LORENA NACIF MARÇAL

IDENTIFICAÇÃO E CARACTERIZAÇÃO DE UM PEPTÍDEO ANTIMICROBIANO DE *Hypsiboas semilineatus*

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de *Magister Scientiae*.

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APROVADA: 24 de janeiro de 2013.

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Evandro Watanabe Silvia Almeida Cardoso

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Leandro Licursi de Oliveira
(Orientador)

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A mente que se abre a uma nova idéia
jamais voltará ao seu tamanho original.

Albert Einstein

SUMÁRIO

RESUMO

MARÇAL, Lorena Nacif, M.Sc., Universidade Federal de Viçosa, janeiro de 2013. **Identificação e caracterização de um peptídeo antimicrobiano de *Hypsiboas semilineatus.*** Orientador: Leandro Licursi de Oliveira. Co-orientadores: Sérgio Oliveira de Paula e Eduardo Rezende Honda.

Os antibióticos revolucionaram o tratamento das doenças infecciosas, especialmente por terem como alvos moléculas e processos que são essenciais à vida bacteriana. Mas, o uso extensivo e indiscriminado destes agentes antibacterianos também introduziu uma grande pressão seletiva nas espécies e a consequente emergência de micro-organismos resistentes. Os métodos tradicionais de descoberta de antibióticos não foram capazes de acompanhar a evolução dessa resistência, fato que alertou para a necessidade de desenvolvimento de novos agentes antimicrobianos. Os peptídeos antimicrobianos (PAM) derivados de animais e plantas têm sido amplamente estudados como substitutos dos compostos antimicrobianos convencionais. A maioria dos PAMs atua ao nível da membrana plasmática do micro-organismo, interferindo na sua estrutura e permeabilidade, mecanismo o qual previne que o micro-organismo alvo desenvolva resistência ao peptídeo. A pele de anuros (sapos, pererecas e rãs) é uma generosa fonte de PAMs citolíticos de amplo espectro, os quais defendem o animal contra infecções e ingestão por predadores. O objetivo deste estudo foi identificar e caracterizar peptídeos antimicrobianos da pele de *Hypsiboas semilineatus*, anuro endêmico da Mata Atlântica, Brasil. A sequência de aminoácidos de um novo peptídeo antimicrobiano derivado de *Hypsiboas semilineatus* foi identificada por ferramentas de clonagem molecular e quimicamente sintetizada. Análises estruturais e físico-químicas revelaram que o PAM sintético, nomeado Hs-1, consiste de uma cadeia linear de 20 resíduos de aminoácidos (FLPLILPSIVTALSSFLKQG) a qual se estrutura em uma alfa-hélice com 2 caudas terminais. Hs-1 tem um peso molecular de 2.144,6 Daltons, pI teórico de 8,75, carga líquida +1 e média total de hidrofobicidade 1,275. As concentrações inibitórias mínimas de Hs-1 foram determinadas pelo método de microdiluição padrão e identificadas como a menor concentração do peptídeo na qual nenhum crescimento visível foi observado. O peptídeo Hs-1 inibiu o crescimento de todas as bactérias gram-positivas analisadas, com um intervalo de

ação de 11-46μM, mas não apresentou efeitos detectáveis contra nenhuma bactéria gram-negativa, o que sugere que Hs-1 possa ter uma ação seletiva para bactérias gram-positivas. Nas mesmas concentrações, Hs-1 não apresentou efeito citotóxico sobre eritrócitos humanos ou leucócitos. Os mecanismos de ação de Hs-1 foram investigados por microscopia eletrônica de transmissão e as imagens obtidas sugerem que Hs-1 possue um efeito bactericida direto, provavelmente por agir ao nível da membrana celular. Estas propriedades qualificam Hs-1 como uma molécula anfipática de bom potencial terapêutico. Mais estudos envolvendo o peptídeo Hs-1 serão necessários para a melhor caracterização de suas propriedades e ações biológicas e para que, finalmente, sua inicialização em ensaios clínicos seja considerada.

ABSTRACT

MARÇAL, Lorena Nacif, M.Sc., Universidade Federal de Viçosa, January, 2013. **Identification and characterization of an antimicrobial peptide of *Hypsiboas semilineatus.*** Adviser: Leandro Licursi de Oliveira. Co-Advisers: Sérgio Oliveira de Paula and Eduardo Rezende Honda.

