RESEARCH ARTICLE

Frog Foam Nest Protein Diversity and Synthesis

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ABSTRACT

Some amphibian species have developed a breeding strategy in which they deposit their eggs in stable foam nests to protect their eggs and larvae. The frog foam nests are rich in proteins (ranaspumin), especially surfactant proteins, involved in the production of the foam nest. Despite the ecological importance of the foam nests for evolution and species conservation, the biochemical composition, the long-term stability and even the origin of the components are still not completely understood. Recently we showed that Lv-RSN-1, a 23.5-kDa surfactant protein isolated from the nest of the frog *Leptodacylus vastus*, presents a structural conformation distinct from any protein structures yet reported. So, in the current study we aimed to reveal the protein composition of the foam nest of *L. vastus* and further characterize the Lv-RSN-1. Proteomic analysis showed the foam nest contains more than 100 of proteins, and that Lv-RSN-1 comprises 45% of the total proteins, suggesting a key role in the nest construction and stability. We demonstrated by Western blotting that Lv-RSN-1 is mainly produced only by the female in the *pars convoluta*

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dilata, which highlights the importance of the female preservation for conservation of species that depend on the production of foam nests in the early stages of development. Overall, our results showed the foam nest of *L. vastus* is composed of a great diversity of proteins and that besides Lv-RSN-1, the main protein in the foam, other proteins must have a coadjuvant role in building and stability of the nest. *J. Exp. Zool. 325A:425–433, 2016.* © 2016 Wiley Periodicals, Inc.

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INTRODUCTION

The foam nest production is a breeding strategy applied by some tunicates, fishes, and amphibian anurans, by which they deposit their eggs in environmentally stable foams to protect the eggs and larvae along their initial developmental stages (Andrade and Abe, '97; Hostache and Mol, '98; Cooper et al., 2005; Castilla et al., 2007) against potential predators, pathogens (Heyer, '69; Downie, '93; Fleming et al., 2009), desiccation (Ryan, '85; Downie, '88), and thermal damage (Gorzula, '77).

Within the 41 different reproductive modes found in anurans (Haddad and Prado, 2005; Iskandar et al., 2014; Seshadri et al., 2014), 10 involve the foam nest construction. This reproductive strategy is present in most genera of the new world family Leptodactylidae, including *Leptodactylus*. The nests can be aquatic, floating on water (e.g., some species from the genus *Leptodactylus*, *Physalaemus*, *Pleurodema*, and *Engystomops*), or terrestrial and placed in excavated basin (e.g., *L. pentadactylus* species group), in subterranean constructed chambers (e.g., genus *Adenomera*, *L. fuscus* species group), or even arboreal (e.g., genus *Chiromantis*, *Ghatixalus*, *Polypedates*, *Rhacopharus*, and *Taruga* (Haddad and Prado, 2005; Meegaskumbura et al., 2014).

The production of foam nests has evolved multiple times and independently in several major clades of anurans (Wells, 2007; Faivovich et al., 2012), showing a more complex evolution trend than previously thought (Heyer, '69).

In spite of the fascinating properties related to the amphibian foam nests and the curiosity to understand how they stand stable for so long (in some cases more than 1 month), only few studies have been published concerning biochemical composition, molecular pathways underlying their syntheses, and long-term stability.

The composition of foam nest was first described by Kabisch et al. ('98), who showed the arboreal foam nest of *Polypedates leucomystax* was composed of 93% of proteins and 7% of sugars. Later, Cooper et al. (2005), Fleming et al. (2009), and Mackenzie et al. (2009) have showed that the foam nest of *Engystomops pustulosus,* which is laid in water, is rich in proteins (named ranaspumins) and it is only maintained by the presence of surfactant proteins. Fleming et al. (2009) also proposed a hypothetical model for the overall structure of the foams, in which surfactant proteins, lectins, and carbohydrates are the components that hold the foams. However, the species studied by these authors have different reproductive modes from *L. vastus* and it is possible that several different adaptations were developed by different species reflecting on the biochemical composition of the nest, especially for the ones build outside the water.

