Molecular cloning of mRNA from toad granular gland secretion and lyophilized skin: identification of Bo8—a novel prokineticin from Bombina orientalis

Tianbao Chen, Yuanzhen Xue, Mei Zhou, Chris Shaw

Molecular Therapeutics Research Group, School of Pharmacy, Queen's University, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland, UK
Pharmaceutical Biotechnology Research Group, School of Biomedical Sciences, University of Ulster, Cromore Road, Coleraine BT52 1SA, Northern Ireland, UK

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Abstract
Prokineticins are small (∼8 kDa), biologically active secretory proteins whose primary structures have been highly conserved throughout the Animal Kingdom. Representatives have been identified in the defensive skin secretions of several amphibians reflecting the immense structural/functional diversity of polypeptides in such. Here we describe the identification of a prokineticin homolog (designated Bo8) from the skin secretion of the Oriental fire-bellied toad (Bombina orientalis). Full primary structural characterization was achieved using a combination of direct Edman microsequencing, mass spectrometry and cloning of encoding skin cDNA. The latter approach employed a recently described technique that we developed for the cloning of secretory peptide cDNAs from lyophilized skin secretion, and this was further extended to employ lyophilized skin as the starting material for cDNA library construction. The Bo8 precursor was found to consist of an open-reading frame of 96 amino acid residues consisting of a putative 19-residue signal peptide followed by a single 77-residue prokineticin (Mr = 7990 Da).

Amino acid substitutions in skin prokineticins from the skin secretions of bombinid toads are confined to discrete sites affording the necessary information for structure/activity studies and analog design.

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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 and prevention of skin colonization/infection by microorganisms [11,24]. 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 [2,13,17]. Rupture of the glandular syncytia, in which the peptide secretions are stored, is immediately followed by extrusion of their contents onto the skin surface. The secretions have been known for some time to be rich in bioactive molecules including biogenic amines, peptides, proteins, alkaloids and heterocyclics [3,12].

Since the isolation of the antimicrobial and hemolytic peptide, bombinin, from European fire-bellied toad (Bombina bombina) skin [10] and subsequently, the magainins from Xenopus laevis skin [29], investigators have discovered a multitude of peptides in many amphibian species, with pep-
differ by only one or a few amino acid substitutions. Re-
hibitory activities[2,3,12,17]. In particular, peptides with
spectrum of pharmacological, antimicrobial and protease in-
tides have been isolated and characterized that display a wide
2.1. Specimen biodata and acquisition of skin secretion
2. Materials and methods
lyophilized skin as starting material for library construction.
match protein A) and frog skin secretion (B. variegata protein Bv8)
in the granular gland secretions of B. orientalis and two novel 
3. Results
2.2. Identification and structural analysis of Bo8

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Materials and methods

2.1. Specimen biodata and acquisition of skin secretion

Specimens of B. orientalis (n = 3) were obtained from commercial sources. The toads were metamorphs (1 cm snout to vent length) on receipt and were maintained to adult size (4 cm snout to vent length) over a 2-year period prior to venom har-

ting. They were maintained in our purpose-designed am-

phibian facility at 20–25 °C and a 12:12 h light/dark cycle and fed multivitamin-loaded crickets three times per week. 

Granular gland secretions were obtained from the dorsal skin 

by transdermal electrical stimulation (6V DC, 4 ms pulse-

width, 50 Hz) through platinum electrodes for two periods

of 15 s duration [28]. The obvious and profuse foamy secre-

tion was washed from the dorsal skin using deionized water, 

snap-frozen in liquid nitrogen and lyophilized. Lympholysate was stored at −20 °C prior to analyses.

