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Cationic Hylin Bioactive Peptides from *Boana pulchella* (Anura: Hylidae): Activity, Structure, and Interaction with Lipid Membranes

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Introduction

Amphibians are an important source of antimicrobial peptides (AMPs), which are an essential component of their innate immune system contributing to the neutralization or elimination of microorganisms [1,2]. Each species produces its own set of AMPs.

The primary mechanism of action of AMPs typically involve nonspecific electrostatic interactions with bacterial membranes, and proceed through the aggregation of AMPs in the bacterial membrane, affecting its integrity and leading to cell death.

The *Boana* genus is one of the most species-rich clades of the hylid family with a widespread distribution in South America [3]. It has been poorly studied in terms of their AMP profile. To our knowledge, no research have explored the potential mechanism of action of AMPs from *Boana* spp., with the exception of some analogues[4,5].

Based on our results, the possible mechanism of action of these novel molecules and the importance of environmental polarity were discussed.

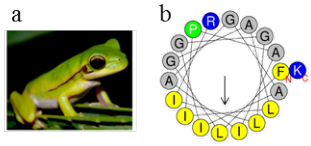

Fig. 1. a) Male adult specimen of Boana pulchella (photo by Andrés Brunetti). b) Helical wheel projection of the AMP hylin-Pul3 identified in the skin secretion of B. pulchella.

Results and Discussion

Our study identified novel peptide sequences from the skin of the tree frog *Boana pulchella* (Figure 1a) using molecular and mass spectrometry analyses. Five unique and novel full-length prepro-peptide cDNAs were identified after cloning, size evaluation, selection of the amplified insert fragments, and sequencing. Precursors showed the typical tripartite structure of AMPs. The analysis of the primary sequences of the peptides identified from the lyophilized crude skin extract of *B. pulchella* by RP-HPLC followed by LC-ESI-MS/MS revealed the presence of several peptides including acidic and amidated C-terminus fragments of the mature peptides identified from mRNAs. This post-transcriptional modification occurs commonly in AMPs and is necessary for membrane binding [6].

Mature peptide sequence alignment with previously described AMPs showed a similarity of 68-84% of four of these peptides with hylin–Prs1 identified from *Boana prasine* [7] and 52-74% with hylin b1, and hylin b2 isolated from *Boana lundii* [8].

Hylin primary sequences are cationic (+1 or +3), with 18 and 20 residues and only Pro7 and Gly11 conserved in all family members. Despite this variability, all hylins exhibit a characteristic amphipathic arrangement with most of the hydrophobic residues (58-68%), such as Leu, Ile, Val, Phe, or Ala, located at the same face (Figure 1b), and the basic amino acids located at the *C*-terminus.

Circular dichroism (CD) was used to evaluate the secondary structure that Fmoc/tert-butyl-synthetized peptides adopt in the presence of neutral and anionic membrane environments. Soluble peptides exhibited a negative band centered at 195–197 nm matching the π-π* transition, typical of unordered conformations. For mature hylin peptides, a high dichroic signal at 195 nm, 208 nm, and 220–230 nm can be observed, compatible with the formation of an α-helical secondary structure upon binding to DMPG:DMPC (1:2) LUVs (Figure 2a) and, to a lesser extent, to pure DMPC. The evaluated fragments hylin-Pul1(12–19) and hylin-Pul2(12–19) did not show significant variations in spectral features when LUVs were added.

Dynamic light scattering (DLS) was used to evaluate the effects of peptides on the size of vesicles mimicking the plasma membrane of eukaryotic and prokaryotic cells. On DMPC membranes, none of the peptides nor any of the fragments were able to increase the size of the vesicles. However, larger particles resulted from the aggregation or fusion of the vesicles in the presence of hylin peptides (Figure 2b**)**.