We have described the protein *Lv*-ranaspumin (Lv-RSN-1) (Hissa et al., 2008), the major protein in the foam nest of the pepper frog *L. vastus* A. Lutz 1930 (Heyer, 2005), endemic from northeastern Brazil (De Sá et al. 2014). Lv-RSN-1 is a monomeric protein with molecular mass of 23.5 kDa, presenting surfactant activity and novel tridimensional conformation (Hissa et al., 2012, Hissa et al., 2014).

In this study, we aimed to access the protein diversity of the foam nest of *L. vastus* and identify the tissue localization of Lv-RSN-1 in the frog, intending to understand the origin of this protein that should be a key component for terrestrial foam nests.

MATERIALS AND METHODS

Collection of Frog and Foam Nest

Foam nests and adult specimens of the frog *L. vastus*, two females and two males, were collected in Campus do Pici, Universidade Federal do Ceará, Brazil during the rainy season with proper regard to Brazilian regulations. Once captured, the frogs were euthanized according International animal ethics standards (American Veterinary Medical Association. AVMA Guidelines for the Euthanasia of Animals: 2013 Edition. Schaumburg, IL, United States of America. 2013. ISBN 978-1-882691-21-0.) and dissected to collect the following organs from the reproductive system: ovaries, ovisac, the enlarged portion of the posterior pars convoluta [also called pars convoluta dilata (Furness et al., 2010), described by Bhaduri and Rudra ('44) and Coe ('74)], and cloaca for female specimens and testis and cloaca of males (Fig. 1). All efforts were made to minimize suffering. After collection, organs were frozen in liquid nitrogen for extraction of total protein. The females examined in this study were mature and had ovaries with large oocytes, indicating that they were collected during the breeding season. All specimens used in this study were deposited in the Collection of Amphibians and Reptiles of

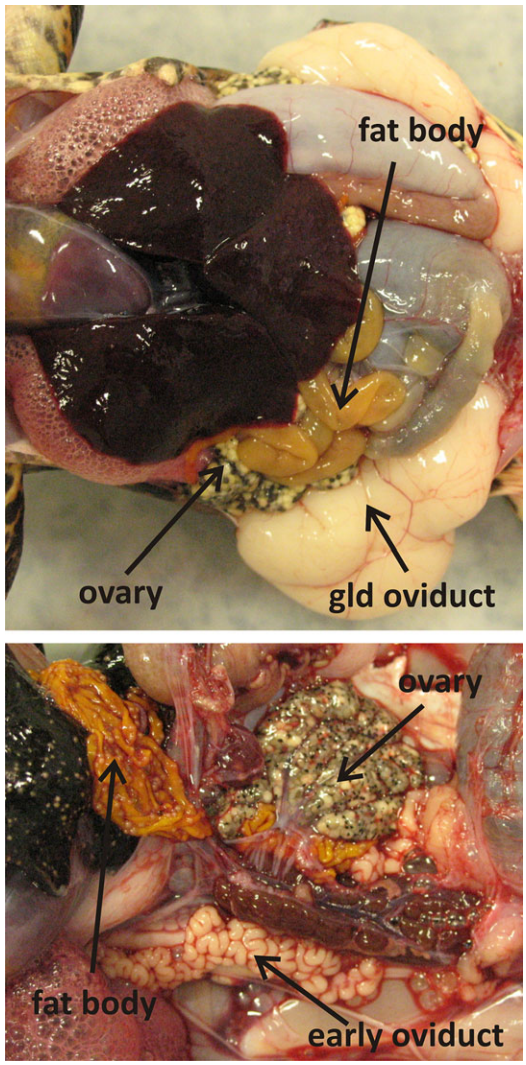

Figure 1. Urogenital female system from *Leptodactylus vastus*, where gld oviduct stands for the glandular region of the oviduct also called *pars convoluta dilata*.

the Federal University of Ceará, NUROF (voucher A4480, A4642, A4550, and A4481). Nests were carefully sampled from the edges of temporary standing water, placed in sterile plastic bags and brought to laboratory, where they were manually cleaned under aseptic conditions, to remove eggs, sand, leafs, and branches. The foam fluid was obtained by drainage and then lyophilized for further uses.

