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Purification and characterization of a novel peptide with antifungal activity from *Bothrops jararaca* venom

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Abstract

Different peptides have been isolated from a wide range of animal species. It is has become increasingly clear that due to the development of antibiotic-resistant microbes, antibacterial and antifungal peptides have attracted the attention in recent years, in order to find new therapeutic agents. In this work, a novel peptide with high inhibitory activity against fungi growth have been isolated from the venom of the Brazilian snake *Bothrops jararaca*. A Sephacryl S-100 gel filtration column was employed for further separation of proteins. The FV fraction with high antifungal activity was named Pep5Bj, pooled and submitted to reverse-phase chromatography in HPLC. The fraction containing the isolated peptide inhibited the growth of different phytopathogenic fungi (*Fusarium oxysporum* and *Colletotrichum lindemuthianum*) and yeast (*Candida albicans* and *Saccharomyces cerevisiae*). The peptide minimal inhibitory concentration is comparable to other known antifungal peptides, like insect defensins and cecropins, found in the last years in a large diversity of animals. We investigate *F. oxysporum* cells membrane permeabilization using SYTOX Green uptake, an organic compound that fluoresces upon interaction with nucleic acids after penetration in cell with compromised plasma membranes. When viewed under fluorescence optical microscopy, *F. oxysporum* cells exposed to Pep5Bj display strong SYTOX Green fluorescence in the cytosol, especially in the nuclei. The SYTOX Green data suggested that this effect is related to membrane permeabilization. The molecular masses of this peptide was obtained by MALDI-TOF spectrometry and corresponded to 1370 Da.
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Keywords: Antimicrobial peptides; *Bothrops jararaca*; Snake venom; Fungi inhibition

1. Introduction

Antimicrobial peptides (AMPs) are an extremely diverse group of small proteins that are considered together because of their native antimicrobial activity. The existence of AMPs has been known for several decades, but only recently has it been recognized that their function is essential to the animal immune response. They participate primarily in the innate immune system and are used as a first line of immune defence by many organisms, including plants, insects, bacteria, and vertebrates (Broekaert et al., 1997; Bulet et al., 1999; Metz-Boutigue et al., 2003).

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As effectors of innate immunity, AMPs directly kill a broad spectrum of microbes, including Gram-positive and Gram-negative bacteria, fungi, and certain viruses. In addition, these peptides interact with the host itself, triggering events that complement their role as antibiotics. Combined, the functions of the AMPs suggest that they are an important and previously under-appreciated component of the animal immunology (Gallo et al., 2002).

Steiner et al. (1981) identified the first antibacterial peptides from the moth *Hyalophora cecropia*, the cecropins. In the following years, such peptides with antibacterial activity have been found in the whole animal and vegetal kingdom, from bacteria, different insect orders to amphibians, mammals and humans (Boman, 1991; Broekaert et al., 1997; Stefanie et al., 2000). Different groups of AMPs can be made based on structural differences (Haeberli et al., 2000), for example: the cecropins (Boman, 1991); the insect and plant defensins (Lehrer et al., 1991; Hofmann et al., 1992; Commue et al., 1992); two proline-rich peptides, the apidaecin and abaecin (Casteels et al., 1989, 1990); different inducible antibacterial polypeptides: attacin (Kockum et al., 1984), sarcotoxin, diptericin (Dimarcq et al., 1988), coleoptericin (Bulet et al., 1991); and magainin, from *Xenopus laevis* (Duclohier et al., 1989; Matsuzaki et al., 1997).

Simultaneously to the finding of native AMPs in different animal classes, analogue and hybrid peptides were synthesized, which are even more potent (Chen et al., 1988). They all share common features like cationic charge and the ability to form amphipathic structures (Maloy and Kari, 1995). They either act as a first line of defence of the immune system to bacterial challenge as an induced response, like insect defensins (Hofmann et al., 1992), or they are constitutively present, like magainins on the skin of *Xenopus laevis* (Zaslof, 1987).

Bothrops jararaca snake is responsible for the majority of the ophidic accidents in Brazil. In humans and experimental animals the envenomation induces systemic haemorrhage and blood incoagulability (Rosenfeld, 1971; Cardoso et al., 1993; Kamiguti and Sano-Martins, 1995; Ribeiro and Jorge, 1997), in addition to intense tissue damage at the site of injection. Symptoms are due to the presence of a variety of toxins in the venom (Rosenfeld, 1971; Gutiérrez and Lomonte, 1989; Ribeiro and Jorge, 1997; Farsky et al., 1999). Snake venoms in general and viperid venoms in particular have long been recognized for the complexity of their molecular composition. More recently, with the advent of proteomics, the extent of the venom complexity has been more clearly illustrated. Kanashiro et al. (2002) have demonstrated that typical viperid venom may contain as many as several thousand proteins with different properties. For example, previous papers on the bactericidal effect of several basic myotoxic PLA_2s from *Bothrops* venoms have been showed by several authors (Páramo et al., 1998; Soares et al., 2000; Rodrigues et al., 2004). One example is a BnpTX-I, from *Bothrops neuwiedi pauloensis* snake venom, that induced neurotoxic effect on mouse neuromuscular preparations and bactericidal activity on *Eschericia coli* and *Staphylococcus aureus* (Soares et al., 2000).

In this paper, we report on the isolation and characterization of a novel peptide from *B. jararaca* venom and the inhibitory activity of this peptide against different fungi.

2. Materials and methods

2.1. Snake venoms

Venoms from adult specimens of *B. jararaca* were provided by the Laboratório de Herpetologia, Instituto Butantan. The venoms were filtered through a 0.22 μm membrane, lyophilized and stored at −20 °C.

2.2. Fungi

Fungal isolates utilized were: *Fusarium oxysporum* and *Colletotrichum lindemuthianum* were kindly supplied by CNPAF/EMBRAPA, Goiania, Goiás, Brazil. *Candida albicans* and *Saccharomyces cerevisiae* (1038) were obtained from the Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, Brazil. The fungi were maintained on Agar Sabouraud (1% peptone, 2% glucose and 1.7% agar–agar).

2.3. Peptides purification

Initially, 50 mg from snake crude venom were submitted to one cycle of gel filtration chromatography in Sephacryl S-100 column (1.5×50 cm) equilibrated and developed with 20 mM Tris–HCl plus 300 mM NaCl, pH 8.0 for separation of proteins. The chromatography was developed at a flow rate of 1.0 mL min^{-1}. Protein peaks (2.0 mL fractions) were collected and recovered after dialysis. The column was calibrated with several proteins of known molecular masses (bovine serum albumin, ovalbumin, soybean trypsin inhibitor and ribonuclease). The peak containing antifungal activity was collected and diluted in 0.1% (v/v) TFA and injected onto an HPLC Vydac C4 reverse phase column. The chromatography was developed at a flow rate of 0.7 mL min^{-1} with 100% solvent A (0.1% TFA in water) for 10 min, 80% solvent B (80% acetonitrile containing 0.1% TFA) over 55 min and finally 100% solvent B over 10 min. Proteins were monitored by on-line measurement of the absorbance at 220 nm.

