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Biochemical Analyses of Proteins from *Duttaphrynus melanostictus* (*Bufo melanostictus*) Skin Secretion: Soluble Protein Retrieval from a Viscous Matrix by Ion-Exchange Batch Sample Preparation

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Abstract
A crucial step in scientific analysis can be sample preparation, and its importance increases in the same rate as the sensitivity of the following employed/desired analytical technique does. The need to analyze complex, viscous matrices is not new, and diverse approaches have been employed, with different success rates depending on the intended molecules. Solid-phase extraction, for example, has been successfully used in sample preparation for organic molecules and peptides. However, due to the usual methodological conditions, biologically active proteins are not successfully retrieved by this technique, resulting in a low rate of protein identification reported for the viscous amphibian skin secretion. Here we describe an ion-exchange batch processing sample preparation technique that allows viscous or adhesive materials (as some amphibian skin secretions) to be further processed by classical liquid chromatography approaches. According to our protocol, samples were allowed to equilibrate with a specific resin that was washed with appropriated buffers in order to yield the soluble protein fraction. In order to show the efficiency of our methodology, we have compared our results to classically prepared skin secretion, i.e., by means of filtration and centrifugation. After batch sample preparation, we were able to obtain reproductive resolved protein chromatographic profiles, as revealed by SDS-PAGE, and retrieve some biological activities, namely, hydrolases belonging to serine peptidase family. Not only that, but also the unbound fraction was rich in low molecular mass molecules, such as alkaloids and steroids, making this sample preparation technique also suitable for the enrichment of such molecules.

Keywords Batch chromatography · Ion-exchange chromatography · Viscous sample · Amphibian skin secretion · Hydrolase

1 Introduction

Animals' poisons and venoms are among Nature's most complex biological fluids. Such molecular richness may be derived from Natural Selection, gene recruitment, maybe a phenotypic response to given environmental conditions or may even be related to the animal's own ontogeny [1–4].

Venoms can be considered chemical weapons that aid the predator on prey hunting or avoid predation, whereas poisons tend to form a protective barrier on the animal's skin, fighting microorganism infection (e.g., antimicrobial peptides, steroids, alkaloids) and predation, by means of its organoleptic properties and/or biologically active molecules that may—ultimately—sedate the predator or induce emesis or pain [5, 6]. Some of the most studied poisonous animals, regarding their skin secretions, are the Amphibians [7–10].

Currently amphibians comprise circa 7600 species, all belonging to the Lissamphibia subclass and further subdivided into three orders (Anura, Caudata and Gymnophiona) being Anura the most representative group with more than 6500 species [11]. Amphibians have evolved a very active skin, rich in glands (mucous and granular) believed to produce and store constitutive and toxic molecules, which take part in several physiological processes [12] or act as a chemical defense [5, 10, 13].

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Collecting small molecules (< 10 kDa) from the amphibian skin is (nowadays) a relatively simple routine: several peptides, alkaloids and steroids have already been identified from the most diverse amphibians [8, 14, 15] based, mainly, on RP-HPLC separation and mass spectrometry and/or NMR analyses. Various studies have been exploring these molecules as potential pharmacological agents against diseases and infections, including antiviral activity—against rabies virus [15]; anti-tumoral molecules [16, 17]; glycemic regulators in obesity and diabetes [18] and drugs that would be active over mild infections and/or the diabetic foot ulcers [19].

On the other hand, the skin secretion of some amphibians may be extremely hard to handle. Konig et al. described the *Dyscophus guineti* skin secretion as being "white, highly viscous, with a glue-like adhesive behavior right after release from then skin glands" [20]. Similar characteristics have also been described by (i) Prates et al. when studying *Leptodactylus lineatus* [21]; (ii) Sciani et al. for *Rhinella* and *Rhaebo* genera [7, 16] and (iii) by Weil and Davis for *Bufo alvarius* [22]. Evans and Brodie explicitly comment that the sticky and adhesive amphibian skin secretion could be a strategy against predation [23].

