

Cloning of the (Thr⁶)-phyllokinin precursor from *Phyllomedusa sauvagei* skin confirms a non-consensus tyrosine *O*-sulfation motif[☆]

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Abstract

Nine bradykinin-related peptides were identified in *Phyllomedusa sauvagei* skin secretion using QTOF MS/MS fragmentation sequencing. The major peptides were (Thr⁶)-bradykinin, (Hyp³, Thr⁶)-bradykinin, (Thr⁶)-phyllokinin and (Hyp³, Thr⁶)-phyllokinin. The phyllokinins occurred in both sulfated and non-sulfated forms. All (Thr⁶)-substituted bradykinins/phyllokinins could be generated from a common precursor by differential post-translational processing and modification. The open-reading frame of the cloned precursor cDNA consisted of 62 amino acid residues with a single bradykinin/phyllokinin coding sequence located at the C-terminus. Structural features included a Glu-Arg processing site at the N-terminus of the bradykinin/phyllokinin domain and the absence of an acidic amino acid residue adjacent to the C-terminal Tyr residue in the phyllokinins. However, the neutral amino acid residue (Ile) at position –1 and the basic amino acid residue (Arg) at position –2 from the Tyr residue, constitute a sulfation motif previously identified only in a protochordate.

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1. Introduction

Bradykinins and related peptides have long been known to occur in a plethora of molecular forms in the defensive skin secretions of anuran amphibians [5,13]. Bradykinin was first detected in large quantities in skin extracts of the European common frog, *Rana temporaria* [2], and soon after, molecular variants such as (Val¹, Thr⁶)-bradykinin and C-terminally extended molecular forms, such as ranakinin N (bradykinyl-Val-Ala-Pro-Ala-Ser), bombinakinin O (bradykinyl-Gly-Lys-Phe-His) and phyllokinin (bradykinyl-Ile-Tyr(SO₃H)), were reported [1,17,22]. Since then, many additional structural variants have been reported from additional species including (Leu⁸)-bradykinins from *Rana palustris*, (Thr⁶)-bradykinin from *Bombina orientalis*, (Ala³, Thr⁶)-bradykinin and (Val¹, Thr³, Thr⁶)-bradykinin from *Bombina variegata* and a most unusual N-terminally decapeptide extended form, named bombinakinin M from *Bombina maxima* [4,7,10,11]. In the latter reports on bombinid toads, the structures of the corresponding skin

precursor cDNAs were also determined, representing the first series of such data from amphibian skin.

Phyllokinin was first isolated and structurally characterized from skin extracts of the Brazilian leaf frog, *Phyllomedusa rhodei*, and was reportedly detected by bioassay in the skin extracts of many additional species from this group [1]. This peptide is essentially bradykinin with a C-terminal dipeptide extension, -Ile-Tyr, and of interest is the fact that the Tyr residue in *O*-sulfated, a post-translational modification that is of apparent importance to the biological activity of this peptide [1]. Sulfation of Tyr residues is a relatively common post-translational modification in proteins but is a rare phenomenon in bioactive peptides occurring most notably in the vertebrate regulatory peptide, cholecystokinin (CCK) [14], the amphibian skin peptide, caerulein [3], the protochordate CCK analog, cionin [15] and the insect neuropeptide, sulfakinin [12]. In CCK and caerulein, the Tyr residues that undergo post-translational sulfation, are adjacent to acidic amino acid residues (aspartyl (D) in cholecystokinin and glutamyl (E) in caerulein), a structural feature that was considered to be a strict requirement for this enzymatic modification.

Here we report for the first time that the skin secretion of a phyllomedusid frog contains a variety of molecular forms of both bradykinins and phyllokinins exhibiting several

[☆] The nucleotide sequence of the cloned precursor has been deposited in EMBL under the accession number AJ549500.

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post-translational modifications, including *O*-sulfation of the C-terminal Tyr residue in the phyllokinins. All of the (Thr⁶)-substituted peptides identified could be generated by differential processing and post-translational modification of a single precursor protein containing a single copy of coding sequence. The amino acid sequence motif identified (neutral residue at the –1 position, basic residue at the –2 position) upstream of the sulfated Tyr residue, has been previously documented for the precursor of the protochordate peptide, cionin, but is documented here in a vertebrate peptide precursor for the first time. Although this motif is present in phyllokinin itself, whose structure has been known for some time, until cloning of the precursor was achieved, no definitive statements as to its organization or to the local sulfation site environment could be made.

