Available online at www.sciencedirect.com
SCIENCE DIRECT®
Peptides 24 (2003) 853–861
PEPTIDES
www.elsevier.com/locate/peptides

Cloning of maximakinin precursor cDNAs from Chinese toad, *Bombina maxima*, venom☆

Tianbao Chen[a,b], Anthony J. Bjourson[a], Stephen McClean[a], David F. Orr[a], Edmund J. O'Kane[a], Pingfan Rao[b], Chris Shaw[a,*]

[a] *Pharmaceutical Biotechnology Research Group, School of Biomedical Sciences, University of Ulster, Cromore Road, Coleraine BT52 1SA, Northern Ireland, UK*
[b] *Institute of Biotechnology, Fuzhou University, Fuzhou 350002, Fujian Province, PR China*

Received 24 February 2003; accepted 6 May 2003

Abstract

Using a novel technique that we have developed for cloning of amphibian skin secretion peptide cDNAs from lyophilized samples, we report here that maximakinin (DLPKINRKGP-bradykinin) is encoded by two different cDNAs, named BMK-1 and BMK-2, containing either four tandem repeat sequences or a single copy. The open reading frames of both precursor cDNAs were found to be 152 and 116 amino acid residues, respectively. These data provide evidence that the structural diversity of peptides in amphibian skin secretions arising from molecular evolutionary events, can be mediated by parallel diversity in encoding mRNAs that in itself may reflect serial gene duplications.

Keywords: Amphibian; Venom; Bradykinin; cDNA; 3′ and 5′ RACE

1. Introduction

Amphibian skin secretions contain a wide spectrum of biologically-active compounds, such as biogenic amines, peptides, alkaloids and bufogenins [10,11,13]. The source of these species-specific molecular cocktails accumulated in the skin, are the highly-specialized poison or granular glands, that, in response to stress or predator attack, exude this complex and often noxious defensive secretion onto the surface of the skin [13,17]. These secretions often contain a plethora of peptides among which bradykinin or structural variants have been identified [4,13]. Structural homologues of vertebrate regulatory peptides found in defensive skin secretions of anuran amphibians often display enhanced bioactivity and receptor binding when compared with autologous mammalian peptide ligands [4,14]. Many bradykinins found in the skin secretions of amphibians have been identified using the isolated uterus and ileum bioassay models that are highly selective for the peptide [13]. Commonly, amphibian bradykinin is found in a slightly modified form with extensions at the C or N terminus of the nonapeptide [2,9]. These include (Thr^6)-bradykinin and (Thr^6)-bradykinyl-IAPEIV in *Rana rugosa* and (Val^1, Thr^6)-bradykinin and (Val^1, Thr^6)-bradykinyl-VAPAS in *Rana nigromaculata* [6,8]. Additional structural variants, such as phyllokinin from *Phyllomedusa sauvagei*, has an Ile Tyr (SO_3H) C-terminal extension [1], Ranakinin N from *R. nigromaculata* has a C-terminal -Val-Ala-Pro-Ala-Ser extension [2].

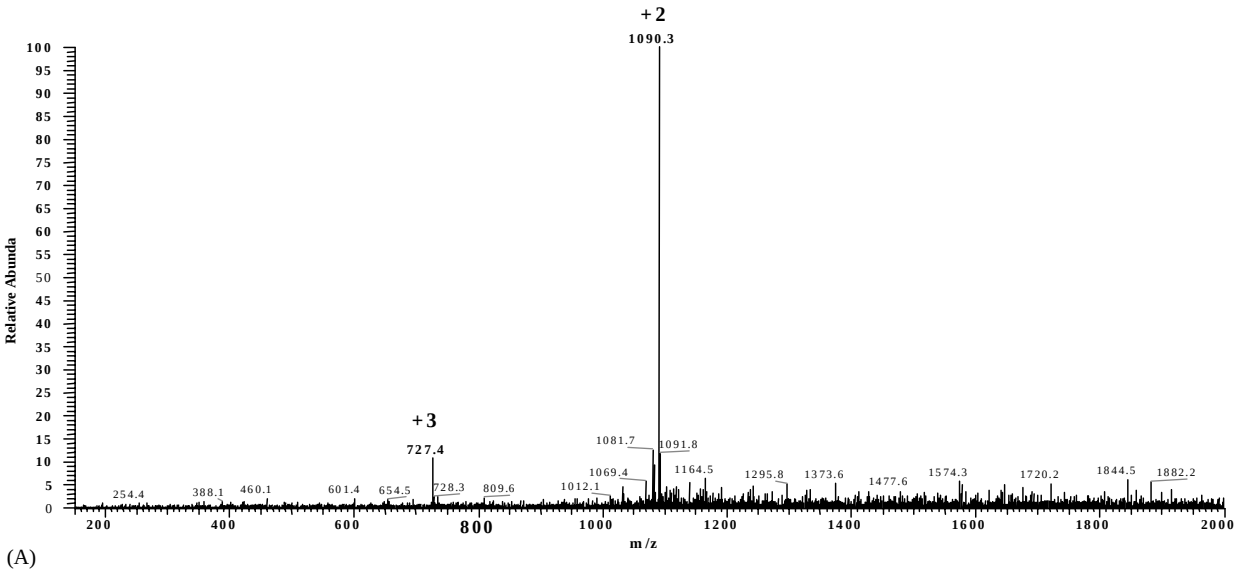
Members of the amphibian genus *Bombina*, are known to secrete a wide variety of peptides that have been characterized on the basis of different pharmacological, antimicrobial and inhibitory effects on regulatory enzymes and proteins, making these an attractive resource for pharmacologically- and therapeutically-relevant compound discovery [8,13,17,19,20,23]. Recently, novel bradykinins, that are active on mammalian arterial and small intestinal smooth muscle, have been structurally characterized and cloned from the skin of both *Bombina orientalis* and *Bombina variegata* [6,7]. Thus members of this group have a high potential for discovery of novel molecular variants of this peptide family.

