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Identification and molecular cloning of novel trypsin inhibitor analogs from the dermal venom of the Oriental fire-bellied toad (*Bombina orientalis*) and the European yellow-bellied toad (*Bombina variegata*)☆

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Abstract

The structural diversity of polypeptides in amphibian skin secretion probably reflects different roles in dermal regulation or in defense against predators. Here we report the structures of two novel trypsin inhibitor analogs, BOTI and BVTI, from the dermal venom of the toads, *Bombina orientalis* and *Bombina variegata*. Cloning of their respective precursors was achieved from lyophilized venom cDNA libraries for the first time. Amino acid alignment revealed that both deduced peptides, consisting of 60 amino acid residues, including 10 cysteines and the reactive center motif, -CDKKC-, can be affirmed as structural homologs of the trypsin inhibitor from *Bombina bombina* skin.
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Keywords: Amphibian; Mass spectrometry; Trypsin inhibitor; cDNA cloning

1. Introduction

The structural diversity of polypeptides secreted from amphibian dermal granular glands is probably reflective of a plethora of different biological functions including the regulation of skin physiology, defense against predators or prevention of skin colonization/infection by microorganisms [10,21]. Granular gland contents are released onto the skin surface following stress or injury to the individual amphibian as a result of neurally-induced contraction of myoepithelial cells surrounding the glands [2,12,17]. The secretions have been known for some time to be rich in exceptional bioactives including biogenic amines, peptides, proteins, alkaloids and heterocyclics [3,11].

Since the isolation of the antimicrobial and hemolytic peptide, bombinin, from European fire-bellied toad (*Bombina bombina*) skin [9] and subsequently, the magainins from *Xenopus laevis* skin [30], investigators have discovered a multitude of peptides in many amphibian species, with peptides from some five hundred species of amphibian from six continents, having been studied over the past few decades. For example, from the skin secretion of *Bombina* species, a variety of peptides have been isolated and characterized that display a wide spectrum of pharmacological, antimicrobial and inhibitory activities [2,3,11,17]. In particular, peptides with broad-spectrum antimicobial and/or hemolytic activities can be grouped into two families, bombinin-like peptides (BLPs) and bombinin Hs [13,25]. Members of each peptide family differ by only one or a few amino acid substitutions. Recently, we have identified bradykinin and (Thr^6)-bradykinin in the venom of *Bombina orientalis* and two novel bradykinin-related peptides (Ala^3, Thr^6)-bradykinin and (Val^1, Thr^3, Thr^6)-bradykinin in the venom of the yellow-bellied toad, *Bombina variegata*, with subsequent cloning of their precursor cDNAs from skin-derived cDNA libraries [5,6].

Peptidic protease inhibitors have been known to be present for some time in many diverse animals from nematodes to humans, and their ubiquitous distribution in microorganisms and plants has been widely documented [19]. To date, only a few protease inhibitor polypeptides have been identified

Abbreviations: HPLC, high performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; mRNA, messenger RNA; cDNA, DNA complementary to RNA; PCR, polymerase chain reaction

☆The nucleotide sequences of two trypsin inhibitor, named BOTI and BVTI, from the skin of *Bombina orientalis* and *Bombina variegata* have been deposited in the EMBL Nucleotide Sequence Database under the accession codes AJ549920 and AJ549921, respectively.

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in amphibian skin secretions such as those of the European fire-bellied toad, *B. bombina* and the Madagascan tomato frog, *Dyscophus guineti* [8,20].

In this study, we present the primary structures of two novel trypsin inhibitor analogs, BOTI and BVTI, identified in and isolated from the dermal venom of an Asian bombinid toad (*B. orientalis*) and a European bombinid toad, *B. variegata*. Partial amino acid sequences were obtained by automated Edman degradation and subsequently their full primary structures were deduced following cloning of their respective precursor cDNAs from libraries made from the dermal venom itself—a novel non-invasive and non-lethal technique (for the toads) recently developed in our laboratory [7].

