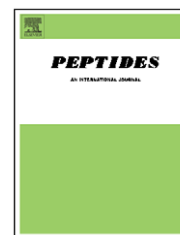


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Components of the peptidome and transcriptome persist in *lin wa pi*: The dried skin of the Heilongjiang brown frog (*Rana amurensis*) as used in traditional Chinese medicine[☆]

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ABSTRACT

Although the ancient practice of traditional Chinese medicine (TCM) utilizes predominantly herbal ingredients, many of which are now the subject of intense scientific scrutiny, significant quantities of animal tissue-derived materials are also employed. Here we have used contemporary molecular techniques to study the material known as *lin wa pi*, the dried skin of the Heilongjiang brown frog, *Rana amurensis*, that is used commonly as an ingredient of many medicines, as a general tonic and as a topical antimicrobial/wound dressing. Using a simple technology that has been developed and validated over several years, we have demonstrated that components of both the skin granular gland peptidome and transcriptome persist in this material. Interrogation of the cDNA library constructed from the dried skin by entrapment and amplification of polyadenylated mRNA, using a “shotgun” primer approach and 3'-RACE, resulted in the cloning of cDNAs encoding the precursors of five putative antimicrobial peptides. Two (ranatuerin-2AMa and ranatuerin-2AMb) were obvious homologs of a previously described frog skin peptide family, whereas the remaining three were of sufficient structural novelty to be named amurins 1–3. Mature peptides were each identified in reverse phase HPLC fractions of boiling water extracts of skin and their structures confirmed by MS/MS fragmentation sequencing. Components of traditional Chinese medicines of animal tissue origin may thus contain biologically active peptides that survive the preparation procedures and that may contribute to therapeutic efficacy.

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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,

[☆] The nucleotide sequences of clones encoding ranatuerin-2AMa, ranatuerin-2AMb, amurin-1, amurin-2 and amurin-3, have been deposited in the EMBL nucleotide sequence database under the accession codes AM233683 through AM233687.

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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 serious 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 hylid 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 amphipathic 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

Skins 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'-GAWYYAYYH-RAGCCYAAADATG-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 Heirarchal 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*). 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 Thermoquest gradient reversed phase HPLC system, fitted with an analytical column (Phenomenex C-5; 0.46 cm × 25 cm), and interfaced with a Thermoquest LCQTM DECA electrospray ion-trap mass spectrometer, was employed. The effluent from the chromatographic column was flow-split with approximately 10% entering the mass

Ranatuerin-2AMa M F T S K K S M L
 1 CGATTGAATC ATTTGAGCCT AAAGATGTT ACCTCAAAGA AATCCATGTT
 L L F F L G T I S L S L C E E E
 51 ACTCCTTTC TTTCTGGGA CCATCTCCTT ATCTCTCTGT GAGGAAGAGA
 R D E D E V I E E E V K R G L L S
 101 GGGATGAAGA TGAAGTTATA GAGGAAGAAG TAAAAAGAGG GCTCTTGAGT
 V F K G V L K G V G K N V A G S L
 151 GTGTTCAAAG GTGTACTCAA GGGAGTGGGC AAGAACGTAG CCGGGTCTTT
 L D Q L K C K I S G G C *
 201 GTTGGATCAA CTAAAATGTA AAATTTCTGG AGGGTGTAA AATCTGAATT
 251 GGAAGTCATC TGATTTGGAA TATCATTTAG CTAACACTA AATGTCTGAT
 301 AACAAATAAA AATATCACAT GAAAAAAAAA AAAAAAAAAA AAAAAAAAAA

Ranatuerin-2AMb M F T S K K S M L L L F F L G
 1 AAAGATGTT ACCTCAAAGA AATCCATGTT ACTCCTTTC TTTCTGGGA
 T I S L S L C Q E E R D A D E D E
 51 CCATCTCCTT ATCTCTCTGT CAGGAAGAGA GAGATGCCGA TGAAGATGAA
 V I E E E V K R G L F S V V K G V
 101 GTTATAGAGG AAGAAGTAAA AAGAGGGCTC TTCAGTGTGG TCAAAGGTGT
 L K G V G K N V A G S L L D Q L
 151 ACTCAAGGGA GTGGCAAGA ACGTAGCCGG GTCTTTGTTG GATCAACTAA
 K C K I S G G C *
 201 AATGTAATAA TTCTGGAGGG TGTTAAAATC TGAATTGGAA GTCATCTGAT
 251 TTGGAATATC ATTTAGCTAA CTAATAAATG TCTGATAACA AATAAAAAATA
 301 TCACATAAAA AAAAAAAAAA AAAAAAAAAA