Abbreviations: HPLC, high performance liquid chromatography; fmoc, *N*-(9-fluorenyl)methoxycarbonyl; LC/MS, liquid chromatography/mass spectrometry; mRNA, messenger RNA; cDNA, DNA complementary to RNA; UPM, universal primer mix; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction

☆ The nucleotide sequences of *Bombina maxima* skin kininogens 1 and 2 have been deposited in the EMBL Nucleotide Sequence Database under the accession codes AJ315488 and AJ440236, respectively. The peptide sequence(s) reported in this paper has been submitted to the Swiss Protein Database under Swiss-Prot accession no. P83055.

* Corresponding author. Tel.: +44-2870-324611; fax: +44-2870-324965.
E-mail address: c.shaw@ulster.ac.uk (C. Shaw).

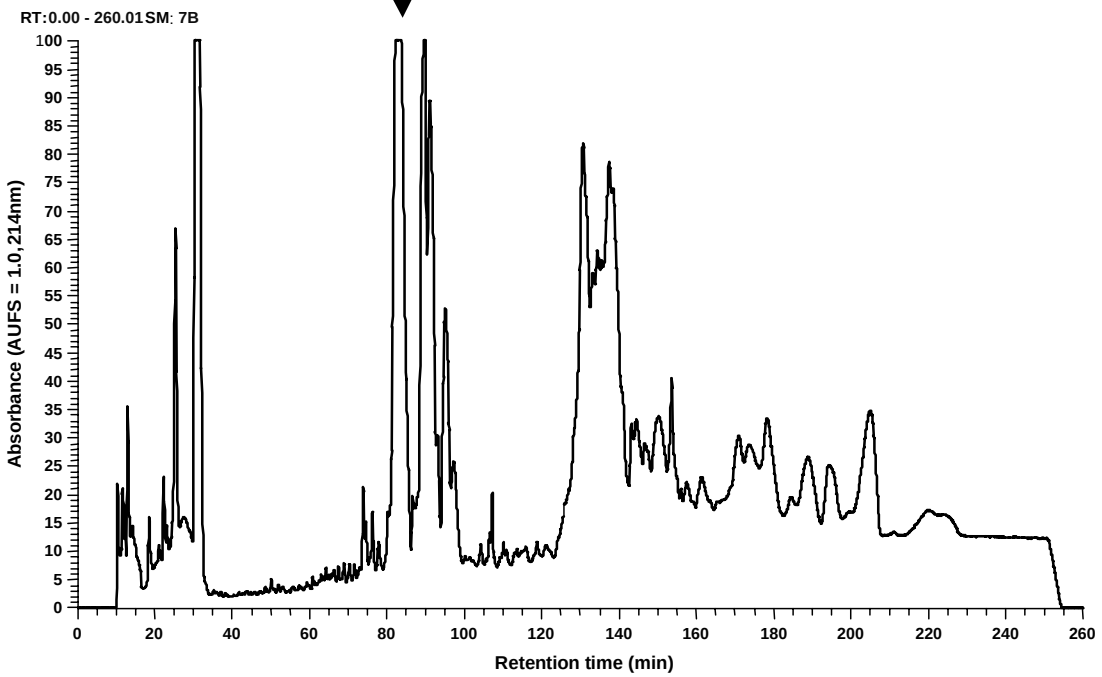
0196-9781/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0196-9781(03)00167-0



(A)

Cycle No.	1	2	3	4	5	6	7	8	9	10
PTH-AA	D	L	P	K	I	N	R	K	G	P
Yield (pmol)	1194.3	787.9	560.0	416.9	585.9	514.6	542.0	299.1	374.5	279.7

Cycle No.	11	12	13	14	15	16	17	18	19	
PTH-AA	R	P	P	G	F	S	P	F	R	
Yield (pmol)	328.7	295.4	361.4	164.0	137.1	48.7	98.0	76.3	14.1	



(B)

Fig. 1. Structural characterization of maximakinin. Electrospray mass spectrum (MS mode) of maximakinin showing the predominant doubly-charged ion (A) and reverse phase HPLC chromatogram (*Y*-axis = relative abundance at $\lambda = 214$ nm in normalized arbitrary absorbance units) of *B. maxima* skin secretion (B). The retention time of maximakinin is indicated (arrow) below corresponding automated Edman degradation data.

Here, we describe for the first time, molecular cloning of two different full length cDNAs, each encoding the novel N-terminally extended bradykinin, DLPKINRKGP-bradykinin, named maximakinin, from a cDNA library constructed from mRNA transcripts present in lyophilized venom of the Chinese large-webbed bell toad, *Bombina maxima*. Maximakinin thus represents the first frog skin-derived bradykinin encoded by two different precursors.

