



## Skin secretion transcriptome remains in chromatographic fractions suitable for molecular cloning

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### ABSTRACT

Traditional sources of mRNA for molecular cloning on amphibian skin secretion studies have been the frog's skin and skin secretions. Here, we demonstrate that mRNA isolated from chromatographic fractions of skin secretions is amenable for molecular cloning assays. We identified precursor sequences of the Arg<sup>0</sup>, Trp<sup>5</sup>, Leu<sup>8</sup>-bradykinin and six antimicrobial peptides of *Pelophylax esculentus* (Ranidae). These results show that both transcriptomic and peptidomic analyses can be performed with a single sample reducing in half the amount of starting skin secretion required. This is a significant advantage when working with endangered or very rare amphibian species, where minimal samples are available.

Molecular cloning is a widely used method for identifying peptides precursor sequences of the amphibian skin secretion [1]. Originally, mRNA isolation required fresh amphibian skin and a complicated isolation process [2]. Later, mRNA was isolated from freeze-dried skin secretions [3], air-dried skins [4], and skin secretion collected directly in mRNA lysis buffer [5]. The latter strategy was advantageous for collecting samples in the field. Recently, mRNA was also obtained from skin secretions stored in aqueous trifluoroacetic acid 0.1% for up to 12 years at  $-20^{\circ}\text{C}$  [6]. The mRNA in the skin secretion is presumably protected by interactions with cationic peptides and proteins [6].

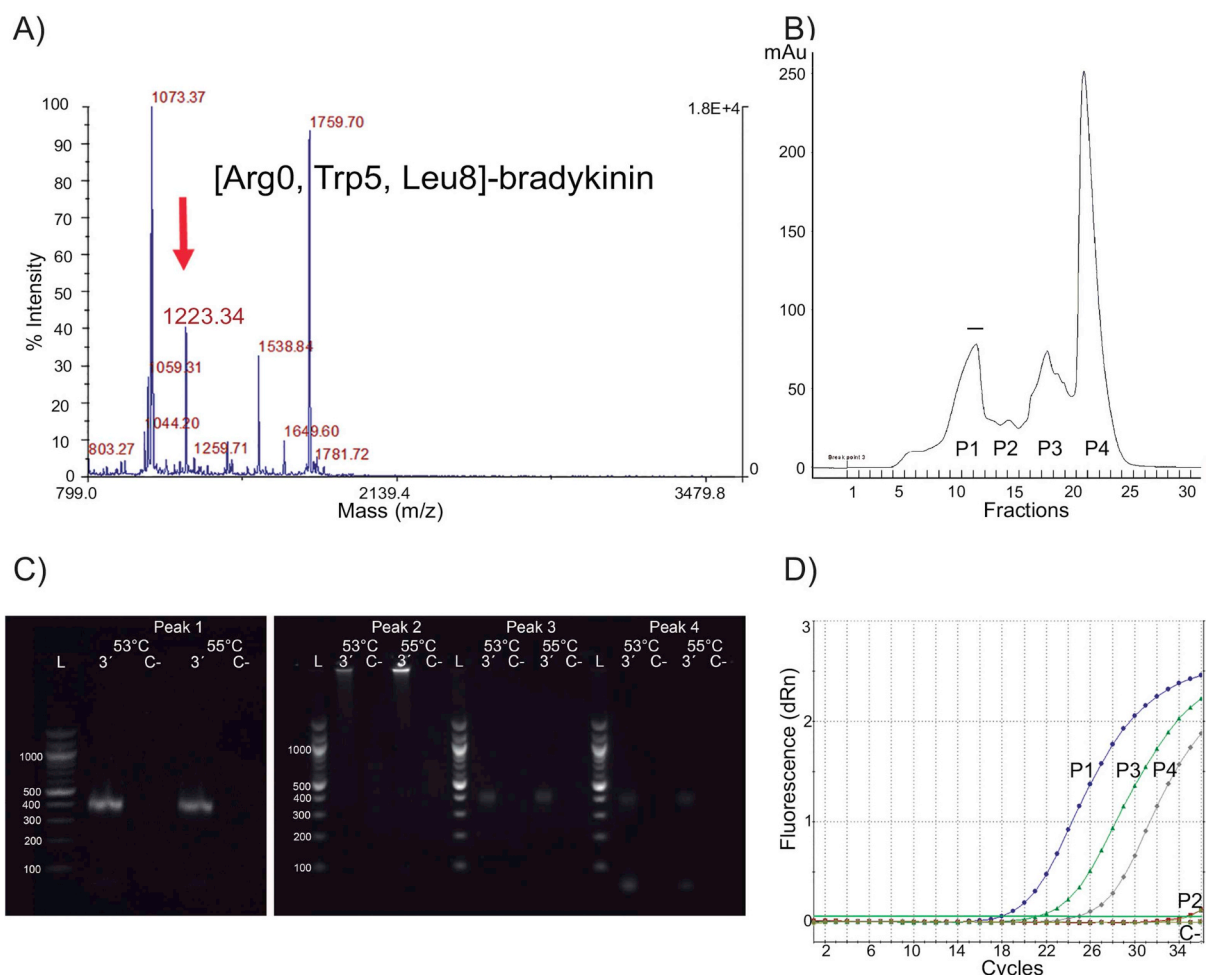
It is a common practice to archive amphibian skin secretions that have been fractionated through chromatographic procedures including fast performance liquid chromatography (FPLC) and reverse-phase high performance liquid chromatography (rpHPLC). FPLC employs a size exclusion column with a spherical composite of cross-linked agarose and dextran as sorbent and a mobile phase composed of acetonitrile, trifluoroacetic acid (TFA), and water; separation is based on molecular weights, with the higher molecular weight compounds eluting first. In contrast, HPLC employs silica columns of C-18, C-4 or C-5 as sorbents, and the mobile phase is a gradient of acetonitrile in acidified water (TFA); separation is based on polarity, with more polar compounds eluting first [7,8]. If viable amounts of poly-A mRNA could be extracted from such archived chromatographic samples, they could be a rich source for molecular cloning research of amphibian secretions. This will be particularly important when dealing with endangered or rare species when fresh material is scarce, but archived chromatographic fractions

