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Research article

Peptidomic analysis of skin secretions of the Mexican burrowing toad *Rhinophrynus dorsalis* (Rhinophrynidae): Insight into the origin of host-defense peptides within the Pipidae and characterization of a proline-arginine-rich peptide

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ABSTRACT

The Mexican burrowing toad *Rhinophrynus dorsalis* is the sole extant representative of the Rhinophrynidae. United in the superfamily Pipoidea, the Rhinophrynidae is considered to be the sister-group to the extant Pipidae which comprises *Hymenochirus, Pipa*, *Pseudhymenochirus* and *Xenopus*. Cationic, α-helical host-defense peptides of the type found in *Hymenochirus*, *Pseudhymenochirus*, and *Xenopus* species (hymenochirins, pseudhymenochirins, magainins, and peptides related to PGLa, XPF, and CPF) were not detected in norepinephrine-stimulated skin secretions of *R. dorsalis*. Skin secretions of representatives of the genus *Pipa* also do not contain cationic α-helical host-defense peptides which suggest, as the most parsimonious hypothesis, that the ability to produce such peptides by frogs within the Pipidae family arose in the common ancestor of (*Hymenochirus* + *Pseudhymenochirus*) + *Xenopus* after divergence from the line of evolution leading to extant *Pipa* species. Peptidomic analysis of the *R. dorsalis* secretions led to the isolation of rhinophrynin-27, a proline-arginine-rich peptide with the primary structure ELRLPEIARPVPEVLPARLPLPALPRN, together with rhinophrynin-33 containing the C-terminal extension KMAKNQ. Rhinophrynin-27 shows limited structural similarity to the porcine multifunctional peptide PR-39 but it lacks antimicrobial and cytotoxic activities. Like PR-39, the peptide adopts a poly-L-proline helix but some changes in the circular dichroism spectrum were observed in the presence of anionic sodium dodecylsulfate micelles consistent with the stabilization of turn structures.

1. Introduction

The phylogenetically ancient family Pipidae comprises four genera: *Pipa*, currently 7 species established in South America, and *Hymenochirus* (4 species), *Pseudhymenochirus* (1 species), and *Xenopus* (29 species) established in sub-Saharan Africa [1]. Until relatively recently, the diploid frog *Xenopus tropicalis* and the tetraploid frog *Xenopus epitropicalis* were assigned to the separate genus *Silurana* but the monophyletic status of *Xenopus* + *Silurana* is well established so that *Silurana* is now generally described as a sub-genus of *Xenopus* [2–4]. The family Rhinophrynidae, which comprises a single species, the Mexican burrowing toad *Rhinophrynus dorsalis* Duméril and Bibron, 1841, is considered to be sister-group to the extant Pipidae and it has been proposed that the two lines of evolution diverged around the time of separation of North America from West Africa following the breakup of Pangaea (approximately 175–190 MYA) [5,6]. The families Pipidae and Rhinophyrinidae are united in the superfamily Pipoidea and both the fossil record and molecular analysis provide strong evidence for the monophyly of the clade [6,7].

Skin secretions of *Hymenochirus boettgeri* [8,9], *Pseudhymenochirus merlini* [10], and a wide range of *Xenopus* species (reviewed in [11,12]) contain extensive arrays of cationic, amphipathic α-helical peptides.

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These peptides display growth inhibitory activity against bacteria and fungi and so may be described as "antimicrobial". However, many also possess immunomodulatory, anti-tumor, anti-viral, and insulin-releasing activities (reviewed in [13,14]) so that they are better described as host-defense peptides (HDPs). Skin secretions from frogs of the *Xenopus* genus have proved to be a particularly rich source of such peptides and five families have been identified on the basis of limited structural similarity: magainin, peptide glycine-leucine-amide (PGLa), xenopsin-precursor fragment (XPF), derived from the post-translational processing of proxenopsin, and both caerulein-precursor fragment (CPF) and caerulein-precursor fragment-related peptide (CPF-RP) derived from the post-translational processing of procaeruleins (reviewed in [11,15]). In contrast, cytotoxic HDPs were not detected in skin secretions of *Pipa pipa* ([11], *Pipa carvalhoi* [16]) and *Pipa parva* (J.M. Conlon, unpublished data),

The New world frog *R. dorsalis* is found in coastal lowland regions (from sea-level to 500 m) in a range of habitats, such as tropical dry forests, grasslands, thorn scrub, and cultivated fields. Although relatively rare in Texas, the species is common and widespread in Mexico and northern Central America and is listed as Species of Least Concern by the International Union for Conservation of Nature (IUCN) Red List [17]. The animal is strictly fossorial, only emerging from its underground burrow to reproduce after the first rains with the result that it is rarely seen in the wild. The present study uses peptidomic analysis (reversed-phase HLPC coupled with MALDI-TOF mass spectrometry and automated Edman degradation) to investigate the occurrence of HDPs in norepinephrine-stimulated skin secretions from *R. dorsalis*.