2.4. Mass spectrometry analysis

The molecular masses of the fractions obtained from reverse phase chromatography were determined by spectrometer analysis. MALDI-TOF spectrometry was performed with a Voyager-DE STR Bioworkstation in

a linear mode using alpha cyano-4-hidoxycinnamic acid as a proton donor matrix.

2.5. Effect of proteins on fungi growth

For the preparation of yeast cell cultures (*C. albicans* and *S. cerevisiae*), inoculum was transferred to Petri dishes containing Agar Sabouraud and allowed to grow at 28 °C for 2 days; after this period the cells were transferred to sterile 0.15 M saline solution (10 mL). Yeast cells were quantified in a Neubauer chamber for further calculation of appropriate dilutions. For the preparation of conidia of *F. oxysporum* and *C. lindemuthianum* the fungal cultures were transferred to Petri dishes containing Agar Sabouraud for 12 days; after this period saline solution (10 mL) was added to the dishes and these were gently agitated for 1 min for spore liberation with the help of a Drigalski loop. Conidia were quantified in a Neubauer chamber for appropriate dilutions.

Quantitative assay for fungal growth inhibition was performed following the protocol developed by Broekaert et al. (1990) with some modifications. To assay the effect of different fractions on the fungi growth, the cells and conidia (20,000 cells mL^{-1} in 1 mL of saline solution) were incubated at 28 °C in 200 μL microplates in the presence of different concentrations from peptide solutions (12.5–200 μg mL^{-1} for total venom and 8 μg mL^{-1} for purified FV fraction). Optical readings at 620 nm were taken at zero time and at each 6 h for the following 44 or 60 h (Broekaert et al., 1990). Cell growth control without addition of peptides was also determined. After period growth the cells were pelleted by centrifugation, washed in 0.1 M Tris–HCl, pH 8.0 and observed in an optical microscope at 400× magnifications. All experiments were run in triplicate and the data were used for a calculation of the reading averages, the standard errors and coefficients of variation.

2.6. Inhibition of the glucose-stimulated acidification of the medium by yeast

The Sabouraud broth (100 mL) *S. cerevisiae* culture was shaken for 16 h at 30 °C with good aeration. The culture (A_{660} = 0.250) was pelleted by centrifugation at 2000×*g* (5 min, 4 °C), followed by three water-washing steps. Inhibitory activity of snake venom was determined by incubation of *S. cerevisiae* cells (4×10^7) with 5 mL of 5 mM Tris–HCl, pH 7.1. Snake venom (FV fraction) was added to final concentrations of 25 μg mL^{-1}. After 30 min time incubation, a 0.5 M glucose solution (1 mL) was added to give a final concentration of 0.1 M and pH measurements were taken at each subsequent minute for the 30 min. Negative (no venom added) was run in order to evaluate the influence of venom on H^+ extrusion by yeast (Gomes et al., 1998).

2.7. SYTOX Green uptake assay

Permeabilization was measured by SYTOX Green uptake as described previously by Thevissen et al. (1999) with some modifications. *F. oxysporum* was grown in the presence of peptide solutions (5 μg mL^{-1}). After 60 h of growth, the cells were pelleted by centrifugation and hyphae were washed in 0.1 M Tris–HCl, pH 8.0. One hundred-microliter aliquots of the suspension of *F. oxysporum* hyphae were incubated with 0.2 μM SYTOX Green in white 96-well microplates for 30 min at 25 °C with periodic agitation. After incubation time, fluorescence was viewed with a fluorescence microscope (Axiophoto Zeiss) equipped with a filer set for fluorescein detection (excitation wavelength, 450–490 nm; emission wavelength, 500 nm).

2.8. Scanning electron microscopy

For scanning electron microscopy, the yeast cells grown for 60 h in Sabouraud broth in the presence or absence of venom fraction were fixed for 30 min at room temperature in a solution containing 2.5% glutaraldehyde, 2.0% formaldehyde in 0.1 M phosphate buffer, pH 7.3. Subsequently, the materials were rinsed three times with the above buffer, post-fixed for 30 min at room temperature with 1.0% osmium tetroxide diluted in the same buffer and rinsed with distilled water. After this procedure, the fungi were dehydrated in acetone, critical point dried in CO_2, covered with 20 nm gold and observed in a Zeiss 962 scanning electron microscope. Cell growth control without addition of peptides was also determined.

3. Results

3.1. Purification and characterization of the venom peptides

Peptide(s) from snake venom were purified using a combination of gel filtration and phase-reverse chromatography. The proteins from snake crude venom was separated in eight different fractions named FI, FII, FIII, FIV, FV, FVI, FVII and FVIII by means of gel filtration chromatography in Sephacryl S-100 column (Fig. 1(A)). The FV fraction, with the strongest antifungal activity (see below), was further fractionated by reverse-phase chromatography. This fraction was loaded on a C4 column and the proteins were eluted using a linear acetonitrile gradient. Only one major peak called FV-RP could be detected when either FV was analysed (Fig. 1(B)).

FV fraction was also submitted to mass spectrometer analysis which shows the presence of one peak and revealed that their molecular masses are equal to 1370 Da (Fig. 2).