Protein Determination

Protein content was determined by the conventional Bradford method using bovine serum albumin as the standard (Bradford, '76).

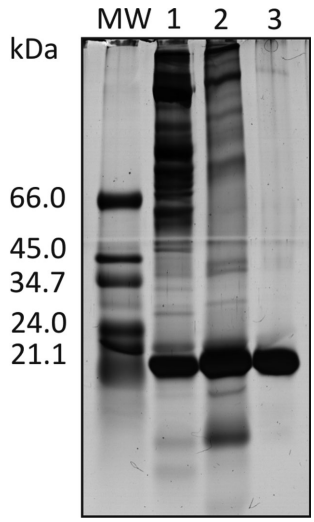

Figure 2. Denaturing tricine-SDS-PAGE showing isolation of Lv-RSN-1. MW, Molecular weight markers (Sigma); 1, foam fluid (3 μg); 2, retained fraction from the DEAE–cellulose chromatography (3 μg); and 3, fraction obtained from the RP-HPLC (3 μg). Gel was revealed by silver staining.

Lv-RSN-1 Purification

The main surfactant protein of the foam fluid was purified by diethyl-aminoethyl (DEAE)–cellulose chromatography followed by reverse phase high-performance liquid chromatography (RP-HPLC). Fifty milligrams of lyophilized foam fluid was dissolved in 5 mL of 0.05 mol L^{-1} Tris–HCl buffer (pH 7.3) and applied to a DEAE–cellulose column (Sigma–Aldrich, Missouri, USA) equilibrated with the same buffer at a 1.5-mL min^{-1} flow rate. After elution of nonretained protein with Tris–HCl buffer pH 7.3, the adsorbed proteins were eluted with the same buffer containing 0.5 mol L^{-1} NaCl. The retained proteins were dialyzed against ultrapure water using 10 kDa membranes and then lyophilized. Two milligrams of the desalted lyophilized retained proteins was dissolved in ultrapure water containing v/v 0.1% of trifluoroacetic acid (TFA) and applied to a RP-HPLC (Shimadzu, Japan) equipped with a Source 15RPC ST 4.6/100 column (GE Healthcare Bio-Science, NJ, USA). The fractions were eluted and manually collected under a 45-min linear gradient (2–98%) at a flow rate of 1.0 mL min^{-1} of acetonitrile containing 0.1% TFA (v/v). Fractions were lyophilized, and purity was routinely monitored by tricine-SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) (Schägger and von Jagow, '87) using molecular-mass markers purchased from Sigma-Aldrich and revealed with silver staining (Blum et al., '87) following mass spectrometry analysis and surfactant activity determination (Fig. 2). The DEAE–cellulose chromatography and RP-HPLC were monitored at 280 nm.

Anti-Lv-RSN-1 Polyclonal Antibody Preparation

For production of polyclonal serum against Lv-RSN-1, preparative tricine-SDS-PAGE gels were performed according to Schägger and Von Jagow ('87). All lanes were run with 10 μg of the foam fluid. After the electrophoresis, the bands relatives to Lv-RSN-1 (around 23 kDa) were removed using razor blades, macerated in sterile saline solution and centrifuged at 10,000×g for 10 min at 4°C. The supernatant was mixed 1:1 with Freund's complete adjuvant and injected into rabbits intramuscularly, after obtaining the preimmune serum. Boosters of the antigen were subcutaneously administered after 14, 21, 28, and 35 days of the primary injection. Blood was collected from the ear vein of the rabbit after each booster. Blood was allowed to clot for 2 hr at room temperature and then at 4°C overnight. Sera were then removed from the clot, centrifuged at 10,000×g for 5 min and stored at −20°C until use in the Western blotting protocol.