2. Materials and methods

2.1. Acquisition of toad venom

Four adult specimens of *B. maxima* were obtained from a commercial source, housed in a vivarium under a 12-h light:12-h dark cycle at 22 °C and fed multivitamin-loaded crickets three times per week. Under these conditions, toads have remained in good health in excess of 4 years. Defensive skin secretions were obtained by two methods and under both sets of conditions, secretions were most pronounced from paired paratoid and tibial glands. Firstly, the dorsal surface was moistened with distilled water followed by three periods of transdermal electrical stimulation (5 V, 100 Hz, 140 ms pulse width), each of 10 s duration [24]. Skin secretions were washed from the dorsal skin with distilled water, snap-frozen in liquid nitrogen and lyophilized. The second and preferred technique involved gently massaging the dorsal skin surface with a latex-gloved finger that was found to be equally effective in terms of dry weight secretion yield (typically 20–25 mg dry weight per toad). Both techniques caused no harm and minimal stress to the animals.

2.2. Isolation and structural characterization of maximakinin

Five milligrams of lyophilized skin secretion were dissolved in 0.5 ml of 0.05/99.5 (v/v) trifluoroacetic acid

```
                                                  M  R  L   W  F  C  L
  1   AAGTTCTCAG TGTCACTTCC AGCTCTGTTC ATGAGACTGT GGTTCTGTCT

        S  L  F   I  V  L   C  L  E  H   F  P  G   T  L  A
 51   AAGTCTCTTC ATTGTCCTGT GCCTGGAGCA TTTTCCAGGA ACCCTGGCAG

      D  E  R  N   V  P  E   S  E  E   K  T  E  Q   F  L  R
101   ATGAAAGGAA TGTTCCAGAG AGTGAAGAAA AAACTGAGCA GTTCCTGAGG

       D  L  P   K  I  N  R   K  G  P   R  P  P   G  F  S  P
151   GATTTGCCTA AGATCAACCG CAAAGGACCA CGTCCACCGG GGTTCTCCCC

        F  R  G   K  F  H   S  Q  T  L   R  D  L   P  K  I
201   TTTTCGAGGA AAATTCCATA GCCAGACCCT GCGGGATTTG CCTAAGATCA

      N  R  K  G   P  R  P   P  G  F   S  P  F  R   G  K  F
251   ACCGCAAAGG ACCACGTCCA CCGGGGTTCT CCCCTTTTCG AGGAAAATTC

       H  S  Q   T  L  R  D   L  P  K   I  N  R   K  G  P  R
301   CATAGCCAGA CCCTGCGGGA TTTGCCTAAG ATCAACCGCA AAGGACCACG

        P  P  G   F  S  P   F  R  G  K   F  H  S   Q  S  L
351   TCCACCGGGG TTCTCCCCTT TTCGAGGAAA ATTCCATAGC CAGTCCCTGC

      R  D  L  P   K  I  N   R  K  G   P  R  P  P   G  F  S
401   GGGATTTGCC TAAGATCAAC CGCAAAGGAC CACGTCCACC GGGGTTCTCC

       P  F  R   G  K  F  H   S  Q  S   H  V  *
451   CCTTTTCGAG GAAAATTCCA TAGCCAGTCC CATGTATGAA ATCAAGCAGT

501   TCAAAACTGC ACACGGACGC CCACCGATCT GCCCTCCGGG TGAACAATGT

551   CCCATTTGGG TTGGGAAGTA GCGTCCCCTG ATATAAATAA GCATTGTTCT

601   GTCACCTTTG TAATACCAGC TCTGACTGAC ATGGTTATTA AACAGCAAAT

651   TTGTGCTCCC AAAAAAAAAA AAAAAAAAAA
```

Fig. 2. Nucleotide sequence of *B. maxima* skin kininogen/preprobradykinin-1 (BMK-1) cDNA encoding four copies of maximakinin (each single underlined). The putative signal peptide (double underlined) and stop codon (asterisk) of the open-reading frame are indicated.

(TFA)/water and clarified of microparticulates by centrifugation. The supernatant was then subjected to liquid chromatography/mass spectrometry (LC/MS) using a gradient formed from 0.05/99.5 (v/v) TFA/water at 0 min, to 0.05/19.95/80.0 (v/v/v) TFA/water/acetonitrile at 240 min at using a flow rate of 1 ml/min. A Thermoquest gradient reversed-phase high performance liquid chromatography (HPLC) system interfaced with a Thermoquest LCQ electrospray ion-trap mass spectrometer was employed. The effluent from the chromatographic column was flow split with approximately 10% entering the mass spectrometer source and 90% directed towards a fraction collector. Dead volume between column and fraction collector was minimal (20 μl). The major peptide present in the secretion (molecular mass 2179 Da) was subjected to structural analysis by either re-infusion of appropriate collected fraction with the spectrometer in ion trap MS/MS mode or by automated Edman degradation using an Applied Biosystems 491 Procise sequencer in pulsed-liquid sequencing mode.