2. Materials and methods

2.1. Specimen biodata and harvesting of skin secretion

Phyllomedusa sauvagei ($n = 3$), were obtained from a commercial source and had been captive bred in the United States. The frogs were metamorphs (2 cm snout to vent length) on receipt and were grown to adult size (8 cm snout to vent length) over a two-year period prior to secretion harvesting. They were maintained in our purpose-designed amphibian facility at 20–25 °C under a 12/12 h light/dark cycle and fed multivitamin-loaded crickets three times per week. Skin secretion was obtained mechanically from the well-developed dorsal paratoid and tibial glands by massaging with the thumb. Of interest was the fact that unlike other frogs, transdermal electrical stimulation [21] failed to initiate release of stored secretion from these glands. The obvious viscous white secretion was washed from the surface of the glands using deionized water, snap-frozen in liquid nitrogen and lyophilized. Lyophilizate was stored at –20 °C prior to analysis.

2.2. Identification and structural analysis of bradykinins and phyllokinins

Five milligrams of lyophilized skin secretion were dissolved in 0.5 ml of 0.05/99.5 (v/v) trifluoroacetic acid (TFA)/water and clarified of microparticulates by centrifugation. The supernatant was then subjected to reverse phase HPLC fractionation using a gradient formed from 0.05/99.5 (v/v) TFA/water to 0.05/29.95/70.0 (v/v/v) TFA/water/acetonitrile in 240 min at a flow rate of 1 ml/min. A Thermoquest gradient HPLC system, fitted with an analytical column (Jupiter C-5, 5 μ particle, 300 Å pore, 250 mm \times 10 mm, Phenomenex, UK), was employed. Fractions (1 ml) were collected at minute intervals. The molecular masses of peptides in each chromatographic fraction were determined using matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS) on

a linear time-of-flight Voyager DE mass spectrometer (PerSeptive Biosystems, MA, USA) in positive detection mode using alpha-cyano-4-hydroxycinnamic acid as the matrix. Internal mass calibration of the instrument with known standards established the accuracy of mass determination as $\pm 0.1\%$. Following determination of sample purity and the molecular masses of MH⁺ ions, peptides ranging from m/z 800 to 1600 were subjected to MS/MS fragmentation and de novo sequencing using a Q-TOF Ultima mass spectrometer (Micromass, Manchester, UK).

2.3. Cloning of bradykinin/phyllokinin cDNA

Five milligrams of lyophilized venom were dissolved in 1 ml of cell lysis/mRNA stabilization solution (DynaL, UK). Polyadenylated mRNA was isolated using magnetic oligo-dT beads as described by the manufacturer (DynaL Biotech, UK). The isolated mRNA was subjected to 3'-RACE procedures to obtain full-length prepro-bradykinin/phyllokinin nucleic acid sequence data using a SMART-RACE kit (Clontech, UK) essentially as described by the manufacturer. Briefly, the 3'-RACE reactions employed an NUP primer (supplied with the kit) and a sense primer (S1, 5'-CCNCCNGGNTTYWSNCCNTTY-3') that was complementary to the internal bradykinin/phyllokinin amino acid sequence, -PPGFT/SPF-, as determined in the previously described part of this study. The products of 3'-RACE reactions were gel-purified and cloned using a pGEM-T vector system (Promega Corp.) and sequenced using an ABI 3100 automated sequencer. Following acquisition of these data, another specific antisense primer (AS1, 5'-CTGTGCTACTTAGATTATAGCATGT-3') was designed to a site in the 3'-untranslated region and was employed in 5'-RACE reactions. Products were likewise gel-purified, cloned and sequenced as described above. According to these obtained data, another specific sense primer (S2, 5'-GAATTACAAGACCAAATATG-3') was designed to a site in the 5'-untranslated region and employed in 3'-RACE reactions. Products were gel-purified, cloned and sequenced as described above (Fig. 1).

3. Results

3.1. Isolation and structural characterization of novel bradykinins/phyllokinins

Bradykinin (1059.45 Da) and two related peptides, (Thr⁶)-bradykinin and (Hyp³, Thr⁶)-bradykinin, with molecular masses of 1074.78 and 1090.63 Da, were identified in fraction 81 (Fig. 2A). Fraction 100 contained phyllokinin (1334.89 Da) and two novel-related peptides, (Thr⁶)-phyllokinin and (Hyp³, Thr⁶)-phyllokinin, with molecular masses of 1351.69 and 1368.10 Da, respectively. All of the phyllokinins were also identified in sulfated forms with molecular masses of 1417.26, 1431.87