Lv-RSN-1 Tissue Localization

The organs obtained from the frogs were macerated with minimum required amount of distilled water for crude extract preparation and then centrifuged at 10,000×g for 20 min. The protein concentration of each extract was measured according to Bradford ('76), and 20 μg of protein was loaded on native tricine-SDS-PAGE. The resolved proteins were electrophoretically transferred (100 V, 1 hr) to polyvinylidene fluoride (PVDF) membrane (0.45 μm pore size; Millipore), according to the procedure described by Towbin et al. ('79). Residual unoccupied protein binding sites were blocked with 5% skimmed milk powder in Tris–HCl 0.05 M, pH 7.4, 0.15 M NaCl, and 0.5% Tween 20 for 1 hr. The PVDF sheet was then incubated for 2 hr at room temperature in the Lv-RSN-1 antiserum diluted 1/500 in the blocking solution and washed four times for 10 min with the same buffer, without the skimmed milk. Subsequently, it was incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (1:2,000) for 2 hr at room temperature. Then, PVDF was washed and the color reaction was developed with 5-bromo-4-chloro-indolyl-phosphate and nitroblue tetrazolium (Sigma-Aldrich, SP, Brazil).

Isoelectric Focusing and Two-Dimensional Electrophoresis

The parameters for isoelectric focusing (IEF) were empirically determined, although some directions were previously published (Gorg et al., '88). IEF was carried out using Ettan™ IPGPhor II™ system (GE Healthcare Bio-Science, NJ, USA). Immobilized pH gradient (IPG) 3–10 and 4–7 linear gel strip (13 cm; GE Healthcare Bio-Science) were first rehydrated overnight at 25°C with the foam fluid proteins diluted in the rehydration buffer containing 7 mol L^{-1} urea, 2 mol L^{-1}thiourea, 10 μg μL^{-1} 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 10 μg μL^{-1} dithiothreitol (DTT), 0.01 μg μL^{-1} bromophenol blue, and 0.6 % (v/v) of ampholites (IPG buffer; GE Healthcare Bio-Science) and covered with mineral oil in a reswelling tray. The IEF was performed in a stepwise mode: 200 V for 1 hr, 500 V for 1.5 hr, 5,000 V for 2.5 hr, and 10,000 V until reaching 18,000 V hr^{-1}. After focusing, each IPG gel strip was placed into a screw-cap tube with 5 mL equilibration buffer (50 mM Tris–HCl, pH 8.8, containing 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 1% (w/v) bromophenol blue containing DTT 1% (w/v). After shaking for 15 min, the first equilibration solution was replaced by the second equilibration solution containing 2.5% (w/v) iodoacetamide. The second dimension of the 2D-SDS-PAGE (Laemmli, '70) was conducted in a vertical system (Hoefer SE 600; GE Healthcare Bio-Science) under 25 mA constant current per gel and 250 V of maximum tension. Gels had identical dimension (140 × 140 × 1 mm) and were performed using 12.5% polyacrylamide gels without a stacking gel, in three replicates. Gels were stained with 0.06% (w/v) Coomassie Brilliant Blue G-250 in 10% (v/v) acetic acid, after a 3-hr-fixation step in 25% (v/v) 2-propanol and 10% (v/v) acetic acid solution. Gels images were scanned using an Imager Scanner (GE Healthcare Bio-Science) with the help of LabScan software. All details of individual gels and comparative analysis were performed using ImageMaster 2D Platinum Software (GE Healthcare Bio-Science).

Mass Spectrometry

To extract the spots corresponding to Lv-Rsn-1, spots were excised from the two-dimensional electrophoresis (2-DE) gel, washed with 50 mM NH_4HCO_3 and acetonitrile, dried, and rehydrated with digestion solution (12.5 ng μL^{-1} of trypsin in 50 mM NH_4HCO_3). After 1 hr, incubation at 4°C, the supernatant of the digestion solution was replaced by 50 mM NH_4HCO_3 and left overnight at 37°C. The digestion solution was further extracted for 30 min with 10 μL of 25 mM NH_4HCO_3 / acetonitrile (1:1, v/v), after that the solutions were dried in SpeedVac (Labconco, Kansas, MO, USA) and dissolved in 10 μL ultrapure water. One microliter of each peptide mixture was then dissolved (1:3, v/v) in the matrix solution (10 mg $mL^{-1}$$\alpha$-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile and 10% (v/v) TFA), spotted onto a MALDI target plate and dried at room temperature for 15 min. The peptide monoisotopic masses were obtained in reflector mode of a MALDI TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) with external calibration, using the Peptide Calibration Standard Mixture (Bruker Daltonics).