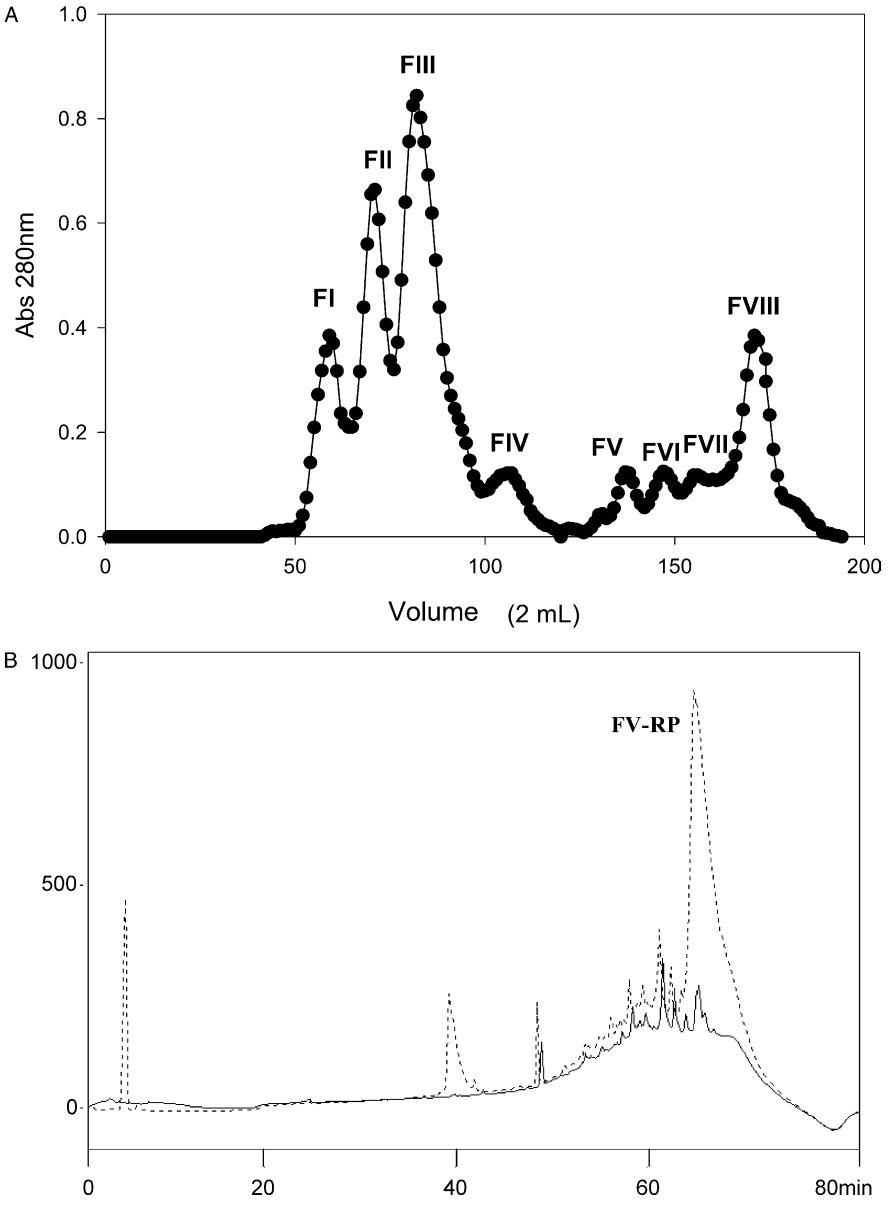

Fig. 1. Purification of the antifungal peptides from snake venom. (A) Sephacryl S-100 chromatography. Column was equilibrated and eluted with 20 mM Tris–HCl buffer plus 300 mM NaCl, pH 8.0. The flow rate was 60 mL h^{-1} and 2.0 mL fractions were collected; (B) RP-HPLC chromatography. PepBj (FV) fraction was load on a reverse-phase column and run in a Shimadzu apparatus. Elution was carried out as described in Section 2. The dark line represents the blanck and the dash line one the protein elution profile at 220 nm.

3.2. Effect of snake venom on fungi growth

Our screening indicated that snake venom proteins display antifungal activities against a wide range of fungi. Fig. 3 shows the patterns of growth for *F. oxysporum* and *C. lindemuthianum* in the presence of crude venom and in control medium. We noticed a high inhibitory effect on growth for both fungi tested in the concentration used the 25 μg mL^{-1}. For and *S. cerevisiae* we decided to analyse the growth inhibition in the presence of different crude venom concentrations (12.5–200 μg mL^{-1}). We can also see a high inhibitory effect on yeast growth using 12.5 μg mL^{-1} with total inhibition of the growth at 200 μg mL^{-1} (Fig. 3(C)). Photomicrographs of the mycelia of the *F. oxysporum* (Fig. 4(A) and (B)) and *C. lindemuthianum* (Fig. 4(C) and (D)) fungi were taken

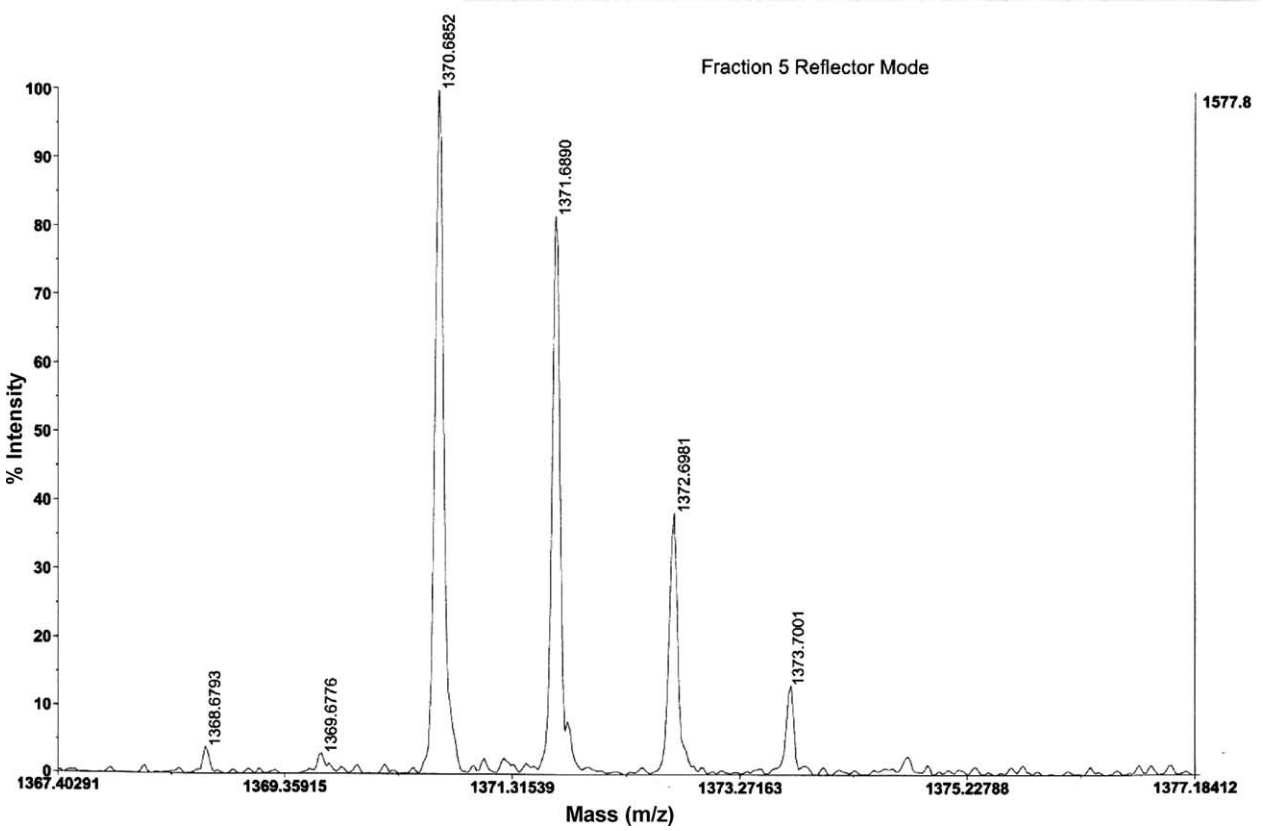

Fig. 2. MALDI-TOF spectrometry of PepBj (FV) fraction: the peptide sample was diluted in a saturated solution of α-cyano-4-hidoxycinnamic acid in acetonitrile. The spectrum was obtained in a Voyager-DE STR operating in linear mode under an acceleration of 20,000 V and laser intensity of 2160. The molecular weight peaks were determined in a similar manner.

after the 44 h growth period. Normal hyphal development (Fig. 4(A) and (C)) was observed in control treatment but in the venom containing fraction there was a noticeable inhibition of conidial germination and hyphal development (Fig. 4(B) and (D)).