Amurin-1 M F I M K K S L L L
 1 TTGATCACT TGAGCCTAAA GATGTTTCATC ATGAAGAAAT CACTGTTACT
 L F F L G T I S L S L C E Q E R
 51 CCTTTTCTTT CTTGGAACTA TCTCCTTGTC TCTCTGTGAG CAAGAGAGAG
 D A D E D E V V E E V K R G L W E
 101 ATGCCGATGA AGATGAAGTT GTGGAAGAAG TAAAAAGAGG TCTCTGGGAA
 S I K N L G K K F A L N I M E K L
 151 AGCATTAAAG ATTTGGGGAA GAAGTTTGCT CTGAATATAA TGGAAAAGCT
 K C K F G G G C L P *
 201 AAAATGTAAA TTTGGTGGAG GATGTCTACC CTGAATCGGA TGCCATCTGA
 251 TGCTGAATAT CATTGGCTA GATCTTTAAA GTCTTATTA AAAAAAAAAA

Amurin-2 M F T L K K S L L L L
 1 ATTCATTGA GCCTAAAGAT GTTCACCTTG AAGAAATCCC TCTTACTCCT
 F F L G T I N L S L C E E E R N
 51 TTTCTTCCTT GGGACCATCA ACTTATCTCT CTGTGAGGAA GAGAGAAATG
 A D E E E R R D D L E E R D V E V
 101 CCGATGAAGA AGAAAGAAGA GATGATCTCG AAGAAAGGGA TGTGAAGTG
 E K R F L S L A L A A L P K L F C
 151 GAAAAACGAT TTCTTTCATT AGCGCTTGCT GCGTTGCCGA AATTGTTTTG
 L I F K K C *
 201 TTTAATATTC AAAAATGTT GAACCTTGGG AATTTGAATT GGAAATCATC
 251 TGATGTAGAA TATCATTTAG CTGAATGCAC ATCGGACGTC TTATAAAAAA
 301 TAAACGTGTG GAGAAAAAAA AAAAAAAAAA AAAAAA

Amurin-3 M F T L K K S L L L L F
 1 CATTGAGCC TAAAGATGTT CACCTTGAAG AAATCCCTGT TACTCCTTTT
 F L G T I N L S L C E E E R N V
 51 CTTCCTTGGG ACAATCAACT TATCTCTCTG TGAGGAAGAG AGAAATGTCG
 D E E R R D D P E E R A V E V E K
 101 ATGAAGAAAG AAGAGATGAT CCCGAAGAAA GGGCTGTGGA AGTGAAAAA
 R I L P I L S L I G G L L G K *
 151 CGAATTTTAC CAATCCTTAG TTGATCGGT GGTTTGTGG GCAAATAACC
 201 AAAAATGTA AACTTTGGA AATGGAATTG GAAATCATCT GATGTGGAAT
 251 ATCATTGAGC TAAATGCACA TCAGATGTCA TAAAAATAA AGATATCGCA
 301 TACATTGCAA AAAAAAAAAA AAAAAAAAAA AAAAA

Fig. 1 – Nucleic acid sequences of cloned cDNAs encoding the novel peptides ranatuierins 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.

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 peptides with masses coincident with those of putative antimicrobial peptide homologs, as deduced from cloned precursor cDNAs, were each subjected to primary structural analysis by automated Edman degradation using an Applied Biosystems 491 Procise sequencer in pulsed-liquid mode or by MS/MS fragmentation sequencing using the LCQ™ DECA.

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) plancyi 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 boylei* [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 peptides 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.

	←-----1-----→	←-----2-----→	3	←-----4-----→
Ranatuerin-2AMa	MFTSKKSMLLFFLGTISLSLC	EEERDEDEVIEEEV	KR	GLLSVFKGVKGVGKNVAGSLLDQLKCKISGGC
Ranatuerin-2AMb	MFTSKKSMLLFFLGTISLSLC	QEERDADEVIEEEV	KR	GLFSVVKGVKGVGKNVAGSLLDQLKCKISGGC
Amurin-1	MFIMKSLLLFFLGTISLSLC	EQERDADEVVEEV	KR	GLWESIKNLGKKFALNIMEKLCCKFGGGCLP
Amurin-2	MFTLKSLLLFFLGTINLSLC	EEERNADEEERRDDLEERDVEVE	KR	FLSLALALPKLFLCLIFKCC
Amurin-3	MFTLKSLLLFFLGTINLSLC	EEERNVDEEERRDDPEERAVEVE	KR	ILPILSLIGLL GK

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.