Antibiotics have revolutionized the treatment of infectious disease because they inhibit specific process that are essential for bacterial life but the indiscriminate and extensive use of these antibacterial agents have also introduced a selective pressure and consequent emergence of resistant pathogens. Traditional methods of antibiotic discovery have failed to keep pace with the evolution of this resistance, which suggests that new antimicrobial agents may be required. The antimicrobial peptides (AMPs) derived from animals and plants have been extensively researched as substitutes for currently used antimicrobial compounds. Most of the AMPs kill microorganisms rapidly by disrupting and permeating the microbial membrane, mechanism that prevents a target organism from developing resistance to the peptide. The anurans' skin (frogs and toads) is a generous source of broad-spectrum cytolytic AMPs, which defend the animal against infections and also protect from ingestion by predators. The objective of this study was to identify and to characterize antimicrobial peptides of the skin of the frog *Hypsiboas semilineatu*s, endemic to Atlantic Forest, Brazil. By molecular cloning tools the amino acid sequence of one new antimicrobial peptide from *Hypsiboas semilineatus* was identified and it was chemically synthesized. The structural and physiochemical analysis of the synthetic AMP, named Hs-1, revealed that it consists of a linear polypeptide chain of 20 amino acid residues (FLPLILPSIVTALSSFLKQG) forming an alpha-helix with 2 terminal tails. Hs-1 has a molecular weight of 2,144.6 Daltons, theorical pI of 8.75, GRAVY of 1.275 and net charge +1. Minimum Inhibitory Concentrations of Hs-1 were determined by a standard microdilution method and were taken as the lowest concentration of peptide where no visible growth was observed. The peptide Hs-1 inhibited the growth of all gram-positive bacteria analysed, with a range of 11-46μM, but it didn't show effect against any gram-negative bacteria, which suggest that Hs-1 may have a selective action for gram-positive bacteria. In this same concentration Hs-1 did not show any cytotoxic effect over human erythrocytes or Leukocytes.

The mechanisms of action of Hs-1 were investigated by transmission electron microscopy and the images obtained suggest that Hs-1 has a direct bactericidal effect probably for acting at membrane level**.** These properties qualify Hs-1 as an amphipathic molecule with good therapeutic potential. Further studies involving peptide Hs-1 will be needed to better characterize their properties and biological actions, and to finally discern about its startup in clinical trials.

Identification and characterization of an antimicrobial peptide of *Hypsiboas semilineatus*

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ABSTRACT

Several cycles of cDNA cloning of the skin of the Brazilian treefrog *Hypsiboas semilineatus* allowed the isolation of a precursor sequence encoding a new antimicrobial peptide (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 a molecular weight of 2,144.6 Daltons. The chemically synthesized Hs-1 showed an antimicrobial activity against all gram-positive bacteria tested, with a range of 11-46μM, but it didn't show effect against any 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 described from *Hypsiboas semilineatus*.

Keywords**:** AMP, Anuran, Anti-bacterial, Gram-positive.

1. INTRODUCTION

The emergence of multiple drug-resistant strains of pathogenic bacteria has become a serious problem of health public that requires novel therapeutic modalities. The most conventional antibiotics act by interfering in a specific manner with bacteria homeostasis, requiring a period of days for disable it. These mechanisms inhibit process that are essential for bacterial growth but also introduces extreme selection pressure for resistant bacteria [1]. 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, producing an enzyme that inactivates the antibiotic or remodeling target molecules [2].

As substitutes or as addition to 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 they represent the first-line defense against invading pathogens, 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 [3], mechanism that prevents a target organism from developing resistance to the peptide, since the membrane redesign is probably a "costly" and improbable solution for most microbial species [4]. Although the exact mechanisms by which the bacterial cell death occurs are not entirely clear, some models like the classic Shai-Matsuzaki-Huang is applicable to a range of cytolytic peptides of frog skin [5-7]. The model assumes that the net positive charge of AMPs allows preferential binding to the negatively charged phospholipids headgroups of bacterium outer surface. This initial interaction is followed by peptide insertion into the membrane with consequent displacement of lipids, alteration of membrane structure and permeabilization of bacterial cell, which may be by pore formation or a detergent-like effect. The AMP activity is not limited to the mechanisms of membrane and/or cell rupture but also extends to intracellular targets [8, 9].

The dorsal skin of anurans is one of the richest natural sources of broad-spectrum antimicrobial peptides [10], which defend the naked skin against

invasion by pathogenic microorganisms and also 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 [11]. As a rule, a given anuran species produces a unique repertoire of antimicrobial peptides, which is composed of peptides with different sizes, sequences, charges, hydrophobicity, three-dimensional structures and spectrum of action [12-14].

Until now several hundred of AMPs were identified in the skins of anurans species belonging to diverse families such as Hylidae [15], Leptodactylidae [16], Ranidae [17] and other. Based on the amino acid sequence and secondary structure, the AMPs are named and classified into families, which can be reviewed in an online database containing a large part of all reported molecules (http://aps.unmc.edu/AP/main.php). These peptides show a marked degree of variability and there are no conserved structural motifs responsible for its activity, but the vast majority of the frog skin AMPs are characterized by a preponderance of cationic and hydrophobic amino acids, which are spatially organized in discrete sectors of the molecule, with propensity to adopt an amphipathic α-helical conformation in the environment of a phospholipids vesicle or in a membrane-mimetic solvent [18, 19]. The cationicity contributes to their preferential binding to the negatively charged outer surface of bacterium, whereas the amphiphilicity permit incorporation into the target membranes. These characteristics confer activity and selectivity for these AMPs [3, 20].