2.3. In vitro cDNA library construction from lyophilized venom

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 5′- and 3′-RACE procedures to obtain full-length kininogen/preprobradykinin nucleic acid sequence data using a SMART-RACE kit (Clontech, UK) essentially as described by the manufacturer. Briefly, the 3′-RACE reactions employed a universal primer mix (UPM) primer (supplied with the kit) and a sense primer (Brady-S1; 5′-AARGGICCIMGICCICCIGGITTY-3′) that was complementary to the internal maximakinin amino acid sequence, -KGPRPPGF-. 3′-RACE reactions were gel-purified and cloned using a pGEM-T vector system (Promega Corporation) and sequenced using an ABI 3100 automated sequencer. The sequence data obtained from these 3′-RACE products were used to design a gene-specific antisense primer (Brady-AS1: 5′-ATATCAGGGGACGCTACTTC-3′) to a region of the 3′-non-translated region. 5′-RACE was carried out using this specific primer in conjunction with the UPM and the generated product was gel-purified, cloned, and sequenced as described above. Following acquisition of these data, another specific sense primer (Brady-S2, 5′-GTCACTTCCAGCTCTGATCATG-3′) was designed to a site in the 5′-untranslated region and was employed in 3′-RACE reactions. Products were gel-purified, cloned and sequenced as described above.

```
                                                  M  R  L  W  F  C  L
  1  TAGTTCTCAG TGTCACTTCC AGCTCTGATC ATGAGACTGT GGTTCTGTCT

       S  F  F  I  V  L  C  L  E  H  F  P  G  T  L  A
 51  AAGTTTTTTC ATCGTCCTGT GCCTGGAGCA TTTTCCAGGA ACCCTGGCAG

     D  E  R  N  N  R  D  Y  T  I  R  T  R  L  H  G  H
101  ATGAAAGGAA TAATCGTGAC TACACCATCA GAACCCGCTT ACATGGCCAT

      H  K  P  S  R  N  N  R  Y  A  I  K  T  S  I  H  G
151  CATAAACCAA GCAGGAATAA CCGTTACGCC ATCAAAACCA GCATACATGG

       H  H  I  P  R  N  V  P  E  S  E  E  K  T  E  Q
201  CCATCATATA CCAAGGAATG TTCCAGAGAG TGAAGAAAAA ACTGAGCAGC

     L  L  R  D  L  P  K  I  N  R  K  G  P  R  P  P  G
251  TCCTGAGGGA TTTGCCTAAG ATCAACCGCA AAGGACCACG TCCACCGGGG

      F  S  P  F  R  G  K  F  H  S  Q  S  L  R  Q  I  P
301  TTCTCCCCTT TTCGAGGAAA ATTCCATAGC CAGTCCCTAC GACAAATTCC

       G  L  G  P  L  R  G  *
351  TGGTTTAGGC CCTCTGCGTG GATAACGAAG CTCAGGGATA AGAATCTGCC

401  CTATGTGTAT GCCATGTTCA CCATAGGCTA AAAAGTAGCG TCCCCTGCTA

451  TAAATAAGCA TTGTTATGTC ACCTCTGTAA TACCAGCTCT GACTGACATG

501  GTTTATTAAA CAGCAGATTT GTGCTCTCTA AAAAAAAAAA AAAAAAAA
```

Fig. 3. Nucleotide sequence of *B. maxima* skin kininogen/preprobradykinin-2 (BMK-2) cDNA encoding a single copy of maximakinin (singe underlined). The putative signal peptide (double underlined) and stop codon (asterisk) of the open-reading frame are indicated.

Fig. 4. Alignment of open-reading frame amino acid sequences of BMK-1 and BMK-2. Identical amino acid residues are shaded. AlignX program of Vector NTI suite (Informax) employed.

3. Results

3.1. Isolation and structural characterization of maximakinin

A major component of the skin secretion (156 μg, 70 nmol/5 mg crude secretion), identified by LC/MS, was a peptide of molecular mass 2180 Da $(M + H)^+$ whose primary structure was established by automated Edman degradation as: DLPKINRKGPRPPGFSPFR (Fig. 1). This novel, N-terminally-extended bradykinin-related nonadecapeptide was named maximakinin, reflecting the specific name of the species of origin, its relatively large molecular mass and its uniqueness. No bradykinin (residues 11–19 of maximakinin) was detected in the skin secretion indicating a lack of cleavage of the Pro^{10}–Arg^{11} bond immediately preceding this sequence. Maximakinin (180 mg) was subsequently synthesized using solid phase *N*-(9-fluorenyl)methoxycarbonyl (fmoc) chemistry on an Applied Biosystems 433A peptide synthesizer, purified by LC/MS and the structure confirmed by MS/MS fragmentation.

3.2. In vitro cDNA library construction from venom

From the *B. maxima* venom cDNA library, two prepromaximakinin cDNAs were consistently cloned. These encoded four tandem repeat sequences of maximakinin (BMK-1) (Fig. 2) and a single copy of peptide (BMK-2) (Fig. 3), respectively. BMK-1 and BMK-2 contained open-reading frames of 152 and 116 amino acid residues. Alignment of open-reading frame amino acid sequences revealed an apparent insert sequence in the N-terminal region of the open-reading frame of BMK-2 (Fig. 4). This insert sequence exhibited a high degree of sequence similarity to a homologous region of BOK-2 (Fig. 5), the precursor of (Thr^6)-bradykinin from the skin of the Oriental fire-bellied toad, *B. orientalis* [6].

