Components of the peptidome and transcriptome persist in lin wa pi: The dried skin of the Heilongjiang brown frog (Rana amurensis) as used in traditional Chinese medicine

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1. Introduction

The treatment of disease for the vast majority of the human population relies upon traditional and ethnic medicines rather than the products of the relatively recently emerged pharmaceutical industry [9,14,18,25]. While many of our mainstream drugs or their lead compounds have been discovered as components of these medicines (aspirin, quinine, artemisinin), the vast majority remain unstudied by contemporary scientific methods for both chemical content and therapeutic efficacy [2,15,19,23]. What at first appears to be a straightforward experimental scheme, that is obtaining material, making an extract and fractionating/identifying components by modern analytical technologies, such as mass spectrometry or NMR,
can often be too simplistic to identify an active molecule. These may be generated only under specific extraction conditions or may only be present in a given plant from a discrete location at a particular phase of its growth cycle (and the latter may occur at a different time each year). Also, the therapeutic effect may be mediated by more than one single chemical entity—a factor that would draw a negative conclusion in a reductionist approach for single actives. This non-standardized aspect of ethnic medicines has been one of the greatest drawbacks to more general utility and of more seriously systematically performed scientific studies. It has also been argued by practitioners of traditional medicine as one of the major reasons why scientific studies fail to produce definitive results in many instances and that cause particular treatments to be subsequently disregarded [5,16,20]. This area of research that should represent the coming together of ancient tried-and-tested wisdom with modern analytical technology must surely instead represent one of the most multivariable and hence pitfall-ridden areas of modern science.

While plant material is the predominant raw material in most traditional medicines [14,15], there is also significant usage of animal-derived materials ranging from urine and venom to complex cocktails of tissue extracts [1,10,17]. This presents an even greater potential barrier to the reduction of molecular complexity in identifying potential actives. The extreme and wide-ranging pharmacological potencies of amphibian skin secretions have long been put to a plethora of usages in the traditional medicines of many native peoples [3,10,17]. In the Orient, toad venom, known as chan’su, has long been used for many conditions especially as a cardiac stimulant [4]. Among some South American tribes, the dried skin secretion of the hyliid frog, Phyllomedusa bicolor, is employed in a “hunting magic” ritual due to its sensory stimulating properties [12].

Amphibian skin bioactive peptide research is the focus of our research group and we have for several years, been modifying analytical techniques to permit parallel peptidome and transcriptome sequencing from ever smaller quantities of biological material, either skin or secretion samples, from frogs [6–8,24].

Here we report that components of the peptidome and transcriptome of the cutaneous granular gland—the source of skin-derived bioactive peptides in amphibians—persists in air-dried skin of the Heilongjiang brown frog (Rana amurensis)—a species used extensively in the traditional medicine of China. Such is the protection from nuclease degradation afforded to endogenous polyadenylated mRNA by endogenous amphibian peptides (a phenomenon established previously [6–8,24]) that a robust cDNA library could be constructed from skin maintained at high ambient temperatures for many months. In addition, the putative bioactive peptides whose primary structures were deduced from cloned cDNAs were identified in HPLC fractionated boiling water extracts of skin prepared as indicated for traditional medicinal purposes.

2. Materials and methods

2.1. Acquisition of material

Skin of the Heilongjiang brown frog (R. amurensis) was purchased in dry form from a number of Traditional Chinese Medicine pharmacies throughout Northern China in Heilongjiang, Jilin and Laoning Provinces. The dried skins were cellophane-wrapped and had been kept at ambient shop temperatures (20–35 °C) for many months.

2.2. Construction of cDNA library from dried skin and subsequent “shotgun” cloning

Pieces from four skins (total 156 mg dry weight) were chopped into small (1–2 mm²) pieces and placed into 1 ml of cell lysis/mRNA stabilization solution for 2 h at 4 °C. Polyadenylated mRNA was isolated from stabilization buffer using magnetic oligo-dT beads as described by the manufacturer (Dynal Biotech, UK) and reverse-transcribed. The cDNA was subjected to 3’-RACE procedures to obtain open-reading frame-encoding nucleic acid sequence data using a SMART-RACE kit (Clontech, UK) essentially as described by the manufacturer. Briefly, the 3’-RACE reactions employed a NUP primer (supplied with the kit) and a degenerate sense primer (S1; 5’-GAWYYAYYHY-RAGCCYAAAADATG-3’) that was designed to a highly conserved domain of the 5’-untranslated region of previously characterized skin peptide-encoding cDNAs from Rana species [22]. The PCR cycling procedure was as follows. Initial denaturation step: 60 s at 94 °C, 35 cycles: denaturation 30 s at 94 °C, primer annealing for 30 s at 54 °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. All putative antimicrobial peptide homolog structures deduced from cDNA clones were subjected to bioinformatic analysis using the BLAST program available on-line through the National Center for Biotechnology Information (NCBI, USA). Secondary structure prediction used the Hierachical Neural Networks program accessible through the Human Proteome Organization (HUPO) web-site.