2. Experimental

2.1. Collection of skin secretions

All experiments with live animals were approved by the Gladys Porter Zoo Scientific and Research Committee and were carried out by authorized investigators. Four adult *R. dorsalis* frogs (1 male, 16 g body weight; 3 female, 20, 20, and 30 g body weight) were collected in the grounds of Rio Grande City High School, Rio Grande City, TX and were housed in a vivarium at the Gladys Porter Zoo, Brownsville, TX.

Each frog was injected via the dorsal lymph sac with norepinephrine hydrochloride (40 nmol/g body weight) and placed in a solution (100 ml) of distilled water for 15 min. The injection did not produce the kind of copious, milky skin secretions often seen when *Xenopus* species are stimulated with norepinephrine but the collection solution became more turbid and foamy. The frog was removed and the collection solution was acidified by addition of trifluoroacetic acid (TFA) (1 ml) and immediately frozen for shipment to Ulster University. The solutions containing the secretions from each frog were pooled and passed at a flow rate of 2 ml/min through 6 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA) connected in series. Bound material was eluted with acetonitrile/water/TFA (70.0:29.9:0.1, v/v/v) and freeze-dried. The material was redissolved in 0.1% (v/v) TFA/water (2 ml).

2.2. Peptide purification

The pooled skin secretions from *R. dorsalis,* after partial purification on Sep-Pak cartridges, were injected onto a semipreparative (1 cm × 25 cm) Vydac 218TP510 (C-18) reversed-phase HPLC column (Grace, Deerfield, IL) equilibrated with 0.1% (v/v) TFA/water at a flow rate of 2.0 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 peak fractions were collected by hand. The peptides within the peaks that were present in major abundance were subjected to further purification. These components were purified to near homogeneity, as assessed by a symmetrical peak shape and mass spectrometry, by chromatography on (1.0 cm × 25 cm) Vydac 214TP510 (C-4) and (1.0 cm × 25 cm) Vydac 219TP510 (phenyl) columns. The concentration of acetonitrile in the eluting solvent was raised from 7% to 35% over 50 min for the more hydrophilic components and from 21% to 49% over 50 min for the more hydrophobic components. The flow rate was 2.0 ml/min.

2.3. Structural characterization

MALDI-TOF mass spectrometry was carried out using a Voyager DE-PRO instrument (Applied Biosystems, Foster City, CA) 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 2–4 kDa range. The accuracy of mass determinations was ± 0.02%. Spectra were recorded using both α-cyano-4-hydroxycinnamic acid and sinapinic acid as matrix solutions. The complete primary structures of peptides in the mass range 1–4 kDa were determined by automated Edman degradation using a model 494 Procise sequenator (Applied Biosystems). For larger peptides/proteins (molecular mass > 4 kDa), only the amino acid sequence at N-terminus (residues 1–10) were determined in order to permit identification. Amino acid composition analyses were performed by the University of Nebraska Medical Center Protein Structure Core Facility (Omaha, NE).

2.4. Peptide synthesis

Rhinophrynin-27 (ELRLPEIARPVPEVLPARLPLPALPRN) was supplied at a purity > 95% by Synpeptide Co., Ltd (Shanghai, China). Its identity and purity were confirmed by electrospray mass spectrometry.

2.5. Antimicrobial and cytotoxicity assays

Reference strains of microorganisms were purchased from the American Type Culture Collection (Rockville, MD, USA). Minimum inhibitory concentrations (MIC) of rhinophrynin-27 against reference strains of *Staphylococcus epidermidis* (ATCC 12228), *Bacillus megaterium* (Bm11), *Escherichia coli* (ATCC 25922) and *Candida parapsilosis* (ATCC 22019) were measured by standard microdilution methods [18,19] as previously described [20]. Hemolyic activity was determined by incubation of washed erythrocytes (2×10^7 cells) from male NIH male Swiss mice (Harlan Ltd, Bicester, UK) with rhinophrynin-27 (31.3–500 μM) for 60 min at 37 °C as previously described [8]. Cytotoxicity of rhinophrynin-27 against human non-small cell lung adenocarcinoma A549 cells was measured as previously described [21]. The effects of the peptide (1–100 μM) on cell viability were determined by measurement of ATP concentrations using a CellTiter-Glo Luminescent Cell Viability assay (Promega Corporation, Madison, WI, USA). All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and EU Directive 2010/63 EU for animal experiments.

2.6. CD spectra

Spectra were obtained using a MOS-500 Circular Dichroism Spectrometer (Bio-Logic, Claix, France). Data points were collected from 260 nm to 185 nm, with an integration time of 2 s per point and a step size of 1 nm, using a 1.0 mm path length rectangular quartz cell. Measurements were carried out at room temperature, 20 °C and 5.5 °C. Rhinophrynin-27 was dissolved in water, in 2,2,2-trifluoroethanol (TFE)-water (25% and 50%, v/v), in 20 mM sodium dodecyl sulfate (SDS) aqueous solution, and in 20 mM dodecylphosphocholine (DPC) aqueous solution at a final concentration of 0.18–0.21 mg/ml. The concentration of 20 mM for detergents was chosen to ensure micelle formation. For each spectrum, three scans were accumulated and then averaged. The baseline was obtained by recording a spectrum of the solvent, and the mean residue molar ellipticity ([θ]MRE),