Due to those peculiarities and also the high content of low molecular mass molecules, protein characterization of skin secretions of some amphibian taxa can be hampered. Only few studies have identified or isolated proteins from *Rhinella* genus. Sousa-Filho et al. [9] performed the dialysis of *Rhinella schneideri* skin secretion to obtain a fraction containing molecules larger than 8 kDa. After some sample preparation strategies, authors studied its biological effects and performed preliminary proteomic analyses [9]. In another study, Anjolette et al. were able to generate two protein fractions from *R. schneideri* skin secretion after two mechanical filtrations and three chromatographic steps [24]. Although noteworthy, both studies provide only a glimpse of the proteome of *Rhinella* sp., as presented by the SDS-PAGE analyses performed by Sciani et al. [7].

According to these authors' understanding, sample preparation is vital for the study of any kind of biological material, particularly of complex matrices such as the *Rhinella* skin secretion. Therefore, the low rate of protein identification problem reported above could be solved by proper sample preparation. In this work we describe an ion-exchange batch processing sample preparation technique that allows viscous or adhesive materials to be further processed by classical liquid chromatography approaches. In this technique, samples were allowed to equilibrate with a specific resin that was washed with appropriated buffers in order to yield the soluble protein fraction. To show the efficiency of our methodology, we have processed in parallel the crude skin secretion by the classical filtration and centrifugation approach and compared the results with our sample preparation technique results. The skin secretion of the Australian bufonide *Duttaphrynus melanostictus* was selected as a model for future studies on the Brazilian bufonides (*Rhinella* sp.), currently under investigation by our group.

2 Materials and Methods

2.1 Reagents

All the employed reagents were purchased from Sigma Co. (St. Louis, MO, USA), unless otherwise stated. Amicon Ultra-4 Centrifugal 3 kDa Filter and Syringe filter (Millex-GV, hydrophobic PVDF 0.22 µm) were purchased from Millipore, USA. QAE—Sephadex A-25 was obtained from Pharmacia Fine Chemicals AB Uppsala, Sweden. pH test strip (pH-Fix 0-14) was obtained from Macherey-Nagel, Germany.

2.2 Skin Secretion Collection

The *D. melanostictus* (*Bufo melanostictus*) lyophilized skin secretion was kindly provided by Venom Supplies Pty ltd., Australia. The skin secretion solutions were prepared depending on the sample processing, as follows.

2.3 Chromatographic Analysis of *D. melanostictus* Skin Secretion

2.3.1 'Classical' Sample Preparation

The centrifuged skin secretion solution was analyzed by ion exchange using an AKTA purifier system (GE Healthcare). Either cationic or anionic media were used for method development.

2.3.1.1 Cation Exchange *Duttaphrynus melanostictus* skin secretion was re-suspended in 2 mL, 50 mM phosphoric acid solution (pH 7.5), centrifuged and the supernatant loaded in a Mono S 5/50 GL column (GE Healthcare) in a two-buffer system: (A) 50 mM sodium phosphate (pH 7.5) and (B) 50 mM sodium phosphate containing 1 M NaCl (pH 7.5.) The column was eluted at a constant flow rate of 2 mL min^{-1} with a 0–100% gradient of buffer B, for 20 min. The eluates were monitored at 220 and 280 nm and 1 mL fractions were automatically collected.

2.3.1.2 Anion Exchange *Duttaphrynus melanostictus* skin secretion was re-suspended in 2 mL, 25 mM Tris–HCl (pH 8), centrifuged and the supernatant loaded in a Mono Q 5/50 GL column (GE Healthcare), in a two-buffer system: (A) 25 mM Tris–HCl (pH 8) and (B) 25 mM Tris–HCl, containing 1 M NaCl (pH 8). The column was eluted at a constant

flow rate of 2 mL min^{-1} with a 0–100% gradient of buffer B, for 20 min. The eluates were monitored at 220 and 280 nm and 1 mL fractions were automatically collected.