2.3. Preparation of skin extract for peptide analysis

Samples from the skins of four frogs (dry weight 202 mg in total) were chopped into small (1–2 mm²) pieces and placed directly into 10 ml of boiling deionized water for 10 min. Following this, the extract was removed from the heat source and permitted to cool to room temperature. Particulates were removed by centrifugation at 1500 × g for 10 min after which the resultant clear supernatant was decanted and lyophilized.

2.4. Fractionation of skin extract and peptide identification

The lyophilized extract of dried frog skin was reconstituted in 1 ml of 0.05% (v/v) aqueous trifluoroacetic acid (TFA) and clarified of microparticulates by centrifugation (1500 × g) for 10 min after which the clear supernatant was decanted and pumped directly onto a reverse phase HPLC column and subjected to LC/MS 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 Thermoset gradient reversed phase HPLC system, fitted with an analytical column (Phenomenex C-5; 0.46 cm × 25 cm), and interfaced with a Thermoset LCQ™ DECA electrospray ion-trap mass spectrometer, was employed. The effluent from the chromatographic column was flow-split with approximately 10% entering the mass...
Fig. 1 – Nucleic acid sequences of cloned cDNAs encoding the novel peptides ranatuerins 2AMa, 2AMb and amurins 1–3 from the skin of the Heilongjiang brown frog, *Rana amurensis*. Putative signal peptides are double-underlined, predicted mature peptides are single-underlined and stop codons are indicated by asterisks. All sequences have been deposited in EMBL under accession codes AM233683 through AM233687.
presented a more difficult nomenclature problem and were structural isomers. The remaining three peptides were named accordingly. These were ranatuerin 2AMa and ranatuerin 2AMb, respectively. In keeping with established nomenclature rules[13], the suffix –AM- represents the species origin (AMurensis) and the a/b designations indicate that the peptides are from the a/b designations from the North American species, Rana berlandieri[13]. Amurin-1 was thus of sufficient primary structural novelty to warrant its unique name. Amurin-2 proved to be likewise of highly original primary structure. The peptides that exhibited the highest degree of primary structural similarity to amurin-2 were brevinins BYb, a and c, respectively, from the North American species, Rana boylii[11]. However, alignments were only possible with these peptides by insertions of gaps in the sequence that differed with each comparison. Additionally, secondary structural prediction analysis of all four peptides (Fig. 4) showed unequivocally that the brevinins were of identical predicted secondary structure but radically different from amurin-2. Thus, on the basis of both primary and secondary structural parameters, amurin-2 was deemed to be of sufficient novelty to warrant its unique name. Amurin-3 was not found to be structurally similar, in terms of amino acid sequence, with any known antimicrobial peptide using the BLAST paradigm employed despite an apparent superficial primary structural resemblance to the temporins[21].

3. Results

3.1. “Shotgun” cloning of skin peptides from the cDNA library

Using the genus-specific degenerate primer described, cDNAs encoding the open-reading frames of precursor proteins of five novel putative antimicrobial peptides were consistently cloned (Fig. 1). The nucleic acid sequences of each novel open-reading frame were confirmed in at least six replicate clones. The topographical organization of each encoded precursor protein is illustrated in Fig. 2. This organization is typical for the defensive skin peptides of amphibian skin granular glands and consists of a putative 22-mer N-terminal signal peptide, an acidic amino acid residue rich “spacer” peptide of variable length, a classical –KR- (-Lys-Arg-) propeptide convertase cleavage site and a C-terminally located and hypervariable active peptide encoding domain. Two of the putative encoded peptides were found to exhibit high degrees of primary structural similarity to members of known amphibian skin antimicrobial peptide families and were named accordingly. These were ranatuerin 2AMa and ranatuerin 2AMb, respectively. In keeping with established nomenclature rules[13], the suffix –AM- represents the species of origin (AMurensis) and the a/b designations indicate that they are structural isomers. The remaining three peptides presented a more difficult nomenclature problem and were named amurins 1–3. Amurin-1 was found to exhibit the highest degree of primary structural similarity to brevinin 2Tc from Rana temporaria following NCBI BLAST analysis (Fig. 3). However, brevinin 2Tc when subjected to the same analysis was found not to be structurally similar to any brevinin (none in the top 108 hits) but rather exhibited the highest degree of primary structural similarity to pelophylaxin-1 from Rana (Pelophylax) planci fukienensis[24], predominantly in C-terminal region, and to ranatuerin 2B from the North American species, Rana berlandieri[13]. Amurin-1 was thus of sufficient primary structural novelty to warrant its unique name. Amurin-2 proved to be likewise of highly original primary structure. The peptides that exhibited the highest degree of primary structural similarity to amurin-2 were brevinins BYb, a and c, respectively, from the North American species, Rana boylii[11]. However, alignments were only possible with these peptides by insertions of gaps in the sequence that differed with each comparison. Additionally, secondary structural prediction analysis of all four peptides (Fig. 4) showed unequivocally that the brevinins were of identical predicted secondary structure but radically different from amurin-2. Thus, on the basis of both primary and secondary structural parameters, amurin-2 was deemed to be of sufficient novelty to warrant its unique name. Amurin-3 was not found to be structurally similar, in terms of amino acid sequence, with any known antimicrobial peptide using the BLAST paradigm employed despite an apparent superficial primary structural resemblance to the temporins[21].

