Short communication

Novel brevinins from Chinese piebald odorous frog (Huia schmackeri) skin deduced from cloned biosynthetic precursors

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1. Introduction

Amphibian defensive skin secretions are highly complex mixtures of biologically active molecules with peptides predominating in many species [1,7]. Within the peptide inventory of such secretions, those peptides that possess antimicrobial properties are often the most abundant and certainly the most studied to date [2–4,14–16]. In the early days of discovery, the rules of nomenclature following structural elucidation were relatively straightforward, reflecting either the generic/specific name of the amphibian (bombinin from bombinid toads, esculentin from Rana esculenta, temporin from Rana temporaria), their skin origin and antiseptic properties (dermaseptins from phyllomedusine frogs) or for other individualized reasons (magainins from Xenopus laevis) [5,13,14]. While antimicrobial peptides have been subsequently isolated from the skin secretions of many additional amphibian genera, members of the family Ranidae have been the subject of the most intensive, comprehensive and systematic investigations [4]. With some 315 species in 17 genera, this is one of the largest, most diverse and widespread groups of frogs in the biosphere [4]. As methods of taxonomy

§ The nucleotide sequences of brevinin-1HS1, brevinin-2HS1, brevinin-2HS2, brevinin-2HS3 and brevinin-1HS2, from the skin secretion of the Chinese piebald odorous frog, Huia schmackeri, have been deposited in the EMBL Nucleotide Sequence Database under accession codes AM901591 through AM901595, respectively.

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increasingly employ more molecular techniques in addition to
the more classical anatomical approaches, there have been
many recent revisions of the taxonomy of this diverse group
[6,12]. Considering the fact that the database of antimicrobial
peptide structures has become very large in recent times with
many structures exhibiting features intermediate between
named structural classes, coupled with the changing taxo-
nomies, the nomenclature adopted for some novel structures
has become a considerable intellectual challenge.

Here, from the Chinese piebald odorous frog, *Huia schmackeri*, we report the primary structures of five novel skin
secretion antimicrobial peptide homologs obtained from their
corresponding biosynthetic precursor structures that were
deduced from cloned cDNAs. From this species, we have
previously reported the primary structures of esculetin 1 and
2 [3] and a brevinin-1 [2], each of which was unequivocal
homolog of previously established peptide families. The five
novel structures elucidated here were more problematic in
naming in that subsequent to publication of the previous
reports on this species, a major peptidomic study on a closely
related species, *Odorrana* (now *Huia*) *grahami*, has been
published [8]. Our strategy described here was to name the
peptides after the best-fit original homologs in databases and
in accordance with this, they represent a novel series of
peptides within the brevinin-1 and brevinin-2 families.

### 2. Materials and methods

#### 2.1. Specimen biodata and secretion harvesting

H. schmackeri (n = 3, respectively) were captured during
expeditions in the People’s Republic of China. All frogs
were adults and secretion harvesting was performed in
the field after which frogs were released. Skin
secretion was obtained from the dorsal skin using
gentle transdermal electrical stimulation as described in
previous studies [2,3]. The stimulated secretions were
washed from the skin using deionized water and divided
into either 0.2% (v/v) aqueous trifluoroacetic acid (for
subsequent peptide detection), or into cell lysis/mRNA
stabilization buffer (Dynal) for subsequent cDNA library
construction.

#### 2.2. “Shotgun” cloning of antimicrobial peptide homolog
cDNAs

Polyadenylated mRNA was isolated from stabilization buffer
using magnetic oligo-dT beads as described by the manu-
facturer (Dynal Biotech, UK) and reverse-transcribed. The
cDNA was subjected to 3′-RACE procedures to obtain full-
length prepro-antimicrobial peptide 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 primer (NUP),
supplied with the kit, and a degenerate sense primer (S1;
5′-GAWYYAYYHRAGCCYAAADATG-3′) that was designed to
a highly conserved domain of the 5′-untranslated region
of previously characterized antimicrobial peptide cDNAs
from *Rana* species [2,3,15,16]. The PCR cycling procedure
was as follows. Initial denaturation step: 60 s at 94 °C;
35cycles: denaturation 30 s at 94 °C, primer annealing for
30 s at 53 °C; extension for 180 s at 72 °C. PCR products were
gel-purified, cloned using a pGEM-T vector system (Promega
Corporation) and sequenced using an ABI 3100 automated
sequencer.