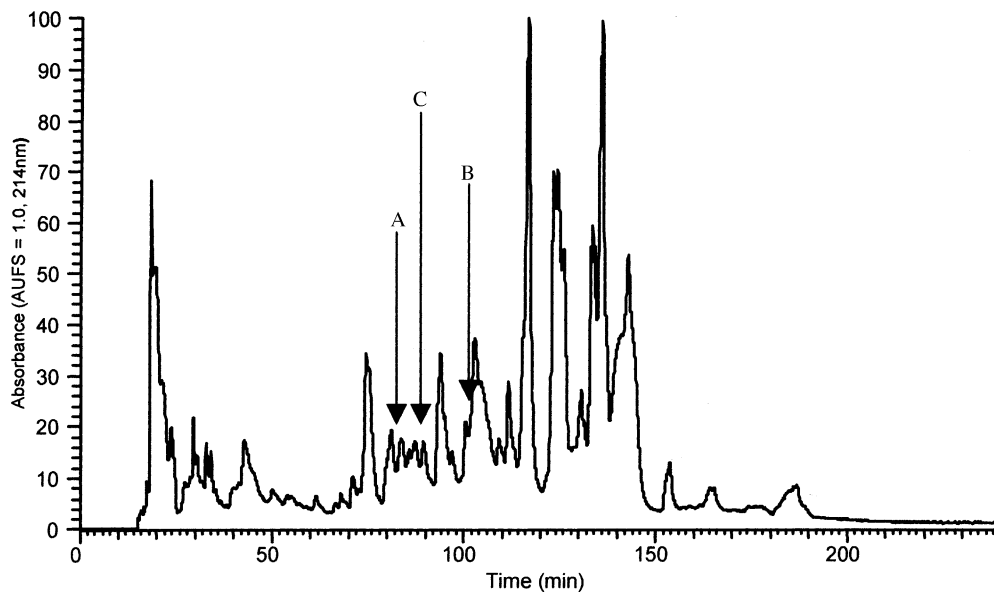


Fig. 1. Reverse phase HPLC chromatogram of *P. savagei* skin secretion. Fractions containing bradykinins (A), phyllokinins (B) and (Val¹, Thr⁶)-bradykinin (C) are indicated by arrows.

Table 1
Bradykinins and phyllokinins identified in the skin secretion of *P. savagei*

Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
(Thr ⁶)-bradykinin	Arg- Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg
(Val ¹ , Thr ⁶)-bradykinin	Val- Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg
(Hyp ³ , Thr ⁶)-bradykinin	Arg- Pro-Hyp-Gly-Phe-Thr-Pro-Phe-Arg
Phyllokinin	Arg- Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Ile-Tyr
(Thr ⁶)-phyllokinin	Arg- Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg-Ile-Tyr
(Hyp ³ , Thr ⁶)-phyllokinin	Arg- Pro-Hyp-Gly-Phe-Thr-Pro-Phe-Arg-Ile-Tyr
(Thr ⁶)-phyllokinin sulfated	Arg- Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg-Ile-Tyr (SO₃H)
(Hyp ³ , Thr ⁶)-phyllokinin sulfated	Arg- Pro-Hyp-Gly-Phe-Thr-Pro-Phe-Arg-Ile-Tyr (SO₃H)

Fully-conserved residues in bold type.

and 1447.79 Da (Fig. 2B). Fraction 90 contained another bradykinin-related peptide, (Val¹, Thr⁶)-bradykinin, molecular mass 1017.19 Da, and despite extensive searching of other fractions, was not detected in post-translationally modified or extended form (data not shown). The primary structures of all peptides detected (summarized in Table 1) were established by MS/MS fragmentation and de novo sequence analysis using a Q-TOF Ultima mass spectrometer (Micromass, Manchester, UK) (Figs. 3 and 4). The (Thr⁶)-substituted bradykinins and phyllokinins were the most abundant in the skin secretion comprising more than 98% of the total bradykinin-related peptide pool. Using the QTOF mass spectrometer in negative mode, sulfated phyllokinins were desulfated in source and the low molecular mass region of the spectrograms was inspected (data not shown). Ions of molecular masses 79.9484 and 96.9474 Da, were observed and identified as SO₃ and HSO₄, respectively, by the elemental analysis software. Tolerances, in the absence of a calibrated mass lock, were −8.4 and −12.2 mDa, respectively.

3.2. In vitro cDNA library construction from skin secretion

From the skin secretion cDNA library, one common bradykinin/phyllokinin cDNA was consistently cloned (identical in 40 different clones over four different experiments). The open-reading frame consisted of 62 amino acid residues containing a putative hydrophobic signal peptide, an acidic amino acid residue-rich prodomain and a single copy of (Thr⁶)-bradykinin/phyllokinin coding sequence at the C-terminus (Fig. 5). Clones encoding additional bradykinin-related peptides were not identified and this may have been due to their low abundance, reflecting that of the mature peptides in the secretion, or to very different gene sequences.