Recently, a peptide named bombinakinin M, of identical structure to maximakinin, was reported from *B. maxima* [16]. However in this study, cloning of precursor cDNA from skin resulted in the identification of a single transcript containing six tandem repeats of coding sequence. In order to validate our data and experimental protocol and to compare

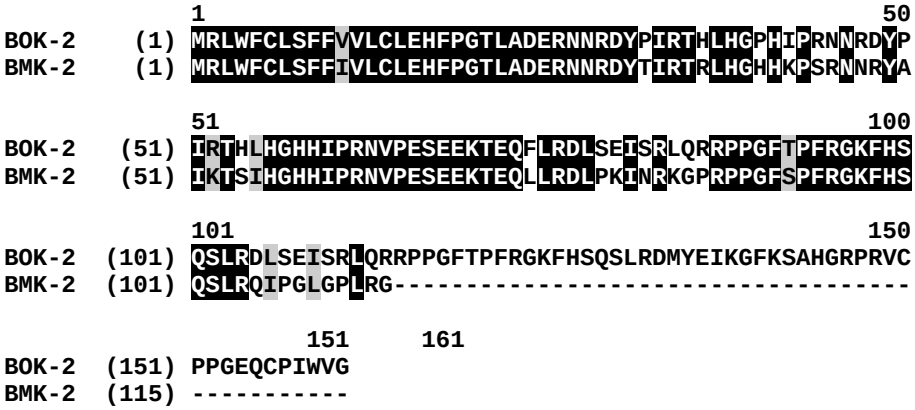


Fig. 5. Alignment of open-reading frame amino acid sequences of BOK-2 (*B. orientalis*) and BMK-2 (*B. maxima*). Identical amino acid residues are shaded. AlignX program of Vector NTI suite (Informax) employed.

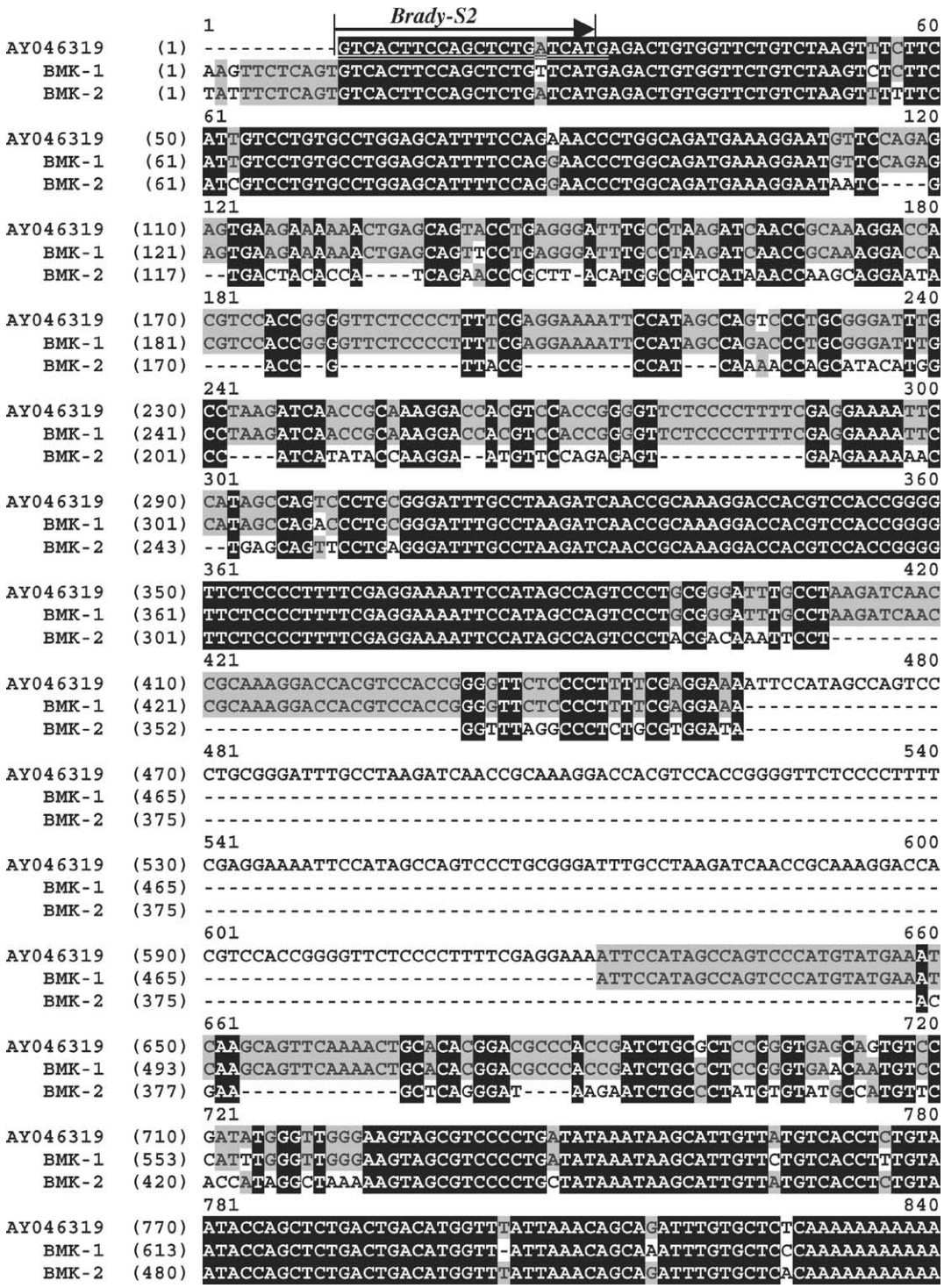


Fig. 6. Alignment of nucleotide sequences of full-length clones of AY046319 (bombinakinin M), BMK-1 and BMK-2. Identical bases are shaded black, consensus bases in two sequences shaded grey. AlignX program of Vector NTI suite (Informax) was employed. The position of the sense primer (Brady-S2) is indicated.