Thermal Stability Analysis of Lv-RSN-1 by Circular Dichroism Spectroscopy

The secondary structure of the purified Lv-RSN-1 (0.2 mg mL^{-1}) was studied by circular dichroism (CD) spectroscopy. The CD spectra were recorded on a Jasco J-815 CD spectropolarimeter (Tokyo, Japan) from 190 to 250 nm using a 1-mm quartz cuvette, at 25°C. Each spectrum was obtained as an average of eight scans and the subtraction of the reference spectrum. The estimation of secondary structure content was performed with the CD-Pro package, with the software *Selcon3*, *Continll,* and *CDSSTR*

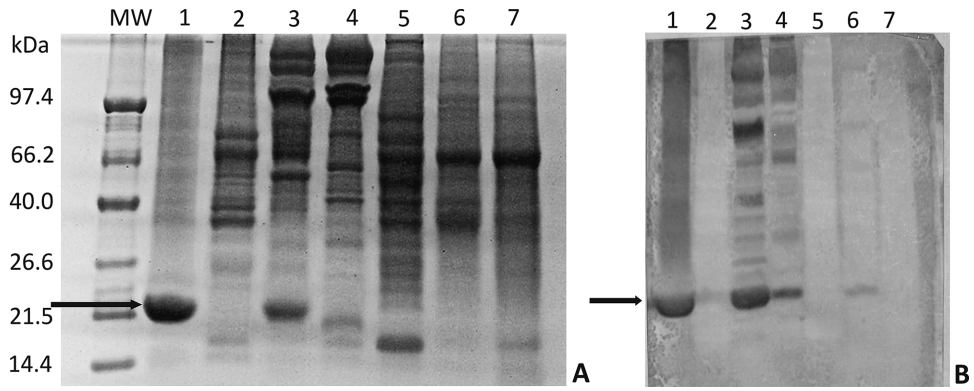

Figure 3. (A) Electrophoresis gel of the proteins of the reproductive systems's organs of the frog *Leptodactylus vastus*. Legend: MW, Molecular weight markers; 1, foam fluid (10 μg of proteins) and organs extract (10 μg of proteins) from: 2, ovary; 3, posterior (glandular) region of the oviduct also called *pars convoluta dilata*; 4, early oviduct; 5, testis; 6, cloaca from female frog; and 7, cloaca from male frog. (B) Western blotting of the existing proteins in the electrophoresis gel in Fig. 3A recognized by anti-*lv*-ranaspumin antibody and revealed with anti-rabbit IgG conjugated to alkaline phosphatase. The arrows represent the protein *lv*-ranaspumin.

using a 56 protein reference set (Provencher and Glockner, '81; Sreerama and Woody, '93; Johnson, '99). To study the protein thermal stability, it was performed a thermo scan in the wavelength 208 nm, in the temperature range from 25 to 95°C with a measurement at each 0.2°C.

RESULTS

Surfactant Protein (Lv-RSN-1) Tissue Distribution in *L. Vastus*

Lv-RSN-1 was detected in the early region of the oviduct, in the glandular part of the oviduct (ovisac), and cloaca. Antibodies did not detect the presence of this protein in the male organs, testis, and cloaca. Figure 3 shows the composition of proteins in the analyzed organs and the detection of the Lv-RSN-1 in these organs.