4. Discussion

The heterogeneity of bradykinin-related peptides in the skin secretion of a phylomedusid frog and the structure

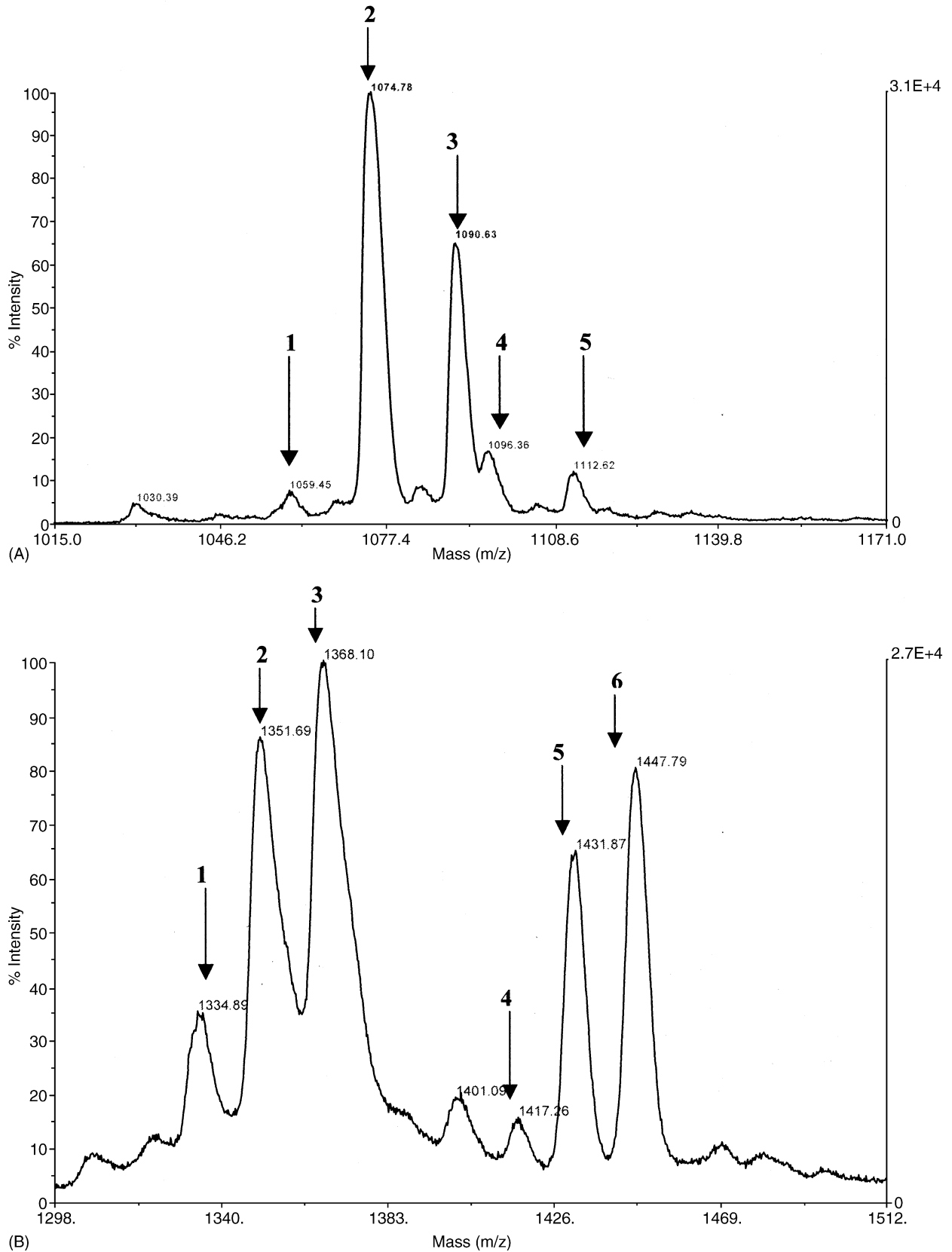


Fig. 2. MALDI-TOF mass spectrograms of fraction 83 (A) containing 1-bradykinin, 2-(Thr⁶)-bradykinin, 3-(Hyp³, Thr⁶)-bradykinin, 4-(Thr⁶)-bradykinin sodium adduct, 5-(Thr⁶)-bradykinin potassium adduct and fraction 100 (B) containing 1-phyllokinin, 2-(Thr⁶)-phyllokinin, 3-(Hyp³, Thr⁶)-phyllokinin, 4-sulfated phyllokinin, 5-sulfated (Thr⁶)-phyllokinin, 6-sulfated (Hyp³, Thr⁶)-phyllokinin.