these previous reports, we designed a specific sense primer (Brady-S2, 5′-GTCACTTCCAGCTCTGATCATG-3′) to a site in the 5′-non-translated region of the preprobombinakinin M cDNA deposited in the EMBL Nucleotide Sequence Database under the accession code AY046319 (Fig. 6), and employed this in a 3′-RACE reaction. This procedure should have without doubt, resolved the six tandem repeat transcript if that were represented. However, only two products were identified, cloned and sequenced and these corresponded exactly in nucleotide

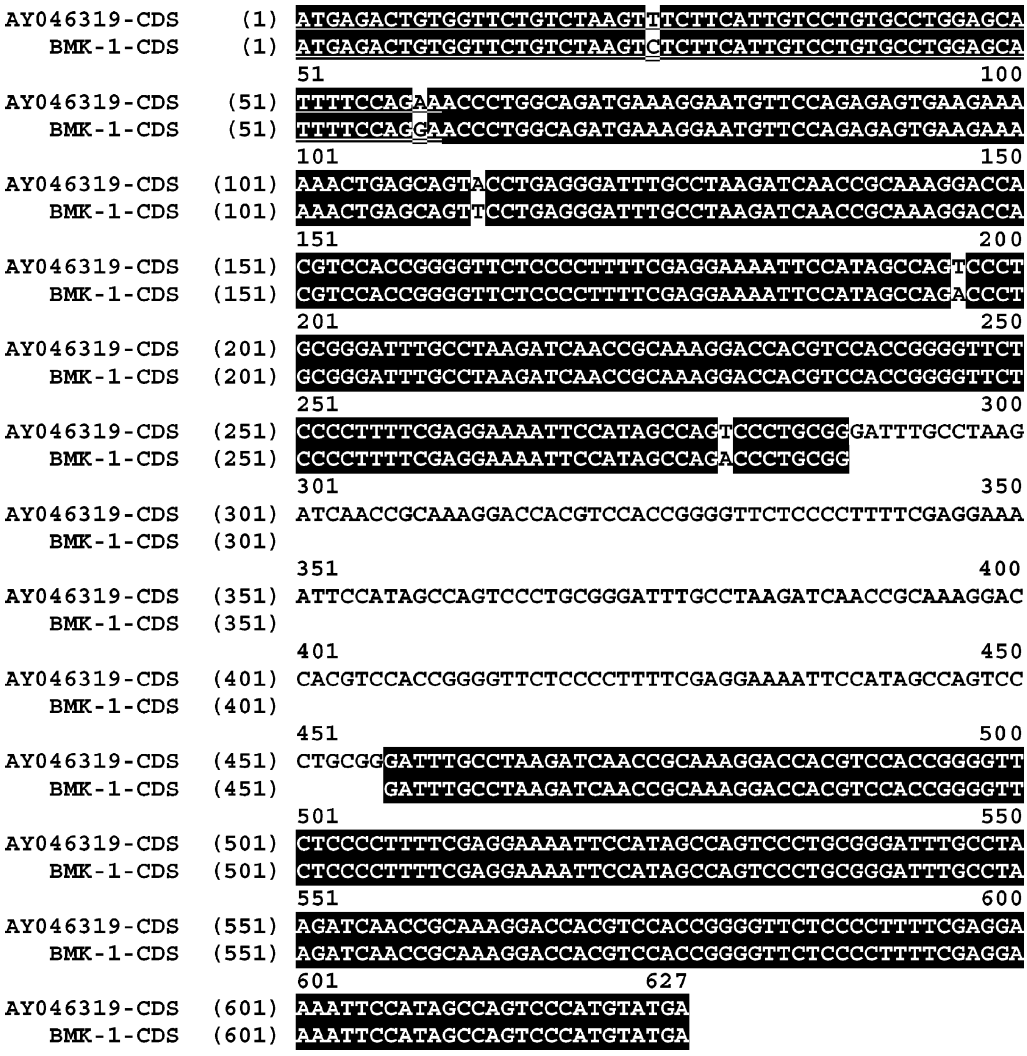


Fig. 7. Alignment of nucleotide sequence of the six tandem-repeat bombinakinin M precursor (AY046319) with that of the four tandem repeat maximakinin precursor (BMK-1) obtained in the present study using an identical sense primer. Identical nucleotides boxed in black.

Fig. 8. Alignment of the open-reading frame amino acid sequences of the six tandem-repeat bombinakinin M precursor (AY046319) and the four tandem-repeat maximakinin precursor (BMK-1) obtained in the present study. Identical amino acid residues boxed in black.

sequence to BMK-1 and BMK-2, respectively (Figs. 7 and 8).

4. Discussion

Amphibian defensive skin secretions remain a largely untapped resource for the peptide biochemist with an interest in the identification, structural characterization and cloning of precursor cDNAs of novel bioactive peptides. While some of these peptides may represent novel analogs of known peptide families, others will exhibit such dramatic structural alterations that very different pharmacological properties may be imparted. For the discerning researcher, however, the most interesting may be the small remainder that represent prototype peptides not encountered before in nature [4,8,12,13,17].

