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)

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A R T I C L E I N F O

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

A B S T R A C T

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*.

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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

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http://dx.doi.org/10.1016/j.toxicon.2015.03.006
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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′-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTTT-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

activity of Hs-1 was also determined. For this, 200 μg/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 incubation with an inoculum (5×10^4 colony forming unit (CFU)) from a log-phase culture of *S. aureus* (ATCC 33591). 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. A parallel incubation of Hs-1 at room temperature was used as control to check for its 100% antimicrobial activity (full activity).

2.7. Transmission electron microscopy (TEM)

The effects of the interaction between Hs-1 and bacteria were observed from the incubation of 5×10^4 CFU of *S. aureus* (ATCC 33591) with 200 μg/mL of synthetic Hs-1 for 2 h and 3 h at 37 °C, followed by observation under TEM, which 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. *S. aureus* (ATCC 33591) was chosen as the standard strain for microscopy as it is the most sensitive strain to Hs-1. The TEM was performed in three independent experiments.

3. Results

3.1. Cloning of AMPs cDNAs

To screening the possible sequences of cDNA coding for AMPs, we used degenerate primers complementary to the highly conserved region of the signal peptide of AMPs previously sequenced from other Hylidae's frog. The agarose gel analysis of the cDNA amplification showed a large band of 300–400 base pairs (data not shown). Following several rounds of cloning and cDNA sequencing, one novel peptide precursor sequence was successfully obtained. The deduced amino acid sequence of the cDNA encoding the putative peptide included an 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 AMP at the C-terminal. A typical Lys–Arg (-KR-) cutting site for trypsin-like proteases, which is responsible for cleavage and release of the mature peptide, is located between acidic region and mature peptide (Fig. 1). An NCBI-Blast and AMP database research revealed that this peptide, named Hs-1, is a novel and an unpublished AMP. The nucleotide sequence of Hs-1 was deposited in GenBank under the accession number KF147143. 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 known AMPs of *Phyllomedusa hypochondrialis*: 54.54% of similarity with Phylloseptin 12, Phylloseptin H9 and Phylloseptin H10 (data not shown).

Table 1
Minimum inhibitory concentration (MIC) against Gram-positive and Gram-negative bacteria of the synthetic peptide Hs-1 identified from the skin of the frog *H. semilineatus*.

Microorganisms	Hs-1 MIC (μM)
Gram-positive bateria	
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 (400 μg/mL).

3.2. Antimicrobial activity

The synthetic peptide Hs-1 was tested for its antimicrobial activity. The 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 ≤ 46.6 μM) against Gram-positive bacteria and *S. aureus* (ATCC 33591) was the most sensitive strain (MIC = 11.7 μM), but Hs-1 showed no detectable effect to any of the tested Gram-negative bacterium. The 50% DMSO solution was not toxic to any bacterial strains (data not shown). The Hs-1 antimicrobial activity and general characteristics were compared with other antimicrobial peptides from *Hypsiboas* species (Table 2).

3.3. Cytolytic effect

The toxicity of Hs-1 was evaluated both in erythrocytes as in leukocytes. In general, Hs-1 had a moderate cytolytic activity (LC_{50} = 82 μM), but had no significant cytolytic activity in the range of its antimicrobial activity (MIC = 11.7–46.6 μM). The Hs-1 showed a greater margin of safety for *S. aureus*, as demonstrated for its TI (TI = 7.3), than for *B. cereus* (TI = 3.7), *B. subtilis* (TI = 3.7) and *L. monocytogenes* (TI = 1.8). The 50% DMSO solution showed no cytotoxicity to both erythrocytes and leukocytes (Fig. 2A and B).

3.4. Structural and physicochemical 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 it is also composed of random coil and beta-turn structures (Figs. 3 and 4A). A Schiffer–Edmudson projection of Hs-1 revealed a spatial

```
atggctttcctgaaaaaatcccttttccttgtactattccttggattggtttccctgtcc   60
 M  A  F  L  K  K  S  L  F  L  V  L  F  L  G  L  V  S  L  S    20
atctgtgaagaagagaaaaaagaagaagaggagaaggaagaggaagaaaatgcggaaagt  120
 I  C  E  E  E  K  K  E  E  E  E  K  E  E  E  E  N  A  E  S    40
aaagaaaagagatttctaccactaattctaccctcaattgtaacagctctcagtagtttt  180
 K  E  K  R  F  L  P  L  I  L  P  S  I  V  T  A  L  S  S  F    60
ttaaaacaggggttgataaaatgtaacgttctatctgtgaggagcacattatcattagttg  240
 L  K  Q  G  *                                                 64
tgccagacatataataaaacatattaaagaagctgttcctcaaaaaaaaaaaaaaaaaa   359
```

Fig. 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 in uppercase 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.

