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Novel surfactant proteins are involved in the structure and stability of foam nests from the frog *Leptodactylus vastus*

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SUMMARY

Many amphibians lay their eggs in foam nests, which allow the eggs to be deposited out of the water. Analysis of some of these foam nests has revealed that they are a rich source of proteins with unusual primary structures and remarkable surfactant activity, named ranaspumins. The aim of this work was to study the foam nests of the frog *Leptodactylus vastus* in order to obtain information regarding their composition and function and to improve the understanding of ranaspumins, which are probably a novel class of surfactant proteins. Analyses of the foam fluid composition showed proteins and carbohydrates that presumably are responsible for providing nutrients for the developing tadpoles. Investigation of the function of foam fluid in chemical defence revealed no significant biological activity that could be associated with recognized defence compounds. However, foam fluid presented UV absorbance, suggesting a role in protection against sun damage, which is considered to be one of the possible causes of recently reported amphibian population declines. The foam nests do not prevent the colonization of microorganisms, such as the observed bacterial community of predominantly Gram-positive bacilli. *L. vastus* foam fluid shows a strong surfactant activity that was associated with their proteins and this activity seems to be due mainly to a protein named *Lv*-ranaspumin. This protein was isolated by ion-exchange chromatography and found to be a 20 kDa monomeric molecule with the following N-terminal sequence: FLEGFLVPKVVPGPTAALLKKALDD. This protein did not show any match to known proteins or structures, which suggests that it belongs to a new class of surfactant protein.

Key words: Amphibia, *Leptodactylus vastus*, foam nest, surfactants, frog proteins.

INTRODUCTION

Many species of vertebrates lay their eggs in stable foam, termed foam nests (Duellman and Treub, 1986; Andrade and Abe, 1997; Downie and Smith, 2003). In amphibians the foam nests are associated with their evolution to terrestrial life (Heyer, 1975) and several functions have been attributed to them, such as protection against potential predators, pathogens (Heyer, 1969; Downie, 1993; Cooper et al., 2005) and thermal damage (Gorzula, 1977), a food source for tadpoles (Tanaka and Nishihira, 1987), desiccation prevention (Downie, 1988) and respiratory advantages to the embryos (Seymour, 1994).

The neotropical frogs of the genus *Leptodactylus* are an example of a group under transition of reproductive and larval development modes from an aquatic to a terrestrial habitat (Heyer, 1969) that have benefited from the capacity to produce foam nests. The species *Leptodactylus vastus* A. Lutz 1930 is endemic to South America, occurring specifically in Northeast Brazil (Heyer, 2005). The female of this frog releases eggs and a proteinaceous fluid, which is whipped up by the male using his legs in a rapid motion that simultaneously fertilizes the eggs. The foam nests are gradually disintegrated and by the end of several weeks they naturally turn into a dense liquid. Similar behaviour has been observed in the frogs *Physalaemus pustulosus* found in Central/South America and parts of the Caribbean (Cooper et al., 2005) and *Polypedates leucomystax* found in Malaysia and adjacent regions of South East Asia (McMahon et al., 2006).

In spite of some interesting properties assigned to amphibian foam nests, little is known about their composition and molecular mechanisms of production and long-term stability. One of the few works on this subject focussed on the foam nest of *P. pustulosus*, which was revealed to be a rich source of proteins with unusual primary structures and remarkable surfactant activity, named ranaspumins (Cooper et al., 2005). More recently, McMahon and colleagues described the crystal structure of the 13 kDa surfactant protein isolated from foam nests of *P. leucomystax*, named ranasmurfin (McMahon et al., 2006). It has been hypothesized that it could be a novel protein since searches with the available partial sequence data did not reveal any match to any known protein or structure.

Taking into consideration the physicochemical characteristics of foam nest proteins and the importance of surfactants produced by vertebrates, particularly due to their biodegradability and biocompatibility, proteins like ranaspumins and ranasmurfin open up huge possibilities of biomedical and industrial applications (Kaneko et al., 1990; Makkar and Rockne, 2003; Cooper et al., 2005). Keeping these clues in mind, the main objective of the present work was to study the foam nests of *L. vastus* in order to add information regarding their composition and function and to improve

the understanding of ranaspumins, which are probably a novel class of surfactant proteins.

