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Primary structures of skin antimicrobial peptides indicate a close, but not conspecific, phylogenetic relationship between the leopard frogs *Lithobates onca* and *Lithobates yavapaiensis* (Ranidae)

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ABSTRACT

The phylogenetic relationship between the relict leopard frog *Lithobates* (*Rana*) *onca* (Cope, 1875) and the lowland leopard frog *Lithobates* (*Rana*) *yavapaiensis* (Platz and Frost, 1984) is unclear. Chromatographic analysis of norepinephrine-stimulated skin secretions from *L. onca* led to the identification of six peptides with antimicrobial activity. Determination of their primary structures indicated that four of the peptides were identical to brevinin-1Ya, brevinin-1Yb, brevinin-1Yc and ranatuerin-2Ya previously isolated from skin secretions of *L. yavapaiensis*. However, a peptide belonging to the temporin family (temporin-ONa: FLPTFGKILSGLF.NH_2) and an atypical member of the ranatuerin-2 family containing a C-terminal cyclic heptapeptide domain (ranatuerin-2ONa: GLMDTVKNAAKNLAGQMLDKLKCKITGSC) were isolated from the *L. onca* secretions but were not present in the *L. yavapaiensis* secretions. Ranatuerin-2ONa inhibited the growth of *Escherichia coli* (MIC = 50 μM) and *Candida albicans* (MIC = 100 μM) and showed hemolytic activity (LC_{50} = 90 μM) but was inactive against *Staphylococcus aureus*. The data indicate a close phylogenetic relationship between *L. onca* and *L. yavapaiensis* but suggest that they are not conspecific species.

1. Introduction

The leopard frogs of North America (excluding Mexico and Central America) traditionally comprise eight species that were formerly assigned to the genus *Rana* but are now reclassified in the genus *Lithobates*: *L. berlandieri*, *L. blairi*, *L. chiricahuensis*, *L. fisheri*, *L. onca*, *L. pipiens*, *L. sphenacephalus*, and *L. yavapaiensis* (Hillis, 1988; Hillis and Wilcox, 2005; Frost, 2009). At this time, the status of a ninth species, *L. subaquavocalis* as a taxon distinct from *L. chiricahuensis* is unclear (Platz, 1993; Goldberg et al., 2004) and *L. fisheri* is almost certainly extinct (Jennings and Hammerson, 2004). There is strong evidence for hybridization of species that occupy the same geographical range (Conant and Collins, 1998; Platz, 1981) so that in some regions, leopard frog populations may be better described as a complex of interbreeding species rather than as a collection of individual species.

Peptides with the ability to inhibit the growth of bacteria and fungi are synthesized in the skins of the majority of species of frogs belonging to the family Ranidae that have been studied to-date (Conlon et al., 2004, 2009b). These peptides represent a component of the system of innate immunity that defends the organism against invasion by pathogenic microorganisms (Hancock, 2001). Antimicrobial peptides from the Ranidae may be grouped together in families on the basis of limited amino acid sequence similarity and at least 13 such families have been proposed (Conlon, 2008). Skin secretions from a single species frequently contain several members of a particular peptide family with varying degrees of antimicrobial potency and selectivity that are presumed to have arisen from multiple duplications of an ancestral gene (Tennessen and Blouin, 2007).

As well as exciting interest as candidates for development into therapeutically valuable anti-infective agents (Rinaldi, 2002), the frog skin antimicrobial peptides have been shown to be of value in investigating phylogenetic relationships among closely related species (Conlon et al., 2004, 2009b). The molecular heterogeneity of the peptides is considerable with very few peptides from one species being found with an identical amino acid sequence in another. Consequently, structural characterization of these dermal peptides can complement other types of molecular phylogenetic investigations, such as

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determinations of nucleotide sequences of orthologous genes, as well as more traditional analyses based upon morphological characters. For example, comparisons of the amino acid sequences of dermal antimicrobial peptides have provided support for the assertions that *Rana aurora aurora* and *Rana aurora draytonii* (Conlon et al., 2006); *Rana tagoi tagoi* and *Rana tagoi okiensis* (Conlon et al., 2010); and *Ascaphus truei* and *Ascaphus montanus* (Conlon et al., 2007) are not conspecific species.

Previous studies have described the isolation and characterization of antimicrobial peptides from skin secretions and/or skin extracts of the North American leopard frogs *L. sphenacephalus* (Conlon et al., 1999), *L. berlandieri* (Goraya et al., 2000), *L. pipiens* (Goraya et al., 2000), *L. blairi* (Conlon et al., 2009a), and *L. yavapaiensis* (Conlon et al., 2009a) and the partial nucleotide sequences of genes encoding antimicrobial peptides have been obtained for *L. chiricahuensis*, *L. pipiens*, and *L. sphenacephalus* (Tennessen and Blouin, 2007). We now extend this work by describing the purification of peptides with growth-inhibitory activity against microorganisms from norepinephrine-stimulated skin secretions from *L. onca.* Nomenclature adopted for antimicrobial peptides from frogs of the Ranidae family follows recent guidelines (Conlon, 2008) so that newly described peptides from *L. onca* belonging to well characterized families are designated ON and isoforms by lower case letters e.g. ranatuerin-2ONa.