During the purification process, we have also tested different proteins fractions from Sephacryl S-100 against the phytopathogenic fungi, *C. lindemuthianum* and *F. oxysporum* and against the yeast *S. cerevisiae* and *C. albicans*, but only those for FV fraction, named now Pep5B, is shown. Fig. 5 shows the growth patterns of these microorganisms in the presence of the 8 $\mu g\,mL^{-1}$ from Pep5B fraction. We observed a notable inhibitory effect of the Pep5B fraction on growth for all fungi tested, especially when observed the yeast growth inhibition (Fig. 5(C) and (D)).

We further analysed through scanning microscopy possible alterations in yeast morphology caused by peptide of the Pep5B fraction. Photomicrographs of the yeast were taken after the 60 h growth period. Normal growth development was observed in control cells treatment (Fig. 6(A) and (C)). However, cultures treated with proteins of the Pep5B fraction showed a notable inhibition of *S. cerevisiae* growth and cell swelling as compared with the control (Fig. 6(B)). For *C. albicans*, we could also see in cultures treated with FV fraction a notable growth inhibition as well morphological alterations with the formation of many pseudo-hyphae in the medium, no observed in control (Fig. 6(D)).

3.3. Effect on the glucose-stimulated acidification of the medium by yeast and permeabilization of F. oxysporum cells

To test whether venom proteins could interact with the fungal plasma membrane we monitored their effect on the glucose-stimulated acidification of the incubation medium by *S. cerevisiae* yeasts. We found that total snake venom was able to inhibit the glucose-stimulated acidification of the incubation medium by fungal cells. The FV fraction, in the case of 25 $\mu g\,mL^{-1}$ demonstrated 60% of the inhibition (Fig. 7). We also tested the ability of Pep5B fraction to permeabilize *F. oxysporum* hyphae. SYTOX

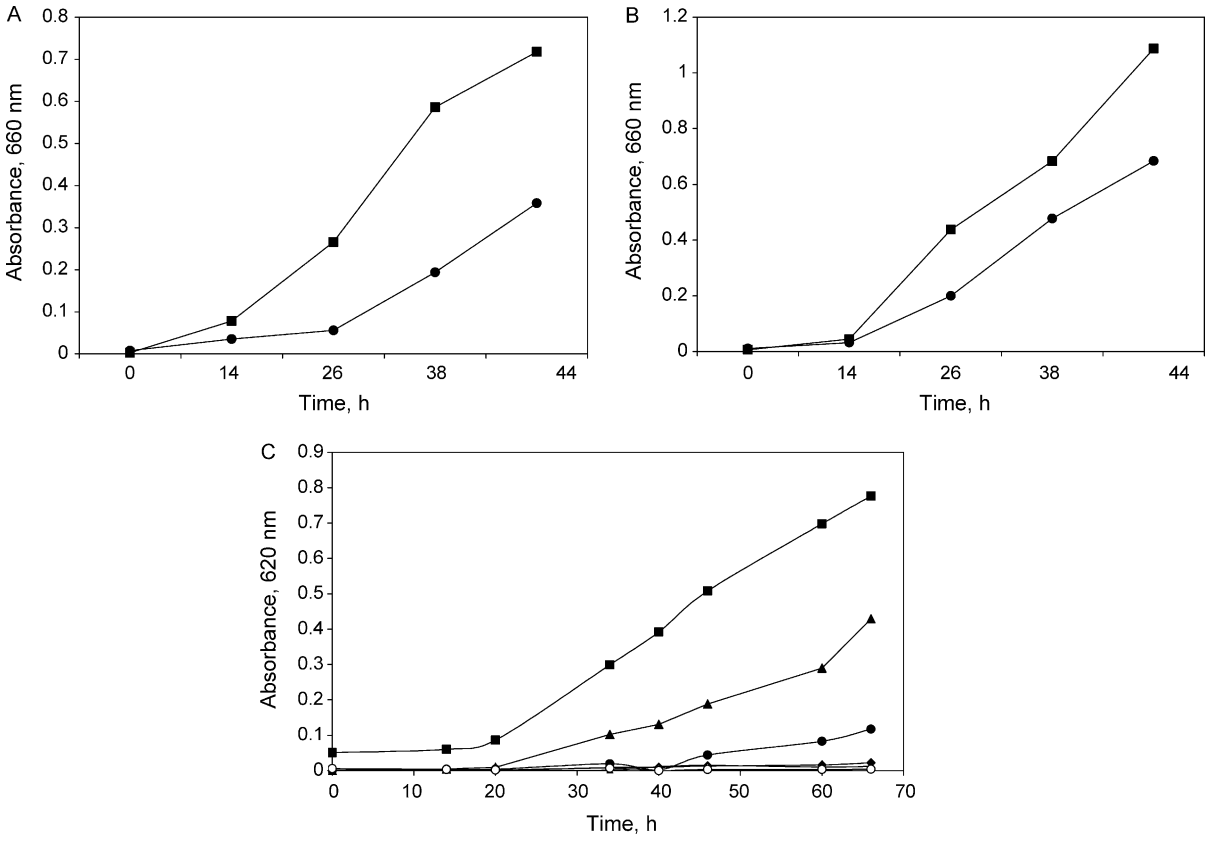

Fig. 3. The effect of total venom on fungal growth. Fungal growth was monitored by measuring the density of fungal culture at 620 nm. (■) Control; (▲) 12.5; (●) 25; (♦) 50; (△) 100; (○) 200 μg mL^{-1}. (A) *Colletotrichum lindemuthinum*; (B) *Fusarium oxysporum*; (C) *Saccharomyces cerevisiae*. Experiments were performed in triplicate and the standard errors (coefficients of variation were less than 20%) were omitted for clarity.

Green permeabilization was assessed 60 h after growth period in presence of peptides and 30 min after the addition of SYTOX Green. When viewed with a fluorescence microscope, *F. oxysporum* cells showed strong SYTOX Green fluorescence in the cytosol, especially in the nuclei (Fig. 8).