The antimicrobial peptides of South American frogs from Hylidae family are derived from precursors, whose the amino-terminal are highly conserved but their carboxyl-terminal domains, that correspond to mature peptide, are strongly diverse [14]. 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 antimicrobial peptide-encoding domain [13, 14].

Molecular cloning of Hylidae frogs cDNAs can be performed to identify and isolate new AMPs. The aim of our study was to identify antimicrobial peptides in the skin of the Brazilian frog *Hypsiboas semilineatus* (Spix, 1824) (Amphibia, Anura, Hylidae) [21] that has never been researched before. We report here the molecular cloning of cDNAs encoding antimicrobial peptides precursors

in *H. semilineatus* and the structural and functional analysis of the new discovered antimicrobial peptide named Hs-1.

2. MATERIALS AND METHODS

2.1. Animal

For this work, we use two specimens of *Hypsiboas semilineatus*. The frogs were captured at forest fragments of Viçosa city, by a specialized team of the João Moogen 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. The experimental protocol used in this study was approved (0006/2011-I) by the Ethics Committee of the School of Biological and Health Sciences, and conforms with the Brazilian College for Animal Experimentation (COBEA) and with actual Brazilian legislation.

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 enteric*a (ATCC 14028) and *Shigella flexneri* (ATCC 12022).

2.2. Screening of cDNAs encoding antimicrobial peptides

After the capture the specimens was immediately euthanized by lethal injection of xylocaine in its 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 synthesized with 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 antimicrobial peptides cDNAs of anurans of Hylidae's family, and the 3'-anchor primer. PCR cycling procedures were carried out as follows: initial denaturation of 94°C for 300s, 35 cycles of 94°C for 60s, 56°C for 45s, 72°C for 60s 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) cloned using the InsTAclone PCR Cloning Kit (Fermentas) and used to transform competent TOP10 *Escherichia coli*. The transformants were amplified with universal M13 primers and the plasmids of the positive clones were extracted with PureYield Plasmid Midiprep System (Promega) and the both strand of the plasmids were sequenced (Macrogen, Korea). The amino acids sequences were deduced from cDNAs sequences and the corresponding peptide of interest was chemically synthesized by PEPTIDES 2.0 (USA). Prior to biological tests the lyophilized peptide was diluted in a solution of DMSO 50%.

2.3. Antimicrobial assays

Minimum inhibitory concentrations (MIC) of synthetic Hs-1 were determined by a standard microdilution method (National Committee for clinical Laboratory Standards, 2003) 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 (50µL) were mixed with an inoculum (50µL of 10^6 CFU/mL) from a log-phase culture of reference strains from the American Type. Reference strains of bacteria (ATCC) were incubated aerobically for 20h at 37°C and the absorbance at 600nm of each well was determined using a microtiter plate reader. Serial dilutions of the broad-spectrum antibiotic gentamicin and of DMSO 50% were used as controls for the antimicrobial assays. The experiments were performed two times with duplicates.

2.4. Cytolytic assays

The cytotoxicity of the peptide was evaluated in human erythrocytes and also 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 saline (NaCl 0.85% - 50µL) were incubated with a 2% suspension of erythrocytes (50µL) in wells of U-shaped bottom plates and incubated for 24h at room temperature. The absorbance of the supernatant was measured at 450 nm. To evaluate the cytotoxicity of the peptide in nucleated cells, 1 x 10^5 leukocytes were exposed to serial dilutions of the peptide in RPMI 1640 and incubated for 24h at 37°C. The leukocyte's viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] method. As controls, a parallel incubation in the presence of 1% v/v Triton-X-100 and saline or RPMI medium were carried out to determine the absorbance associated with respectively 100% and 0% of cytolysis. The cytotoxicity of DMSO 50% was also measured. The LC_{50} value was taken as the mean concentration of peptide producing 50% of cytolysis in two independent experiments with duplicates. To assess selectivity of the peptide, we calculated its therapeutic index (TI = LC_{50}/MIC).

2.5. Structural and physiochemical analysis of Hs-1

The prediction of the secondary structure of Hs-1 was performed using the SOPMA method [22] and the PEP-FOLD server. The 3D maps were visualized with Jmol [23]. The two-dimensional projection of the amino acids in the alpha helix was visualized by the Schiffer-Edmundson helical wheel projection [24]. The peptide parameters, including theorical 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 activity of Hs-1 was also determined. For this, 200µg/mL of Hs-1 in Mueller-Hinton broth was pre-incubated at various temperatures (50, 60, 70, 80, 90 e 100°C) and cooled before it was incubated with an inoculum (5 x 10^4 CFU) from a log-phase culture of *Staphylococcus aureus* (ATCC 33591). Bacteria were incubated aerobically for 20h at 37°C and the absorbance at 600nm of each well was determined using a microtiter plate reader. As control, a parallel incubation of Hs-1 at room temperature was performed for its 100% (full) antimicrobial activity.

