Toxicon 99 (2015) 16–22

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Identification and characterization of an antimicrobial peptide of *Hypsiboas semilineatus* (Spix, 1824) (Amphibia, Hylidae)

Lorena Nacif-Marçal [a], Gracielle R. Pereira [a], Monise V. Abranches [a], Natália C.S. Costa [a], Silvia A. Cardoso [a], Eduardo R. Honda [b], Sérgio O. de Paula [a], Renato N. Feio [c], Leandro L. Oliveira [a, *]

[a] Federal University of Viçosa, Departament of General Biology, Av. P.H. Rolfs s/n, 36570-000 Viçosa, MG, Brazil
[b] Research Center for Tropical Medicine – CEPEM, BR 364, km 4.5, 78900-970 Porto Velho, RO, Brazil
[c] Federal University of Viçosa, Departament of Animal Biology, Av. P.H. Rolfs s/n, 36570-000 Viçosa, MG, Brazil

ARTICLE INFO

Article history:
Received 1 September 2014
Received in revised form
28 February 2015
Accepted 11 March 2015
Available online 12 March 2015

Keywords:
Anuran
Anti-bacterial
Gram-positive
Synthetic peptide

ABSTRACT

The multidrug-resistant bacteria have become a serious problem to public health. In this scenery the antimicrobial peptides (AMPs) derived from animals and plants emerge as a novel therapeutic modality, substituting or in addition to the conventional antimicrobial. The anurans are one of the richest natural sources of AMPs. In this work several cycles of cDNA cloning of the skin of the Brazilian treefrog *Hypsiboas semilineatus* led to isolation of a precursor sequence that encodes a new AMP. The sequence comprises a 27 residue signal peptide, followed by an acidic intervening sequence that ends in the mature peptide at the carboxy terminal. The AMP, named Hs-1, has 20 amino acids residues, mostly arranged in an alpha helix and with a molecular weight of 2144.6 Da. The chemically synthesized Hs-1 showed an antimicrobial activity against all Gram-positive bacteria tested, with a range of 11–46 μM, but it did not show any effect against Gram-negative bacteria, which suggest that Hs-1 may have a selective action for Gram-positive bacteria. The effects of Hs-1 on bacterial cells were also demonstrated by transmission electron microscopy. Hs-1 is the first AMP to be described from *H. semilineatus*.

1. Introduction

The emergence of multiple drug-resistant strains of pathogenic bacteria has become a serious problem to public health that requires novel therapeutic modalities. Most conventional antibiotics act by interfering in a specific manner with bacteria homeostasis, which requires a period of few days for disabling. These mechanisms inhibit process that is not only essential for bacterial growth but also introduces extreme selection pressure for resistant bacteria (Smith and Romesberg, 2007). Besides, under such circumstances the bacteria morphology is normally preserved and a bacterium that is initially sensitive to the drug can develop resistance through mechanisms such as preventing the antibiotic from binding or entering the organism and producing an enzyme that inactivates the antibiotic or remodeling target molecules (Stark et al., 2002).

As substitutes or in addition to the currently used antimicrobial compounds the antimicrobial peptides (AMPs) derived from animals and plants have been widely researched. These peptides are part of the innate immune system of the organisms and represent the first-line of defense against invading pathogens, which is one of the most ancient and efficient components of host defense. Unlike traditional antibiotic agents, most of the AMPs kill microorganisms rapidly by disrupting and permeating the microbial membrane (Dathe et al., 2002), which is a mechanism that prevents a target organism from developing resistance to the peptide because the membrane redesign is probably a “costly” and improbable solution for most microbial species (Zasloff, 2002).

The dorsal skin of anurans is one of the richest natural sources of broad-spectrum AMPs (Rinaldi, 2002), which defend the naked skin against invasion by pathogenic microorganisms and protect from ingestion by predators. The anurans dermal granular glands synthesize and expel an extensive spectrum of bioactive molecules such as neuropeptides, alkaloids, proteins, biogenic amines and huge amounts of AMPs in response to stress or injury (Lazarus and Attila, 1993). As a rule, a given anuran species produces a unique

* Corresponding author.
E-mail address: leandro.licursi@ufv.br (L.L. Oliveira).

http://dx.doi.org/10.1016/j.toxicon.2015.03.006
0041-0101/

repertoire of AMPs, which is composed of peptides with different sizes, sequences, charges, hydrophobicity, three-dimensional (3D) structures, and spectrum of action (Charpentier et al., 1998; Nicolas et al., 2003; Vanhoye et al., 2003).

The AMPs of South American frogs from Hylidae family are derived from precursors whose amino terminal are highly conserved, but the carboxyl terminal domains, which correspond to mature peptides, are strongly diverse (Vanhoye et al., 2003). The conserved region contains a hydrophobic signal peptide, followed by an acidic propiece that ends by a typical prohormone processing signal Lys–Arg and a C-terminal AMP-encoding domain (Nicolas et al., 2003; Vanhoye et al., 2003).

Molecular cloning of Hylidae frog cDNAs can be performed to identify and isolate new AMPs. Therefore, the aim of our study was to identify AMPs from the skin of the Brazilian frog *Hypsiboas semilineatus* (Spix, 1824) (Amphibia, Anura, Hylidae) (Frost, 2013) that has never been researched before. We have reported here the molecular cloning of cDNAs encoding AMPs precursors in *H. semilineatus* and the structural and functional analysis of the new discovered AMP named Hs-1.

