a C-terminal extension of FVPASSL and Thr^6-BK. The Thr^6-BK reported in the present study had an unusual FP-insertion in the N-terminal part and ended in FAPEII, which is very different from the IAPAIV sequence reported in other ranid frogs. Unlike the mammalian bradykinin and its precursor, amphibian BRPs and their precursors are extremely variable, as evident from the present study. This forms the first report of BRPs from *Hylarana temporalis*, endemic to India and Sri Lanka.

Keywords: Bradykinin-related peptides, amphibian, hylaranakinin TE, *Hylarana temporalis*

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Introduction

Bioactive molecules present in the amphibian skin include many peptides that can generally be classified as having either regulatory or hormonal functions, or antimicrobial activity.[1] An important family of bioactive compounds having regulatory or hormonal functions within amphibians are the bradykinin-related peptide (BRPs) which are counterparts of mammalian bradykinin.[2] Bradykinin is a peptide that mediates a wide range of physiological phenomena in mammals including vasodilation, hypotension, smooth muscle contraction, pain and inflammation.[3,4]

The BRPs produced by frog skin generally differ from the canonical mammalian bradykinin in primary structure, mainly due to modifications of both the NH_2- and COOH-terminals.[3] Bradykinin and BRPs occur in the defensive secretions of anurans independently of a kallikrein–kinin system, which is the means by which bradykinin is produced in mammalian tissues.[5,6] Previous studies addressing the cloning of amphibian skin kininogens (pre-pro-bradykinins) in a variety of species[2,7] have revealed a plethora of different precursor structures with a few encoding single BRP domains and others encoding multiple domains. Some species produce multiple splice variants while others have single transcripts. Within the multi-domain precursors, the primary structures of the encoded BRPs can be either identical or highly variable. Prediction of the BRP precursor structure within a given species, based on the information present in contemporary databases, is thus impossible. The molecular cloning of the skin-derived biosynthetic precursor of BRPs, bombinakinin M/maximakinin from the giant fire bellied toad *Bombina maxima*, was the first study reporting the relationship of the amphibian skin kininogens to those in higher vertebrates, especially in mammals. A considerable amount of data now exists for the skin kininogen structures from representative bombinid toads, ranid frogs and some hylids.[3,4,8–11]

Studies on natural peptides from amphibians have so far dealt with only a few species. Therefore, it can be expected that many more skin peptides exist in nature. Although the diversity of amphibians is very rich in India, studies on the natural peptides of frogs are limited to a single species *R. tigerina*.[12] Hence, the present work is designed to identify the BRPs present in the skin secretions of the Indian bronzed frog *Hylarana temporalis*, currently considered endemic to India and Sri Lanka.

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) frogs of *H. temporalis* were captured from different locations along the Western Ghats bordering the State of Kerala, India. The skin secretions were obtained by mild transdermal electrical stimulation[13] and collected by washing the dorsal region with nuclease free water. The frogs were released promptly to the same habitat after secretion harvesting. The solutions collected were immediately snap-frozen with liquid nitrogen and subsequently lyophilized.

Construction of cDNA library

Polyadenylated mRNA was isolated from lyophilized skin secretion in a stabilization buffer using magnetic oligo (dT) beads as directed by the manufacturer (Dynal Biotech, UK). A PCR-based cDNA library was constructed using the SMART™ cDNA Amplification Kit (Clontech, UK) in strict accordance with the manufacturers' instructions. The first strand of cDNA was synthesized using MMLV RT by SMART II™ A Oligonucleotide Primer 5′ AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′ and 3′CDS Primer A 5′ AAGCAGTGGTATCAACGCAGAGTAC(T)30V N-3′ (N = A,C,G or T; V = A, G, C), which were 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 that encodes antimicrobial peptides

Two sense primers, including a degenerate primer (S1; 5′-GAWYYAYYHRAGCCYAAADATG 3′) and a specific primer were designed from a highly conserved domain of the signal peptide and untranslated 5′ regions of peptide-encoding cDNAs from ranid frogs.[14] The antisense primer used in the PCR reactions was 3′CDS Primer A as described above. 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 10s, 50 °C for 30s, 72 °C for 40s; and followed by a final extension at 72 °C for 10 min. PCR products were gel purified and cloned using the pGEM-T easy vector system (Promega Corp.). Plasmids were isolated, purified and sequenced using an ABI 3730 automated sequencer. The primary structure of skin kininogen from *H. temporalis* was subjected to homology searches using BlastX (for short nearly exact matches) available as an on-line database at NCBI (National Centre for Biotechnology Information).