2.6. Transmission electron microscopy (TEM)

The effects of the interaction between Hs-1 and bacteria were observed from the incubation of 5 x 10^4 CFU of *Staphylococcus aureus* (ATCC 33591) with 200µg/mL of synthetic Hs-1 for 2h and 3h at 37°C followed by the microscopy that was performed under standard operating conditions following negative staining with 2% Uracil in a Zeiss EM 109 transmission electron microscope. Cultures without peptides were used as negative control. The TEM was performed in three independent experiments.

3. RESULTS

3.1. Cloning of antimicrobial peptides cDNAs

To screening the possible sequences of cDNA coding for antimicrobial peptides we have used degenerate primers complementary to the highly conserved region of the signal peptide of antimicrobial peptides previously sequenced from other Hylidae's frog. The agarose gel analysis of the cDNA amplification resulted in a large band between 300 and 400 base pairs (data not shown). Following several rounds of cloning and cDNA sequencing one novel peptide precursor sequence was successfully obtained. This sequence was represented in 7 clones of a total of 54 clones sequenced. The deduced amino acid sequence of the cDNA encoding the putative peptide includes a N-terminal region encompassing 27 residues of the signal peptide followed by an acidic region containing several glutamic acid residues that terminates in a single copy of the mature antimicrobial peptide at the C-terminal. A typical-Lys-Arg (-KR-) cutting site for trypsin-like proteases, that is responsible for cleavage and release of the mature peptide, is located between acidic region and mature peptide (Figure 1). An NCBI-Blast and AMP database research revealed that this peptide, named Hs-1, is a novel and an unpublished antimicrobial peptide. The sequence analysis of Hs-1 in online *Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php)* revealed high levels of sequence similarity to other already described antimicrobial peptides of *Phyllomedusa hypochondrialis*: 54.54% of similarity with Phylloseptin 12, Phylloseptin H9 and Phylloseptin H10.

Figure 1. Nucleic and amino acid sequences of cDNA encoding the putative Hs-1 of *Hypsiboas semilineatus*. The nucleotides are in lowercase letters and the predicted amino acid sequence is given in capital letters above the nucleotide sequence. Numbers on the right indicate the positions of the nucleotides and amino acids. The putative signal peptides (solid-line), the acidic region (italic), the mature peptide (bold) and the stop codon (asterisk) are indicated.

3.2. Antimicrobial activity

The synthetic peptide Hs-1 was tested for its antimicrobial activity. The Minimum Inhibitory Concentrations (MIC) of the peptide Hs-1 against Gram-positive and Gram-negative bacteria are shown in Table 1. Hs-1 displayed relatively high potency (MIC≤ 46.6μM) against Gram-positive bacteria and *S. aureus* ATCC 33591 was the most sensitive strain (MIC=11.7μM), but Hs-1 showed no detectable effect to any Gram-negative bacterium tested. The DMSO50% solution was not toxic to any bacterial strains (Data not shown).

Table 1. Minimum Inhibitory Concentrations (MIC) against Gram-positive and Gram-negative bacteria of the synthetic peptide Hs-1 identified in the skin of the frog *Hypsiboas semilineatus*.

Microorganisms	**Hs-1 MIC (μM)**
Gram-positive bacteria	
Staphylococcus aureus ATCC 33591	11.7
Bacillus cereus ATCC 14579	23.3
Bacillus subtilis ATCC 23858	23.3
Listeria monocytogenes ATCC 7644	46.6
Gram-negative bacteria	
Citrobacter freundii ATCC 8090	NA
Enterobacter sakazakii ATCC 29004	NA
Escherichia coli ATCC 29214	NA
Moraxella catarrhalis ATCC 25238	NA
Proteus vulgaris ATCC 13315	NA
Salmonella enterica ATCC 14028	NA
Shigella flexneri ATCC 12022	NA

NA - not active at 187 μM (400μg/mL).

3.3. Cytolytic effect

The toxicity of Hs-1 was evaluated both in erythrocytes as in leukocytes. In general, Hs-1 had a moderate cytotoxicity activity (LC_{50} = 85.8µM), but had no significant cytolytic activity in the range of its antimicrobial activity (MIC = 11.7 – 46.6µM). The Hs-1 showed a good therapeutic index for *S. aureus* (TI = 7.3) but a lower for *B. cereus* (TI = 3.7), *B. subtilis* (TI = 3.7) and *L. monocytogenes* (TI = 1,8). The DMSO50% solution showed no cytotoxicity to both erythrocytes or leukocytes (Data not shown).

