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Original article

Structure and function of a novel antioxidant peptide from the skin of tropical frogs

Eder Alves Barbosa[a,b], Ana Oliveira[c], Alexandra Plácido[d], Renato Socodato[e], Camila C. Portugal[e], Ana Carolina Mafud[f,g], Alicia S. Ombredane[h], Daniel C. Moreira[i], Nuno Vale[j], Lucinda J. Bessa[k], Graziella A. Joanitti[h,l], Cláudia Alves[k], Paula Gomes[k], Cristina Delerue-Matos[d], Yvonne Primerano Mascarenhas[f], Mariela M. Marani[m], João B. Relvas[e], Manuela Pintado[c], José Roberto S.A. Leite[e,i,*]

[a] Laboratório de Espectrometria de Massa, EMBRAPA Recursos Genéticos e Biotecnologia, Brasília, Brazil
[b] Laboratório de Síntese e Análise de Biomoléculas, Instituto de Química, Universidade de Brasília, Brasília, Brazil
[c] Centro de Biotecnologia e Química Fina, CBQF, Universidade Católica Portuguesa, Rua Arquiteto Lobão Vital Apartado, 2511, Asprela, Porto, Portugal
[d] LAQV/REQUIMTE, GRAQ, Instituto Superior de Engenha do Porto, ISEP, Porto, Portugal
[e] Glial Cell Biology Lab, Instituto de Investigação e Inovação em Saúde and Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal
[f] Instituto de Física de São Carlos, IFSC, Universidade de São Paulo, USP, São Carlos, SP, Brazil
[g] Dept Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, Basel 4051, Switzerland
[h] Laboratório de Nanobiotecnologia, Instituto de Biologia, Campus Darcy Ribeiro, UnB, Brasília, DF, Brazil
[i] Área de Morfologia, Faculdade de Medicina, Universidade de Brasília, UnB, Brasília, DF, Brazil
[j] UCIBIO/REQUIMTE, Laboratório de Farmacologia, Departamento de Ciências do Medicamento, Faculdade de Farmácia da Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal
[k] LAQV/REQUIMTE, Departamento de Química e Bioquímica, Faculdade de Ciências da Universidade do Porto, Rua do Campo Alegre, 687, 4169-007 Porto, Portugal
[l] Campus Ceilândia, Centro Metropolitano, UnB, Ceilândia, Brasília, DF, Brazil
[m] IPEEC-CONICET, Consejo Nacional de Investigaciones Científicas y Técnicas, Puerto Madryn, Argentina

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ABSTRACT

The amphibian skin plays an important role protecting the organism from external harmful factors such as microorganisms or UV radiation. Based on biorational strategies, many studies have investigated the cutaneous secretion of anurans as a source of bioactive molecules. By a peptidomic approach, a novel antioxidant peptide (AOP) with *in vitro* free radical scavenging ability was isolated from *Physalaemus nattereri*. The AOP, named antioxidin-I, has a molecular weight $[M+H]^+ = 1543.69$ Da and a TWYFITPYIPDK primary amino acid sequence. The gene encoding the antioxidin-I precursor was expressed in the skin tissue of three other Tropical frog species: *Phyllomedusa tarsius, P. distincta* and *Pithecopus rohdei.* cDNA sequencing revealed highly homologous regions (signal peptide and acidic region). Mature antioxidin-I has a novel primary sequence with low similarity compared with previously described amphibian's AOPs. Antioxidin-I adopts a random structure even at high concentrations of hydrophobic solvent, it has poor antimicrobial activity and poor performance in free radical scavenging assays *in vitro*, with the exception of the ORAC assay. However, antioxidin-I presented a low cytotoxicity and suppressed menadione-induced redox imbalance when tested with fibroblast in culture. In addition, it had the capacity to substantially attenuate the hypoxia-induced production of reactive oxygen species when tested in hypoxia exposed living microglial cells, suggesting a potential neuroprotective role for this peptide.