are available. However, it was unknown whether the mRNA typically degraded during chromatographic separation due to the mRNA's labile nature or if it co-eluted with peptides. Here, using the well-characterized skin secretion of the Eurasian edible frog (*Pelophylax esculentus*, a hybridogenetic associate of *Pelophylax lessonae* and *P. ridibunda*, Ranidae) as a model, we show that chromatographic fractions of amphibian skin secretions can be used as a source of mRNA suitable for molecular cloning.

The skin secretion (10 mg) of *Pelophylax esculentus* was initially fractionated by size exclusion chromatography employing an FPLC system fitted with a Superdex 10/300 GL column. The sample was separated with a mobile phase composed of 0.05/30/70 (v/v/v) TFA/acetonitrile/H<sub>2</sub>O at a flow rate of 0.5 ml/min for 80 min. Detection was set to 254 nm (to detect nucleic acids) and to 280 nm (to detect proteins). Fractions were collected every minute. The peptide elution pattern was concentrated between fractions 5 to 25 and these fractions were pooled according to four peaks in the chromatogram: Peak 1 (fraction 10–12); Peak 2 (fractions 14–15); Peak 3 (fractions 17–20); and, Peak 4 (fractions 21–24) (see Fig. 1A). For each fraction, a 200  $\mu\text{l}$  aliquot was vacuum-concentrated and later reconstituted in sterile PBS (8  $\mu\text{l}$ ). Later, aliquots (2  $\mu\text{l}$ ) of each fraction were placed in 1 mm<sup>3</sup> holes made in agar plates seeded with 10<sup>6</sup> CFU/ml of bacteria (*Escherichia coli*, *Staphylococcus aureus*) or yeast (*Candida albicans*) and incubated overnight at 37  $^{\circ}\text{C}$ . As a result, antimicrobial activity (recorded as growth inhibition zone) was located in peak 1 against *E. coli*, *S. aureus*, and *C. albicans*. Individual fractions were analysed in a 500–7500 mass

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**Fig. 1.** Analysis of FPLC fractions of *Pelophylax esculentus* skin secretion. A) MALDI TOF MS analysis of FPLC fraction 18 from pool Peak 3 containing [Arg<sup>0</sup>, Trp<sup>5</sup>, Leu<sup>8</sup>]-bradykinin [M+H]<sup>+</sup> of 1223.34. B) FPLC chromatogram where Peak 1 (P1) corresponds to fractions 10–12, Peak 2 (P2) to fractions 14–15, Peak 3 (P3) to fractions 17–20 and Peak 4 (P4) to fractions 21 to 24. The dash over peak 1 indicates antimicrobial activity. C). 3'RACE PCR of Peaks 1–4. D) qPCR of Peaks 1–4.

range by MalDI-TOF MS employing 2  $\mu$ l of sample and 1  $\mu$ l of  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) matrix (10 mg/ml). The Arg<sup>0</sup>, Trp<sup>5</sup>, Leu<sup>8</sup>-bradykinin was identified in fraction 18 from pool P3 based on its monoisotopic molecular mass  $m/z$  1223.34 (Fig. 1B).