Profile of 2D Gels of *L. vastus* Foam Nest

On average, about 146 spots were visualized using Coomassie brilliant blue G-250 staining and package Image Master 2D Platinum Software in the gel corresponding to the pH 3–10 IPG strip for the first dimension. The Lv-RSN-1 appeared as a large spot with molecular mass around 23.5 kDa (Fig. 4A) and pI around 5. The Lv-RSN-1 corresponded to almost 45% of all proteins in the gel. On the pH range from 4.0 to 7.5 spots were visualized, and interestingly the large spot corresponding to Lv-RSN-1 was divided into five spots (Fig. 4B). All Lv-RSN-1 species presented molecular mass ranging from 23.8 to 24.1 kDa; however, their pI values ranged from 4.6 to 5.1. Three main spots (a, b, and c) were excised, hydrolyzed by trypsin and analyzed by mass spectrometry.

MS Spectra

Mass spectrometry (MS) showed very similar spectrum profile between the three protein components a, b, and c excised from the 2D gel using a pH range of 4–7 and digested with trypsin (Fig. 5). This result leads to the assumption that those have very similar amino acid residues sequence.

Thermal Stability

CD analysis showed that the protein is stable regarding temperature increase as its CD spectra was maintained in the temperature range from 25 to 95°C.

DISCUSSION

Since 1974, Coe ('74) had already suggested that the oviduct was responsible for the production of the foam fluid that leaves the cloaca and is whipped to a foam by the male. Kabisch et al. ('98) have added that the foam gland was located in the posterior part of the oviduct, 4–5 mm distant from the cloaca. A few years later, Furness et al. (2010) examined the oviduct modifications in foam-nesting frogs from many species of *Leptodactylus* and reported an enlarged portion of the pars convoluta (called of *pars convoluta dilata*); this morphological trait is characteristic of all foam-nesting species. In the meantime, Fleming et al. (2009) when working with *E. pustulosus* have demonstrated by transcriptomic analysis the presence of the mRNA encoding ranaspumins in different tissues (ovary, liver, kidney, among others), and the complete set of the six ranaspumins they studied was only found in the glandular part of the oviduct. However, the presence of the mRNA does not confirm that the protein will be translated and that it will be in its active state.

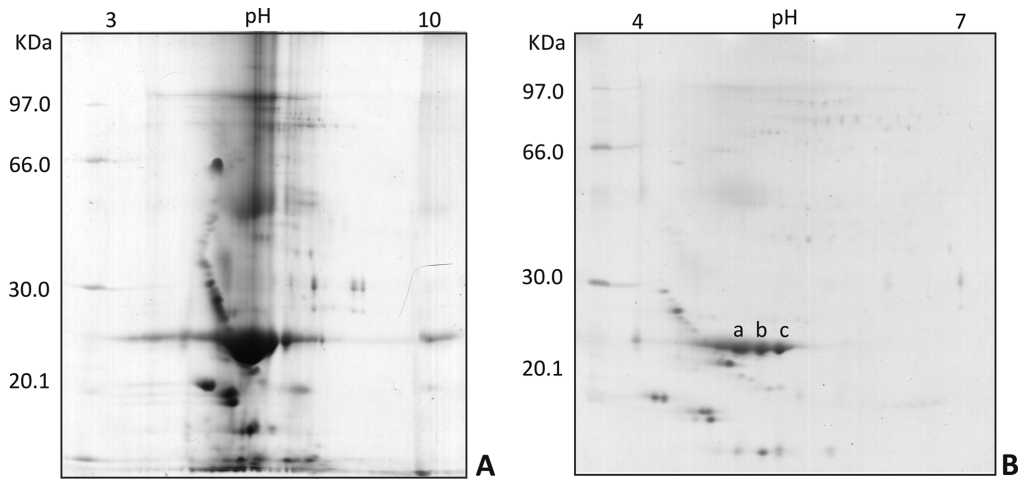

Figure 4. Coomassie brilliant blue G250-stained 2D-SDS-PAGE (12.5%) of foam fluid proteins. Gel separations were performed using the pH range of 3–10 (A) or 4–7 (B). It was added 370 μg of proteins in A and 92 μg in B. The protein spots a, b, and c were used for mass spectrometry.