The relict leopard frog *L. onca* is one of the rarest frogs in North America and was once believed to be extinct. The species has disappeared from large areas of its former distribution and its present range is confined to a few scattered sites in the border region of Arizona, Nevada, and Utah. A rough estimate of total population size is 2500 adults (Jaeger et al., 2004). Population declines have arisen from loss of habitat to agriculture and water development, introduction of the bullfrog *L. catesbeianus* and non-native predatory fish, and encroachment of exotic vegetation (Bradford et al., 2004). The life history of the species has not been well studied but adult frogs prefer fast-flowing streams and geothermal springs where dense vegetation does not dominate and reach a size of approximately 42 mm snout to vent length at the age of reproductive maturity (Bradford et al., 2009).

2. Materials and methods

2.1. Collection of skin secretions

All experiments with live animals were approved by the Animal Research Ethics Committee of U.A.E. University and the IACUC committee of the University of Missouri-St Louis and were carried out by authorized investigators. Five specimens of *L. onca* (body weights 19–34 g; sexes unknown) were collected in the Black Canyon region of Lake Mead National Recreation Area, Nevada, USA under permits from the United States Department of the Interior and Department of Wildlife of the State of Nevada. Each animal was injected via the dorsal lymph sac with norepinephrine hydrochloride (40 nmol/g body mass; 200 μL) and placed in a solution of distilled water (100 mL) for 15 min. The frog was removed and the collection solution was acidified by addition of trifluoroacetic acid (TFA) (1 mL) and immediately frozen for shipment to U.A.E. University. All animals were released unharmed at the exact site of collection. The secretions were pooled and passed at a flow rate of 2 mL/min through 6 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA, USA) connected in series. Bound material was eluted with acetonitrile/water/ TFA (70.0:29.9:0.1, v/v/v; 10 mL) and freeze-dried.

2.2. Antimicrobial and hemolysis assays

Purification of the peptides was monitored by incubating lyophilized aliquots of chromatographic effluent in Mueller-Hinton broth (50 μL) with an inoculum (50 μL of 10^6 colony forming units/mL) from a log-phase culture of reference strains of *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) in 96-well microtiter cell-culture plates for 18 h at 37 °C in a humidified atmosphere of air. After incubation, the absorbance at 630 nm of each well was determined using a microtiter plate reader. Incubations with *Candida albicans* (ATCC 90028) were carried out in RPMI 1640 medium for 48 h at 35 °C. In order to monitor the validity and reproducibility of the assays, incubations with bacteria were carried out in parallel with increasing concentrations of ampicillin and incubations with *C. albicans* in parallel with amphotericin B, as previously described (Conlon et al., 2009a). Minimum inhibitory concentrations (MIC) of the purified peptides were measured by standard microdilution methods (Clinical and Laboratory Standards Institute, 2008a,b) and was taken as the lowest concentration of peptide where no visible growth was observed.

Purified peptides (1–200 μM) were incubated in duplicate with washed human erythrocytes (2×10^7 cells) from a healthy donor in Dulbecco's phosphate-buffered saline, pH 7.4 (100 μL) for 1 h at 37 °C. After centrifugation (12,000 *g* for 15 s), the absorbance at 450 nm of the supernatant was measured. A parallel incubation in the presence of 1% v/v Tween-20 was carried out to determine the absorbance associated with 100% hemolysis. The LC_{50} value was taken as the mean concentration of peptide producing 50% hemolysis in two independent experiments.

2.3. Peptide purification

The pooled skin secretions, after partial purification on Sep-Pak cartridges, were redissolved in 0.1% (v/v) TFA/water (2 mL) and injected onto a (2.2×25-cm) Vydac 218TP1022 (C-18) reversed-phase HPLC column (Grace, Deerfield, IL, USA) equilibrated with 0.1% (v/v) TFA/water at a flow rate of 6.0 mL/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min and to 63% (v/v) over 60 min using linear gradients. Absorbance was monitored at 214 nm and 280 nm, and fractions (1 min) were collected. The abilities of freeze-dried aliquots (50 μL) of the fractions to inhibit the growth of *S. aureus* and *E. coli* were determined as described in the previous section. Fractions associated with antimicrobial activity were successively chromatographed on a (1×25-cm) Vydac 214TP510 (C-4) column and a (1×25-cm) 238EV510 Everest (C-18) column (Grace, Deerfield, IL, USA). The concentration of acetonitrile in the eluting solvent was raised from 21% to 56% over 50 min and the flow rate was 2.0 mL/min.

2.4. Structural characterization

The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems model 494 Procise sequenator (Foster City, CA, USA). MALDI-TOF mass spectrometry was carried out using a Voyager DE-PRO instrument (Applied Biosystems) that was operated in reflector mode with delayed extraction and the accelerating voltage in the ion source was 20 kV. The instrument was calibrated with peptides of known molecular mass in the 2000–4000 Da range. The accuracy of mass determinations was ±0.02%.

3. Results

3.1. Purification of the peptides

The pooled skin secretions from *L. onca*, after concentration and partial purification on Sep-Pak C-18 cartridges, were chromatographed on a Vydac C-18 preparative reversed-phase HPLC column (Fig. 1). For purposes of comparison, the elution profile under the same conditions of chromatography of partially purified skin secretions from *L. yavapaiensis* is shown in Fig. 2. The prominent peaks designated 1–6 in Fig. 1 were collected and subjected to further purification. Under the conditions of assay, peaks 2, 4 and 5 were