MATERIALS AND METHODS

Collection and processing of foam nests

Foam nests of the frog *L. vastus* (Fig. 1) were collected in the field (Pici Campus, Federal University of Ceara, Brazil) during the rainy season (March and April) with proper regard to Brazilian ecological concerns (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis, IBAMA). The nests (six samples) were carefully sampled from the edges of temporary standing water, placed in sterile plastic bags and brought immediately to the laboratory, where they were manually cleaned and the eggs removed under aseptic conditions. Foam fluid was obtained by drainage and stored in sterile flasks under freezing conditions until use in physicochemical and biological analyses.

Protein and carbohydrate determination

Protein content was determined by conventional assay using bovine serum albumin as the standard (Bradford, 1976), and total carbohydrate by using the phenol/sulphuric acid method with glucose as the standard (Dubois et al., 1956).

Absorption spectrum

The ultraviolet-visible spectrum of foam fluid (1.0 mg ml^{-1} protein) was determined in 1 cm pathlength quartz cuvettes using a spectrophotometer (Genesys 10UV, Spectronic Unicam, Rochester, NY, USA) at a wavelength range of 210 to 700 nm.

Microbiota associated with foam nests

The colonization of the foam nests by bacteria was evaluated by cell viable counting. For the assay, 1.0 g of foam nests collected aseptically was immediately dissolved in 9.0 ml of sterile saline and the suspension was homogenized by vortexing for 2 min. The resultant solution was serially diluted (10^{-1}, 10^{-2}, up to 10^{-6}) and 0.1 ml of each dilution was spread onto Müller–Hinton Agar (Difco Laboratories, Detroit, MI, USA) in duplicate. Simultaneously, samples of pond water from the same environment as the foam nests were analysed. The plates were incubated at 30°C and observed for development of colonies over 48 h. The test was repeated twice. The macromorphology of colonies was analysed and cell morphology studied in slide preparations stained by the Gram procedure.

Antimicrobial activity

The antibacterial susceptibility test was determined by the disc diffusion method on Müller–Hinton agar, as described by Bauer and colleagues (Bauer et al., 1966) against the following bacteria from American Type Culture Collection (ATCC): *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 25319), *Salmonella choleraesuis* (ATCC 10708), *Bacillus subtilis* (ATCC 6633), *Chromobacterium violaceum* (ATCC 12472), *Klebsiella pneumoniae* (ATCC 10031) and *Enterobacter aerogenes* (ATCC 13048). The antifungal activity test was done as described by Bormann and colleagues (Bormann et al., 1999). Briefly, an agar plug containing mycelia of the test fungus was placed in the centre of a Petri dish containing 15 ml of Sabouraud agar (Difco) and incubated until the colony reached a diameter of 3–4 cm. A 6 mm sterile paper disc that had absorbed 15 µl of foam fluid was placed at the growing front of the hyphae, and the test plates were incubated at 30°C and monitored for inhibition of hyphal growth. The tests were done against the fungi *Trichoderma viride*, *Penicillium herguei*, *Colletotrichum lindemuthianum*, *Fusarium solani*, *Aspergillus fumigatus*, *Pythium oligandrum*, *Phomopsis* sp., *Rhizoctonia solani*, *Mucor* sp. and *Neurospora* sp. The tests were done in duplicate and repeated twice. All microorganisms were obtained from the culture collections of the Microbial Ecology and Biotechnology Laboratory, Biology Department of the Federal University of Ceara, Brazil.

Haemagglutinating activity

Haemagglutinating activity of the foam fluid was carried out essentially as described by Moreira and Perrone (Moreira and Perrone, 1977). Serial 1:2 dilutions of the foam fluid were mixed in small glass tubes with untreated or trypsin-treated rabbit, rat and mouse erythrocytes (2% suspension prepared in 0.15 mol l^{-1} NaCl). The enzyme-treated cells were obtained by incubation of trypsin (0.1 mg) with 25 ml of the 2% suspension for 60 min at 4°C. After washing six times, a 2% suspension was prepared in 0.15 mol l^{-1} NaCl. The degree of agglutination was monitored visually after the tubes had been left to stand at 37°C for 30 min and at room temperature for an additional 30 min. The haemagglutination titre was defined as the minimal amount (µg) of protein per millilitre able to induce visible erythrocyte agglutination. The assay was performed in duplicate and repeated twice.