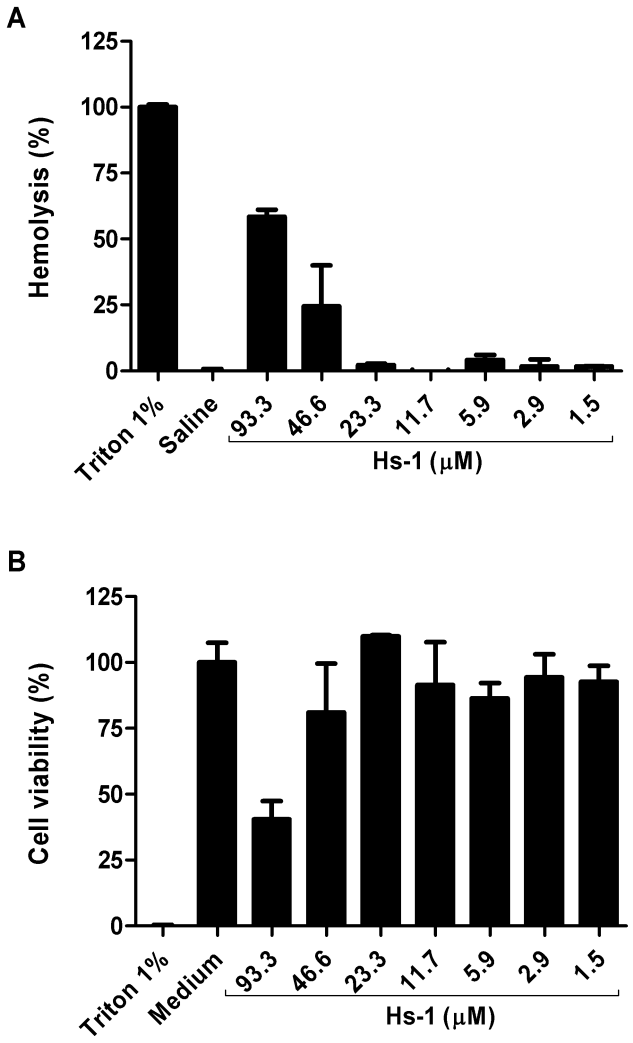

Figure 2. Cytolytic effects of Hs-1 on human erythrocytes and leukocytes. A) Red blood cells were treated with different concentrations of Hs-1 and its cytotoxicity was evaluated in comparison with triton X-100 (100% hemolysis) and saline (0% hemolysis). **B)** Leukocytes were treated with different concentrations of Hs-1 and its effects on viability were evaluated in comparison with triton X-100 (0% viability). RPMI medium was considered as 100% viability. Assays were performed in triplicate and the results represent the mean ± SEM.

3.4. Structural and physiochemical properties of Hs-1

Hs-1 consists of a polypeptide chain of 20 amino acid residues FLPLILPSIVTALSSFLKQG. Secondary structural prediction analysis showed that Hs-1 is structured primarily in an alpha-helix and has 2 terminal tails composed of random coil and beta turn structures (Fig.3; Fig.4A). A Schiffer-Edmudson projection of Hs-1 revealed a spatial segregation of polar and apolar residues onto the opposite faces of the helix (Fig.4B), confirming the amphipathic character of the helix. According to ProtParam, Hs-1 has a molecular weight of 2,144.6 Daltons, theorical pI of 8.75, GRAVY of 1.275 and net charge +1. In the thermostability screening assay the peptide Hs-1 was thermostable in temperatures up to 60°C (data not shown).

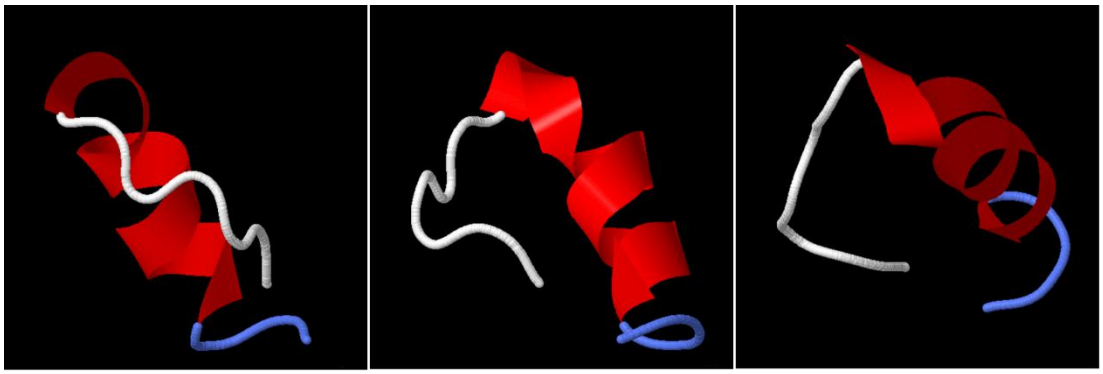

Figure 3. 3D representation of Hs-1 secondary structure. Alpha-helix, random coil and beta turn structures are respectively in red, white and blue. The alpha-helix is represented in different angles: lateral (left), frontal (middle) and superior (right).