2. Materials and methods

2.1. Specimen biodata and venom acquisition

Specimens of *B. orientalis* ($n = 3$) and *B. variegata* ($n = 3$) were obtained from commercial sources. The frogs were metamorphs (1 cm snout to vent length) on receipt and were grown to adult size (4 cm snout to vent length) over a 2-year period prior to venom 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. Dermal venom was obtained from the dorsal skin by transdermal electrical stimulation (6 V dc, 4 ms pulse-width, 50 Hz) through platinum electrodes for two periods of 15 s duration [28]. The obvious foamy secretion was washed from the dorsal skin using deionized water, snap-frozen in liquid nitrogen and lyophilized. Lyophilizate was stored at −20 °C prior to analyses.

2.2. Identification and structural analysis of BOTI and BVTI

A 10 mg sample of each lyophilized venom was dissolved in 0.5 ml of 0.05/99.5 (v/v) trifluoroacetic acid (TFA)/water and clarified of microparticulates by centrifugation. The supernatants were then subjected separately to LC/MS 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 reversed phase HPLC system, fitted with an analytical column (Jupiter C-5, 5 μm particle, 300 Å pore, 250 mm × 10 mm, Phenomenex, UK) and interfaced with a Thermoquest LCQ^{TM} 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 molecular masses of polypeptides in each chromatographic fraction were further analyzed 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 ±0.1%. The major polypeptides with masses of approximately 6.4 kDa, that were identified in the venom, were each subjected to primary structural analysis by automated Edman degradation using an Applied Biosystems 491 Procise sequencer in pulsed-liquid mode. The limit for detection of phenylthiohydantoin (PTH) amino acids was 0.1 pmol.

2.3. Cloning of B. orientalis (BOTI) and B. variegata (BVTI) trypsin inhibitor cDNAs

A 10 mg sample of each lyophilized venom was dissolved separately in 1 ml of cell lysis/mRNA protection buffer supplied by Dynal Biotec, UK. Polyadenylated mRNA was isolated by the use of magnetic oligo-dT beads as described by the manufacturer (Dynal Biotec, UK). The isolated mRNA was subjected to 5′- and 3′-rapid amplification of cDNA ends (RACE) procedures to obtain full-length trypsin inhibitor analog nucleic acid sequence data using a SMART-RACE kit (Clontech, UK) essentially as described by the manufacturer. Briefly, the 3′-RACE reactions employed a nested universal (NUP) primer (supplied with the kit) and a sense primer (S: 5′-AAYTTYGTITGYCCICCIGG-3′) that was complementary to the amino acid sequence, -FVCPPGQ-, of both BOTI and BVTI. The 3′-RACE reactions were 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 was used to design a specific antisense primer (AS: 5′-CAAAAGTGCTAATTTATTAACAAT-3′) to a conserved site within the 3′-non-translated region of both BOTI and BVTI cDNAs. 5′-RACE was carried out using this specific primer in conjunction with the NUP RACE primer and resultant products were purified, cloned and sequenced.

3. Results

3.1. Identification and structural analyses of BOTI and BVTI

Two novel components, resolved in the dermal venoms by LC/MS, were polypeptides having molecular masses of 6446 Da (BOTI) and 6418 Da (BVTI), respectively (Figs. 1–2). Each was present in similar levels in respective venoms (5.85 μg (900 pmol)/10 mg venom). The sequences of the first 20 amino acid residues of each polypeptide were established by automated Edman degradation and were found to be identical: NFV-PPGQSFQT-ASS-PKT-. Blank cycles were assumed to represent cysteinyl residues. This N-terminal sequence data was submitted to automatic alignment using the NCBI-BLAST search system that revealed