Bradykinin in mammals, including man, is synthesized as a small domain (nine amino acid residues (-RPPGFSPFR-)) within a much larger precursor protein (kininogen) in hepatocytes and actively secreted into the circulation [3]. Bradykinin is released to mediate its plethora of biological effects by the action of circulating and tissue bound proteases (kallikreins) at sites of inflammation or tissue damage. A question to be addressed here was to ascertain the structural relatedness of amphibian skin kininogens with their mammalian counterparts. This was made an even more intriguing venture by nature of the fact that a circulating kallikrein/kininogen system has not been demonstrated in either frog or toad plasma using standard methodologies despite several attempts in different species [3,12].

The N-terminally extended bradykinin, named maximakinin, was identified in the defensive skin secretion of *B. maxima*. This peptide, by nature of its N-terminal extension sequence, provided a much better basis for degenerate primer design for the purpose of precursor cloning by PCR than the bradykinin sequence itself in which the most highly-degenerate amino acids (in terms of codon base sequence) predominate. Molecular cloning of bioactive peptide precursors from amphibian skin provides much useful information on endogenous propeptide convertase specificities, co-encoded peptides and can be a vital step in the initiation of studies designed to map genomic organization of respective genes [4,21,22,25,26]. However, all such previous studies required sacrifice of specimens and removal of dorsal skin for the purpose of cDNA library construction. Amphibians are a group of vertebrates that are suffering unexplained global declines due to a combination of factors, including habitat destruction and pollution on one hand, and pathogenic viral and fungal diseases on the other [15]. These factors, however, do not explain all observed population crashes [18]. The acquisition of amphibian species for dermal venom studies is not simple and every effort has to be made to address the well-being of rare captive specimens. We were thus faced with the problem of obtaining structural information on dermal venom gland peptide precursor mRNAs without recourse to specimen sacrifice. We thus attempted to obtain this information by a novel and highly-speculative route—cloning from the skin secretion itself—essentially using the secretion as a surrogate skin tissue for this purpose. It worked extremely well. We successfully cloned two different cDNAs containing either four tandem repeats or a single copy of maximakinin from lyophilized venom of *B. maxima*. A previous study on this species identified the same peptide, which was named bombinakinin M, although bombinakinin O, from *B. orientalis*, is actually a C-terminally extended bradykinin (bradykinyl-GKFH). In this study, cloning of the peptide precursor from a skin cDNA library, resolved a single transcript encoding six tandem repeats. This transcript was never encountered in any of our cloning experiments that consistently yielded the four tandem repeat and single copy transcripts.

In order to fully validate our data, we designed a specific sense primer to a site within the previously published bombinakinin M cDNA sequence in the 5′-non-translated region (that was incidentally identical in base sequence to the analogous domain in each of our transcripts) and performed 3′-RACE reactions. Again, consistently over three experiments, we resolved only four tandem repeat and single copy transcripts. This strategy would without doubt have amplified a six copy transcript if such were present in our cDNA library. What could be the possible reasons for this discrepancy? Firstly, as the previous group screened a phage display library with an inappropriate primer (actually a degenerate primer for the bombesin precursor of this species), they may have missed these additional transcripts as their study was not systematic as here. Secondly, as we have cloned from secreted venom, the six tandem repeat transcript may not be a component of expression in the venom gland cells but rather may be found in some other skin cell or somatic cell contaminant of the skin preparation used in skin cDNA library construction. Thirdly, as several very closely-related species of *Bombina* occur in the region of China from which *B. maxima* hails, we may inadvertently be studying two such closely-related species or different local populations of the one species exhibiting minor genetic variations.

Although the precursor structures of amphibian skin-derived bradykinins have only been deduced to date from three species of bombinid toad (*B. orientalis*, *B. variegata* and *B. maxima*), there exists some striking differences and similarities. Each species appears to produce two transcripts. In *B. orientalis*, there are two separate precursors encoding bradykinin (BOK-1) and (Thr^6)-bradykinin (BOK-2), containing four and two tandem repeats of each peptide, respectively. In *B. variegata*, there are likewise two separate precursors although in this instance, they each encode a single copy of (Ala^3, Thr^6)-bradykinin (BVK-1) and (Val^1, Thr^3, Thr^6)-bradykinin (BVK-2). In the present study, *B. maxima* was found to continue the trend of having two separate bradykinin precursors but each encoded the same peptide, maximakinin, in quadruplicate (BMK-1) and

single copies (BMK-2), respectively. Another interesting comparative feature was the presence of a homologous 36 amino acid residue insert in the N-terminal regions of both BOK-2 (*B. orientalis*) and BMK-2 (*B. maxima*), both eastern Asian species. Additionally, BMK-2 encoded a second peptide (pGlu-Ile-Pro-Gly-Leu-Gly-Pro-Leu-Arg amide) at the C-terminus that was identified in LC/MS fractions of venom and whose structure was confirmed (no data shown).

This simple technology described here in cloning precursor cDNAs from lyophilized venom is robust has been applied previously to other species of both amphibian and reptile [5] and the discovery of venom cloning provides an elegant means of establishing a global functional genomic repository for amphibian venom gland research and other molecular applications, without interfering with already fragile native populations and ecosystems.