4. Discussion

In the last years, several AMPs have been found in different venoms from different animals and these are traditionally linked to defence mechanisms (Gallo et al., 2002). In this study, the low molecular proteins from snake venom, referred to here as FV-RP, derived from the FV fraction by Sephacryl S-100, were purified after reverse-phase chromatography (Fig. 1). When screening new peptides in snake crude venom by MALDI-TOF-MS, we found one principal short peptide with molecular weight of 1370 Da (Fig. 2). One supposition was that they might be fragments coming from proteins or polypeptides since the existence of proteases in the crude venom (Dai et al., 2002). However, the characterization of two cytotoxic short peptides IsCT and IsCT2, from scorpions' venom, and especially the further gene analysis results showed that some of them are original biological peptides in the crude venom (Dai et al., 2002). The values for the isolated peptide lie in the range of molecular masses found by several authors for these peptides isolated from other venom (Haeberli et al., 2000; Konno et al., 2001; Dai et al., 2002)

It is generally accepted that different venoms have several thousand proteins with different properties. However, it was recently shown that the peptides present in venoms from wasp, spider and scorpions were identified, for the first time, as a novel class of antifungal proteins (Haeberli et al., 2000; Konno et al., 2001; Moerman et al., 2002). In our study, the FV fraction that contain one major peptide, named PepBj, displayed inhibitory activity against several fungi tested, *C. lindemuthianum*, *C. musae*, *F. oxysporum* and *S. cerevisiae*. The peptide minimal inhibitory concentration is comparable to other known antifungal peptides, like insect defensins and cecropins, found in the last years in a large diversity of animals (Sitaram and Nagaraj, 1999; Moerman et al., 2002). We also

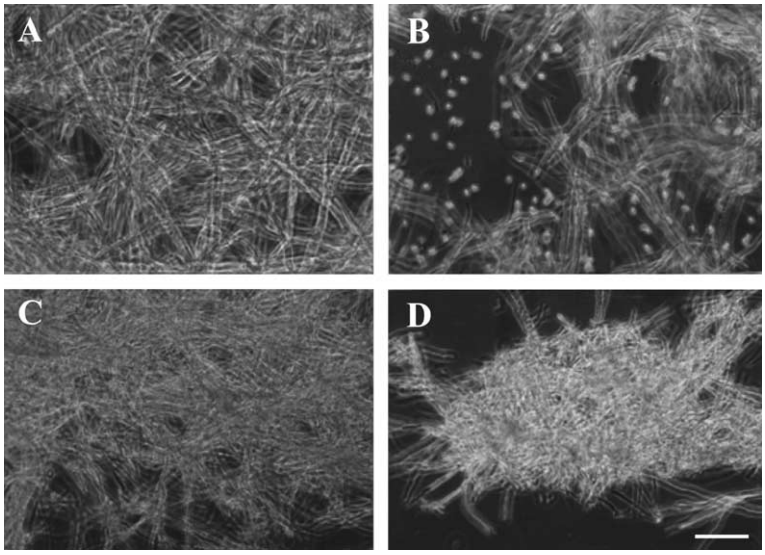

Fig. 4. Light micrographs of *Fusarium oxysporum* (A, B) and *Colletotrichum lindemuthinum* (C, D) mycelia after 44 h of fungi growth in the (A, C) control medium or presence of (B, D) total venom (25 μg mL^{-1}). Magnification bars = 15 μm.

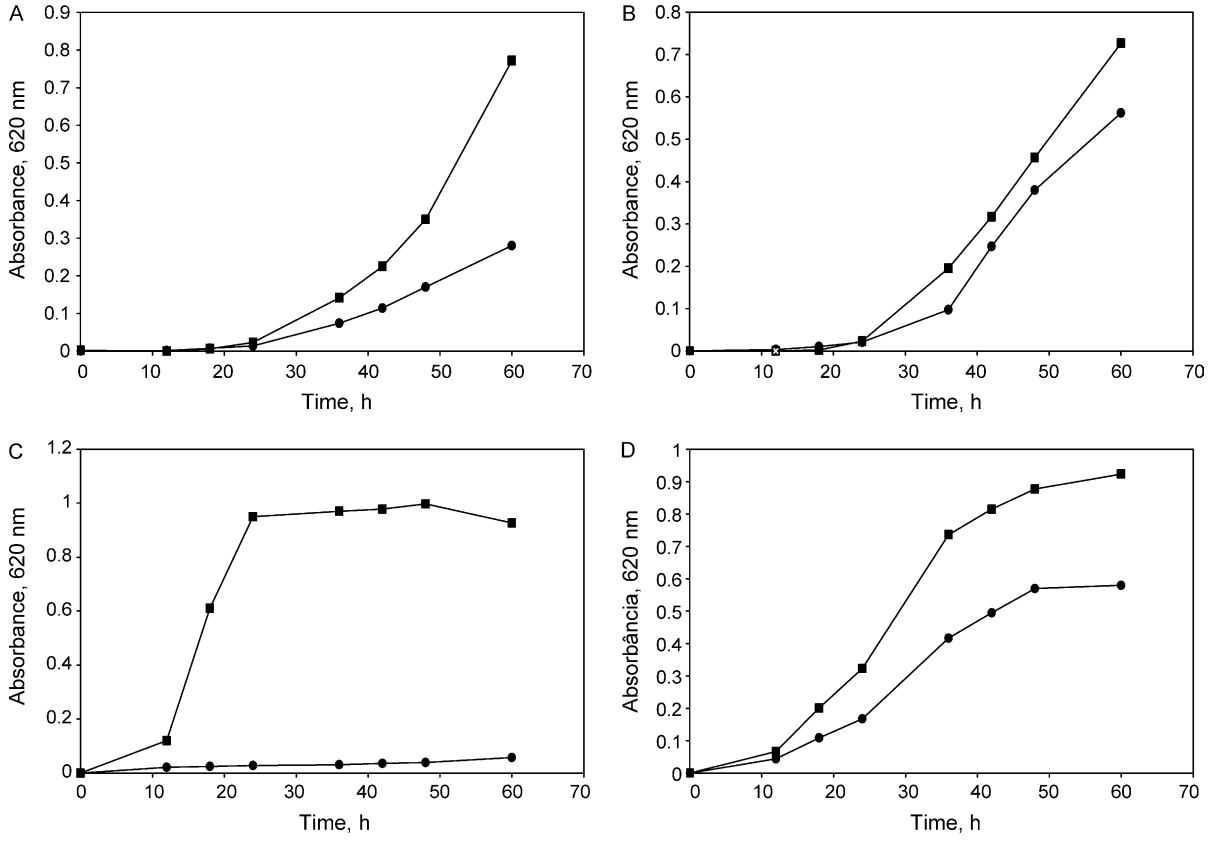

Fig. 5. The effect of peptides (FV fraction) on fungal growth. Fungal growth was monitored by measuring the density of fungal culture at 620 nm. (■) Control; (●) 10 μg mL^{-1}. (A) *Colletotrichum lindemuthinum*; (B) *Fusarium oxysporum*; (C) *Saccharomyces cerevisiae*; (D) *Candida albicans*. Experiments were performed in triplicate and the standard errors (coefficients of variation were less than 20%) were omitted for clarity.