2.2. Identification and structural analysis of Bo8

Ten milligrams of lyophilized skin secretion were dis-

solved in 0.5 ml of 0.05/99.5 (v/v) trifluoroacetic acid (TFA)/water and clarified of microparticulates by centrifu-
gation. The supernatant was then subjected to LCMS us-
ing 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 Thermosquest gradient re-

versed phase HPLC system, fitted with an analytical column
(Jupiter C-5, 5 μm particle, 300 Å pore, 250 mm × 10 mm, 
Phenomenex, UK), interfaced with a Thermosquest 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 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 α-cyano-4-hydroxy cinnamic acid as the matrix. Internal mass calibration of the instru-
m ent with known standards established the accuracy of mass determination as 0.1%. The major polypeptide, with 
a mass of approximately 8 kDa, that was identified in the 

venom, was subjected to primary structural analysis by au-
tomated 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. Molecular cloning of Bo8 cDNA

Five milligrams of lyophilized skin secretion were dis-
solved in 1 ml of cell lysis/mRNA stabilization solution (Dy-
nal, UK). Polyadenylated mRNA was isolated using mag-
netic oligo-dT beads as described by the manufacturer (Dynal 
Biotech, UK) and reverse transcribed. The cDNA was sub-
jected to 3′-RACE procedures to obtain full-length prepro-
Bo8 nucleic acid sequence data using a SMART-RACE Kit (Clontech, UK) essentially as described by the manufac-
turer. Briefly, the 3′-RACE reactions employed an UPM 
primer (supplied with the kit) and a sense primer (5′: TGAYTTTACTAYTCA GTWWATCATG-3′) that was de-
signed to a segment of the 5′ untranslated region of Bv8 
cDNA from B. variegata (EMBL Accession no. AF168790) 
and Bm8a cDNA from B. maxima (EMBL Accession no.
AJ440423). The PCR cycling procedure was carried out as follows: initial denaturation step: 60 s at 94 °C, 35 cycles: de-
naturation 30 s at 94 °C, primer annealing for 30 s at 56 °C; extension for 180 s at 72 °C. PCR products were gel-purified and cloned using a pGEM-T vector system (Promega Corporation) and sequenced using an ABI 3100 automated sequencer.

Alternatively, skin that had been removed from euthanized frogs was snap-frozen in liquid nitrogen, lyophilized and stored at −20 °C under vacuum for 12 months, was employed for cDNA library construction. A 10 mg dry weight of lyophilized skin was ground to a powder in liquid nitrogen and dissolved in 2 ml of cell lysis/mRNA stabilization buffer (Dynal, UK). All subsequent procedures were performed as described above.

3. Results

3.1. Identification and structural analyses of Bo8

The novel prokineticin described here, resolved in the skin secretion by LC/MS and further analyzed by MALDI–TOF MS, was a small protein having a molecular mass (MH+) of 7990.1 Da (Figs. 1 and 2). The sequence of 23 N-terminal amino acid residues of the polypeptide was established by automated Edman degradation as: AVITGAXDRDVQXGS-GTXAAASA. Blank cycles (X) were assumed to represent cysteinyl residues. This N-terminal sequence was submitted to automatic alignment using the NCBI-BLAST search sys-

![Fig. 1. Reverse phase HPLC chromatogram of B. orientalis skin secretion. The retention time of Bo8 is indicated by an arrow.](image1)

![Fig. 2. MALDI–TOF mass spectogram of Bo8 (m/z = 7990.1 (MH+)).](image2)
tem that revealed a high degree of identity with the corre-
sponding region of Bv8 and Bm8, from B. variegata and B.
maxima, respectively [7,23]. This alignment also confirmed
that cysteinyl residues were in fact coincident with the blank
cycles in our automated Edman degradation.