The structural diversity of peptides in amphibian defensive skin secretions probably reflects different roles, either in the regulation of physiological functions of the skin or in defence against specific predators [4,14].

Acknowledgments

Tianbao Chen is in receipt of a Vice Chancellor's Research Studentship at the University of Ulster and we thank the Fujian Provincial Science and Technology Department, People's Republic of China, for financial support (2001I010).

References

[1] Anastasi A, Bertaccini G, Erspamer V. Pharmacological data on phyllokinin (bradykinyl-isoleucyl-tyrosine-*O*-sulphate) and bradykinyl-isoleucyl-tyrosine. Br J Pharmacol 1966;27:479–85.
[2] Anastasi A, Erspamer V, Bertaccini G. Occurrence of bradykinin in the skin of *Rana temporaria*. Comp Biochem Physiol 1965;14:43–52.
[3] Bhoola KD, Figueroa CD, Worthy K. Bioregulation of kinins: kallikreins, kininogens and kinases. Pharmacol Rev 1992;44:1–80.
[4] Charles LB, Michael Z. Peptides from frog skin. Annu Rev Biochem 1990;59:395–414.
[5] Chen T, Bjourson AJ, Orr DF, Kwok H, Rao P, Ivanyi C, et al. Unmasking venom gland transcriptomes in reptile venoms. Anal Biochem 2002;311:152–6.
[6] Chen T, Orr DF, Bjourson AJ, McClean S, O'Rourke M, Hirst DG, et al. Bradykinins and their precursor cDNAs from the skin of the fire-bellied toad (*Bombina orientalis*). Peptides 2002;23:1547–55.
[7] Chen T, Orr DF, Bjourson AJ, McClean S, O'Rourke M, Hirst DG, et al. Novel bradykinins and their precursor cDNAs from European yellow-bellied toad (*Bombina variegata*) skin. Eur J Biochem 2002;269:4693–700.
[8] Clarke BT. The natural history of amphibian skin secretions, their normal functioning and potential medical applications. Biol Rev Camb Phil Soc 1997;72:365–79.
[9] Conlon JM, Aronsson U. Multiple bradykinin-related peptides in the skin of the frog, *Rana temporaria*. Peptides 1997;18:361–5.
[10] Daly JW, Myers CW, Whittaker N. Further classification of skin alkaloids from Neotropical poison frogs (Dendrobatidae), with a general survey of toxic/noxious substances in the Amphibia. Toxiconomics 1987;25:1023–95.
[11] Daly JW, Spoande TF. Amphibian alkaloids: chemistry, pharmacology and biology. In: Pelletier SW, editor. Alkaloids: chemical and biological perspectives, vol. 4. New York: Wiley; 1986. p. 1–274.
[12] Duellman WE, Trueb L. Biology of amphibians. Baltimore/London: Johns Hopkins University Press; 1994.
[13] Erspamer V. Bioactive secretions of the integument. In: Heatwole H, Barthalmus GT, editors. Amphibian biology, vol. 1. The integument. Chipping Norton: Surrey Beatty & Sons; 1993 [chapter 9].
[14] Erspamer V, Melchiorri P, Falconieri-Erspamer GF, Montecucchi PC, De Castiglione R. *Phyllomedusa* skin: a huge factory and store-house of a variety of active peptides. Peptides 1985;3:7–12.
[15] Houlahan JE, Findlay CS, Schmidt BR, Meyer AH, Kuzmin SL. Quantitative evidence for global amphibian population declines. Nature 2000;404:752–5.
[16] Lai R, Liu H, Hui Lee W, Zhang Y. A novel bradykinin-related peptide from skin secretions of toad *Bombina maxima* and its precursor containing six identical copies of the final product. Biochem Biophys Res Commun 2001;286:259–63.
[17] Lazarus LH, Atilla M. The toad, ugly and venomous, wears yet a precious jewel in his skin. Prog Neurobiol 1993;41:473–507.
[18] Keisecker JM, Blaustein AR, Belden LK. Complex causes of amphibian population declines. Nature 2001;410:681–4.
[19] Mangoni ML, Grovale N, Giorgi A, Mignogna G, Simmaco M, Barra D. Structure–function relationships in bombinins H, antimicrobial peptides from *Bombina* skin secretions. Peptides 2000;21:1673–9.
[20] Mignogna G, Simmaco M, Kreil G, Barra D. Antibacterial and haemolytic peptides containing D-alloisoleucine from the skin of *Bombina variegata*. EMBO J 1993;12:4829–32.
[21] Richter K, Egger R, Kreil G. D-Alanine in the frog skin peptide dermorphin is derived from L-alanine in the precursor. Science 1987;238:200–2.
[22] Regoli D, Barabe J. Pharmacology of bradykinin and related kinins. Pharmacol Rev 1980;32:1–46.
[23] Simmaco M, Mignogna G, Barra D. Antimicrobial peptides from amphibian skin: what do they tell us? Biopolymers 1998;47:435–50.
[24] Tyler MJ, Stone DJM, 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.
[25] Wechselberger C, Severini C, Kreil G, Negri L. A new opioid peptide predicted from cloned cDNAs from skin of *Pachymedusa dacnicolor* and *Agalychnis annae*. FEBS Lett 1998;429:41–3.
[26] Zasloff M. Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc Natl Acad Sci USA 1987;84:5449–53.