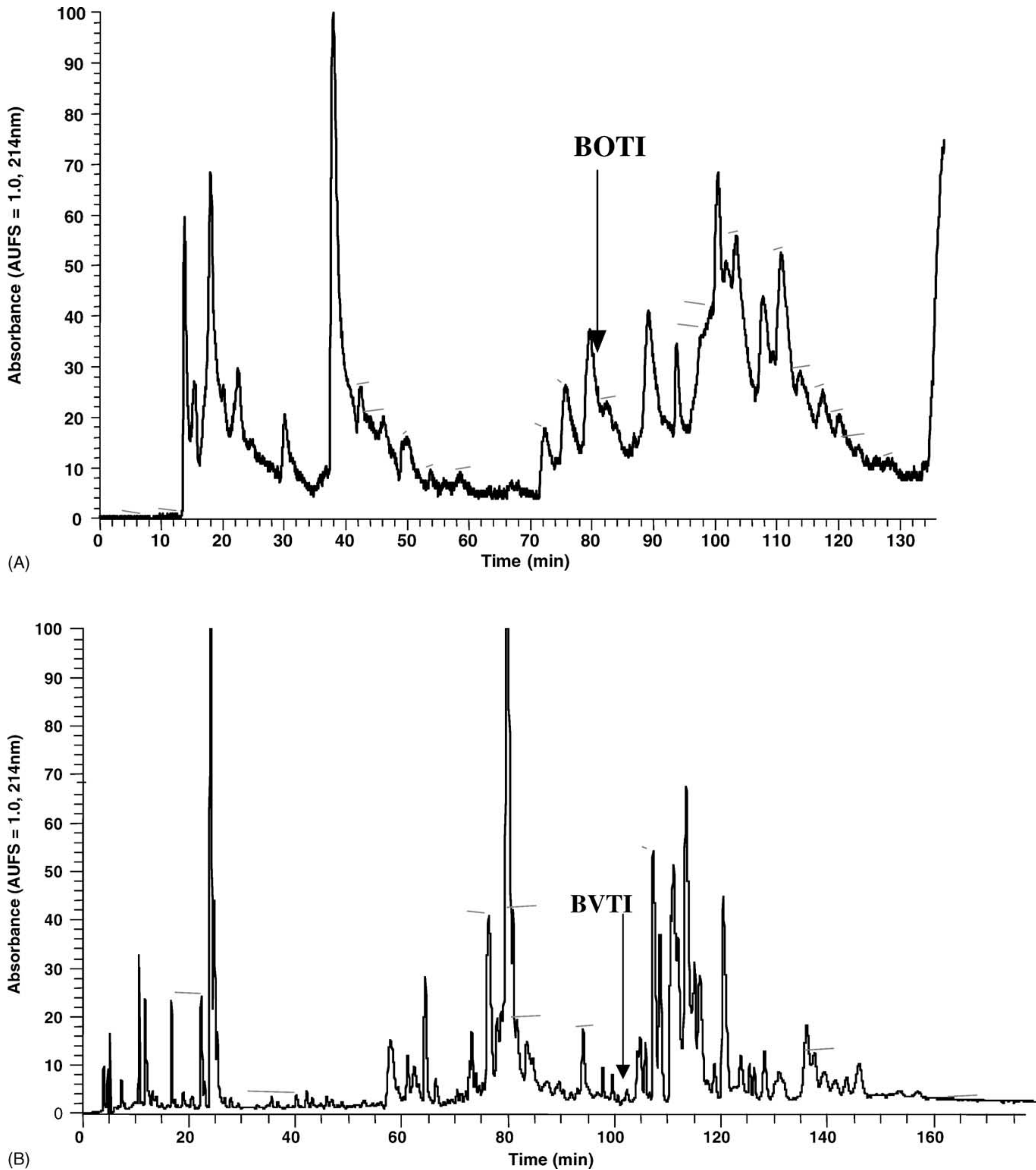

Fig. 1. Reverse phase HPLC chromatograms of *Bombina orientalis* venom (A) and *Bombina variegata* venom (B). The retention times of BOTI and BVTI are indicated by arrows on respective chromatograms.

a 95% identity with that of BSTI, a trypsin inhibitor from the skin of the European fire-bellied toad, *B. bombina* [20]. This alignment also confirmed that cysteinyl residues were coincident with the blank cycles in our automated Edman degradation data.