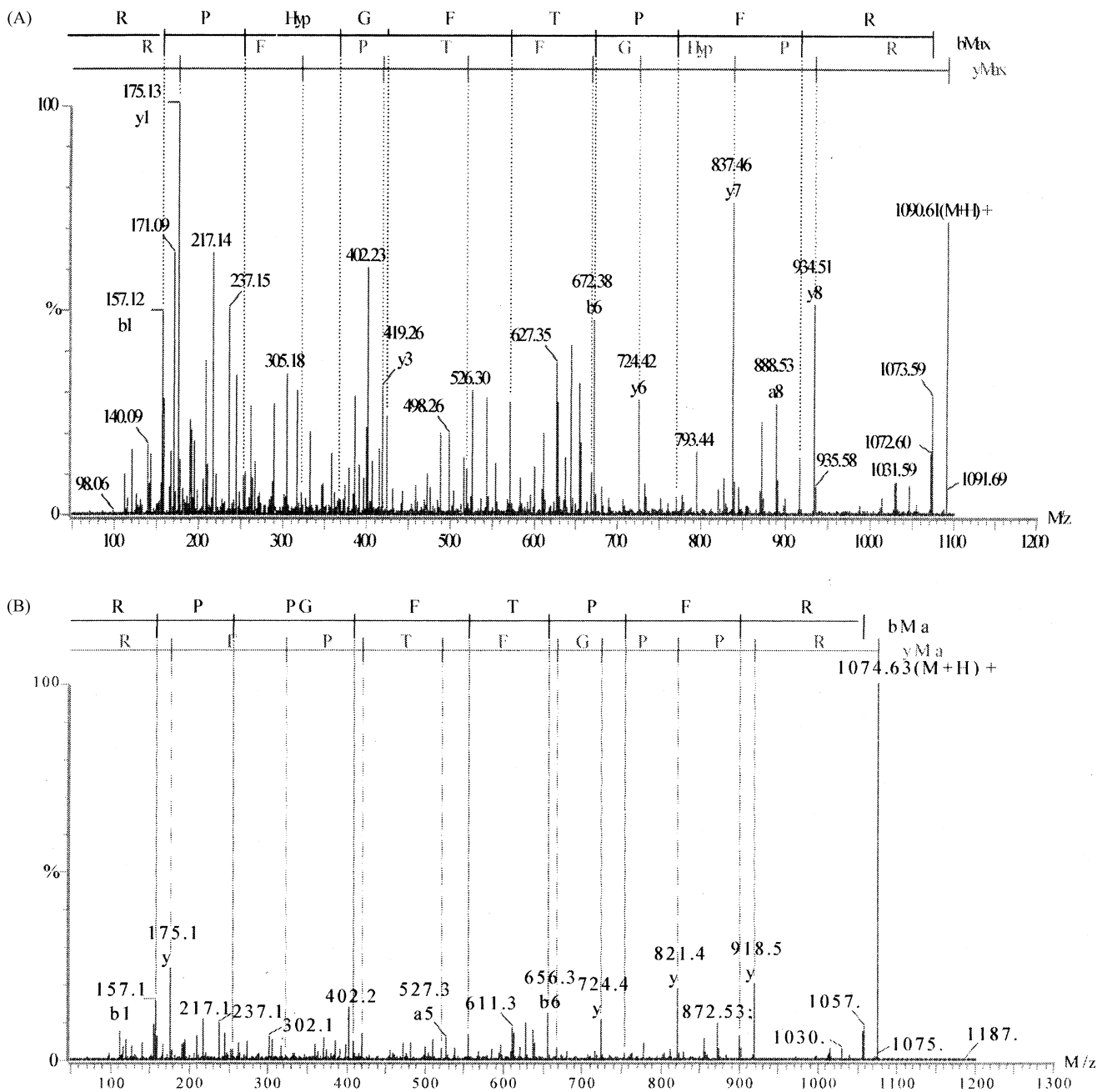


Fig. 3. QTOF MS/MS fragmentation spectra and associated b- and y-ion sequence assignments of (A) (Hyp³, Thr⁶)-bradykinin and (B) (Thr⁶)-bradykinin.