Haemolytic activity

The assay for haemolytic activity of the foam fluid was done using rabbit erythrocytes according to a previous publication (Nakajima et al., 2003). Erythrocytes were isolated from heparinized blood by centrifugation at 1000 ***g*** for 5 min and washed three times with phosphate-buffered saline (PBS), pH 7.4. A 1000 µl sample of cell suspension adjusted to 1×10^{9} cells ml^{-1} in PBS, pH 7.4, was incubated at 37°C for 30 min with an equal volume of foam fluid. The incubation media were centrifuged at 1000 ***g*** for 5 min and aliquots (200 µl) of supernatant were diluted in water (2000 µl). The

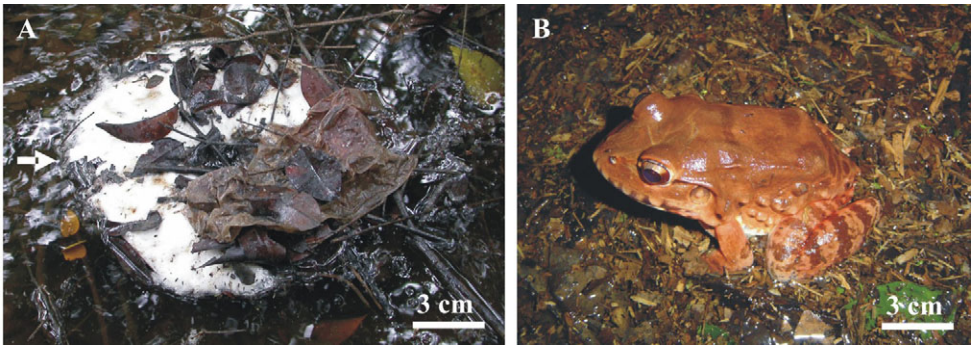

Fig. 1. (A) Foam nests of *Leptodactylus vastus* (B).

absorbance of the diluted solution was measured at 420 nm. The absorbance values obtained after treating erythrocytes with PBS and 2% SDS were taken as 0% and 100%, respectively. The test was performed in duplicate and repeated twice.

Larvicidal activity

The larvae toxicity assay was run against *Aedes aegypti* according to Thangam and Kathiresan (Thangam and Kathiresan, 1991), with some modifications. Third stage larvae were collected with a Pasteur pipette placed on filter paper for removal of excess water and transferred (10 per test) with a tiny brush into test tubes containing 5 ml of 20% foam fluid (v/v). Larvae were exposed to the samples at room temperature and mortality/survival registered after 24 h. Each test was run in triplicate and distilled water was used as the control.

Toxicity activity

Foam fluid toxicity was assessed in mice (Swiss strain from the animal facilities of the Federal University of Ceara, Brazil) by intraperitoneal injection of fluid (30 mg protein kg^{-1} body weight) according to Litchfield and Wilcoxon (Litchfield and Wilcoxon, 1949). The animals were kept under observation until 48 h after inoculation for description of symptoms or death quantification.

Surfactant activity

The surfactant activity was evaluated by the emulsification activity (Iqbal et al., 1995). Briefly, the foam fluid (1.0 mg ml^{-1}) or the purified protein (0.1 mg ml^{-1}) was combined with the same volume of kerosene in a 20 ml screw-capped tube and was homogenized using a vortex for 2 min and left to stand for 24 h. The emulsification index (EI, %) was calculated using the equation EI=(height of emulsion layer/height of oil plus emulsion layer)×100. The capacity of the foam fluid to reduce the water surface tension was also measured according to the Du Nouy ring method (ASTM D971, ASTM International Standards Worldwide; http://www.astm.org/) using pure water at 30°C for calibration. Both the surface tension and EI measurements were performed in triplicate and each experiment was repeated twice.