Table 2
General characteristics of antimicrobial peptides derived from *Hypsiboas* species.

Organism	Peptide	Sequence	Length	Mass	MIC (μM)		Ref.
					S. aureus	*E. coli*	
H. semilineatus	Hs-1	FLPLILPSIVTALSSFLKQG	20	2.144.6	11.7	ND	This work
H. albopunctatus	Hylin a1	IFGAILPLALGALKNLIK	18	1864.19	8	32	Castro et al., 2009
H. biobeba	Hylin-b1	FIGAILPAIAGLVHGLINR	19	1945.3	ND	ND	Castro et al., 2005
H. biobeba	Hylin-b2	FIGAILPAIAGLVGGLINR	19	1865.2	ND	ND	Castro et al., 2005
H. punctata	Hylaseptin-P1	GILDAIKAIAKAAG	14	1311.80	6.1–8	24.4–32	Prates et al., 2004
H. raniceps	Rsp-1	AWLDKLKSLGKVVGKVALGVAQNYLNPQQ	29	ND	20	5	Magalhães et al., 2008
H. pulchellus	P1-Hp-1971	TKPTLLGLPLGAGPAAGPGKR-NH2	21	1971.1	8	16	Siano et al., 2014
H. pulchellus	P2-Hp-1935	KLSPSLGPVSKGKLLAGQR-NH2	19	1935.2	66	3	Siano et al., 2014
H. pulchellus	P3-Hp-1891	RLGTALPALLKTLLAGLNG-NH2	19	1891.2	17	17	Siano et al., 2014

ND-Not determined.

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 2144.6 Da, theorical pI of 8.75, GRAVY of 1.275, and a net charge +1. In the thermostability screening assay, the peptide Hs-1 was thermostable in temperatures up to 60 °C (data not shown).

3.5. TEM

To investigate the possible mechanisms of action of Hs-1, the peptide was incubated with *Staphylococcus aureus* and monitored under TEM. Control treatment of *S. aureus* with no peptide exhibited intact electron-dense cells with a normal shape (spherical) and smooth surface (Fig. 5A and D). In contrast, bacteria treated by Hs-1 showed altered morphology and irregular surfaces. With 2 h of exposition to the AMP, bacteria presented damage that was indicative of cell death, such as swelling, leakage of cellular contents, and blebbing of the plasma membrane (Fig. 5B and C). After 3 h of exposure, the damage cited above was more pronounced and some cells collapsed as well (Fig. 5E and F). These micrographs suggest that Hs-1 has a direct bactericidal effect, probably for acting at the membrane level.