of the (Thr⁶)-bradykinin/phyllotoxin precursor protein, deduced from cloned cDNA, is reported for the first time. The most abundant bradykinin-related peptides identified were structural modifications of the core sequence of (Thr⁶)-bradykinin that was identified with and without hydroxylation of the Pro residue at position 3 from the N-terminus. Both of these peptides were identified with -Ile-Tyr extensions at their C-terminals (phyllotoxins), the terminal Tyr residue being either *O*-sulfated or not. Although canonical bradykinin and phyllotoxin, the latter in both sulfated and non-sulfated forms, were identified in the

skin secretion, they represented less than 2% of the total bradykinin pool. An interesting minor bradykinin-related peptide identified was the (Val¹, Thr⁶)-bradykinin variant that has only previously been identified in skin secretions from frogs of the genus *Rana* [2]. The most structurally-modified bradykinin variant in *P. sauvagei* skin secretion was found to be a phyllotoxin that displays a Thr for Ser substitution at position 6, hydroxylation of Pro-3, an -Ile-Tyr extended C-terminus and *O*-sulfation of this C-terminal Tyr residue. While hydroxylation of Pro residues is a stable post-translational modification, *O*-sulfation of

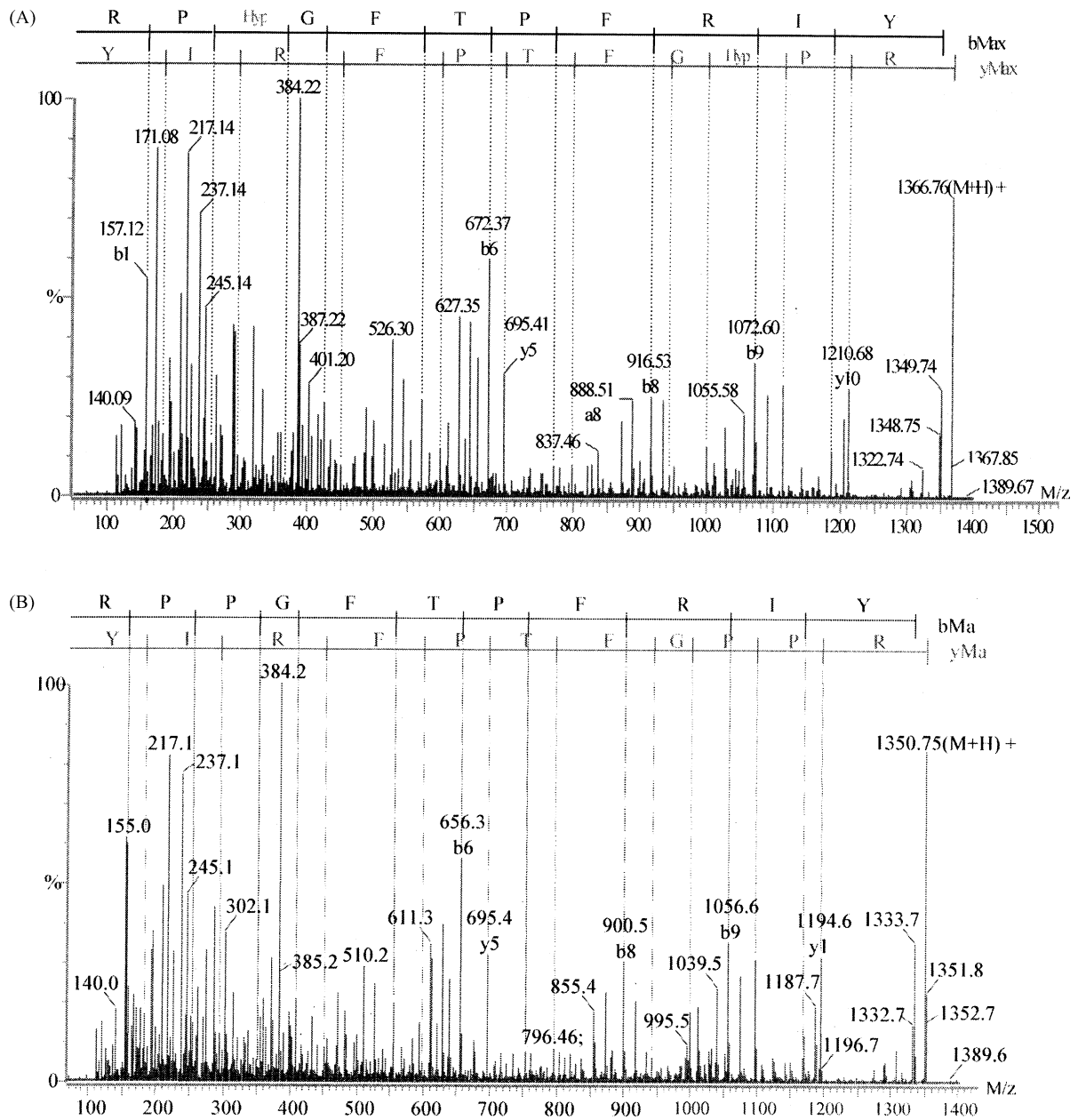


Fig. 4. QTOF MS/MS fragmentation spectra and associated b- and y-ion sequence assignments of (A) (Hyp³, Thr⁶)-phyllokinin and (B) (Thr⁶)-phyllokinin.