Isolation and characterization of the surfactant protein of the foam fluid

The surfactant protein of the foam fluid was purified by CM-Sepharose chromatography. Briefly, 16 mg of lyophilized foam fluid was dissolved in 2.0 ml of 0.05 mol l^{-1} Tris-HCl buffer pH 7.0 and applied to a CM-Sepharose fast flow column equilibrated with the same buffer. After elution of non-retained proteins with 0.05 mol l^{-1} Tris-HCl buffer pH 7.0, the adsorbed proteins were eluted with the same buffer containing 0.5 mol l^{-1} NaCl. The chromatography was monitored at 280 nm and the surfactant activity followed through an emulsification activity test. The protein fractions were subjected to Tricine-SDS-PAGE according to Schägger and von Jagow (Schägger and von Jagow, 1987). Protein bands were stained with 0.05% Coomassie Brilliant Blue R-250.

N-terminal sequence analysis

The N-terminal amino acid sequence of the surfactant protein was determined on a Shimadzu PPSQ-10 automated protein sequencer (Kyoto, Japan) performing Edman degradation. The sequence was determined from protein blotted on polyvinylidene fluoride (PVDF) after Tricine-SDS-PAGE. Phenylthiohydantoin (PTH) amino acids were detected at 269 nm after separation on a reversed phase C_{18} column (4.6 mm×2.5 mm) under isocratic conditions, according to the manufacturer's instructions. The sequence was compared with available amino acid sequence databases. The sequence was subjected to automatic alignment, which was performed using the NCBI-BLAST search system (Altschul et al., 1997).

RESULTS

L. vastus foam nest characterization

The foam nests of *L. vastus* contain proteins (1.0±0.2 mg ml^{-1}) and carbohydrates (0.3±0.02 mg ml^{-1}). The absorption spectrum analysis of the foam fluid showed a peak of absorption at 280 nm, confirming the presence of proteins in the fluid. The foam nests were colonized by a bacterial community estimated at 3.0–4.0($\times 10^7$) cfu ml^{-1} (cfu, colony-forming units) predominantly formed by Gram-positive bacilli. The bacterial count for pond water samples was about the same.

Biological activity

The foam fluid did not show antimicrobial activity, acute toxicity to mice or larvicidal action. As for haemagglutinating activity, the foam fluid showed only traces of agglutination in trypsin-treated rat erythrocytes but it was negative for rabbit and mouse erythrocytes. In addition, trypsin-treated rat and rabbit erythrocytes showed only slight haemolysis when in contact with foam fluid.

Surfactant activity

The whole foam fluid (1.0±0.2 mg protein ml^{-1}) was able to emulsify kerosene and this activity was quite stable, persisting for over a month, even when the tubes containing the fluid and the hydrocarbon were placed upside down (data not shown). The emulsification index was 62%. The foam fluid also reduced the surface tension of the water from 74 to 52 mN m^{-1}.

Purification and characterization of a surfactant protein

The foam fluid was separated into two fractions by ion-exchange chromatography. The non-retained fraction eluted with 0.05 mol l^{-1} Tris-HCl pH 7.0 showed a well-defined and dense protein band of approximately 20 kDa, whereas the retained fraction, eluted with 0.5 mol l^{-1} NaCl in the same buffer, was characterized by exclusion of the 20 kDa band (Fig. 2). Both fractions showed surfactant activity and the 20 kDa protein at 0.1 mg ml^{-1} presented an emulsification index of 57%. The N-terminal amino acid sequence of the 20 kDa protein had the following composition: FLEGFLVPKVVPGPTAALLKKALDD.