1. Introduction

Amphibian's skin constitutes a water and gas permeable thin barrier between the organism and the environment. Physiological skin roles include gas exchange, excretion, thermoregulation, and reproduction [1]. Additionally, the amphibian skin presents an important defensive function against predators, bacteria and fungi. These are particularly relevant in the light of the natural history of amphibians. Most amphibians inhabit moist or aquatic habitats and the constantly humid skin represents an adequate media for bacterial and fungal growth. Over the last decades, one of the most investigated features of the amphibian's skin was related to the innate immune system. Amphibian

* Correspondence to: Universidade de Brasília (UnB), Faculdade de Medicina, Campus Universitário Darcy Ribeiro, Asa Norte, Brasília, DF CEP 70910-900, Brasil.
E-mail addresses: jrsaleite@gmail.com, jrleite@pq.cnpq.br (J.R.S.A. Leite).

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species developed a protection mechanism based on the production and storage of antimicrobial peptides by specialized granular glands spread throughout the skin that protects skin tissue against microorganism infection [2]. These glands also produce a set of biomolecules having distinct structures and well-established activities *e.g.* vasodilatation, hypotension [3,4] and endopeptidase inhibition [5]. This molecular arsenal protects these animals not only from microorganisms but also from predators and other abiotic threats [6].

By employing a biorational approach (*i.e.* predict the presence of compounds with potential pharmacological applications based on organisms' interactions with the environment) a great number of bioactive molecules from amphibian skin have been described, most of them with, but not limited to, antimicrobial activity [7]. Many amphibians transit between aquatic and terrestrial habitats, which expose the animal's skin to wide variations in O_2 availability. Moreover, during day light the delicate amphibian skin, lacking an external protective layer and with a thin stratum corneum, is exposed to UV radiation. Both, UV exposure and drastic changes in O_2 exposure have been linked with altered reactive oxygen species (ROS) generation. Therefore, it could be predicted, using a biorational approach, that amphibian skin secretion would have a molecular mechanism to minimize the threat from these sources. Pheomelanin, a melanin type protein, present in the skin of *Hymenochirus boettgeri,* is an example of a protector molecule against UV light damage in amphibians [8]. Indeed, a collection of peptides with antioxidant activity in chemical systems isolated from the skin secretion of Asiatic frogs has been recently reported [9–11]. In general, a great variability of structures was found among the species studied [9,10,12], suggesting that these peptides did not arise from a common ancestor molecule.

In biological systems, antioxidant networks manage the steady state of reactive species [13]. Reactive species, including ROS, are important signals that participate in many physiological processes [14–16]. There are several cellular pathways that lead to ROS production, including mitochondrial electron transport chain, NADPH oxidases and several other enzymatic reactions (*e.g.* oxidases) [13]. The rate of ROS formation from such sources responds intra and extracellular stimuli. For example, hypoxia exposure [17] and reperfusion after an ischemic event [18] elicit an overproduction of mitochondrial ROS. Thus, the constantly produced ROS and their control by antioxidants constitute a redox signaling mechanism, in which reversible oxidation of endogenous thiols has a central role [19]. In some situations, excess ROS may overcome the management capacity of antioxidants, leading to a redox imbalance. Due to their reactivity, excess ROS may cause irreversible oxidative damage to biomolecules, which may have signaling functions, but also cause loss of function [20]. The disruption of signaling pathways and accumulation of oxidatively modified molecules have been linked to several neurodegenerative disorders, such as Alzheimer's disease [21], Parkinson's disease [22], Huntington disease [23] and Amyotrophic Lateral Sclerosis [24].

In this work, we predicted the presence of peptides with antioxidant activity in the cutaneous secretion from the tropical frog *Physalaemus nattereri* (Steindachner, 1863). We used chromatography, tandem mass spectrometry, molecular biology and *in silico* techniques followed by *in vitro* assays to characterize antioxidin-I, a peptide isolated from the tropical frog *P. nattereri* with potential antioxidant activity. We demonstrated the expression of this peptide in other tropical frogs of the Phyllomedusa and Pithecopus genus: *Phyllomedusa tarsius*, *P. distincta* and *Pithecopus rohdei*. Then, aiming at the potential pharmaceutical use of this peptide, we conducted viability and antioxidant assays in cell culture. We tested the ability of the peptide to mitigate menadione-induced redox imbalance in fibroblasts and the capacity to suppress ROS levels in hypoxia-exposed microglia.