Results

Molecular cloning of bradykinin related peptides

The skin secretion of *H. temporalis* served as a source for isolating mRNA that encodes the biosynthetic precursors for skin peptides. Polyadenylated mRNA thus isolated was used to construct a cDNA library. This library was subjected to 3′RACE procedures using the primers described and full-length pre-pro-bradykinin nucleic acid sequence data was obtained successfully. The pre-pro-bradykinin obtained in the present study was denoted as hylaranakinin TE in accordance with nomenclature rules recently suggested for antimicrobial skin peptides[15] in an attempt to establish a universally understood system. The complete nucleotide sequence of hylaranakinin TE and the deduced amino acid sequence are shown in Figure 1a, b. The deduced open reading frame consisted of 115 amino acid residues and encoded two copies of BRPs. The polypeptide begins with a putative signal peptide of 22 amino acid residues, followed by a spacer region and mature peptide regions that encode for two BRPs: a canonical bradykinin R-9-R (RPPGFSPFR) and Thr^6-BK (a variant of canonical bradykinin with Thr substitued). The Thr^6-BK reported in the present study had an unusual FP-insertion in the N-terminal part. Moreover, we uncovered a C-terminal extension FVPASSL for R-9-R and found that the Thr^6-BK ends in FAPEII, which is very different from the IAPAIV sequence reported in other ranid frogs (Fig. 2). A BLAST search in the EMBL protein sequence database revealed that the skin kininogen obtained from the present study exhibited 89% structural similarity to the skin BRP (kininogen-1) precursor from *Odorrana schmackeri*.[7]