DISCUSSION

In spite of the ecological and physiological importance of frog foam nests, little is known about their production and composition. Analyses of the foam fluid composition of the frog *L. vastus* showed proteins and carbohydrates, which presumably are responsible for providing nutrients to the developing tadpoles. The foam nests do not prevent the colonization of microorganisms, such as the observed bacterial community of predominantly Gram-positive bacilli, which accounted for 3.0–4.0($\times 10^7$) cfu ml^{-1}. The foam production resembles that of beaten egg white but the frog foam nests are considerably less viscous and show long-term stability. *L. vastus* foam nests last for weeks, which is particularly important as this frog species lives in environments where the rainfall is unpredictable and the tadpoles, which ecloded about 7 days after oviposition, may remain in the nests until they are washed away by rain (Vieira et al., 2007). The foam nests also make it possible for *L. vastus* to lay the eggs at the beginning of the rainy season, even before the

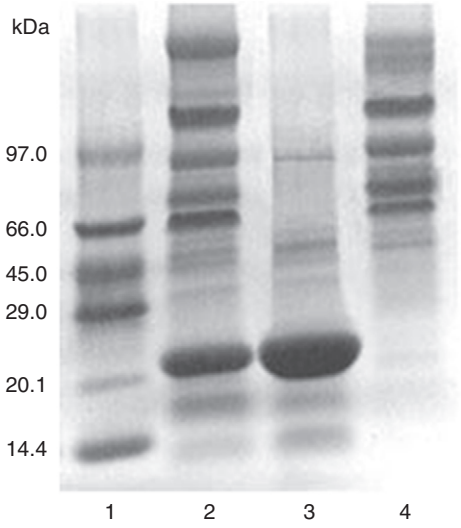

Fig. 2. Denaturing Tricine-SDS-PAGE of the foam fluid and fractions obtained during isolation of the surfactant 20 kDa protein. Lane 1, molecular mass standards; lane 2, foam fluid (20 μg); lanes 3 and 4, non-retained and retained fractions, respectively, in CM-Sepharose (20 μg). Molecular mass markers were phosphorylase b (97.0 kDa), bovine serum albumin (67.0 kDa), egg white albumin (45.0 kDa), carbonic anhydrase (29.0 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa).

complete formation of the water ponds in the margins of which the foam nests are produced, as the tadpoles can remain in the foam nests until the water volume increases and washes the tadpoles to the pond.

Investigation of the role of foam nests in chemical defence revealed no significant biological activity that could be associated with some recognized defence compounds such as lectins, enzymes, haemolysins, antimicrobial compounds or toxins, commonly detected in secretions released by vertebrates (Bols et al., 1986; Conceição et al., 2007; Che et al., 2008) and invertebrates (Canicatti et al., 1992; Becerril et al., 1996; Melo et al., 2000; Derby, 2007).

The foam nests of *L. vastus* seem to be involved in the physical protection of eggs and tadpoles. This protection is ensured by its unusual stability, which allows the eggs to be suspended in the water or to develop on land, giving good access to moisture, oxygen and a food source, the latter being the foam itself or the non-fecundated eggs (Vinton, 1951; Muedeking and Heyer, 1976). Foam nests may float on the surface of warm tropical pools, providing access to atmospheric oxygen for the newly hatched tadpoles (Seymour and Loveridge, 1994) and preventing the eggs from sinking to areas with lower oxygen content (Seymour and Roberts, 1991). Terrestrial incubation of typically aquatic eggs provides increased access to oxygen but also increased risk of desiccation and sun damage (Martin and Strathmann, 1999). In this particular case, the UV absorbance of the foam nests may be fundamental to the prevention of desiccation and sun damage since the foams absorb maximally at 280 nm, protecting eggs and embryos against UV injury. As a matter of fact, this was one of the first hypothesized functions for foam nests (Gorzula, 1977; Downie, 1988). The UV absorbance of foam nests is particularly important since the decline in amphibian populations has been widely reported in recent times and several studies have suggested that UV radiation may be one of the contributing factors (Blaustein and Belden, 2003).

The foam nest of the frog *L. vastus* is characterized by long-term stability and surfactant activity. These properties probably provide desiccation resistance either by slowing evaporative water loss or by drawing water towards the developing eggs.

Cooper and colleagues have already described the foam nest properties of the common frog from Central/South America and parts of the Caribbean, *P. pustulosus* (Cooper et al., 2005). Those authors have also reported some physicochemical properties of the crude foam fluid of this frog, and have named the mixture of proteins in the 10–40 kDa mass range ranaspumin. They presented evidence of the extraordinary surfactant activity of ranaspumin and its involvement in foam nest stabilization. Nevertheless, the authors did not associate any protein in particular with the surfactant activity. In the present work, the foam fluid of *L. vastus* showed a different profile comprising several proteins with molecular masses in the range 14 to over 97 kDa.