2. Material and methods

2.1. Isolation and characterization of the antioxidin-I

Adult *Phyllomedusa distincta*, *P. tarsius*, *Pithecopus rohdei* (first assessed under the generic name Phyllomedusa, but has been transferred to the genus Pithecopus by Duellman et al. [25]) and *Physalaemus nattereri* specimens were captured manually in the Brazilian Atlantic forest of Santa Catarina and São Paulo, Tropical forest of Amazônia and Cerrado of Goiás, respectively, under the license N°240/2005-CGFAU from the Instituto Brasileiro e dos Recursos Renováveis – IBAMA (Process No. 034/06-COFAN). The cutaneous secretion from *Physalaemus nattereri* was obtained by electrical stimulation (6 V), collected with Milli-Q water in 50 mL tubes, filtrated (Millipore filters, 0.22 μm) and immediate frozen and lyophilized.

The *P. nattereri* dry secretion (1 mg) was dissolved in Milli-Q water (500 μL) and subjected to an HPLC system (Shimadzu Co., LC-20 CE model), using a Vydac C18 reverse phase column (2018 TP). The fractions were eluted with a linear gradient of 0.1% (v/v) TFA/acetonitrile ranging from 5% to 60% over 60 min and 75–95% over 5 min at a flow rate of 1 mL/min. Fractions were monitored at 216 and 280 nm, collected in tubes and dried under vacuum centrifugation.

Dried fractions were re-dissolved in Milli-Q water. The amount of solvent, ranged from 10 to 100 μL, was adjust according to the absorbance value previously obtained for each fraction. One microliter aliquot of the chromatographic fractions was dissolved in α-cyano-4-hydroxicinamic acid matrix solution (1:3, v/v) and then applied on the MALDI plate. The molecular mass of the peptides was determined by MALDI-TOF/MS using the UltraFlex Xtreme mass spectrometer (Bruker Daltonics) in the positive reflected mode, controlled by the FlexControl software. The ions of interest were fragmented in the LIFT mode (MS/MS), aiming the *de novo* sequencing of peptides. Before each analysis, the spectrometer was calibrated using a mixture of peptides. The spectra were analyzed manually using the FlexAnalysis software (Bruker Daltonics). Amino acid sequencing data were compared with cDNA sequencing analyses.

2.2. cDNA sequencing

Three specimens from each amphibian species were euthanized by infusion of 2% liquid lidocaine hydrochloride solution (100 μL) directly into the brain [26]. The dorsal cutaneous tissues were isolated by dissection. The inguinal glands of *P. nattereri* were dissected separately from dorsal skin. Tissues for RNA extraction were immediately frozen in liquid nitrogen and stored at −80 °C. The cDNA characterization and analysis were performed as previously reported (Brand et al., 2006). Briefly, the isolated tissues were pulverized in liquid nitrogen and approximately 10 mg of the resulted powder were used for extraction of the total RNA using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. One microgram of RNA was subjected to cDNA synthesis with the enzyme ImProm II (Promega), according to the manufacturer's protocol. Degenerate oligonucleotides (Table S1) were used for PCR reactions.

PCR products were run by electrophoresis in 1% agarose gel for analysis of the amplified fragments. Those having molecular weights between 250 and 400 bp were isolated and purified with Wizard SV Gel and PCR Clean-Up System (Promega), following manufacturer's instructions. The purified DNA samples were subjected to the pGEM-T Easy (Promega) vector binding reaction, according to manufacturer's instructions, and transfected into *E. coli* (DH5α) by electroporation. After selecting the clones of interest, the plasmids were isolated and submitted to automatic nucleotide sequencing (Sanger sequencing method).