A) SOPMA prediction

```
Hs-1    FLPLILPSIVTALSSFLKQG
        ccccccchhhhhhhhhhhtt
```

Alpha-helix (h): 60%
Beta turn (t): 10%
Random coil (c): 30%

B) Schiffer-Edmundson projection

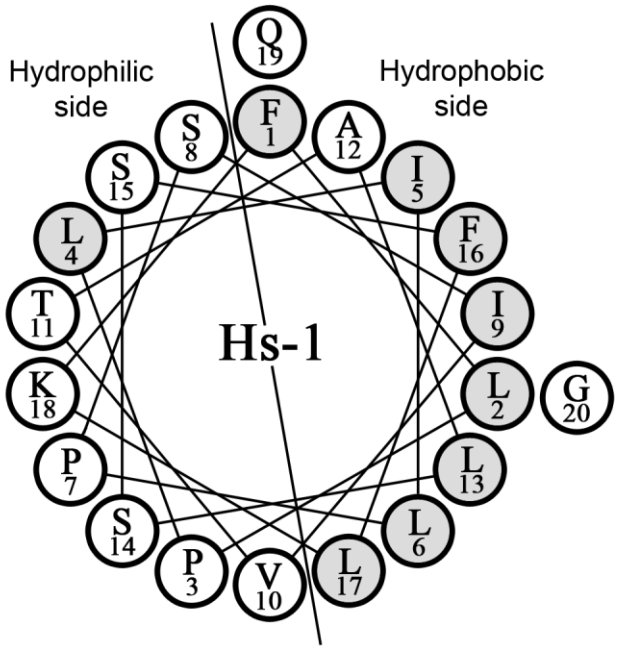

Figure 4. SOPMA prediction and Schiffer-Edmundson projection of Hs-1. (A) The SOPMA prediction using window with: 17, similarity threshold: 8 and number of states:4. **(B)** A Shiffer-Edmundson helical wheel projection illustrating the amphipathic nature of the alpha-helix. Hydrophobic residues are in gray circles and hydrophilic residues are in white circles. Each amino acid residue is numbered according to their position in the primary sequence of the peptide Hs-1.

3.5. Transmission electron microscopy

To investigate the possible mechanisms of action of Hs-1, the peptide was incubated with *S. aureus* and monitored by TEM. Control treatment of *S.aureus* with no peptide exhibited intact electron-dense cells with normal shaped (spherical) and smooth surface (Fig.5A and 5D). In contrast, bacteria treated by Hs-1 showed altered morphology with roughness and irregular surfaces. With 2 hours of exposition to the AMP, bacteria have already presented damage indicative of cell death: swelling, leakage of cellular contents, blebbing of the plasma membrane (Fig 5B-C). After 3 hours of exposure, that damage cited above were more pronounced and even some cells are collapsed (Fig 5E-F). These

micrographs suggest that Hs-1 had a direct bactericidal effect probably for acting at membrane level.