Later, another 10 mg of skin secretion were fractionated by a rpHPLC system fitted with a Jupiter C-18 (Phenomenex) column employing a linear gradient formed from 100% buffer A 99.95/0.05 (v/v) H<sub>2</sub>O/TFA to 100% buffer B 80/19.95/0.05 (v/v/v) acetonitrile/H<sub>2</sub>O/TFA at a flow rate of 1 ml/min for 100 min. Detection was set at 260 and 280 nm. The peptide elution profiles were concentrated in the range of 16–90 fractions and the fractions were pooled in four zones: Zone 1 (fractions 15–29); Zone 2 (fractions 30–50); Zone 3 (fractions 51–70); and, Zone 4 (fractions 70–95).

The pooled chromatographic fractions (Peaks 1–4 and Zones 1–4) were freeze-dried and polyadenylated mRNA was extracted from each pool employing the magnetic Dynabeads Oligo (dT)s mRNA extraction kit, as described by the manufacturer (DynaL Biotec, UK). Isolated mRNA was subjected to 3'-rapid amplification of cDNA by using the SMART-RACE kit (Clontech, UK). The 3'RACE used a nested universal primer (NUP) provided with the kit and the sense degenerate primer 5'GAWCYAYYHRAGCCYAAADATG3'. The 3'RACE reactions were purified and cloned using the pGEM-T vector system (Promega Corporation) and sequenced using an ABI 3100 automated sequencer. Sequences were analysed by Mega 6.0, aligned by ClustalW and compared with databases in NCBI employing the tool BLAST [9,10].

The mRNA isolation was evaluated throughout the 3'RACE PCR

product by conventional PCR, real time PCR, and sequencing of the cloned products. From conventional 3'RACE PCR, FPLC pools produced consistent 400 bp bands from Peaks 1, 3 and 4 (but not from Peak 2), with the strongest band being from Peak 1 (Fig. 1C). By contrast, Zones 1, 2 and 4 of the HPLC pools generated no bands, while Zone 3 generated a weak 300 bp band. To confirm the negative results of the 3'RACE PCR reactions and to determine the relative quantification of the positive results, we ran qPCR employing the same 3'cDNA and primers described above and the Kapa Sybr Fast qPCR Kits following manufacture instructions. According to the cycle thresholds (Ct) values and amplification curves generated, FPLC peaks 1, 3 and 4 and HPLC zones 1–4 had cDNA and therefore mRNA. FPLC peak 1 and HPLC zone 3 were the most concentrated (Ct 18 and Ct 29, respectively). Among the FPLC pools, Peak 1 (Ct18) was followed by Peak 3 (Ct 21), and then Peak 4 (Ct 25) (Fig. 1D). Among HPLC zones, the most copious pool was Zone 3 (Ct29), followed by Zone 4 (Ct 31), Zone 2 (Ct 32), and finally Zone 1(Ct 33).

The 3'RACE PCR products of FPLC peaks 1, 3, and 4 (conventional PCR) were cloned and sequenced. Nucleotide sequences of precursors of the previously reported Arg<sup>0</sup> Trp<sup>5</sup> Leu<sup>8</sup>-bradykinin, temporin-Ea, brevinin-1E, brevinin-1R, esculentin-1A, brevinin-2Ef, and brevinin-2E-like were identified in FPLC peaks 1, 3 and 4 and submitted to Genbank (accession numbers MH556886-MH556892; Table 1). On the other hand, the 3'RACE PCR product of HPLC zone 3 did not produce any informative sequence to be fully identified probably due to the small size of the cloned products (~200 bp).

**Table 1**  
Peptide sequences identified by molecular cloning in FPLC chromatographic fractions of *Pelophylax esculentus*.