(A)	
Ranatuering 1AMa	GLLSVFKGVLKGVGKNAVAGSLLDQLKCKISGGC
Ranatuering 1T	GLLSGLKKVKGKHAVAKNAVAVSLMDSLKCKISGDC
	**** * * * * * * * * * * * * * * * * *
Ranatuering 1AMb	GLFSVVKGVLKGVGKNAVAGSLLDQLKCKISGGC
Ranatuering 1T	GLLSGLKKVKGKHAVAKNAVAVSLMDSLKCKISGDC
	** * * * * * * * * * * * * * * * * *
(B)	
Amurin-1	GLWESIKNLGKKFALNIMEKLCCKFKGGGCLP
Brevinin-2Tc	GLWETIKNFGKKFTLNILHKLKCKIGGGC
	**** ** * * * * * * * * * * * * * * *
Brevinin-2Tc	GLWETIKNFGKKFTLNI-LHKLKCKIGGGC
Pelophylaxin 1	GILTDTLKGAAKNAVAGVLLDKLCKITGGC
	* * * * * * * * * * * * * * * * *
Brevinin-2Tc	GLWETIKNFGKKFTL--NILHKLKCKIGGGC
Ranatuering-2B	GLLDTIKGVAK--TVAASMLDKLCKISG-C
	** * * * * * * * * * * * * * * * * *
(C)	
Amurin-2	FLS-LA-LAA-L-PKLFCLIFKKC
Brevinin-1BYb	FLPILASLAAKLGPKLFCLVTKKC
	** * * * * * * * * * * * * * * *
Amurin-2	FLSLALAAL----PKLFCLIFKKC
Brevinin-1BYa	FLPI-LASLAAKFGPKLFCLVTKKC
	** * * * * * * * * * * * * * * *
Amurin-2	FL---SLALAAL-PKLFCLIFKKC
Brevinin-1BYc	FLPILASLA-ATLGPKLLCLITKKC
	** * * * * * * * * * * * * * * *

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.

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* skin (*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

Table 1 – Identification and structural characterization of the predicted mature peptides encoded by the five novel cDNAs cloned from a library generated from the dried skin (*lin wa pi*) of the Heilongjiang brown frog—*Rana amurensis*

Peptide	HPLC fraction	Mass obs./calc. (Da)	Primary structure
Ranatuering-2AMa	115	3288.05/3286.96	GLLSVFKGVLKGVGKNAVAGSLLDQLKCKISGGC
Ranatuering-2AMb	119	3273.25/3272.93	GLFSVVKGVLKGVGKNAVAGSLLDQLKCKISGGC
Amurin-1	128	3422.20/3421.16	GLWESIKNLGKKFALNIMEKLCCKFKGGGCLP
Amurin-2	136	2236.45/2235.87	FLSLALAALPKLFCLIFKKC
Amurin-3	153	1221.44/1220.59	ILPILSLIGLLamide

Peptides were identified in reverse phase HPLC fractions of a boiling water extract of dried skin. Disulfide-bridged domains (*Rana* boxes) are underlined.

Amurin-2	FLSLALAALPKLFCLIFKKC chhhhhhhhhhhhhhhccc
Brevinin 1BYb	FLPILASLAALKGPKLFCLVTKKC ccchhhhhhhhhcccceeeeeeccc
Brevinin 1BYa	FLPILASLAAKFGPKLFCLVTKKC ccchhhhhhhhhcccceeeeeeccc
Brevinin 1BYc	FLPILASLAATLGPPLLCLITKKC ccchhhhhhhhhcccceeeeeeccc

c = coil; h = helix; e = extended sheet

Fig. 4 – Comparison of the predicted secondary structures of amurin-2 with the closest homologs established by primary structural comparisons. While all the brevinins from *Rana boylei* (BYb, BYa and BYc) are obviously homologous both by primary and secondary structural comparisons, amurin-2 is quite different.

and transcriptomes of the granular glands (the source of bioactive amphibian skin peptides) actually are [6–8,24]. The protective effect of endogenous amphipathic peptides on the endogenous polyadenylated mRNAs that encode granular gland peptides (including the amphipathic 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 frog 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 identities with known peptides, following BLAST analysis, were very low and thus indicative of sequence novelty. In view of this, these peptides were likewise named in accordance with previously established nomenclature rules after the species of origin as amurins 1 through 3. Although their bioactivity profile was not determined, the amurins, as a consequence of their predicted helical nature and limited primary structural similarity to 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.

In addition to the demonstration of robustness of skin peptide-encoding cDNAs within the dried frog skin, the predicted peptides from each clone, incorporating appropriate post-translational modifications, were identified in reverse phase HPLC fractions of skin extract prepared by boiling in water as recommended by the traditional medicine physician. Many additional uncharacterized peptides were also present. Taken together, these data demonstrate that putative biologically active frog skin peptides survive both the drying/storage process and the subsequent solubilization from skin by boiling in water. The extract, when ingested or applied topically to the skin or mucus membranes, thus has the potential to mediate or modulate a wide spectrum of biological effects.

In common with many traditional medicines derived from plants and an increasing number derived from animal tissues, there is accumulating evidence that a complex array of putative and proven biologically active substances occur in extracts or potions prepared following ancient regimes. The *lin wa pi* as employed in traditional Chinese medicine can thus be added to this list.

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