Since the anionic medium yielded better results, the chromatographic condition was optimized, namely: the buffer pH was adjusted to 8.5. The other chromatographic parameters were preserved. All collected fractions were concentrated using an Amicon Ultra-4 centrifugal filter (3 kDa) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.3.2 'New' Sample Preparation

Ion exchange (IEX) batch chromatography was performed as an alternate approach to column chromatography due to the poor solubilization of the crude skin secretion. Initially, 0.5 g of QAE—Sephadex A-25 resin was re-suspended in 12.5 mL of 25 mM ammonium bicarbonate buffer (pH 8.5) for 18 h, at room temperature. After this period, the resin was centrifuged (500 g, for 5 min), and the supernatant was discarded. The resin was washed again with 12.5 mL, 25 mM ammonium bicarbonate (pH 8.5), for 30 min, centrifuged (500 g, 5 min) and the supernatant was discarded. This process was repeated twice.

In sequence, 103.1 mg *D. melanostictus* skin secretion were dissolved in 20 mL, 25 mM ammonium bicarbonate buffer (pH 8.5), under constant agitation, followed by sonication. The skin secretion solution was then mixed to the resin, under mild agitation for 1 h at room temperature. Next, the mixture was centrifuged (500 g, 5 min) and the supernatant was removed and termed 'unbound fraction' (UBF). This step was repeated twice, and the supernatants were pooled as UBF.

Following the removal of the UBF, the resin was washed with 40 mL 25 mM ammonium bicarbonate, containing 2 M NaCl (pH 8.5). This step was conducted as described for the UBF, i.e., homogenization (1 h) and centrifugation (500 g, 5 min). However, the supernatant was collected and termed 'salt-displaced fraction' (SDF). This step was repeated twice, and the supernatants were pooled as SDF.

Finally, 40 mL acetic acid acidified ammonium bicarbonate (pH ~ 3, as estimated by a pH test strip) were added. The procedure was repeated twice: homogenization (1 h), centrifugation (500 g, 5 min) and supernatant collection was termed 'acid-displaced fraction' (ADF).

In order to remove any possible resin particle present in the fractions, UBF, SDF and ADF were mechanically filtered (0.22 µm syringe filters) prior to lyophilization.

2.3.2.1 Desalting SDF and ADF were desalted by using a HiPrep 26/10 desalting column (GE Healthcare) coupled to an AKTA avant 25 preparative system (GE Healthcare). Each fraction was re-suspended in 10 mL, 25 mM Tris–HCl (pH 8.5) and two 5 mL runs were performed. The column was eluted at a constant flow rate of 10 mL min^{-1} with 25 mM Tris–HCl buffer (pH 8.5) and monitored at 220 and 280 nm. Protein peaks were collected and lyophilized.

2.3.2.2 Chromatographic Analysis Following desalting, SDF and ADF were concentrated using an Amicon Ultra-4 centrifugal filter (3 kDa), lyophilized and re-suspended in 2 mL 25 mM Tris–HCl (pH 8.5) and individually loaded into a Mono Q 5/50 GL column, in a two-buffer system: (A) 25 mM Tris–HCl (pH 8.5) and (B) 25 mM Tris–HCl, 2 M NaCl (pH 8.5). The column was eluded at a constant flow rate of 1 mL min^{-1} with 0 to 50% gradient of buffer B, for 20 min. The eluates were monitored at 220 and 280 nm. One mL fractions were automatically collected during the gradient phase. All fractions were concentrated using an Amicon Ultra-4 centrifugal filter (3 kDa) and analyzed by SDS-PAGE.

2.4 SDS-PAGE

Polyacrylamide gels electrophoresis (10, 12 or 15%) containing sodium dodecyl sulfate were performed according to Laemmli (1970) [25], under reducing conditions.

2.5 Hydrolase Activities

Zymograms for the detection of peptidasic and/or hyaluronidasic activities were performed according to Prezotto-Neto et al. [26], using three different substrates: casein (2 mg mL^{-1}), gelatin (2 mg mL^{-1}) and hyaluronic acid (170 µg mL^{-1}). *Bothrops jararaca* (40 µg) and *Crotalus viridisviridis* (10 µg) venoms were used as positive control for hyaluronidase activity and casein activities, respectively.