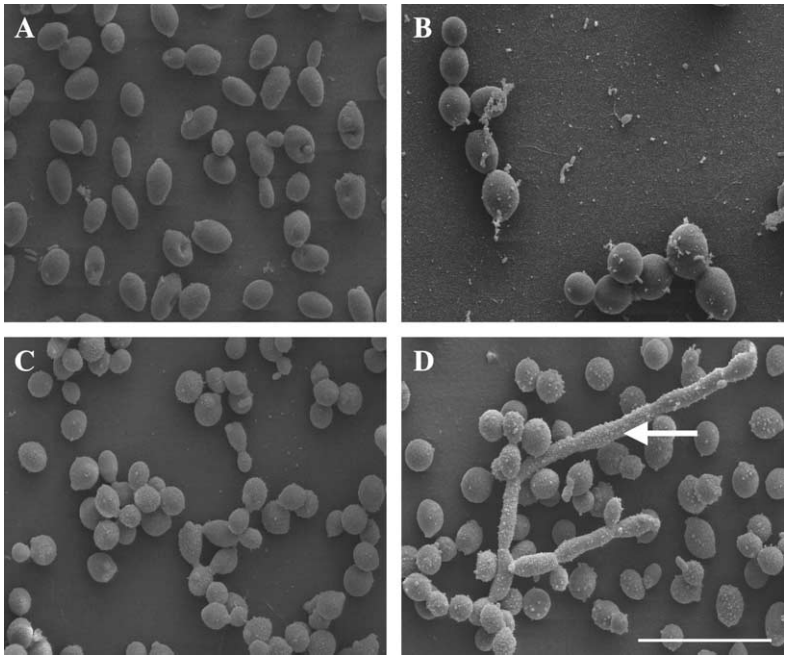

Fig. 6. Scanning electron microscopy of *Saccharomyces cerevisiae* (A, B) and *Candida albicans* (C, D) cells after 60 h of fungi growth in the (A, C) control medium or presence of (B, D) FV fraction (10 μg mL^{-1}). Magnification bars: (A and B) 10; (C and D) 5 μm.

showed from optical and scanning electron microscopy that peptides derived from FV fraction induced various hyphal morphological alterations in these fungi. The total venom fraction also inhibited the glucose-stimulated acidification of the medium by *F. oxysporum* by up to 40%. We have observed that the partial inhibitory effect of the peptides on the glucose dependent acidification of the medium by fungi cells correlated well with the inhibition of fungal growth. This fact can be taken as an indication that the fungi growth inhibition by this fraction may be mediated by either an inhibition of the H^+-ATPase of the fungal plasma membrane or an increase in the H^+ permeability of this membrane. At present, the information on the mechanism of action of antifungal proteins is very limited.

Peptide antibiotics which are hydrophobic in nature such as gramicidin A and alamethicin have been extensively used to delineate peptide–membrane interactions (Sitaram and Nagaraj, 1999). Their small size and easy availability have made it possible to study lipid–peptide interactions in depth by a variety of biophysical techniques. Different peptides were found to have the ability to modulate ion flux across membranes and hence became popular models for understanding how ion channel proteins function (Sitaram and Nagaraj, 1999). Since the early 1980s, it has become evident that peptides are an important constituent of the host-defence mechanism against microbes in species right across the evolutionary scale from insects to mammals (Steiner et al., 1981, James et al., 1994; Konno et al., 2001; Gallo et al., 2002). The amino acid composition of these peptides,

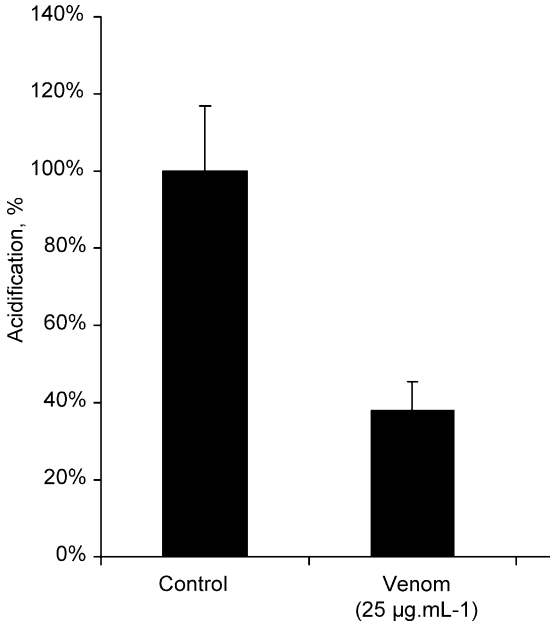

Fig. 7. The effect of FV Fraction on the glucose dependent acidification of the medium by *Fusarium oxysporum* cells. (■) Control; (●) FV fraction (25 μg mL^{-1}). Glucose was added after 30 min of cell incubation with the FV fraction to a final concentration of 100 mM.