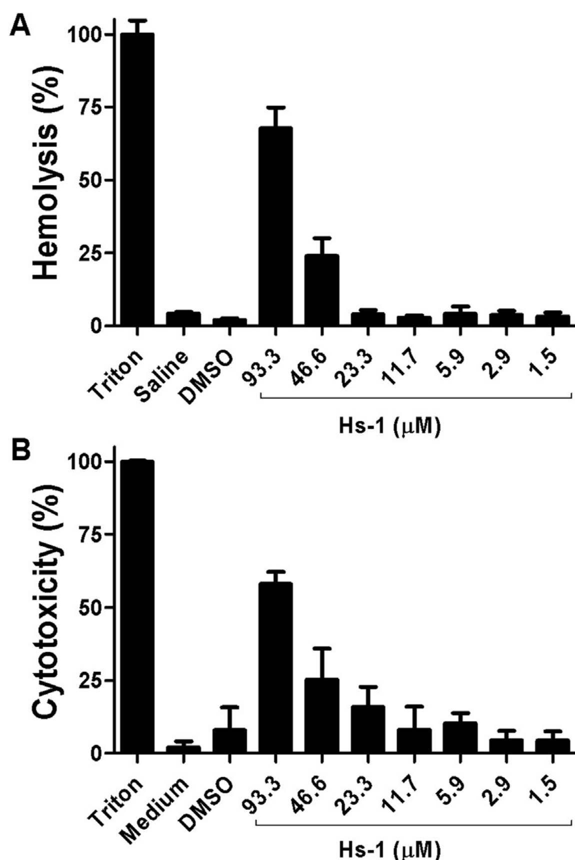

Fig. 2. Cytolytic effects of Hs-1 on human erythrocytes and leukocytes. A) Red blood cells were treated with different concentrations of Hs-1, and its cytotoxicity was evaluated in comparison with Triton X-100 (100% hemolysis) and saline (0% hemolysis). B) Leukocytes were treated with different concentrations of Hs-1, and its cytotoxicity was evaluated in comparison with Triton X-100 (100% cytolysis) and Roswell Park Memorial Institute medium (RPMI) medium (0% cytolysis). Assays were performed in triplicate, and the results represent as mean ± stand error of mean (SEM).

4. Discussion

This work reports the identification and characterization of Hs-1, an AMP from the skin of *H. semilineatus*. This treefrog species is endemic to Atlantic Forest (Frost, 2013), a rich biome present in Brazil, and, until now, no records of AMPs derived from this species are available. The interest in antimicrobial peptides of amphibians belonging Hylinae subfamily has increased recently including antimicrobial peptides from skin secretions of the genus *Hypsiboas* (Prates et al., 2004; Castro et al., 2005; Magalhães et al., 2008; Castro et al., 2009; Siano et al., 2014).

The cDNA cloning was the method utilized here for the screening of AMPs. All peptides are made from precursor genes and most of them can be cloned. As nature rarely provides enough material for all the analysis required for proper characterization of a peptide, molecular cloning is a fast and efficient alternative for obtaining AMPs' sequence. The deduced amino acid sequence of the cDNA identified in this work has the same overall architecture as that of other precursors of Hylidae's AMPs: 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 AMP (Charpentier et al., 1998; T Chen et al., 2005; Nicolas et al., 2003; Thompson et al., 2007; Vanhoye et al., 2003; Zhou et al., 2006).

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 (Rinaldi, 2002). The Hs-1 was chemically synthesized without the C-terminal amidation and its activity could have been underestimated.

The antimicrobial assays suggest that Hs-1 has a selective activity against Gram-positive bacteria, because all Gram-positive strains tested were sensitive to it, whereas all the Gram-negative strains were resistant. The MIC values (11–46 μM) are very good if compared to AMPs from *Hypsiboas* group (Table 2) and other

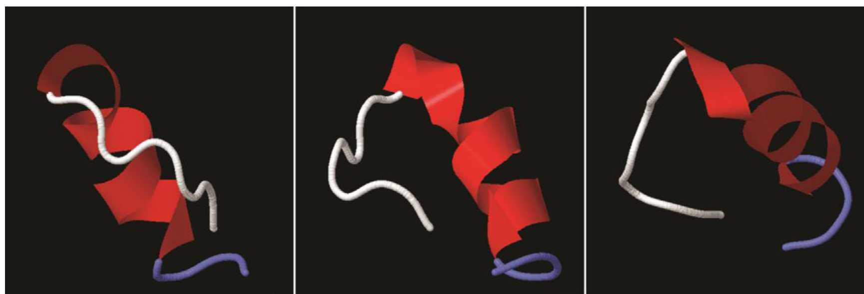

Fig. 3. Three-dimensional (3D) representation of Hs-1 secondary structure. Alpha helix, random coil, and beta-turn structures are respectively in red, white, and blue. Hs-1 is represented in different angles: lateral (left), frontal (middle), and superior (right). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

A) SOPMA prediction

```
Hs-1    FLPLILPSIVTALSSFLKQG
        cccccchhhhhhhhhhhhtt
```