3 Results

3.1 Chromatographic Analysis of *D. melanostictus* Skin Secretion

3.1.1 'Classical' Sample Preparation

Ion exchange chromatography was performed in order to assess which chromatographic condition was more suitable to analyze *D. melanostictus* skin secretion solution. Figure 1 shows that most of the proteins in the skin secretion solution eluted in the unbound fraction of either the cationic (Fig. 1a, F1) or the anionic (Fig. 1c, F1–F3) exchange chromatographies, as depicted by the SDS-PAGE analyses (Fig. 1b, d, respectively). Nevertheless, the anion exchange

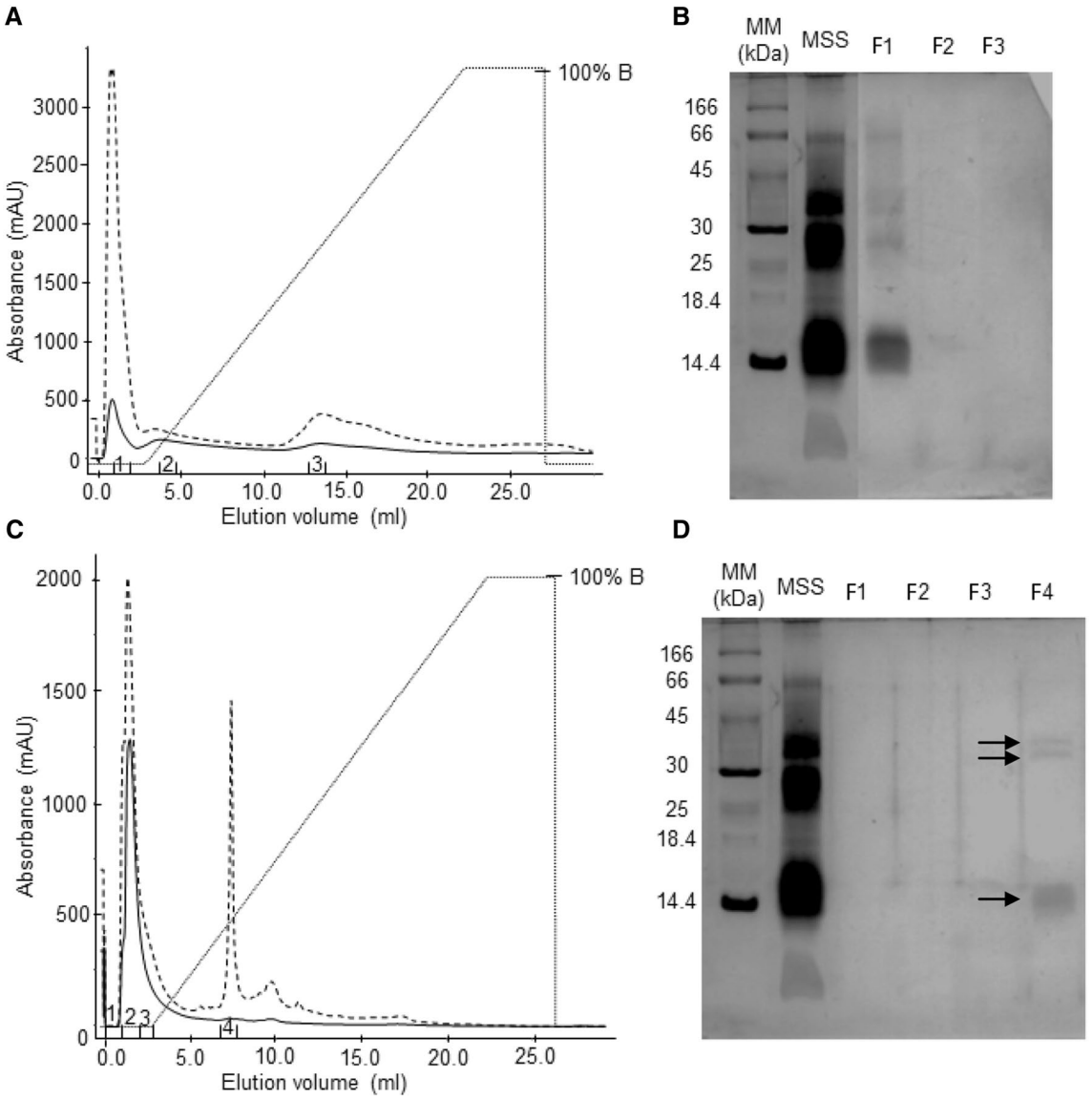

Fig. 1 Preliminary ion exchange chromatography of *D. melanostictus* skin secretion. **a** Cation exchange chromatography, according to "Materials and Methods" section, under a linear gradient of NaCl (dotted line), UV monitoring at 220 (dashed line) and 280 (solid line) nm. 1 mL fractions were automatically collected (X-axis numbering). *1–3* Indicate fractions selected for SDS-PAGE analyses. **b** 15% SDS-PAGE of *D. melanostictus* skin secretion (MSS) and fractions F1 (2 min), F2 (5 min) and F3 (14 min). **c** Anion exchange chromatography, according to "Materials and Methods" section, under a linear gradient of NaCl (dotted line), UV monitoring at 220 (dashed line) and 280 (solid line) nm. 1 mL fractions were automatically collected (X-axis numbering). *1–4* Indicate fractions selected for SDS-PAGE analyses. (D) 15% SDS-PAGE of MSS and fractions F1 (1 min), F2 (2 min), F3 (3 min) and F4 (8 min) collected after anionic exchange. Gel images were digitally composed for better visualization

chromatography yielded an apparently more resolved chromatographic profile (gradient phase), generating three peaks (Fig. 1c; ~7.5′, 9.5′ and 11′).