![Fig. 1 – Nucleotide sequences of cloned cDNAs encoding the open-reading frames of the novel brevinin homologs described in the present study from the skin secretion of the Chinese piebald odorous frog, *Huia schmackeri*. Putative signal peptides are double-underlined, mature peptides are single-underlined and stop codons are marked by asterisks.](image-url)
2.3. Detection of antimicrobial peptide homologs in skin secretion

The acidified skin secretion washings were clarified of microparticulates by centrifugation (1500 × g) for 10 min. The clear supernatant was then pumped directly onto a reverse phase HPLC column and peptides were eluted using a gradient formed from 0.05/99.5 (v/v) TFA/water to 0.05/19.95/80.0 (v/v/v) TFA/water/acetonitrile in 240 min at a flow rate of 1 ml/min. A Cecil CE4200 Adept (Cambridge, UK) gradient reverse phase HPLC system, fitted with an analytical column (Phenomenex C-5, 0.46 cm × 25 cm) was employed and fractions were collected automatically at 1 min intervals. Dead volume between column and fraction collector was minimal (20 μl). The molecular masses of polypeptides in each chromatographic fraction were 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%. Peptides with masses coincident with those of cloned precursor-deduced mature peptides were identified.

3. Results

3.1. “Shotgun” cloning of antimicrobial peptide homolog cDNAs

Five novel antimicrobial peptide homolog-encoding cDNAs were consistently cloned from the *H. schmackeri* skin secretion library and each encoded a single copy of respective mature peptides (Fig. 1). Bioinformatic investigations were performed using the US National Center for Biotechnology Information (NCBI) on-line portal and deduced mature peptide sequences were analyzed using the BLAST program. Table 1 summarizes the top hits obtained for each peptide following this analysis and indicates that all peptides are brevinin-1 or brevinin-2 homologs. Each peptide was named in accordance. The domain topology of each deduced prepropeptide is shown in Fig. 2 and the construct of each was entirely consistent with that observed and reported previously for several other related species of ranid frog [2–4,11,13,15,16].

3.2. Detection of mature antimicrobial peptide homologs in reverse phase HPLC fractions of skin secretion

Each putative mature antimicrobial peptide homolog was detected in reverse phase HPLC fractions of the same skin secretion.

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Table 1 – Percentage identities of *Huia schmackeri* skin antimicrobial peptides (brevinins) with those present in the NCBI database

<table>
<thead>
<tr>
<th>Peptidin</th>
<th>Percentage Identity</th>
<th>Common Name</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brevinin-1HS1</td>
<td>100%</td>
<td>Odorranina grahini odorra-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>nina-P1b</td>
<td><em>Odorana grahami</em></td>
</tr>
<tr>
<td>Brevinin-2HS1</td>
<td>93%</td>
<td>Odorranina grahini palustrin-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>OG2</td>
<td><em>Odorana grahami</em></td>
</tr>
<tr>
<td>Brevinin-2HS2</td>
<td>87%</td>
<td>Odorranina grahini odorra-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>nina-C8</td>
<td><em>Odorana grahami</em></td>
</tr>
<tr>
<td>Brevinin-1HS2</td>
<td>87%</td>
<td>Rana versabilis brevinin-1V</td>
<td><em>Rana versabilis</em></td>
</tr>
<tr>
<td>Brevinin-2HS2/3</td>
<td>87%</td>
<td>Rana versabilis brevinin-1V</td>
<td><em>Rana versabilis</em></td>
</tr>
</tbody>
</table>

The top hits are given in each case.