3.2. Cloning of homologous trypsin inhibitor cDNAs from venom

Two trypsin inhibitor analog cDNAs were consistently cloned from the venom-derived libraries (one from each)

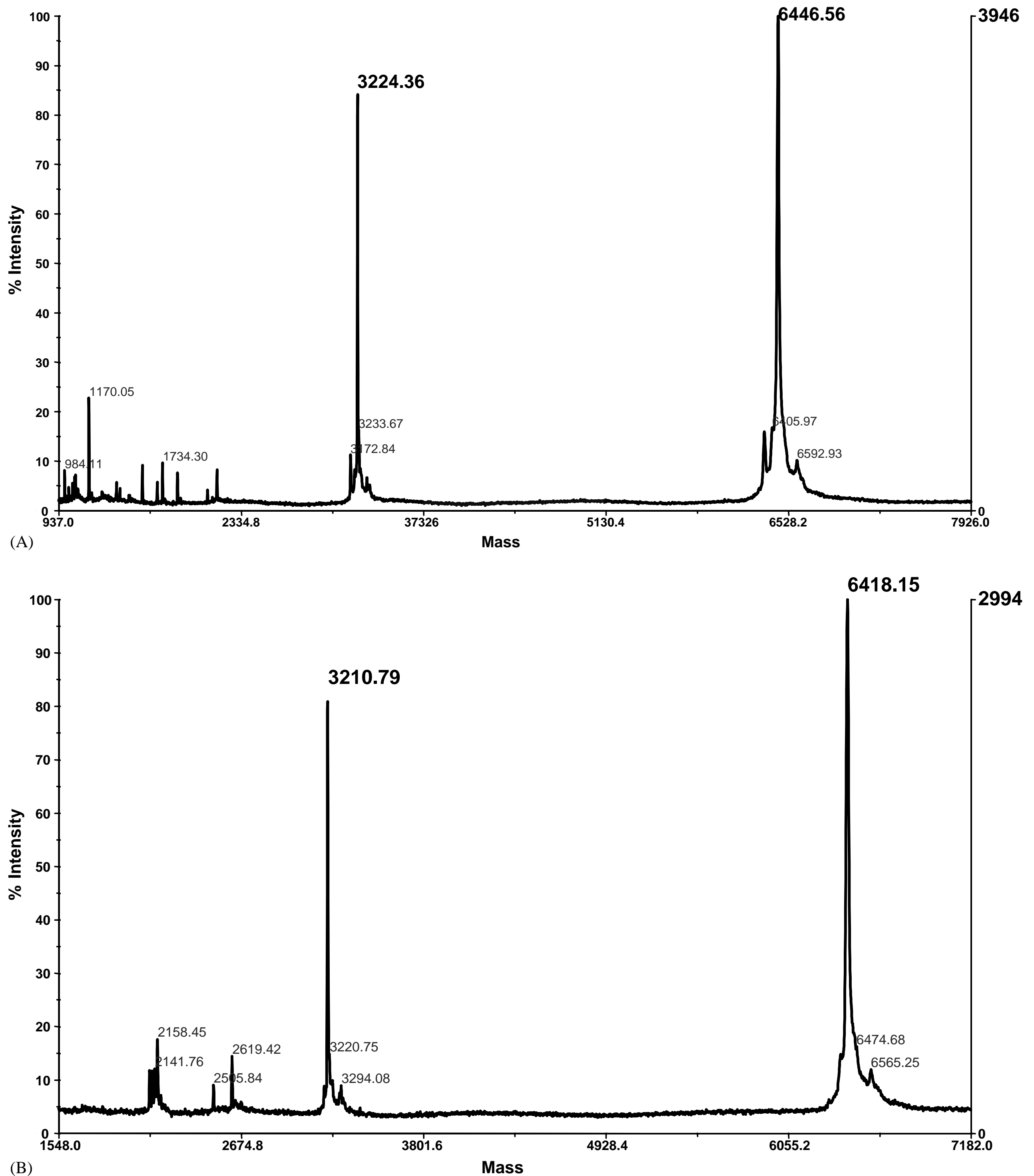

Fig. 2. MALDI-TOF mass spectrograms of (A) BOTI (m/z 6446.56 (MH^+), m/z 3224.36 (MH^{2+})) and (B) BVTI (m/z 6418.15 (MH^+), m/z 3210.79 (MH^{2+})).

