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Phylloseptins: a novel class of anti-bacterial and anti-protozoan peptides from the *Phyllomedusa* genus

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Abstract

Six novel peptides called phylloseptins (PS-1, -2, -3, -4, -5, and -6) showing anti-bacterial (PS-1) and anti-protozoan (PS-4 and -5) activities were isolated from the skin secretion of the Brazilian tree-frogs, *Phyllomedusa hypochondrialis* and *Phyllomedusa oreades*. Phylloseptins have a primary structure consisting of 19–21 amino acid residues (1.7–2.1 kDa). They have common structural features, such as a highly conserved N-terminal region and C-terminal amidation. Phylloseptin-1 (FLSLIPHAINAVSAIAKHN-NH_2) demonstrated a strong effect against Gram-positive and Gram-negative bacteria (MICs ranging from 3 to 7.9 μM), without showing significant hemolytic activity (<0.6% at the MIC range) towards mammalian cells. Atomic force microscopy experiments indicated that the bacteriolytic properties of these peptides might be related to their disruptive action on the cell membrane, characterized by a number of bubble-like formations, preceding every cell lysis. PS-4 and PS-5 showed anti-protozoan activity with IC_{50} at about 5 μM for *Trypanossoma cruzi*.

Keywords: Antimicrobial peptides; Phylloseptin; *Phyllomedusa oreades*; *Phyllomedusa hypochondrialis*; Atomic force microscopy; Trypanocidal activity

1. Introduction

Bacteria, fungi, and protozoa coexist with all living animals for millions of years. However, considering the number and diversity of these microorganisms and their constant interactions with animals, lethal microbial invasion to the hosts is quite rare [20]. This is mainly due to the highly specific cell-mediated immune responses, and to the broad-spectrum antimicrobial peptides from their innate immune systems. The different families of antimicrobial peptides, which have been classified on the basis of their amino acid sequences, biological activity and secondary structures, are often found in many insects [42], plants [14], amphibians [45], mammals [3,15,32], and microorganisms [6,8]. In amphibians, amphiphilic α-helical antimicrobial peptides such as magainins [17], bombinins [22], buforins [34], and dermaseptins [4,10,30,35] have been a subject of intense research regarding their biosynthesis, activity towards microorganisms, mechanisms of action, and potential clinical applications. In spite of all the effort put on these research lines up to now, it is clear that predicting peptide activity, specificity and 3-D structure from the amino acid sequence alone is not a simple task [23], most peptides without disulfide bonds show no

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defined structure in water, and it is only in a membrane or in a hydrophobic environment that they may fold into α-helices or β-structures [5,19]. It is known that the net positive charge associated with the hydrophobic nature of the polypeptide chain, found in the great majority of the antimicrobials, is the cornerstone feature for the activity and specificity of these molecules. In fact, plasma membrane recognition and binding are the initial steps of the peptide–membrane interaction and a detailed knowledge of these processes and their driving forces are important prerequisites to understand the mechanism of selective lysis of bacterial, fungal and protozoan membranes, which have been reported so far [24,38]. The three-dimensional structures of many antimicrobial peptides demonstrate an amphiphilic motif, where the hydrophilic face is formed by polar and positively charged residues (typically, lysine and arginine) and the hydrophobic face are constituted by non-polar amino acids. This topological arrangement is often induced by the contact of the peptide with hydrophobic environments such as organic solvents, SDS vesicles and phospholipid micelles [36,44]. The lytic activity, on the other hand, appears to be related to the physicochemical properties of the peptide conferred by its amino acid composition and sequence.