Tyr residues is not, with peptides displaying such a feature in the natural state being easily desulfated by low pH conditions. An interesting aspect of this fact is that desulfation of modified Tyr residues in peptides renders the resultant desulfated peptide more hydrophobic under standardised reverse phase HPLC conditions. As a consequence, if desulfated phyllokinins were present in the defensive skin secretion when taken, they can readily be resolved from the sulfated peptides by reverse phase HPLC. If however, desulfated phyllokinins were generated from sulfated phyllokinins during storage as HPLC fractions (containing trifluoroacetic acid) for even short periods, they should be detected in the same fractions. In this study, the latter sit-

uation was found to be the case strongly suggesting that the phyllokinins in the skin secretion were sulfated in the natural state and desulfated as a result of a storage artefact (sulfated phyllokinin becomes desulfated when stored in 0.1% (v/v) aqueous trifluoroacetic acid after 4 weeks). This was in fact the case as confirmed by lack of identification of desulfated phyllokinins in other HPLC fractions and by rechromatography of original mixed fractions from which both sulfated and desulfated phyllokinins could be resolved (data not shown). This fact is not overtly surprising as desulfated phyllokinin is four to seven times less potent than phyllokinin in lowering mammalian blood pressure [1].

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                                M D I L K K S L F
1  CTTTCTGAAT TACAAGACCA AATATGGATA TCCTGAAGAA ATCTCTTTTC
   GAAAGACTTA ATGTTCTGGT TTATACCTAT AGGACTTCTT TAGAGAAAAG
   L V L F L G L V S F S I C E E E K
51  CTTGTACTTT TCCTTGGATT GGTCTCCTTT TCTATCTGTG AAGAAGAGAA
   GAACATGAAA AGGAACCTAA CCAGAGGAAA AGATAGACAC TTCTTCTCTT
   R D T E E E E N D D E I E E E S
101 AAGAGATACT GAAGAGGAAG AGAATGACGA TGAAATAGAG GAAGAAAGTG
   TTCTCTATGA CTTCTCCTTC TCTTACTGCT ACTTTATCTC CTTCTTTCF
   E E K K R E A P E R P P G F T P F
151 AAGAGAAGAA AAGAGAGGCT CCAGAAAGAC CTCCCGGATT CACTCCTTTT
   TTCTCTTCTT TTCTCTCCGA GGTCTTTCTG GAGGGCCTAA GTGAGGAAAA
   R I Y *
201 AGAATTATT AATACATTAA GAAAGTGTA CATGCTATAA TCTAAGTAGC
   TCTTAAATAA TTATGTAATT CTTTCACATT GTACGATATT AGATTATCGC
251 ACAGTTATCA ATGATTATGC TAAAAACATA TAAAGCATA TTTAATGAAA
   TGTCATAGT TACTAATACG ATTTTGTAT AATTTCGTAT AAATTACTTT
301 AAAAAAAAAA
   TTTTTTTTTT

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Fig. 5. Nucleotide sequence of full-length cDNA (both strands) encoding (Thr⁶) bradykinin/phyllokinin from *P. sauvagei* skin secretion. The putative signal peptide is single underlined and the (Thr⁶)-bradykinin/phyllokinin sequence, located at the C-terminus of the open-reading frame, is double underlined. The stop codon is indicated by an asterisk.