of *B. orientalis* and *B. variegata* (sequencing of 40 clones, each sequence represented at least 10 times) and both open-reading frames (BOTI and BVTI) consisted of 84 amino acid residues (Figs. 3–4). Alignment of BOTI, BVTI and BSTI [20] nucleotide sequences (Fig. 5) and open-reading frame amino acid sequences (Fig. 6), using the AlignX programme of the Vector NTI Bioinformatics suite (Informax), revealed a very high degree of primary

BOTI

```
                                         M   K  L  A  T   V  I  L
  1  CAGCCTAGAA AAAGAAAATT AAAATAATGA AATTAGCCAC AGTAATCCTT
     GTCGGATCTT TTTCTTTTAA TTTTATTACT TTAATCGGTG TCATTAGGAA
      I  M  A   I  V  L  P   C  L  F   Y  K  E   M  E  A  N
 51  ATTATGGCAA TTGTCCTGCC GTGCTTGTTT TATAAGGAAA TGGAAGCTAA
     TAATACCGTT AACAGGACGG CACGAACAAA ATATTCCTTT ACCTTCGATT
      F  V  C   P  P  G   Q  S  F  Q   T  C  A   S  S  C
101  TTTTGTTTGC CCACCTGGAC AGAGCTTCCA AACATGTGCT TCCAGCTGCC
     AAAACAAACG GGTGGACCTG TCTCGAAGGT TTGTACACGA AGGTCGACGG
     P  K  T  C   E  T  R   N  K  V   V  L  C  D   K  K  C
151  CAAAGACCTG TGAAACAAGA AATAAAGTTG TGCTCTGTGA TAAGAAGTGC
     GTTTCTGGAC ACTTTGTTCT TTATTTCAAC ACGAGACACT ATTCTTCACG
      N  Q  R   C  D  C  V   S  G  T   V  L  K   S  K  G  S
201  AATCAAAGAT GTGACTGTGT CTCTGGAACT GTGCTGAAAT CAAAGGGTTC
     TTAGTTTCTA CACTGACACA GAGACCTTGA CACGACTTTA GTTTCCCAAG
       S  E  C   V  H  P   S  K  C  *
251  ATCTGAATGT GTGCACCCCA GTAAATGTTA ATTACAGACA AAATTGTTAA
     TAGACTTACA CACGTGGGGT CATTTACAAT TAATGTCTGT TTTAACAATT
301  TAAATTAGCA CTTTTGATTG TGCCCTAAGA ATAAAAAAAA AAAAAAAAAA
     ATTTAATCGT GAAAACTAAC ACGGGATTCT TATTTTTTTT TTTTTTTTTT
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Fig. 3. Nucleotide sequence of cDNA encoding *Bombina orientalis* trypsin inhibitor (BOTI). The putative signal peptide (double-underlined), mature peptide (single-underlined) and stop codon (asterisk) are indicated.

BVTI

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                                         M   K  L  T  T   V  I  L
  1  CAGCCTAGAA AAAGAAAATT AAAATAATGA AATTAACCAC AGTAATCCTT
     GTCGGATCTT TTTCTTTTAA TTTTATTACT TTAATTGGTG TCATTAGGAA
      I  M  A   I  V  L  P   C  L  F   Y  K  E   I  E  A  N
 51  ATTATGGCAA TTGTCCTGCC GTGCTTGTTT TATAAGGAAA TAGAAGCTAA
     TAATACCGTT AACAGGACGG CACGAACAAA ATATTCCTTT ATCTTCGATT
      F  V  C   P  P  G   Q  S  F  Q   T  C  A   S  S  C
101  TTTTGTTTGC CCACCTGGAC AGAGCTTCCA AACATGTGCT TCCAGCTGCC
     AAAACAAACG GGTGGACCTG TCTCGAAGGT TTGTACACGA AGGTCGACGG
     P  K  T  C   E  T  R   N  K  L   V  L  C  D   K  K  C
151  CAAAGACCTG TGAAACAAGA AATAAACTTG TGCTCTGTGA TAAGAAGTGC
     GTTTCTGGAC ACTTTGTTCT TTATTTGAAC ACGAGACACT ATTCTTCACG
      N  Q  R   C  G  C  I   S  G  T   V  L  K   S  K  G  S
201  AATCAAAGAT GTGGCTGTAT CTCCGGAACT GTGCTGAAAT CAAAGGGTTC
     TTAGTTTCTA CACCGACATA GAGGCCTTGA CACGACTTTA GTTTCCCAAG
       S  E  C   V  H  P   S  K  C  *
251  ATCTGAATGT GTGCACCCCA GTAAATGTTA ATTACAGAAA AAATTGTTAA
     TAGACTTACA CACGTGGGGT CATTTACAAT TAATGTCTTT TTTAACAATT
301  TAAATTAGCA CTTTTGATTG TGCCCTAAGA ATAAAAAAAA AAAAAAAAAA
     ATTTAATCGT GAAAACTAAC ACGGGATTCT TATTTTTTTT TTTTTTTTTT
```