For more than two decades the worldwide resurgence of infectious diseases due to evolution of antibiotic-resistant strains is increasing at an alarming pace, as antibiotics progressively loose their effectiveness. In this work, we report a novel family of antimicrobial and anti-protozoan peptide from the skin secretion of two *Phyllomedusa* species (*Phyllomedusa oreades* and *Phyllomedusa hypochondrialis*) named phylloseptins. These new peptides (PS-1, -2, -3, -4, -5, and -6) have molecular mass ranging from 1.7 to 2.1 kDa, and PS-1 showed MICs at about 3 μM for pathogenic bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus aureus*), and PS-4 and -5 anti-*Trypanosoma cruzi* activity and PS-1 and -2 had negligible effects on red blood cells.

2. Materials and methods

2.1. Amphibians

Frogs skin secretions were obtained from adult specimens of *P. hypochondrialis* ($n = 6$, captured in Brasília, Brazil) and *P. oreades*, a recently described [11] species of the *Phyllomedusinae* sub-family ($n = 4$, captured in Serra da Mesa, Goiás, Central Brazil). Frogs were collected according to the Instituto Brasileiro do Meio Ambiente e dos Recursos Renováveis, IBAMA, under the license number 097/96-DIFAS (Process no. 0637/91A.C).

2.2. Peptide purification

Frog secretions were obtained by mild electric stimulation of the skin granular glands of *P. hypochondrialis* and *P. oreades* for a few seconds and freshly collected in distilled water. The water-soluble secretions from each species were filtered by gravity through filter paper, frozen, and lyophilized (Centrivap Concentrator, Labconco). Peptide separation was performed by application (5 mg aliquots each time) of the crude extract to a semi-preparative Vydac reverse-phase (RP) chromatographic column, C_{18}, 5 μm (218TP510; 10 mm × 250 mm) in HPLC system (Shimadzu Co.) Peptides were purified by using linear gradients, initially 0–80% acetonitrile containing 0.1% TFA (trifluoroacetic acid) for 70 min, followed by 80–100% of the same solvent for 20 min. The experiments were monitored at 216 and 280 nm. Fractions were collected manually and lyophilized. The isolated fractions were submitted to another chromatographic step using a Vydac 218TP54, C_{18}, 5 μm (4.6 mm × 250 mm) analytical column, with optimized gradients of acetonitrile in 0.1% TFA over 60 min.

2.3. Purity and molecular mass determination

The molecular mass and homogeneity of PS-1, PS-2, PS-3, and PS-6 (*P. hypochondrialis*), and PS-4 and PS-5 (*P. oreades*) were determined by MALDI-TOF/MS in a 4700 Proteomics Analyzer with TOF–TOF optics (Applied Biosystems, Framingham, MA). Approximately 20 nmol of lyophilized peptide was dissolved in Milli-Q water and mixed with a saturated matrix solution of α-cyano-4-hydroxycinnamic acid (1:3, v/v) and spotted onto a MALDI-TOF/TOF sample plate, at room temperature.

2.4. Sequence analysis

De novo sequencing of the phylloseptins were performed by MS/MS experiments using argon as collision gas on a Q-TOF Ultima (Waters-Micromass, Manchester, UK) operating in W mode and in a 4700 Proteomics Analyzer with TOF–TOF optics (Applied Biosystems, Framingham, MA). The MS and MS/MS spectra were carried out in reflector mode with external calibration, using the 4700 calibration mixture kit (Applied Biosystems). Peptide de novo sequencing was performed by precursor ion fragmentation, using N_2 as CID gas and the collision cell pressure was kept at 1.8×10^{-6} Torr. Leucine, isoleucine and lysine residues present in each one of the phylloseptin polypeptide chains were assigned based on the high energy fragmentation ions and confirmed by automated Edman degradation on a *PPSQ-23* protein peptide sequencer (Shimadzu Co., Japan). Peptide sequence alignments and similarity searches were performed using the FASTA 3 program on the ExPASy molecular server (http://www.expasy.ch/). Secondary structure prediction was performed using SOPMA, also at this server [21,40].

2.5. Hemolytic assay

Hemolytic assays were performed using human red cells (blood type O^-) in liquid medium, as reported previously