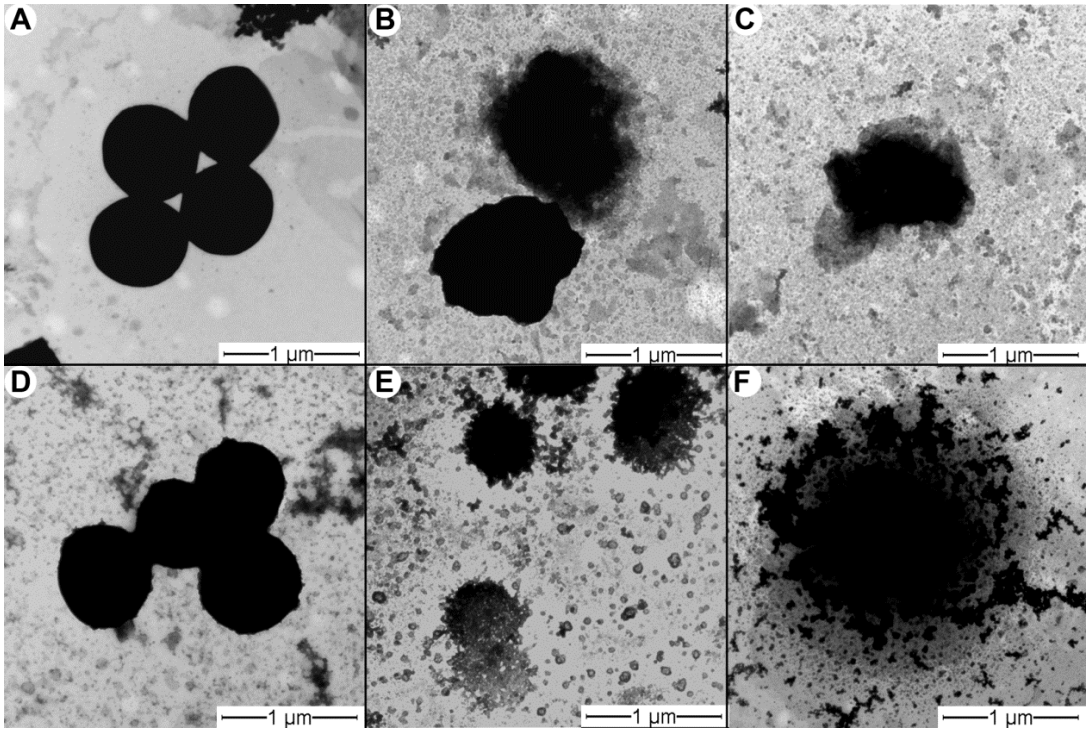

Figure 5. Ultrastructure of *Staphylococcus aureus* treated with Hs-1. Bacteria were exposed to 200µg/mL of Hs-1 for 2h **(B-C)** and 3h **(E-F)**. Control Bacteria were not exposed to the peptide **(A,D)**. The scale bar is represented.

4. DISCUSSION

The Brazil covers a variety of biomes and holds the largest biodiversity on the planet. However, due to the unknowing of both the number of existing species as the role that they play in natural ecosystems and its potential use to humans, many species are still poorly documented. The Brazilian fauna is extremely rich in anurans species, many of which are endemic. It is clear the need for studies that shed light to the characteristics and particularities of these species.

In recent years, the antimicrobial peptides derived from anurans' skin have become a pivotal model for the discovery of new antibiotics agents. The frog dermal glands synthesize and expel a plethora of biologically active components thought to be involved in the defense of the naked skin against microbial invasion and to aid in wound repair. This work reports the identification and characterization of Hs-1, an AMP from the skin of *Hypsiboas semilineatus.* This treefrog specie is endemic to Atlantic Forest, a rich biome present in Brazil and until now no records of AMPs derived from this species are available.

The cDNA cloning was the method utilized here for the screening of AMPs. All peptides are made from precursor genes that in most of the time can be cloned. As nature rarely provides enough material to all analysis required for a proper characterization of a peptide, the molecular cloning is a fast and efficient alternative for obtaining antimicrobial peptides' sequence. The deduced amino acid sequence of the cDNA indentified in this work has the same overall architecture that other precursors of Hylidae's antimicrobial peptides: a well conserved N-terminal region of approximately 50 residues encompassing the signal peptide and an acidic propiece followed by a markedly variable C-terminal domain that corresponds to the mature antimicrobial peptide [12-14, 25-27] These extensive conservation of the N-terminal sequence and the great similarities of the transcript organization support the hypothesis that the antimicrobial peptides of anurans originate from a common ancestral gene that have undergone for repeated duplications over the years [14].

The mature peptide of Hs-1 ends in a glycine residue suggesting that this is an amide donor for C-terminal amidation during post-translational modification. This kind of modification occurs commonly in AMPs and it normally increases the antimicrobial activity of a given peptide by increasing the overall positive

charge [10]. The Hs-1 was chemically synthesized without the C-terminal amidation and its activity could have been underestimated. Further tests will be done to verify this issue.

Hs-1 showed high similarity (54.54%) with AMPs of the Phylloseptins family. Phylloseptins were characterized in the skin secretion of *Phyllomedusa azurea* (family Hylidae, subfamily Phyllomedusinae) and this family comprises cationic peptides that have a primary structure consisting of 19-21 amino acid residues (1.7-2.1 kDa) and a broad spectrum of antimicrobial activity with low hemolytic effect [28]. The physiochemical properties of Hs-1 satisfy the requirements of the family, but its spectrum of activity is more selective. To date, the description of AMPs of the subfamily Hylinae is scarce and many species have been classified as non-producing of AMPs [18, 29]. The few studies that highlighted the presence of AMPs in species representative of Hylinae made their own classification of the AMP family, based on the species name [30-33]. For now Hs-1 is the only AMP representative of *H. semilineatus* (family Hylidae, subfamily Hylinae) and still does not characterize a new family *per si* and neither is similar enough to be classified in an existing family.

The antimicrobial assays suggest that Hs-1 has a selective activity against Gram-positive bacteria, since all Gram-positive strains tested were sensitive to it whereas all the Gram-negative were resistant. The MIC values (11-46µM) are very good if compared to other anurans AMPs [19, 30, 34-36], especially for *S. aureus* (11.7µM) that is a clinically important pathogen, the major involved in nosocomial infections. Because of high incidence, morbidity, and antimicrobial resistance, *S. aureus* infections are a growing concern to public health [37]. The *S. aureus* ATCC 33591 used in this work is a methicillin-resistant strain and we believe that it can give us a clue about the possible action of Hs-1 in other multi-resistant strains.