2. Materials and methods

2.1. Animal

We used two specimens of *H. semilineatus* in this study. The frogs were captured from the forest fragments of Viçosa city by a specialized team of João Moojen Museum of Zoology of the Federal University of Viçosa, Brazil, according to the Brazilian Environmental Agency (IBAMA – Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renovavéis) under the license 10504-1.

2.2. Bacterial strains

The references strains of bacteria used in the biological assays are from the American Type Culture Collection (ATCC; Rockville, MD, USA) and include the Gram-positive bacteria methicillin-resistant *Staphylococcus aureus* (ATCC 33591), *Bacillus cereus* (ATCC 14579), *Bacillus subtilis* (ATCC 23858), *Listeria monocytogenes* (ATCC 7644) and the Gram-negative bacteria *Citrobacter freundii* (ATCC 8090), *Enterobacter sakazakii* (ATCC 29004), *Escherichia coli* (ATCC 29214), *Moraxella catarrhalis* (ATCC 25238), *Proteus vulgaris* (ATCC 13315), *Salmonella enterica* (ATCC 14028) and *Shigella flexneri* (ATCC 12022).

2.3. Screening of cDNAs encoding AMPs

After capturing them, the specimens were immediately euthanized by lethal injections of xylocaine in the ventral region. The specimens were processed in two independent experiments. The dorsal skins were removed surgically and homogenized in TRIzol reagent (Invitrogen). Total RNA was isolated directly from the skin homogenate and cDNA was synthesized using the enzyme M-MLV reverse transcriptase (SIGMA) and an oligo(dT)-anchor primer (5′-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTT-3′). The cDNA amplification reactions employed a degenerate 5′-primer (5′-ATGGCTTTCCTGAARAARTCBCTTTTY-3′) that was designed based on the highly conserved 5′-signal regions of previously characterized AMPs cDNAs of anurans of Hylidae's family, and the 3′-anchor primer. Polymerase chain reaction (PCR) cycling procedures were performed as follows: initial denaturation of 94 °C for 300 s, 35 cycles of 94 °C for 60 s, 56 °C for 45 s, 72 °C for 60 s and one cycle of 72 °C for 10 min. The PCR products were gel-purified using a Wizard SV Gel and PCR Clean-up System (Promega) and they were cloned using the InsTAclone PCR Cloning Kit (Fermentas) and used to transform competent TOP10 *E. coli*. The transformants were amplified with universal M13 primers, the plasmids of the positive clones were extracted with PureYield Plasmid Midiprep System (Promega), and both the strands of the plasmids were sequenced (Macrogen, Korea). The amino acids sequences were deduced from cDNAs sequences and PEPTIDES 2.0 (USA) chemically synthesized the corresponding peptide of interest. Prior to biological tests, the lyophilized peptide was diluted in a solution of 50% Dimethyl sulfoxide (DMSO) to a 1 mg/mL final concentration.

2.4. Antimicrobial assays

Minimum inhibitory concentration (MIC) of synthetic Hs-1 were determined by a standard microdilution method (Ferraro and Wikler, 2009) using 96-well microtiter cell-culture plates and were taken as the lowest concentration of peptide where no visible growth was observed. Serial dilutions of the peptide in Mueller–Hinton broth (50 μL) were mixed with an inoculum (50 μL of 10^6 CFU/mL) from a log-phase culture of reference strains from ATCC. The bacteria were incubated aerobically for 20 h at 37 °C and the absorbance at 600 nm of each well was determined using a microtiter plate reader. Serial dilutions of the broad-spectrum antibiotic gentamicin and of 50% DMSO were used as controls for the antimicrobial assays. The experiments were performed three times with triplicates.

2.5. Cytolytic assays

The cytotoxicity of the peptide was evaluated in human erythrocytes and in leukocytes from a healthy donor. The blood was collected in citrate buffer 3.8% (pH 7.4) and separated by centrifugation. To determine the hemolytic activity, serial dilutions of the peptide Hs-1 in 0.85% saline (50 μL) were incubated with a 2% suspension of erythrocytes (50 μL) in wells of U-shaped bottom plates and incubated for 24 h at room temperature. The absorbance of the supernatant was measured at 450 nm. To evaluate the cytotoxicity of the peptide in nucleated cells, 1×10^5 leukocytes were exposed to serial dilutions of the peptide in Roswell Park Memorial Institute medium (RPMI) 1640 and incubated for 24 h at 37 °C. The leukocyte's viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method (Levitz and Diamond, 1985). A parallel incubation in the presence of 1% v/v Triton X-100 and saline or RPMI medium were used as controls to determine the absorbance associated with respectively 100% and 0% of cytolysis, respectively. The cytotoxicity of 50% DMSO was also measured. The median toxic dose (LC_{50}) value was taken as the mean concentration of peptide producing 50% of cytolysis (Conlon et al., 2007a, 2008). To assess selectivity of the peptide, we calculated its therapeutic index ($TI = LC_{50}/MIC$) (Y Chen et al., 2005; Conlon et al., 2008). The experiments were performed three times with triplicates.

2.6. Structural and physiochemical analysis of Hs-1

The prediction of the secondary structure of Hs-1 was performed using the self-optimized prediction method with alignment (SOPMA) method (Combet et al., 2000) and the PEP-FOLD server. The 3D schematic representations were visualized with Jmol (www.jmol.org). The two-dimensional projection of the amino acids in the alpha helix was visualized by the Schiffer–Edmundson helical wheel projection (Schiffer and Edmundson, 1967). The peptide parameters, including theoretical molecular weight, pI, net charge and grand average of hydropathicity (GRAVY) were computed by ProtParam (http://web.expasy.org/cgi-bin/protparam/protparam). The thermal stability of the antimicrobial