Peptide	First isolated in	Accession number	Mature sequence	Cloned from FPLC pools				Accession number from this study
				P1	P2	P3	P4	
[Arg <sup>0</sup> Trp <sup>5</sup> Leu <sup>8</sup> ]-bradykinin	<i>Pelophylax esculentus</i>	CBV36943	RRPPGWSPLR	x				MH556886
Temporin-ECa.	<i>Euphlyctis cyanophlyctis</i>	COHJB9.1	FLPGLLAGLLa	x				MH556887
Brevinin-1E	<i>P. esculentus</i>	P32412	FLPLLAGLAANFLPKIFCKITRKC	x		x	x	MH556888
Brevinin-1R	<i>P. ridibundus</i>	P86027	FFPAIFRLVAKVVPISICSVTKKC			x		MH556889
Esculentin-1A	<i>P. esculentus</i>	P40843.1	GIFSKLAGKLLKNLLISGLKKNVGEVGMDDVVRTGDIAGCKIKGEC			x		MH556890
Brevinin-2Ef	<i>P. esculentus</i>	CAA54843.1	GIMDTLKNLAKTAGKALQSLVKMASCCLSGQC	x		x	x	MH556891
Brevinin-2E-like	<i>P. esculentus</i>	AAB27056	GIMDTLKNLAKTAGKALQSLNTASCCLSGQC			x		MH556892

In the present study, it has been demonstrated that poly(A)<sup>+</sup> mRNA can be isolated from FPLC and HPLC fractions of *Pelophylax esculentus* skin secretions. These results are surprising. Firstly, FPLC is a size-exclusion chromatography, so it was expected that, if present, all mRNA molecules would be eluted only in the first fractions due to their large size. However, mRNA was also eluted in the latter two Peaks (3 and 4) where some antimicrobial peptides were identified (Table 1). These results suggest an interaction between the mRNA and the peptides present in these fractions, resulting in their co-elution. The apparent absence of mRNA in fractions corresponding to Peak 2 are also noteworthy, especially since this result correlates with the lower absorbance at 260 nm and the absence of peptides in those fractions (results not shown). mRNA was also isolated from HPLC fractions, predominantly from Zone 3 but also from Zones 2 and 4. These results contrast with our expectations of finding mRNA mainly in the final HPLC fractions corresponding to nonpolar compounds. Given that the mRNA was located at various concentrations over the range of fractions, the HPLC results also suggest an interaction between peptides and mRNA molecules.

In terms of mRNA quality, the 3'-RACE products of FPLC Peaks 1, 3, and 4 were cloned and sequenced, and several peptide precursor transcripts were identified. For instance, the previously reported Arg<sup>0</sup> Trp<sup>5</sup> Leu<sup>8</sup>-bradykinin, brevinin-1E, brevinin-2E, brevinin-2Ef, and esculentin-1A (full open reading frames) were identified in FPLC peaks 1, 2 and 3. Notably, brevinin-1E and brevinin-Ef were identified in the three peaks, implying that mRNA is randomly distributed. On the other hand, 3'-RACE PCR performed with HPLC Zones was negative, except for Zone 3 that produced a weak 300 bp band; however, the cloned sequences were too small (~200 bp) to be fully identified. They contained the 3'non-translated region and poly A tail of antimicrobial peptides, but in most cases the sequences corresponded to mitochondrial or ribosomal RNA. Therefore, although mRNA was successfully extracted and cDNA was produced, the data indicated that RNA is chopped or partially degraded in HPLC fractions and therefore not suitable to obtain full precursor sequences. These results confirm the occurrence of mRNA in chromatographic fractions of FPLC and HPLC.

An important point to be noted in these trials is that we used 20 mg of skin secretion for FPLC fractionation and only 10 mg for HPLC. So, the weak amplification found in zone 3 (HPLC) could be due to the lower amount of starting material (half) used in comparison with FPLC. However, considering that we typically use 5 mg of skin secretion to build a cDNA library, we anticipate that 10 mg should be enough starting material for HPLC fractionation analysis.

These findings support the hypothesis of an interaction between peptides and mRNA in the chromatographic fractions of amphibian skin

secretions, and confirm the feasibility of analysing transcriptomes from archived chromatographic fractions of rare species. Moreover, our findings indicate that both proteomic and transcriptomic analysis could be performed with the same starting material. Both features are especially advantageous when working with endangered species for which samples are very scarce and difficult to obtain due to the legislation that protects them or their rarity in the field.

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