The preliminary 15% SDS-PAGE analyses performed to evaluate the presence of proteins in *D. melanostictus* skin secretion, as well as in the fractions F1–3 or F1–4 collected during the cationic and anionic ion exchange chromatography are presented in Fig. 1b, d, respectively. The cationic exchange fraction 1 (unbound) shows the presence of proteins ranging between 14 and 66 kDa. Fractions 2 (5 min) and 3 (14 min), actually belonging to much broader 'peaks' at 4–6 and 13–19 min respectively, present faint or no sign of protein bands. On the other hand, the anionic exchange chromatography was more effective in separating proteins of *D. melanostictus* skin secretion solution, as observed by the chromatographic profile and by the presence of three protein bands in the SDS-PAGE sample 4 (arrows, Fig. 1d).

Anionic exchange chromatography was, therefore, selected for *D. melanostictus* skin secretion solution analyses and some level of optimization was performed. Figure 2a presents the chromatographic profile obtained after altering the pH (from 8 to 8.5) and by increasing the sample mass (from 10 to 28 mg). More chromatographic peaks could be detected during the gradient phase and the 10% SDS-PAGE analyses of such peaks showed the presence of proteins in samples 5 (10 min), 6 (11 min), 7 (12 min) and 8 (14 min) (Fig. 2b). Nevertheless, the unbound fraction was still predominant.

Previous knowledge on amphibian skin secretions [7, 8, 15, 27] has shown that some animal skin secretions are rich in molecules featuring low molecular mass. Accordingly, we had performed reversed phase-high performance liquid chromatography (C18-RP-HPLC) of some of the ion exchange fractions as well as of the crude skin secretion of *D. melanostictus* solution and compared the results (Supplemental material 1, Fig. 1a–i). Furthermore, we performed mass spectrometry analysis of this skin secretion solution (Supplemental material 2), and it became clear that the ion exchange chromatography was also separating the polar low molecular mass components of the skin secretion.