A

Hylaranakinin- TE

```
    M  F  T  L  K  K  S  M  K  L  L  L  F  L  R  T  I  S  L  S
  1 ATGTTCACCTTGAAGAAATCTATGAAGCTCCTTCTCTTTCTCCGGACCATCTCCTTATCT
    L  C  E  Q  E  R  D  A  D  E  D  E  N  E  R  E  T  K  V  E
 61 CTCTGTGAGCAAGAGAGGGATGCTGATGAAGATGAAAATGAACGAGAAACTAAAGTGGAA
    D  V  K  R  A  G  N  S  G  K  V  I  L  K  R  P  P  G  F  S
121 GATGTTAAAAGAGCGGGAAACTCGGGAAAGGTCATATTAAAACGCCCACCTGGGTTTAGC
    P  F  R  F  V  P  A  S  S  L  K  R  D  A  D  E  D  E  N  E
181 CCATTTCGCTTTGTACCAGCGTCTTCTCTCAAGAGAGATGCTGATGAAGATGAAAATGAA
    R  E  A  K  V  E  D  V  K  R  A  G  Y  S  R  M  I  R  F  P
241 CGGGAAGCTAAAGTGGAAGACGTTAAAAGAGCTGGATACTCGAGAATGATCAGATTCCCA
    R  P  P  G  F  T  P  F  R  F  A  P  E  I  I  *
301 CGCCCACCTGGGTTTACCCCATTTCGCTTTGCACCAGAAATCATTTAACCTTGAGCTAAA
361 AATGATCTGATGTGGGATGTTCTGTAGCTAAATGCACAACAGATGTCTAATAAAAAATAA
421 AATGGTCATAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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B

MFTLKKSMKLLLFLRTISLSLCEQER-DADEDENERETKVEDV-KR-AGNSGKVILK - **RPPGFSPFR**-FVPASSL

KR-DADEDENEREAKVEDV-KR-AGYSRMIR-FP- **RPPGFTPFR**-FAPEII

Figure 1. A) Nucleic acid sequences and translated open reading frames of *Hylarana temporalis* skin kininogen (hylaranakinin TE). Putative signal peptide residues are underlined, mature bradykinin residues are in bold and underlined, and the stop codon is indicated by an asterisk. **B**) Domain architecture of *Hylarana temporalis* skin kininogen (Hylaranakinin TE). The signal peptide is underlined; mature bradykinin is represented in bold.

Bradykinin	RPPGFSPFR		ref-2
Amolopkinin	RAPVPPGFTPFR	(IN)	ref-2
Amolopkinin-W1	RVALPPGFTPFR	(IN)	ref-20
Amolopkinin-W2	RAALPPGFTPFR	(IN)	ref-20
(Leu^1,thr^6,trp^8)-BK,	LPPGFTPWR	(SUB)	ref-20
Bombinakinin M	DLPKINRKGPRPPGFSPFR	(NE)	ref-2
Bombinakinin O	GPRPPGFSPFRGKFH	(NE & CE)	ref-2
Ranakinin-R	RPPGFTPFRIAPEIV	(CE)	ref-2
Preprobradykinin-2(TO)	RPPGFSPFR		ref-19
Preprobradykinin(SK)	LPPGFTPWR	(SUB)	ref-19
Skin kininogen -1	RVISLPAGLSPLR	(IN & SUB)	ref-21
(Val^1, thr^6)-BK	VPPGFTPFR	(SUB)	ref-20
Ranakinin-N	RAEAVPPGFTPFR	(IN)	ref-22
Hylaranakinin-TE	AGNSGKVILKRPPGFPFRFVPASSL	(NE & CE)	This report
	AGYSRMIRFPRPPGFTPFRFAPEII	(NE & CE)	This report

Figure 2. Sequence comparison of amphibian BRPs. The amino acid sequences that are identical to the first line (bradykinin) are highlighted.
Abbreviations: IN, insertion; SU, substitution; NE, N-terminal extension; CE, C-terminal extension.

Discussion

BRPs represent one of the most extensively studied peptide families that are present in the defensive skin secretions in amphibians. In the present study, the skin secretions of *Hylarana temporalis* were investigated for the presence of BRPs and to clone their precursor cDNA using validated molecular cloning techniques. After the successful molecular cloning of precursors (skin kininogen) cDNAs and translation of the open reading frames, two copies of BRPs were confirmed as fully mature peptides within the skin secretion. The deduced pre-pro-bradykinin was named hylaranakinin TE. Accumulated data from several studies has established that the organisation of the biosynthetic precursors of amphibian skin peptides is both highly ordered and highly conserved between different taxa.[16] In general, the hydrophobic amino acid residue-rich signal peptide is followed by a region rich in acidic amino acid residues that terminates with a single copy of the bioactive peptide. Alignment of either nucleic acid sequences or open reading frame amino acid sequences of the 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 nontranslated 3′ and 5′ regions. This permitted the design of general PCR primer sets that are class, family or genus specific within the amphibians.[14] Thus with a skin secretion-derived cDNA library from an unstudied species and a general primer set, one can "shotgun amplify" and clone peptide transcripts in the absence of primary structural data for a specific peptide.[14]

The nucleotide and open reading frame of skin kininogen from the present study on *Hylarana temporalis* is illustrated in Figure 1. The acidic spacer is a hexadecapeptide and is highly conserved in its primary structure and in length across other reported ranid species.[2] In the repeat domains found in *H. temporalis* skin kininogen, a classical double basic residue propeptide convertase cleavage site (-KR-) is located immediately after the acidic spacer domain. The bradykinin nonapeptide domains are flanked by decapeptide AGNSGKVILK and AGYSRMIRFP in the N-terminus of R-9-R and Thr^6-BK, respectively, which is quite different from the octapeptide AGYS/ARMIR mostly seen in other reported ranid BRPs.[2] The Thr^6-BK is essentially a copy of canonical bradykinin with site substitution as well an unusual insertion (-FP-) at the N-terminus. In other ranids, the 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 present study, the C-terminal contains a phenylalanine residue that has not been reported to date in any of the BRPs from amphibians. The domain is flanked by hexapeptide FVPASS and the open reading frame terminates in FAPEII. The most striking feature of the present study is the heterogeneity of the precursor site and organisation within a species. *Hylarana guentheri* has 5–6 domains coding for three BRPs[17] where as *H. temporalis* contains only two domains encoding BRPs. Even though the BLAST search showed 89% structural similarity to the kininogens from *Odorrana schmackeri* skin, it has a single kininogen containing seven tandem repeat domains encoding canonical bradykinin.[7]

All known BRPs are bradykinins with extensions from its N-terminus or its C-terminus by amino acid residue segment. Bradykinin is a hydrolysis product produced by limited proteolysis of kallikrein on kininiogens. There are three types of kininogens in mammals: high molecular weight and, low molecular weight kininogens and T-kininogens.[18] All BRPs and their precursors from mammals are highly homologous. It suggests that mammals share a similar or the same mode of bradykinin biosynthesis. Unlike the mammalian bradykinin and its precursor, amphibian BRPs and their precursors are extremely variable, as evident from the present study (Fig. 2). This and related studies[2] suggest that amphibians may not share a similar mode of biosysnthesis of bradykinin. A large number of BRPs with diverse structures were identified from amphibian skin, but their role in the biological system is not clear. However, there are speculations that the potent pain- and inflammation-inducing properties of BRPs make them ideal deterrents to mammalian predators.[19]

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Disclosure

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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