The *L. vastus* female frogs examined in the present study also exhibited the enlargement of the pars convoluta dilata, and this specific region was strongly recognized by anti-Lv-RSN-1 antibody, confirming indeed, using a different technique, that this site may be the primary locale of the production and/or storage of the ranaspumins responsible for the production of foam nest. The anterior part of the ovisac and the cloaca was also recognized by the antibody, allowing the assumptions that these two organs may also produce small quantity of the proteins, or the protein was there due to remnants of the protein transportation. This is the first immunocytochemically localization of a protein isolated from a foam nest. Therefore, given the above results, it has no doubt that *pars convolute dilate* is the main site where the foam fluid is synthesized and this morphological trait is characteristic of all foam-nesting species.

The anterior part of the ovisac and the cloaca of *L. vastus* were also recognized by the Lv-RSN-1 antibody, allowing the assumptions that these two organs may also produce small quantity of the protein or are transit areas.

As an attempt to better characterize the proteins composition, the foam fluid was subject to a 2D electrophoresis. The obtained gels show a great diversity of proteins, with molecular weight ranging from 5 to 100 kDa. The Lv-RSN-1, with 23.5 kDa, appeared as the major spot (45%) in the gel with the pH range from 3 to 10, showing that this surfactant protein is very important in the construction of the nest and constitutes foam nest's main component.

The foam nest of *E. pustulosus* showed the existence of six major proteins appearing in the 2D-SDS-PAGE gels. Two presented an amino acid sequence unlike any other existent in protein data bank, and the other four presented partial similarities to other proteins previously reported, such as lectins. Among the set of six proteins, the Ranaspumin-2 (Rsn-2) showed a strong but unstable surface activity. Fleming et al. (2009) hypothesized a model for the arrangement of the ranaspumins explaining the long durability of the foam nests. The model proposed is composed of a layer of the surfactant protein, a layer of carbohydrates, and lectins stabilizing the foam nest.

Taking into account that egg deposition in foam is a case of convergence evolution in which amphibian foam nests are believed to have evolved independently at least seven times (Altig and McDiarmid, 2007; Furness et al., 2010; Faivovich et al., 2012), more studies are needed to confirm whether the model proposed by Fleming et al. (2009) was evolutionarily shared by other foam-nesting amphibian. It is important to emphasize that Lv-RSN-1 showed no sequence or conformation similarity to the proteins isolated from *E. pustulosus*'s foam nest and that the *L. vastus*'s foam nest presented at least 146 protein spots, a larger number than the six main spots found in the *E. pustulosus*'s foam nest. Also, the amount of carbohydrates found in *L. vastus*'s foam nest is at least five times less than the amount found for *E. pustulosus*'s and there was no evidence of lectins in the *L. vastus*'s foam nest (Hissa et al., 2008).

2D gels and MS analysis confirmed that only Lv-RSN-1 is composed of at least five species of proteins. Regarding to the MS spectra obtained from the three analyzed spots, it was noticed that the three proteins exhibited very similar spectra suggesting