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

B) Schiffer-Edmundson projection

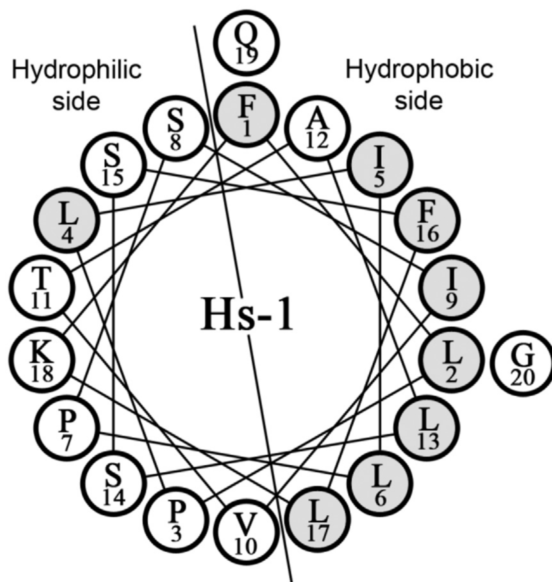

Fig. 4. Self-optimized prediction method with alignment (SOPMA) prediction and Schiffer–Edmundson projection of Hs-1. (A) The SOPMA prediction using window with: 17, similarity threshold: 8, and number of states: 4. (B) A Shiffer–Edmundson helical wheel projection illustrating the amphipathic nature 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.

anurans AMPs (King et al., 2005; Lai et al., 2002; Wang et al., 2012a, 2012b). The selectivity of Hs-1 is a great sign for its future application because Gram-positive bacteria account for causing majority of the antibiotic-resistance infections (Chen et al., 2007; Rice, 2006).

The ability of a peptide to induce cell death is the result of a complex interrelationship of factors involving conformation, charge, hydrophobicity and amphipathicity (Giangaspero et al., 2001). 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 (Tossi et al., 2000). According to SOPMA prediction, 60% of the amino acids residues of Hs-1 are structured in alpha helix and it is highly amphipathic, as 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. However, 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 (Conlon et al., 2007b), are well-represented in Hs-1, especially the apolar leucine that balances the hydrophobic side.

The selectivity of Hs-1 to Gram-positive bacteria can be explained for 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 structural requirements for activity against Gram-negative bacteria are less stringent (Giangaspero et al., 2001). Several strains of Gram-negative bacteria are susceptible to both non-helical and scrambled peptides, which suggests that antimicrobial activity against Gram-negative bacteria are mainly modulated by electrostatic interactions (Dathe et al., 1997; Giangaspero et al., 2001). Hs-1 exhibits one single positively charged amino acid (lysine), therefore, this low cationicity probably limits its activity against Gram-negative bacteria.

Hs-1 showed high similarity (54.54%) with AMPs of the Phylloseptins family. Phylloseptins were characterized in the skin secretion of *Phyllomedusa azurea* (Hylidae, Phyllomedusinae) and this family comprised 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 (Leite et al., 2005). The physicochemical properties of Hs-1 satisfy the requirements of the family, but its spectrum of activity is more selective. Even at *Hypsiboas* antimicrobial peptides group, Hs-1 is the only selective AMP identified until now. Recently, Siano et al. (2014) identified twenty-three novel sequences of peptides from skin secretions of *Hypsiboas pulchellus*, a treefrog that also occurs in Brazil. From these sequences three were chemically synthesized and characterized (P1-Hp-1971, P2-Hp-1935, P3-Hp-1891). In contrast with Hs-1, P1-Hp-1971, P2-Hp-1935 and P3-Hp-1891 are broad spectrum and they have good activity against *E. coli*, probably due their high cationicity (+4, +5 and +3 respectively). Against *S. aureus*, only P1-Hp-1971 showed a better activity than Hs-1 (8 μM versus 11.7 μM). The mass and size of Hs-1 are very similar to *H. pulchellus* antimicrobial peptides, which range from 1891.2 to 1971.1 Da and 19 to 21 amino acids, but in respect to structure Hs-1 is more similar to P3-Hp-1891 that adopts an amphipatic helical structure in the presence of 2,2,2-trifluoroethanol and anionic liposomes. In fact, a blast