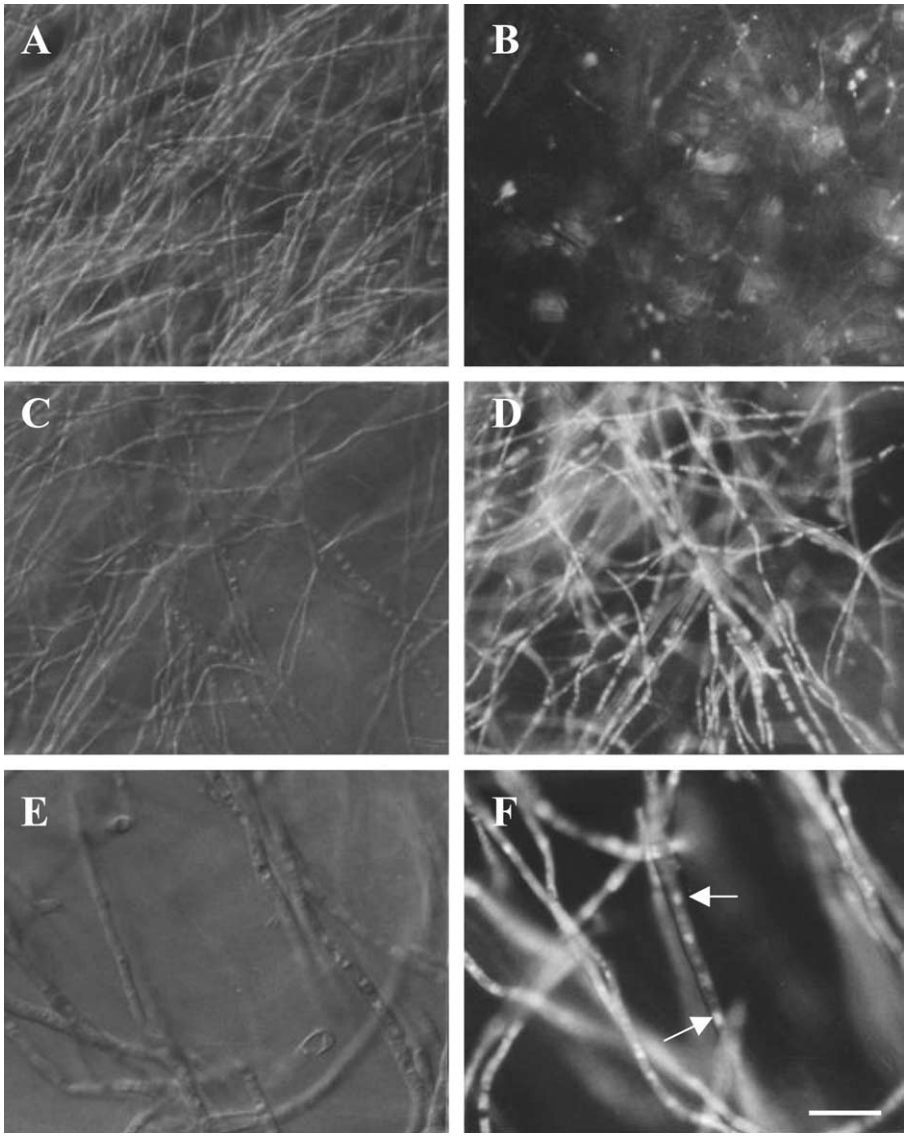

Fig. 8. Fluorescence microscopy of fungal cells in the presence of SYTOX Green and FV fraction. *F. oxysporum* cells were suspended in buffer containing 0.2 μM SYTOX Green and treated for 30 min in the absence (A, B) or presence (C–F) of FV fraction (10 μg mL^{-1}). Section of presented in bright-field (A, C, E) and viewed with fluorescence (B, D, F). Magnification bars: (A–D) 5 μm; (E and F) 15 μm. Arrows represents cells showed strong fluorescence in the cytosol, especially in the nuclei.

along with the presence of cationic amino acids, suggested that they would have the ability to associate with membrane lipids (Sitaram and Nagaraj, 1999). When used an assay based on the uptake of SYNTOX Green to investigate membrane permeabilization in fungi we found that the peptides present in FV fraction were also capable to induce the membrane permeabilization, displaying strong SYTOX Green fluorescence in the cytosol, especially in the nuclei.

The biological activity of each individual natural peptide is likely to be specific for the defensive role that the peptide must play in different cell types. Because resident microflora, potential pathogens, and environmental stresses are very different in each tissue, the expression pattern and regulation of the AMPs in each organ provide insight into their function and importance (Gallo et al., 2002). Structural analyses such as amino acid sequence and circular diachronic studies as well as conductance measurements and pharmacological studies of the action on the different pathogens will reveal more about the mode of action of the AMPs of snake venoms.

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References

Boman, H.G., 1991. Antibacterial peptides: key components needed in immunity. Cell 65, 205–207.

Broekaert, W.F., Terras, F.R.G., Cammue, B.P.A., Vanderleyden, J., 1990. An automated quantitative assay for fungal growth inhibition. FEMS Microbiol. 69, 55–60.

Broekaert, W.F., Cammue, B.P.A., De Bolle, M.F.C., Thevissen, K., De Samblanx, G., Osborn, R.W., 1997. Antimicrobial peptides from plants. Crit. Rev. Plant Sci. 16, 297–323.

Bulet, P., Cociancich, S., Dimarcq, J.-L., Lambert, J., Reichhart, J.-M., Hoffmann, D., Hetru, C., Hoffmann, J.A., 1991. Insect immunity: isolation from a coleopteran insect of a novel inducible antibacterial peptide and of new members of the insect defensin family. J. Biol. Chem. 266, 24520–24525.

Bulet, P., Hetru, C., Dimarcq, J.-L., Hoffmann, D., 1999. Antimicrobial peptides in insects; structure and function. Dev. Comp. Immunol. 23, 329–344.

Cammue, B.P.A., De Bolle, M.G.C., Terras, F.R.G., Proost, P., Damme, J., Van Rees, S.B., Vanderleyden, J., Broekaert, W.F., 1992. Isolation and characterization of a novel class of plant antimicrobial peptides from *Mirabilis jalapa* L. seeds. J. Biol. Chem. 267, 2228–2233.

Cardoso, J.L.C., Fan, H.W., Franca, F.O.S., Jorge, M.T., Leite, R.P., Nishioka, S.A., Avila, A., Sano-Martins, I.S., Tomy, S.C., Santoro, M.L., Chudzinski, A.M., Castro, S.C.B., Kamiguti, A.S., Kelen, E.M.A., Hirata, M.H., Mirandola, R.M.S., Theakston, R.D.G., Warrell, D.A., 1993. Randomized comparative trial of three antivenoms in the treatment of envenoming by lance-headed vipers (*Bothrops jararaca*) in São Paulo Brazil. Q. J. Med. 86 (5), 315–325.

Casteels, P., Ampe, C., Jacobs, F., Vaeck, M., Tempst, P., 1989. Apidaecins: antibacterial peptides from honeybees. EMBO J. 8, 2387–2391.

Casteels, P., Ampe, C., Riviere, L., Damme, J.V., Elicone, C., Flemming, F., Jacobs, F., Tempst, P., 1990. Isolation and characterization of abaecin, a major antibacterial response peptide in the honey-bee (*Apis mellifera*). Eur. J. Biochem. 187, 381–386.

Chen, H.-C., Brown, J.H., Morell, J.L., Huang, C.M., 1988. Synthetic magainin analogues with improved antimicrobial activity. FEBS Lett. 236, 462–466.

Dai, L., Corzo, G., Naoki, H., Andriantsiferana, M., Nakajima, T., 2002. Purification, structure–function analysis, and molecular characterization of novel linear peptides from scorpion *Opisthacanthus madagascariensis*. Biochem. Biophys. Res. Commun. 293, 1514–1522.

Dimarcq, J.-L., Keppi, E., Dunbar, B., Lambert, J., Reichhart, J.-M., Hoffmann, D., Rankine, S.M., Fothergill, J.E., Hoffmann, J.A., 1988. Insect immunity. Purification and characterization of a family of novel inducible antibacterial proteins from immunized larvae of the dipteran *Phormia terranovae* and complete amino-acid sequence of the predominant member, diptericin A. Eur. J. Biochem. 171, 17–22.

Duclohier, H., Molle, G., Spach, G., 1989. Antimicrobial peptide magainin I from *Xenopus* skin forms anion-permeable channels in planar lipid bilayers. Biophys. J. 56, 1017–1021.