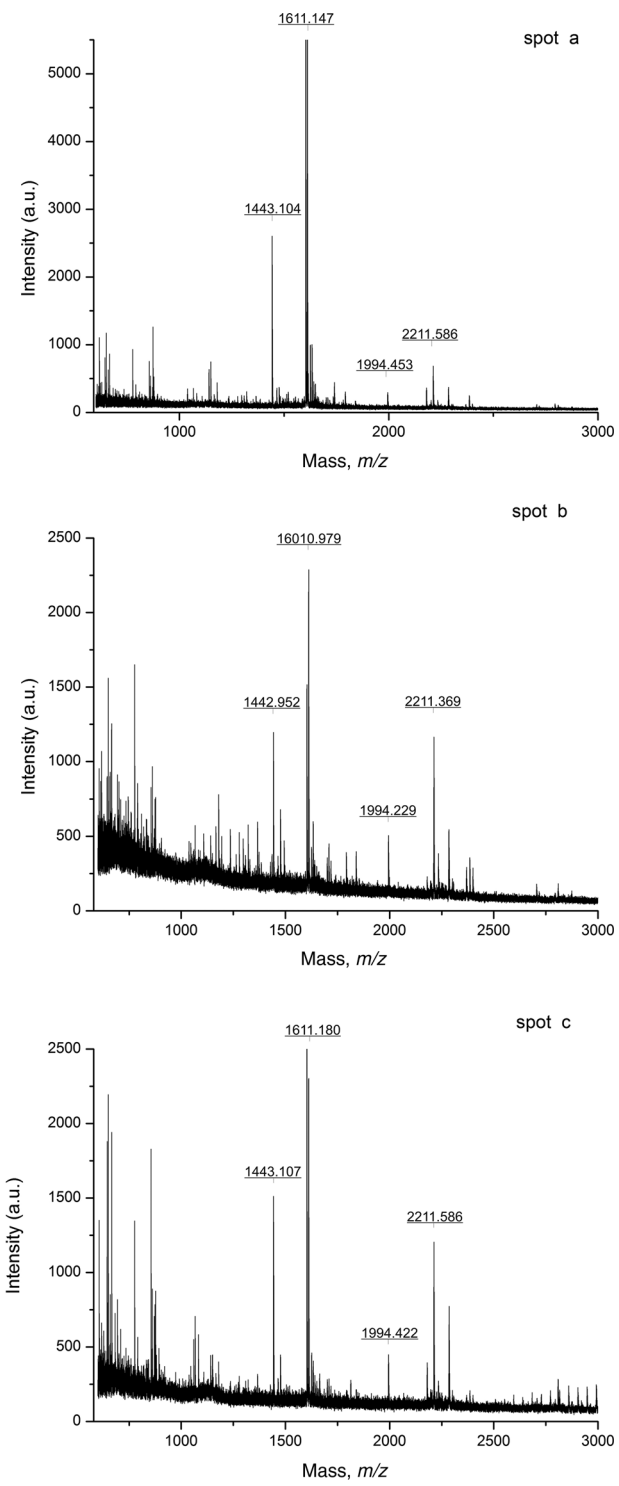

Figure 5. MALDI/TOF spectra (obtained in a reflector mode) of the three *lv*-ranaspumin species (a, b, and c) after digestion by trypsin.

that they have the same or a very similar amino acid sequence with microheterogeneities. The different isoelectric points can be due to few modifications in the amino acid sequence as confirmed by Hissa et al. (2014) and Hissa et al. (2012) showing the presence of at least four isoforms in the protein amino acid sequence of Lv-RSN-1. It is also important to mention that Lv-RSN-1 has no glycosylation or phosphorylation.

The surfactant proteins from foam nests are believed to have distinct hydrophobic and hydrophilic structural domains adsorbing naturally at the air/water interface, thus allowing the formation of the foams. Those proteins require a high-energy input for the formation of biofoams to defeat the unusually high surface tension of water; they should to be soluble, should have a high activity at low concentrations, and should be harmless to cell membrane of biological tissues, being therefore very singular in nature (Cooper and Kennedy, 2010).

The Lv-RSN-1 is a very stable protein, mainly due to the presence of at least four disulfide bridges. The purified protein retained its activity to reduce the surface tension even after heated at 100°C for 10 min. In the current study, we confirmed that the Lv-RSN-1 has maintained its secondary conformation in the temperature range from 25 to 95°C, confirming its thermal resistance and corroborating its environmental role in the eggs protection. *L. vastus* is a frog from the Brazilian semiarid region, in which the environment where they deposit the nests can reach up to 35°C and the proteins present in the nests are the main protection to the eggs against heat and UV radiation.

Ranaspumins are intriguing proteins not only due to the exclusive and notable surfactant properties but also regarding to its ecological roles in the foam nests. The results of this study have shown that our knowledge of the diversity, structure, and function of ranaspumins is still far from complete and offer new challenges for future studies on frog foam nests.

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