3.2. Cloning of Bo8 cDNA from skin secretion and
lyophilized skin

The Bo8-encoding precursor cDNA was successfully
cloned from the skin secretion library using the RACE proto-
col described. The 3’-RACE product (approximately 500 bp)
contained the entire open reading frame of the Bo8 precursor
(Fig. 3). This was repeatedly and consistently cloned from
both the skin secretion-derived library and the lyophilized
skin-derived library (sequencing of 40 clones from each).
The open-reading frame consisted of 96 amino acid residues
(Fig. 4). Alignment of Bo8, Bv8 (EMBL Accession no.
AF168790) and Bm8a (EMBL Accession no. AJ440230) nu-
cleotide sequences (Fig. 5) and open-reading frame amino
acid sequences (Fig. 6), using the AlignX program of the
Vector NTI Bioinformatics suite (Informax), revealed a very
high degree of primary structural similarity of both nucleic

![Fig. 3. Gel electropherogram of Bo8 RT-PCR product generated from a
lyophilized skin secretion library of B. orientalis. Lane 1: 100 bp ladder
 calibration; lane A: 3’-RACE product from degenerate primer; lane B: non-
template control.](image)

![Fig. 4. Nucleotide sequence of cDNA encoding Bo8. The putative signal peptide (double-underlined), mature peptide (single-underlined) and stop codon
 (asterisk) are indicated.](image)
acid and amino acid sequences. The NCBI-BLAST search revealed that Bv8 and Bm8a showed 96% and 92% sequence identity, respectively, with Bo8. An interesting observation was that the putative signal peptides in all three proproteins were 100% identical in primary structure. Also, one notable characteristic motif of these proteins is the presence of 10, completely conserved cysteinyl residues.
4. Discussion

In amphibians, the granular glands of the skin synthesize and store many biologically active compounds, some of which are neuropeptides and hormones similar to those isolated from higher vertebrates, including mammalian sources [12,17]. The skin secretions containing these molecular cocktails 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 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 understand their bioactivity, can be a long-term project using conventional protein chemistry [1,20,22]. However, being armed with molecular mass data and a short segment of N-terminal sequence, is sufficient for initiation of cDNA cloning studies that can effect much more rapid primary structural characterization (and perhaps establishment of microstructural diversity) of skin secretion polypeptides and proteins. Likewise, simply establishing 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 [4,25]. However, the parallel protein sequencing, molecular mass determination and corresponding cDNA cloning described here permits unequivocal structural assignment.

Bo8 represents the B. orientalis homolog of Bv8 and Bm8a (94% and 92% sequence identity, respectively), originally isolated from B. variegata and B. maximus skin secretions [7,23]. Bo8 was found to exhibit a high degree of structural similarity to protein A from the venom of the black mamba, D. polyedus polyedus. Although black mamba protein A was of unknown function for many years following its discovery, bioactivity studies in parallel with Bv8 established that both polypeptides induced hyperalgesia in mouse models of pain induction. More recent research has identified the structural and neural distribution of the mammalian counterpart of these polypeptides and has established that they are neurotrophic, act via a specific MAP kinase-activating receptor and are encoded near syntenic breakpoints on mouse chromosomes 6 and human 3p21 [15,21]. An interesting observation is that the putative signal peptides in all three amphibian skin prokinetics are identical in primary structure. In general terms, signal peptides of secreted proteins are often not highly conserved even within a single species. Although the prokinetics of B. orientalis, B. variegata and B. maximus, exhibit a high degree of primary structural identity, including a core of 10, identically sited cysteinyl residues, there are 12 common sites of amino acid substitution present. Interestingly between these three species, all amino acid substitutions occur in a limited number of sites leaving large intervening domains of total identity. The present data set may thus provide the pharmacologist with an insight into conserved structure/function requirements and thus may provide the starting point for an analog design program.

This simple technology described here in cloning defensive skin peptide precursor cDNAs from lyophilized skin secretion is robust has been applied to other species of amphibian and to reptile and scorpion venoms [7–9]. For the first time, we report successful cloning of polyadenylated mRNAs encoding such peptides from lyophilized amphibian skin. The resultant data generated from both cDNA libraries were identical, substantiating the robustness of the technologies and extending the possibility of precursor cDNA cloning of skin peptides when deep-frozen or lyophilized specimens are all that is available. However, the non-invasive and life-sparing nature of the skin secretion cloning technique renders this the method of choice for ethical reasons and, as it can be adapted for field use, obviates any interference with already fragile native populations of amphibians and their associated ecosystems.

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References