Farsky, S.H.P., Goncalves, L.R.C., Cury, Y., 1999. Characterization of local tissue damage evoked by *Bothrops jararaca* venom in the rat connective tissue microcirculation: an intravital microscopic study. Toxicon 37, 1079–1083.

Gallo, R.L., Murakami, M., Ohtake, T., Zaiou, M., 2002. Biology and clinical relevance of naturally occurring antimicrobial peptides. J. Allergy Clin. Immun. 110, 823–831.

Gomes, V.M., Okorokov, L.A., Rose, T.L., Fernandes, K.V.S., Xavier-Filho, J., 1998. Vicilins (7S Globulins) inhibit yeast growth and glucose-stimulated acidification of the medium by yeast cells. Biochim. Biophys. Acta 1379, 207–216.

Gutiérrez, J.M., Lomonte, B., 1989. Local tissue damage induced by *Bothrops* snake venoms. Mem. Inst. Butantan 51, 211–223.

Haeberli, S., Kuhn-Nentwig, L., Schaller, J., Nentwig, W., 2000. Characterisation of antibacterial activity of peptides isolated from the venom of the spider *Cupiennius salei* (Araneae: Ctenidae). Toxicon 38, 373–380.

Hofmann, J.A., Dimarcq, J.-L., Bulet, P., 1992. Les peptides antibacteriens inductibles des insectes. Médecine/Science 8, 432–439.

James, S., Gibbs, B.F., Toney, K., Bennett, H.P., 1994. Purification of antimicrobial peptides from an extract of the skin of *Xenopus laevis* using heparin-affinity HPLC. Characterization by ion-spray mass spectrometry. Anal. Biochem. 217, 84–90.

Kamiguti, A., Sano-Martins, I., 1995. South American snake venoms affecting haemostasis. J. Toxicol. Toxins Rev. 14, 359–374.

Kanashiro, M.M., Petretski, J.H., Dias Da Silva, W., Machado, O.L.T., Alves, E.W., Kipnis, T.L., 2002. Biochemical and biological properties of phospholipase A2 from *Bothrops atrox* snake venom. Biochem. Pharmacol. USA 64, 1179–1186.

Kockum, K., Faye, I., Hofsten, P., Lee, J.Y., Xanthopoulos, K.G., Boman, H.G., 1984. Insect immunity. Isolation and sequence of two cDNA clones corresponding to acidic and basic attacins from *Hyalophora cecropia*. EMBO J. 3, 2071–2075.

Konno, K., Hisada, M., Fontana, R., Lorenzi, C.C.B., Naoki, H., Itagaki, Y., Miwa, A., Kawai, N., Nakata, Y., Yasuhara, T., Neto, J.R., Azevedo Jr.., W.F., Palma, M.S., Nakajima, T., 2001. Anoplin, a novel antimicrobial peptide from the venom of the solitary wasp *Anoplius samariensis*. Biochim. Biophys. Acta 1550, 70–80.

Lehrer, R.I., Ganz, T., Selsted, M.E., 1991. Defensins: endogenous antibiotic peptides of animal cells. Cell 64, 229–230.

Maloy, W.L., Kari, U.P., 1995. Structure–acitvity studies on magainins and other host defense peptides. Biopolymers 37, 105–122.

Matsuzaki, K., Sugishita, K., Harada, M., Fujii, N., Miyajima, K., 1997. Interactions of an antimicrobial peptide, magainin 2, with outer and inner membranes of Gram-negative bacteria. Biochim. Biophys. Acta 1327, 119–130.

Metz-Boutigue, M.H., Kieffer, A.E., Goumon, Y., Aunis, D., 2003. Innate immunity:involvement of new neuropeptides. Trends Microbiol. 11, 585–592.

Moerman, L., Bosteels, S., Noppe, W., Willems, J., Clynen, E., Schoofs, L., Thevissen, K., Tytgat, J., Van Eldere, J., Walt, J.V.D., Verdonck, F., 2002. Antibacterial and antifungal properties of a-helical, cationic peptides in the venom of scorpions from southern Africa. Eur. J. Biochem. 269, 4799–4810.

Páramo, L., Lomonte, B., Pizarro-Cerda, J., Bengoechea, J.A., Gorvel, J.P., Moreno, E., 1998. Bactericidal activity of Lys49 and Asp49 myotoxic phospholipases A2 from *Bothrops*n asper snake venom-synthetic Lys49 myotoxin II-(115–129)-peptide identifies its bactericidal region. Eur. J. Biochem. 253, 452–461.

Ribeiro, L.A., Jorge, M.T., 1997. Bites by snakes in the genus *Bothrops*: a series of 3239 cases. Rev. Soc. Bras. Med. Trop. 30, 475–480.

Rodrigues, V.M., Marcussi, S., Cambraia, R.S., Araújo, A.L., Malta-Neto, N.R., Hamaguchi, A., Ferro, E.A.V., Homsi-Brandeburgo, M.I., Giglio, J.R., Soares, A.M., 2004. Bactericidal and neurotoxic activities of two myotoxic phospholipases A2 from *Bothrops neuwiedi pauloensis* snake venom. Toxicon 44, 305–314.

Rosenfeld, G., 1971. Symptomathology, pathology and treatment of snake bites in South America. In: Bücherl, W., Buckley, E. (Eds.), Venomous Animals and Their Venoms, V2. Academic Press, New York, pp. 345–384.

Sitaram, N., Nagaraj, R., 1999. Interaction of antimicrobial peptides with biological and model membranes: structural and charge requirements for activity. Biochim. Biophys. Acta 1462, 29–54.

Soares, A.M., Guerra-Sá, R., Borja-Oliveira, C., Rodrigues, V.M., Rodrigues-Simioni, L., Rodrigues, V., Fontes, M.R.M., Gutiérrez, J.M., Giglio, J.R., 2000. Structural and functional characterization of BnSP-7, a myotoxin Lys49 phospholipase A2 homologue, from *Bothrops neuwiedi pauloensis* venom. Arch. Biochem. Biophys. 378, 201–209.

Stefanie, H., Lucia, K.-N., Johann, S., Wolfgang, N., 2000. Characterisation of antibacterial activity of peptides isolated from the venom of the spider *Cupiennius salei* (Araneae: Ctenidae). Toxicon 38, 373–380.

Steiner, H., Hultmark, D., Engström, A., Bennich, H., Boman, H.G., 1981. Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature 292, 246–248.

Thevissen, K., Terras, F.R.G., Broekaert, W.F., 1999. Permeabilization of fungal membranes by plant defensins inhibits fungal growth. Appl. Environ. Microbiol. 62, 5451–5458.

Zaslof, M., 1987. Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc. Natl Acad. Sci. USA 84, 5449–5453.