The ability of a peptide to produce cell death is the result of a complex interrelationship of factors involving conformation, charge, hydrophobicity and amphipathicity [38], but two common and functionally important requirements are a net cationicity that facilitates interaction with negatively charged microbial surfaces and the ability to assume amphipathic structures that permit incorporation into microbial membranes. These characteristics do not impose a rigorous primary or secondary structural organization but the alpha-helical peptides are among the

most abundant and widespread in nature [20]. According to SOPMA prediction, 60% of the amino acids residues of Hs-1 are structured in alpha-helix and it is highly amphipathic, like demonstrated by the Schiffer-Edmundson helical wheel projection. The amphipathicity of the helix is almost 100%, it has only some residues (Leu4, Gln19 and Gly20) occupying sides of opposite polarity. But, if we join the Schiffer-Edmundson helical with the SOPMA prediction we can see that these residues do not compose the helix indeed. Helix-stabilizing amino acid residues such as leucine, alanine and lysine [39], are well represented in Hs-1, especially the apolar leucine that balance the hydrophobic side. On the other hand glycine and proline that are typically constraints on the formation of an alpha-helix are present in random coil and beta-turn structures of N- and C-terminal regions of Hs-1. In proline, the nitrogen atom is part of a rigid ring that block the rotation of the residue and glycine has more conformational flexibility than the other amino acid residue. Thus a proline residue introduces a kink in an alpha-helix and glycine prevents its conformational stability.

The selectivity of Hs-1 to Gram-positive bacteria can be explained for that amphipathic alpha-helix that predominates in Hs-1. It has been assumed that a stabilized amphipathic alpha-helical conformation is an absolute requirement for antimicrobial activity against Gram-positive bacteria whereas the structural requirements for activity against Gram-negative bacteria are less stringent [38]. Several strains of Gram-negative are susceptible to both non-helical and scrambled peptides which suggest that antimicrobial activity against Gram-negative are mainly modulated by electrostatic interactions [38, 40]. Hs-1 exhibits one single positively charged amino acid (Lysine) so this low cationicity probably limits its activity against Gram-negative bacteria.

The transmission electron microscopy showed that Hs-1 has a direct antibacterial killing and the target site is the cytoplasmic membrane. This physical integrity of the lipid bilayer is disrupted and many cellular damage can be visualized. This is in agreement with the mode of action proposed and observed for the vast majority of anurans antimicrobial peptides [35, 41] however we do not have conclusive information about the exact mechanism by which the membrane is disrupted (membrane thinning, transient poration or micellization in a detergent-like). The antimicrobial killing capacity of Hs-1 probably occurs between 2h and 3h of exposure since the cellular lesions of *S. aureus* observed

after 2 hours of exposure to Hs-1 continued to accumulate with 3 hours of treatment. Indeed, in many instances cellular damage occurs at the same rate as that of the killing but also has been demonstrated that cellular injuries lags behind the time required for antimicrobial killing [8].

Despite the impact of antimicrobial activity, the toxicity tests are a critical step for the ranking of a new peptide as a potential therapeutic agent. Host toxicity is preponderantly evaluated in terms of hemolytic activity, although the susceptibility of erythrocytes is not necessarily extendable to other animal cells [20]. In this context, we tested Hs-1 in erythrocytes and in the nucleated leukocytes. Although Hs-1 did not exhibit a significant cytolytic activity in the range of MIC, we observed that the LC_{50} values are close of the MICs indicating a poor therapeutic index for *Bacillus.sp* and *Listeria.sp.*, on the other hand the high therapeutic index for *S.aureus* suggests that Hs-1 has a potential applicability for treatment of nosocomial infections.

It has been postulated that hemolysis depends primarily on amphipathic structure formation [38, 40]. Clearly the amphipathic structure of Hs-1 explains its cytotoxicity to mammalian cells. Additionally, Hs-1 also presents a considerable hydrophobicity (GRAVY 1.275), and a positive correlation between increasing hydrophobicity and mammalian cell toxicity has been demonstrated for several AMPs [38, 40, 42].

Selected amino acid substitutions can be a strategy to potentialize naturally-occurring AMPs. This kind of "design of peptides" has been extensively done [43] and it enables not only the optimization of the product as well a better cost planning. A simple strategy for optimization of Hs-1 can be the increasing of its cationicity while decreasing the amphipathicity of the helix by the substitution of apolar residues on the hydrophobic of the face by residues of Lysine [39]. Being more cationic the initial attraction of Hs-1 to the negatively charged microbial surfaces can increase whereas it prevents the interaction with the predominantly zwitterionic phosphatydilcholine and sphingomyelin phospholipids of the outer leaflet of membrane of mammalian cells.

5. CONCLUSION

Hs-1 is the first antimicrobial peptide reported of *Hypsiboas semilineatus*. This peptide consists of a predominant amphipathic alpha-helix and has a selective action for gram-positive bacteria probably for acting at membrane level**.** Despite its moderate hemolytic activity Hs-1 has a good therapeutic index against *S. aureus* and may be used as a template for development of alternative therapeutic agents.

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