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Fig. 3 – Region of reverse phase HPLC chromatogram of *Huia schmackeri* skin secretion indicating elution positions (determined by MALDI-TOF MS) of (1) brevinin-1HS1, (2) brevinin-2HS1, (3) brevinin-2HS2, (4) brevinin-2HS3 and (5) brevinin-1HS2. Absorbance wavelength (A) employed was 214 nm.

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Fig. 2 – Domain topology of biosynthetic precursors encoding the five novel brevinin homologs from *Huia schmackeri* skin secretion. Domain 1 – putative signal peptide, domain 2 – acidic amino acid residue-rich spacer peptide, domain 3 – prepeptide convertase cleavage site and domain 4 – mature peptide. Conserved residues are indicated by asterisks and gaps have been inserted to maximize alignments.
secretion sample used in cDNA library construction [Fig. 3], by use of MALDI-TOF MS. These data are summarized in Table 2.

### 4. Discussion

Antimicrobial peptides represent the most intensively studied group of peptides in amphibian defensive skin secretions with several hundred structures represented in on-line protein/peptide databases. The reasons for this are several-fold. They tend to be the most abundant peptides in many secretions leading to a relative ease of isolation and primary structural analysis. This has been greatly facilitated by the continuous advances in chromatographic and mass spectrometric hardware and software. Due to the continuing emergence and prevalence of multiple drug-resistant strains of pathogenic bacteria and the apparent efficacy of these peptides in killing such in vitro, they possibly represent an array of templates or leads for the generation of new classes of antibiotics [4,9,10], although definitive efficacy in vivo has yet to be reported. Additionally, they may represent a molecular tool-chest with which one may define new drug targets or more simply as instruments to unravel membrane structure and complexity.

In the early days of this research, as in many parallel fields such as endogenous regulatory peptide discovery, nomenclature of peptides was largely empirical and was testament in many instances to the artistic licence of scientific investigators, a good example being the magainins (derived from the Hebrew word magen meaning shield) that were identified in X. laevis skin secretion [14]. Peptide nomenclature is thus not a trivial issue as this can lead to much confusion particularly among non-specialists but also among specialists when databases of peptide families become large and individuals are discovered that have structural attributes falling between established criteria. The naming of entirely novel structures does not present a problem in the first instance, but in time, as many more analogs are characterized, these will inevitably arise. This is essentially a problem of molecular taxonomy not entirely dissimilar to that encountered by those who name species.

Here we describe five antimicrobial peptide homologs identified in skin secretions of the Chinese piebald odorous frog, *H. schmackeri*, each of which is typified by a C-terminal disulfide – bridged loop, the so-called Rana box [10] (Table 1). The primary structures of the biosynthetic precursors encoding each peptide were deduced from cloned cDNAs and the domain topology of each is shown in Fig. 2. The conserved propeptide includes a putative 22 amino acid residue signal peptide followed by an acidic amino acid residue-rich spacer peptide of between 17 and 22 residues that is flanked by a typical -Lys–Arg–(–KR–) propeptide convertase processing motif for cleavage and release of each respective mature peptide located at the C-terminal region of each open-reading frame (Fig. 2). Once primary structures of each peptide had been established then identity and uniqueness was determined by using the BLASTp programme available through NCBI. All of the peptides identified in the present study were found to be either brevinin-1 or brevinin-2 homologs and, by adopting original terminologies, were named in accordance. The names appended to the five novel peptides described in the present study represent the most logical within the framework of the body of current literature. We contend, on this basis, that the discoverers of novel peptides in this class perform careful due diligence of both the existing literature and of comprehensive on-line databases, such as those available through NCBI or EMBL, as a necessary prerequisite to naming and publication and that accession numbers be obtained to establish both novelty and priority. This may serve to archive such structures in a most readily accessible format for a wide spectrum of researchers many of whom may not be specialists in this particular field.

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### References