The common (Thr⁶)-bradykinin/phyllokinin precursor structure, as deduced from cloned cDNA, was unusual in a number of respects. Unlike other frog skin bradykinin precursors that have been deduced from cloned cDNA [7,10,11], the N-terminal residue of (Thr⁶)-bradykinin/phyllokinin (Arg) was not generated by an Arg-X cleavage but rather by a Glu-X cleavage. However, a typical paired basic amino acid propeptide convertase cleavage site (-Lys-Arg-) is present in the precursor several residues upstream and a putative scheme may involve cleavage at this site with subsequent aminopeptidase processing to the N-terminal Arg residue in the mature peptide(s). A similar situation is present within the precursor of tryptophyllin-1 from another phyllomedusid frog, *Pachymedusa dacnicolor*, (EMBL Accession no. AJ507318) where an Asp residue resides between the N-terminal Lys residue of the mature peptide and the typical -Lys-Arg- processing site. Of additional interest is that the Pro residue at position 3 in tryptophyllin-1 is also hydroxylated leading to the speculation that this motif may facilitate this specific post-translational modification. Most other frog skin peptide precursors exhibit standard propeptide convertase cleavage patterns although the vast majority of these encode antimicrobial peptides and hence may not be representative. The C-terminals of the bradykinins identified are generated by Arg-X cleavages from the precursor but the phyllokinins are not processed at this site as the dipeptide (-Ile-Tyr) extension resides at the termination of the open-reading frame. Curiously the C-terminal Tyr residue that is *O*-sulfated does not occur in juxtaposition to an acidic amino acid residue and departs from the original proposed consensus rules for generation of this modification [16,19]. However, although this consensus appears to hold for vertebrate cholecystokinins, amphibian skin caeruleins and arthropod sulfakinins, it does

not hold true for the protochordate CCK analog, cionin [3,14,15,18,20]. Since both Tyr residues in natural cionin are *O*-sulfated, despite the absence of juxtaposed acidic amino acid residues, this led the authors to conclude that the protochordate tyrosyl-protein sulfotransferase had a different substrate specificity to those in vertebrates and insects. However, in a subsequent report from the same group [16], expression of cionin in mammalian cell lines resulted in full sulfation of its tyrosyl residues. Hence, a different specificity for the protochordate sulfotransferase could be ruled out leading to the conclusion that the parameters for *O*-sulfation of bioactive peptides remain poorly understood. This would be an assertion consistent with the data presented in this study. New consensus features for tyrosine *O*-sulfation have been reported [6] and the structures reported here are consistent with the presence of a neutral residue (Ile) in the -1 position and a basic residue (Arg) in the -2 position from the modified tyrosine. Although this motif is present in phyllokinin, whose structure has been known for some time, the structure of the precursor is described here for the first time and this encodes a single copy of (Thr⁶)-phyllokinin. Of the five bradykinin precursors cloned from amphibian skin to date, three contain multiple copies of coding sequence [7,10,11]. In the skin secretion of *P. sauvagei*, we have identified both sulfated and non-sulfated phyllokinins although we contend that desulfation is most likely an experimental artefact. The possibility could thus still have existed that sulfated and non-sulfated phyllokinins were encoded by separate precursors or that a multi-copy precursor could have different processing/modification site motifs. The cloning data described in this report unequivocally demonstrate a single phyllokinin precursor encoding a single, C-terminally located copy of coding sequence thus substantiating the assertion that the sulfation motif described

here is real and in accordance with that previously described for the protochordate peptide, cionin [16]. However, one alternative but speculative explanation for the mechanism of phyllokinin *O*-sulfation observed here, would be that the C-terminal Tyr residue, as a consequence of the conformational effects induced by the three Pro residues in the bradykinin domain, is placed close to the acidic residue rich region in the presequence. This might provide the necessary environment for expression of sulfotransferase activity.

Despite exhaustive sequencing of clones, none were found to contain cDNA sequence encoding either bradykinin, phyllokinin or (Val¹, Thr⁶)-bradykinin. There are two possible explanations for this. The first is that the transcript abundance is extremely low reflecting the fact that these peptides are of relatively low abundance in the skin secretion accounting for some 2% of the total bradykinin-related peptide pool. A second possibility is that the nucleotide sequence and hence primer recognition of these precursor cDNAs is very different from that successfully cloned and reported in this study.

A significant feature of the present study regards the use of live animals in research. Here, we have obtained the skin secretion from three frogs (*P. sauvagei*) by gentle skin massage and obtained peptide structural data and corresponding cloning of precursor cDNA from the same lyophilized sample [7–9]. The frogs remain alive, visibly unaffected by the procedure and can be re-used at monthly intervals for secretion acquisition. We have used this procedure on the same animals for two years. In contrast, the original report on the structure and bioactivity of phyllokinin used the dissected skin from 3555 freshly-killed *P. rhodei* [6]. This effectively illustrates the contribution that the application of modern technology can bring to maintenance of biodiversity and that the acquisition of robust scientific data can be achieved in the absence of sacrificing living representatives of animal groups that are under threat of extinction in the global environment. This often happens before the biochemical complexity and the potential biotechnological and biomedical applications of their unique molecules can be realized.

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