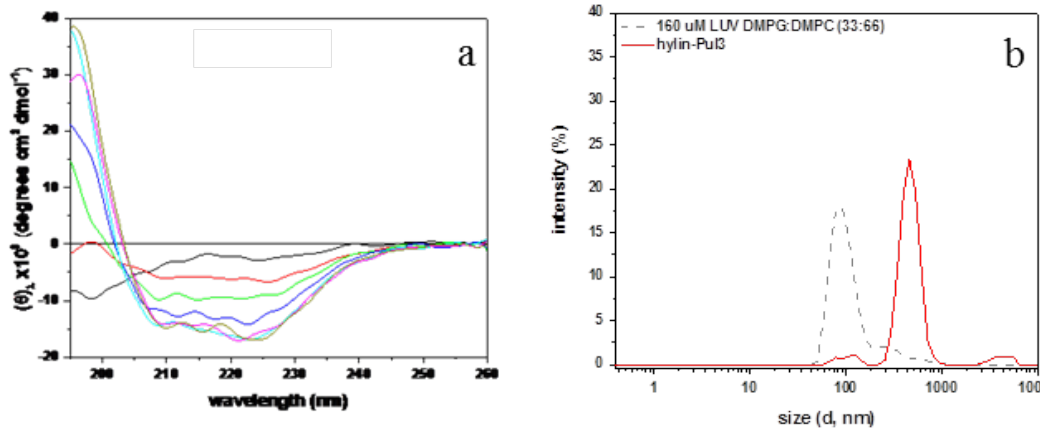

Fig. 2. a) Far-UV CD spectra of the most active peptide, hylin-Pul3,in 10 mM Tris-HCL with 0.15 M of NaCl at pH 7.4 buffer with LUVs DMPG:DMPC (1:2) are shown as follows: soluble peptides 40 μM (black lines) and six peptide:lipid molar ratios 1:1 (red), 1:2 (green), 1:4 (blue), 1:8 (cyan), 1:16 (magenta), and 1:32 (dark yellow).b) Dynamic light scattering of hylin-Pul3 interacting with LUV DMPG:DMPC (33:66) membranes. Size distributions by scattered intensity of particles after peptide addition to LUVs is shown.

Hemolytic activity and antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* were used to characterize the activity of peptides and fragments. Results of antimicrobial assays of purified synthetic hylin-Pul1, hylin-Pul2, hylin-Pul3, hylin-Pul4, prasin b-Pul, and some of their fragments are summarized in Table 1. Hylin-Pul3 exhibited the most potent antimicrobial activity against the Gram-positive bacteria *S. aureus*, with a MIC concentration of 14 μM. Hemolysis assay of hylin-Pul3 showed less than 2% hemolytic activity on human red blood cells (RBCs) when assessed at concentrations corresponding to *S. aureus* MIC, indicating a selective effect towards bacteria cells.

Table 1. Antimicrobial activity determination of peptides from the skin of B. pulchella.

Name	*Sequence*	*MIC*			
		E. coli		*S. aureus*	
		μg/ml	*μM*	*μg/ml*	*μM*
hylin-Pul1	FLGALIPAITGLIGGLINR-NH_2	200	102	200	102
ylin-Pul1(1-11)	FLGALIPAITG	≥ 800	N/D	≥ 800	N/D
hylin-Pul1(12-19)	LIGGLINR-NH_2	≥ 800	N/D	≥ 800	N/D
hylin-Pul2	FLGALIPAAIGLISGLIKK-NH_2	200	102	400	205
hylin-Pul2(12-19)	LISGLIKK-NH_2	≥ 800	N/D	≥ 800	N/D
hylin-Pul3	FLGALIPAIAGAIGGLIRK-NH_2	200	108	25	14
hylin-Pul3(1-11)	FLGALIPAIAG	≥ 800	N/D	≥ 800	N/D
hylin-Pul3(12-19)	AIGGLIRK-NH_2	≥ 800	N/D	≥ 800	N/D
hylin-Pul4	FFGALIPAVAGAIGGFFRK-NH2	100	52	50	26
prasin b-Pul	GALEIFKKYRLPKCF-NH_2	200	110	400	221

N/D: not determined

The adoption of a cationic amphipathic α-helix conformation allows an efficient interaction with bacterial outer membranes, as observed for several AMPs. Here, the adoption of such structure by the novel hylin peptides is greatly influenced by their environment. This was evidenced by disordered conformations of all mature hylin-Pul peptides in aqueous solution and a shift to helical conformations in the presence of LUVs mimicking prokaryotic and eukaryotic membranes. Noteworthy, higher fractional helicity values (*f*H) were observed when peptides interacted with vesicles containing anionic head groups (DMPG:DMPC).

Taken together, far-CD and dynamic light scattering data indicate that, although peptides in helical conformation co-absorb and are selectively bound regardless of membrane composition, the subsequent hydrophobic interaction can alter membrane structure to different degrees depending on membrane composition [9].

In conclusion, we present the identification, synthesis, and biological activities of novel hylin peptides isolated from the skin secretion of *Boana pulchella*. Their antimicrobial activity, as well as their interaction with lipid vesicles, indicates a nonspecific mode of interaction with bacterial membranes. Thus, the hylin peptides from *Boana pulchella* show the typical mechanism of action of α-helix amphibian peptides in bacterial membranes. Our findings highlight the role of membrane composition in inducing a stable conformation and activity of these AMPs against bacteria.

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