Fig. 4. Nucleotide sequence of cDNA encoding *Bombina variegata* trypsin inhibitor (BVTI). The putative signal peptide (double-underlined), mature peptide (single-underlined) and stop codon (asterisk) are indicated.

structural similarity of both nucleic acid and amino acid sequence between the trypsin inhibitors. In addition, using the observed molecular masses of each polypeptide and the N-terminal Edman sequencing data, it was possible to deduce the sites of propeptide convertase cleavage in respective precursors that generate the trypsin inhibitor analogs present in the venom of both species. The NCBI-BLAST search found that BOTI and BVTI showed 91 and 96% sequence identity, respectively, with BSTI. An interesting observation was that the sites of amino acid substitutions in all of these three trypsin inhibitors were identical and confined to residue positions 9, 27, 39, 41 and 50 in the mature polypeptides.

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

Fig. 5. Alignment of nucleotide sequences of cDNAs encoding BOTI, BVTI and BSTI. Identical bases in all three shaded in black. Consensus bases in two sequences shaded grey. Gaps inserted to maximise alignment.

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 [2,3,11,12,17].

Primary structural studies on peptides and proteins from amphibian skin secretions, often an important prerequisite to understanding their bioactivity, can be a long-term project using conventional protein chemistry [1,8,20]. However, being armed with molecular mass data and a short segment of N-terminal sequence, is sufficient to initiate cDNA cloning studies that can effect much more rapid primary structural characterization (and perhaps establishment of micro-structural diversity) of skin secretion polypeptides and proteins [7,18]. Likewise, simply establishing their precursor cDNA sequence may not always facilitate deduction of final, post-translationally processed products. This may be possible but tentative, by comparing deduced structures with analogs from related species [4,22]. However, the parallel protein sequencing, molecular mass determination and corresponding cDNA cloning described here permits unequivocal structural assignations.

Protein inhibitors of proteases are ubiquitous. They are present in multiple forms in numerous tissues of animals and plants as well as in microorganisms. They are grouped primarily as serine, cysteine, aspartic or metallo-protease inhibitors. Serine protease inhibitors function by binding to their cognate enzyme in a substrate-like manner, forming a stable complex. They are of broad interest because

Fig. 6. Alignment of translated open-reading frame amino acid sequences of BOTI, BVTI and BSTI. Identical amino acid residues shaded in black.

```
                1                                                50
BOTI     (1)   MKLATVILIMAIVLPCLFYKEMEANFVCPPGQSFQTCASSCPKTCETRNK
BMTI     (1)   MELTPVIPIMAIVLPCLFYKEMEANVVCPPGQSFQTCASSCPKTCETRKK

                51                               84
BOTI    (51)   VVLCDKKCNQRCDCVSGTVLKSKGSSECVHPSKC
BMTI    (51)   VVLCDKKCNLGCYCVPGTVLKSKGSSECVNPSKC
```

Fig. 7. Alignment of translated open-reading frame amino acid sequences of BOTI and BMTI. Identical amino acid residues shaded in black.