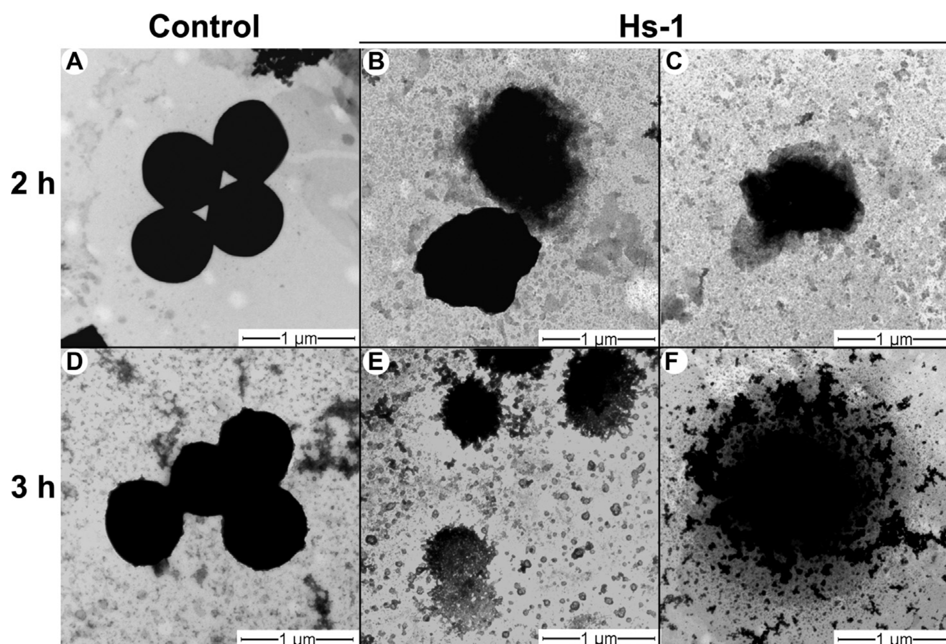

Fig. 5. Ultrastructure of *Staphylococcus aureus* treated with Hs-1. Bacteria were exposed to 200 μg/mL of Hs-1 for 2 h (B–C) and 3 h (E–F). Control bacteria were not exposed to the peptide (A, D). The scale bar is represented.

comparison between Hs-1 and P3-Hp-1891 shows some identity, unlike of P1-Hp-1971 and P2-Hp-1935 that shows no significant similarity. Likewise, comparing Hs-1 to others AMPs from *Hypsiboas* spp. it only has similarity with Hylin a1. For now, Hs-1 is the only AMP representative of *H. semilineatus* (Hylidae, Hylinae).

TEM showed that Hs-1 has a direct antibacterial killing and that 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 AMPs (Hou et al., 2011; Wang et al., 2012a).

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 but the susceptibility of erythrocytes is not necessarily extendable to other animal cells (Tossi et al., 2000). In this context, we tested Hs-1 in erythrocytes and in nucleated leukocytes. Hs-1did not exhibit a significant cytolytic activity in the range of MIC, but we observed that the LC_{50} values were close to MICs indicating a poor TI for *Bacillus* spp. and *Listeria* spp. On the other hand, Hs-1 showed a high TI for *Staphylococcus aureus* (TI = 7.3). The TI is an important parameter for the characterization and optimization of the safety and efficacy of a drug candidate (Muller and Milton, 2012).

World Health Organization (WHO) recognizes antimicrobial resistance as a growing global health threat and also considers, among other factors, that the evolution of this resistance is associated with the dearth of new antimicrobial Agents in the development pipeline (WHO, 2012). Here, we have identified and characterized Hs-1, a selective AMP that presents a great cidal activity against Gram-positive bacteria and that also has a preferential affinity for the multidrug-resistant *S. aureus* ATCC 33591. Predominantly structured as an amphipathic alpha helix, Hs-1 is the first AMP reported from *H. semilineatus* and may be used as a template for the development of alternative therapeutic agents intended for many purposes, including treatment of nosocomial infections caused by MRSA, preservation and conditioning of food (with a focus on *B. ereus* infection) and treatment of chronic sources of infection such as bacterial biofilms.

Acknowledgments

We thank Heliomar Cazelli de Oliveira Filho, Karla Veloso Gonçalves and the Núcleo de Microscopia e Microanálise of the Federal University of Viçosa for the excellent technical assistance. This work was supported by grants from CNPq (470365/2010-2 and 552459/2011-19) and FAPEMIG (PPM-00436-11). L. N. Marçal was supported by fellowship from CAPES.

Conflict of Interest

The authors declare that they have no conflict of interest.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2015.03.006.

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