The foam fluid (1.0±0.2 mg protein ml^{-1}) of *L. vastus* showed strong and stable emulsification and was particularly effective in reducing the water surface tension, which dropped from 74 to 52 mN m^{-1}. Cooper and colleagues have obtained similar results for water surface tension with foam fluid from *P. pustulosus* (Cooper et al., 2005), distinguishing the superiority of this property when compared with those of common globular proteins such as bovine serum albumin (BSA), which at the same concentration, 1.0 mg ml^{-1}, reduced the water surface tension to 65 mN m^{-1}. This is a significant finding considering that surfactants produced by vertebrates are of great interest due to their biodegradability and biocompatibility, with a huge potential for biomedical and industrial utilization (Kaneko et al., 1990; Makkar and Rockne, 2003; Rodrigues et al., 2006).

Chemicals with surface-active properties are synthesized by an amazing variety of living bodies, from plants (e.g. saponins) and microorganisms (e.g. glycolipids, lipopeptides/lipoproteins, phospholipids, neutral lipids, substituted fatty acids, lipopolysaccharides and hydrophobins) to higher complexity animals (e.g. bile acids) for intra- and extracellular activities, which may range from emulsification of food for the transport of material across cell membranes to recognition of cells and defence (Ivshina et al., 1998; Hofmann, 1999; Wang et al., 2003). Thus, frog foam nests appear to be new sources of surface-active compounds that need to be explored for better characterization, understanding of their biological role and potential use.

As an attempt to identify and better characterize the proteins responsible for the surfactant activity, the foam fluid of *L. vastus* was subjected to ion-exchange chromatography, which allowed us to separate its proteins into two fractions. A well-defined and dense protein band of approximately 20 kDa appeared in the non-retained fraction, which showed a strong emulsification activity (EI 57%) at a concentration of 0.1 mg ml^{-1} similar to that shown by the whole foam fluid tested at 1.0 mg ml^{-1} (EI 62%). The mixture of proteins in the retained fraction also showed surfactant activity, confirming the participation of more than one protein with this function in the foam of *L. vastus*. Thus, all these proteins presumably have a role in the construction of the foam nests and it may be hypothesized that the 20 kDa protein isolated in the present work represents a major function in foam nest stabilization.

Partial sequencing of the 20 kDa band revealed an N-terminal amino acid sequence consisting of 25 amino acid residues in the following order: FLEGFLVPKVVPGPTAALLKKALDD. Using the NCBI-BLAST search system program, no matches in current databases were observed, indicating it to be a novel protein. The protein sequence data reported in this work will appear in the UniProt Knowledgebase under the accession number P85507. These data

are of paramount importance since they associate the surfactant activity with a component of protein nature originating from a vertebrate. This protein will hereafter simply be referred to as *Lv*-ranaspumin (ranaspumin from *L. vastus*).

Lv-ranaspumin seems to be different from ranasmurfin, another surfactant protein, which has been isolated from the foam nests of *P. leucomystax* (McMahon et al., 2006). These proteins differ in their molecular masses – 20 and 13 kDa for *Lv*-ranaspumin and ranasmurfin, respectively. Moreover, ranasmurfin can occur in dimeric form while *Lv*-ranaspumin occurs only as a monomer. In addition, the 26 kDa dimer of *P. leucomystax* is strongly blue due to a chromogen in its structure whereas *Lv*-ranaspumin did not show any colour. Coincidently, neither protein revealed any match to any known protein or structure in the databases. This reinforces the notion that *Lv*-ranaspumin as well as ranasmurfin are novel proteins and therefore may show different and interesting properties, to which further studies must be dedicated.

In conclusion, the foam nests of *L. vastus* are rich sources of surfactant proteins and *Lv*-ranaspumin is the major surfactant component of the foam. This 20 kDa protein did not show any match to any known protein or structure, which suggests that it belongs to a new class of surfactant protein.

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