serine proteases play key roles in such functions as peptide hormone release, blood coagulation and complement fixation, and constitute pathogenic factors in numerous diseases, including some cancers, pulmonary emphysema and inflammatory processes such as glomerulonephritis and acute pancreatitis [15,24]. Serine protease inhibitors have been classified into at least 10 families according to primary structural homology [16]. Variability at the reactive site within a family can lead to differences in specificity for proteases within a given mechanistic class. Two novel trypsin inhibitor analogs, named BOTI and BVTI, have been identified in the dermal venom of *B. orientalis* and *B. variegata*, and subsequently their precursor cDNAs were cloned from libraries made from the same venom samples using a recently-described technique developed in our laboratory [7]. A comparison of the primary structures of BOTI and BVTI with those of other known serine protease inhibitors, revealed that both BOTI and BVTI have significant sequence similarity to BSTI, originally isolated from the skin of a related toad, *B. bombina* [20]. BSTI was itself found to exhibit a high degree of primary structural similarity to ATI from the obligate parasitic nematode, *Ascaris lumbricoides* var *suum* [14]. ATI-type serine protease inhibitors are characterized by the presence of five disulfide bonds in a single small protein domain of 61–62 amino acid residues [14]. ATI-type inhibitors form tight enzyme–substrate complexes. One peptide bond of the inhibitor is hydrolyzed, but the resulting enzyme–product complex does not dissociate. The scissile bond providing the base of the inhibitory specificity is between residue positions 31–32 in these ATI-type inhibitors. The reactive site of BSTI comprises the sequence of residues 30–34, -Cys-Asp-Lys-Lys-Cys-, which would imply that the -KK- doublet is indeed the reactive center of the inhibitor [23]. The location of this site and its primary structure in both BOTI and BVTI, are completely conserved.

Protein-based protease inhibitors serve to protect the host against the range of extrinsic proteases produced by invasive microorganisms [26]. Such enzymes play direct roles in the pathogenesis and virulence of most strains of opportunistic bacteria by damaging host tissues and evading host defenses [27]. It has been speculated, therefore, that the trypsin inhibitor in the skin secretion of the Madagascan tomato frog, *D. guineti*, may be employed as a surface anti-infective agent, as this species does not apparently, produce antimicrobial peptides unlike many other frogs [16]. However, the presence of trypsin inhibitors in the dermal venom of toads from the genus *Bombina*, a group that co-secrete large amounts of broad-spectrum antimicrobial peptides (bombinins), would tend to question this concept [3]. An alternative explanation would be that such enzyme inhibitors prevent degradation of other bioactive peptides in the amphibians skin secretion by endogenous predator proteases prior to delivery of the toxic payload to target receptors.

An interesting finding in the present study was the extremely high degree of primary structural conservation between the skin trypsin inhibitors of *B. bombina*, *B. variegata* and *B. orientalis*, with only five common sites of amino acid substitution observed. Both *B. bombina* and *B. variegata* have overlapping distributions in Europe and in fact the close relationship of these species is reflected in zones of hybridization where both species occur sympatrically [29]. *B. orientalis*, as its name suggests, is found in the Far East, specifically in North East China and Korea. Despite the effects of zoogeographical isolation of this species, the present data would substantiate a close evolutionary relationship with its European congenerics. However, recently the sequence of a skin trypsin inhibitor [EMBL Accession no. AF411088] has been deduced from another Oriental bombinid toad, *Bombina maxima*, that occurs in a discrete region of South West China with several closely-related species of even more restricted distributions. Comparison of the primary structures of *B. orientalis* and *B. maxima* skin trypsin inhibitors produced an interesting finding (Fig. 7). Although exhibiting a high degree of primary structural identity, including the defining inhibitory core motif sequence, there are more amino acid substitutions evident (seven versus five) between the mature peptides and all but one (residue 39) of the substituted sites are different. These data might imply that the *B. bombina*/*B. variegata*/*B. orientalis* group represent a different taxon to the *B. maxima* group, although within the limits of interpretation of a single venom polypeptide sequence. However, molecular aspects are being employed more frequently in the field of taxonomy as an adjunct to the more classical approach of phenotypic characteristics such as skeletal structure. The present data may thus provide the amphibian taxonomist with an additional insight into the relationships of bombinid toads and may provide the starting point for a more systematic approach to generic assignations within this currently single taxon.

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