3.2. Identification and structural confirmation of putative mature peptides in chromatographic fractions of frog skin extract

The molecular masses of the putative novel peptide encoded within the open-reading frames of the five cloned precursor proteins were deduced incorporating known and consistent post-translational modifications—disulfide bridges between the cysteinyl residues in the ranatuerins and amurins 1 and 2 (–2 amu) and C-terminal amidation of amurin 3 (–1 amu). The MALDI-TOF MS data obtained following reverse phase HPLC fractionation of the skin extract was interrogated with these data and respective encoded peptides were identified by mass coincidence in chromatographic fractions (Table 1). The identity of each peptide was confirmed by MS/MS fragmentation sequencing using the LCQ DECA electrospray ion trap mass spectrometer.

Fig. 2 – Domain topology of respective granular gland peptide precursors. 1, the putative signal peptide domain. 2, the variable length acidic “spacer” peptide domain. 3, KR (-Lys-Arg-) represents the conserved classical propeptide convertase processing site. 4, the hypervariable bioactive peptide-encoding domain. Mature peptide sequences are in bold typeface.
4. Discussion

In the present study, we have unequivocally demonstrated that at least some elements of both the granular gland peptidome and transcriptome are persistent in air-dried and ambient temperature-stored *R. amurensis* (lin wa pi) as employed in traditional Chinese medicine. By applying contemporary analytical chemical technologies to the quest for actives in traditional medicines many novel drug leads may be uncovered that can be subjected to objective efficacy assessing procedures for the treatment of human disease in a much more standardized manner [2,4,10,19,23]. This study has extended our work on amphibian skin peptides over the past three years, specifically those aspects that have been directed towards refining the use of live animals in our research and in determining just how robust the peptidomes are.

![Fig. 3 – (A–C) Primary structural comparisons of each novel peptide from *Rana amurensis* with the corresponding highest probability homolog in contemporary protein/nucleic acid databases established by BLAST analysis. Fully conserved amino acid residues are indicated by asterisks. Gaps have been inserted to maximize alignments.](image)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>HPLC fraction</th>
<th>Mass obs./calc. (Da)</th>
<th>Primary structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ranatuerin 1Ma</td>
<td>115</td>
<td>3288.05/3286.96</td>
<td><strong>GLLSVFKGVLKGVKNGVAGSLLDLKCKISGC</strong></td>
</tr>
<tr>
<td>Ranatuerin 1T</td>
<td>119</td>
<td>3273.25/3272.93</td>
<td><strong>GLFSVVKGLKGVKNGVAGSLLDLKCKISGC</strong></td>
</tr>
<tr>
<td>Ranatuerin 1Mb</td>
<td>128</td>
<td>3422.20/3421.16</td>
<td><strong>GLWSIKLGGKFLALNIMELKCKFGGCLP</strong></td>
</tr>
<tr>
<td>Amurin-1</td>
<td>136</td>
<td>2236.45/2235.87</td>
<td><strong>FLSLALAAKFLGFLCIFKCC</strong></td>
</tr>
<tr>
<td>Amurin-2</td>
<td>153</td>
<td>1221.44/1220.59</td>
<td><strong>FLSLSAAAKFLGFLCIFKCC</strong></td>
</tr>
<tr>
<td>Amurin-3</td>
<td>136</td>
<td>2236.45/2235.87</td>
<td><strong>FLSLSAAAKFLGFLCIFKCC</strong></td>
</tr>
</tbody>
</table>

Peptides were identified in reverse phase HPLC fractions of a boiling water extract of dried skin. Disulfide-bridged domains (Rana boxes) are underlined.
and transcriptomes of the granular glands (the source of bioactive amphibian skin peptides) actually are [6–8,24]. The protective effect of endogenous amphibatic peptides on the endogenous polyadenylated mRNAs that encode granular gland peptides (including the amphibatic peptides themselves) was demonstrated in a previous publication [6–8,24], has been indirectly evidenced in many species since this initial report [6], and here is extended to a degree of robustness that permits access to the transcriptome in skin that has been air-dried and kept at ambient temperature for prolonged periods. The data presented in the current study have unequivocally proven that sufficient polyadenylated mRNA is preserved under the unlikely experimental conditions described, to facilitate PCR-mediated amplification that ultimately led to the obtaining of the nucleotide sequences of five skin secretion peptides. Two of these were obvious members, as judged by primary structural similarities, of an existing family of structurally related antimicrobial skin peptides, the ranatuerins, and were appended systematic names in accordance with previously suggested nomenclature rules [13]. However, the classification of the remaining three peptides was more contentious as the degrees of primary (and in one instance, secondary) structural similarities with known families of frog skin antimicrobials, are likely to possess at least some antimicrobial potential. This is also strengthened by the observation that the antimicrobial activity of this skin preparation, as determined by preliminary screening of reverse phase HPLC fractions from this species, is coincident with fractions containing all of these peptides but with some other unidentified components (data not shown). This aspect was not pursued as it did not represent the core message of the study.

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REFERENCES