3.1.2 'New' Sample Preparation

Initial sample preparation approaches revealed that after a one-step batch ion exchange chromatography it was possible to significantly improve the chromatographic results obtained with *D. melanostictus* skin secretion solution (Supplemental material 3, Figs. 3a, b, 4a, d). We used two criteria to evaluate such improvement: the decrease in the intensity of the chromatogram absorbance at 220 nm and the reduction of the low molecular mass molecules present in the acid displaced fraction 2 (ADF 2) (Supplemental material 4, Fig. 4a–d). These initial attempts set ground for the development of the here described method that allows the proper analyses of proteins form complex matrices, such as amphibian skin secretion.

Figure 3 presents the chromatographic profile of the anionic-ion-exchange-based separation of the *D. melanosticus* skin secretion solution following the IEX batch sample preparation with NaCl (SDF, Fig. 3a) or acetic acid (ADF, Fig. 3c) media displacement. One-minute fractions were manually collected and 12% SDS-PAGE analyses were performed for SDF and ADF (Fig. 3b, d), respectively. We observed significant decrease of the unbound fraction after the batch processing, in comparison to the unprocessed—or 'classically processed'—sample (Fig. 2a). Supplemental material 5, Fig. 5 presents the C18-RP-HPLC comparison of the unbound molecules for each batch processing step. Both SDF and ADF exhibit similar chromatographic profiles after anionic exchange chromatography, varying the signal intensity (Fig. 3a, c). However, the SDS-PAGE analyses (Fig. 3b, d) of the collected peaks indicate that SDF was more efficient in separating the proteins present in the skin secretion, as indicated by the presence of more proteins bands (lanes 2–8, Fig. 3b). On the other hand, ADF protein yield was smaller, as indicated by fainter SDS-PAGE bands (lanes 2–5, Fig. 3d).

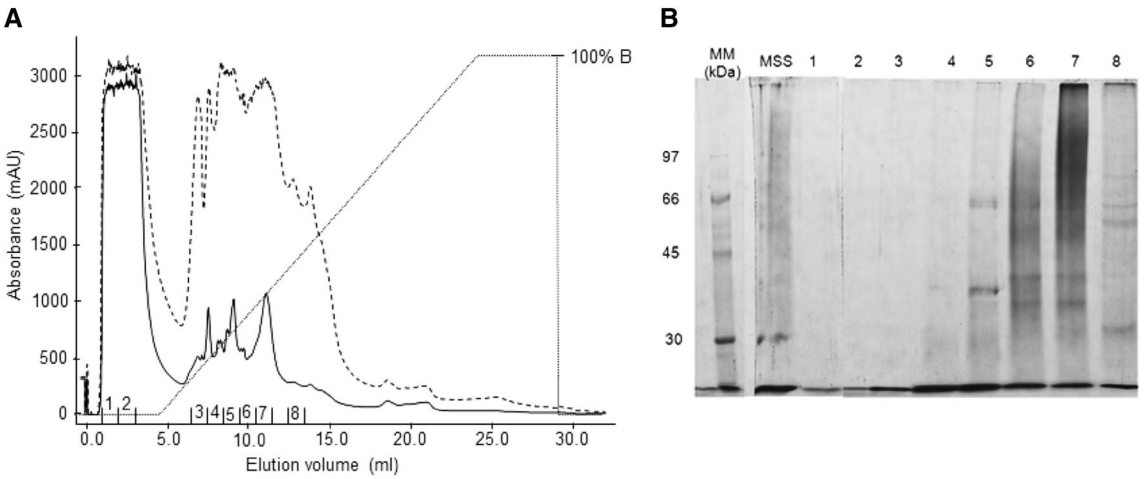

Fig. 2 **a** Anion exchange chromatography, according to "Materials and Methods" section, under a linear gradient of NaCl (dotted line), UV monitoring at 220 (dashed line) and 280 (solid line) nm. 1 mL fractions were collected automatically (X-axis numbering). *1–8* Indicate fractions selected for SDS-PAGE analyses. **b** 10% SDS-PAGE profile of *D. melanostictus* skin secretion (MSS) and samples 1 (2 min), 2 (3 min), 3 (8 min), 4 (9 min), 5 (10 min), 6 (11 min), 7 (12 min) and 8 (14 min) collected after anionic analysis. Gel images were digitally composed for better visualization

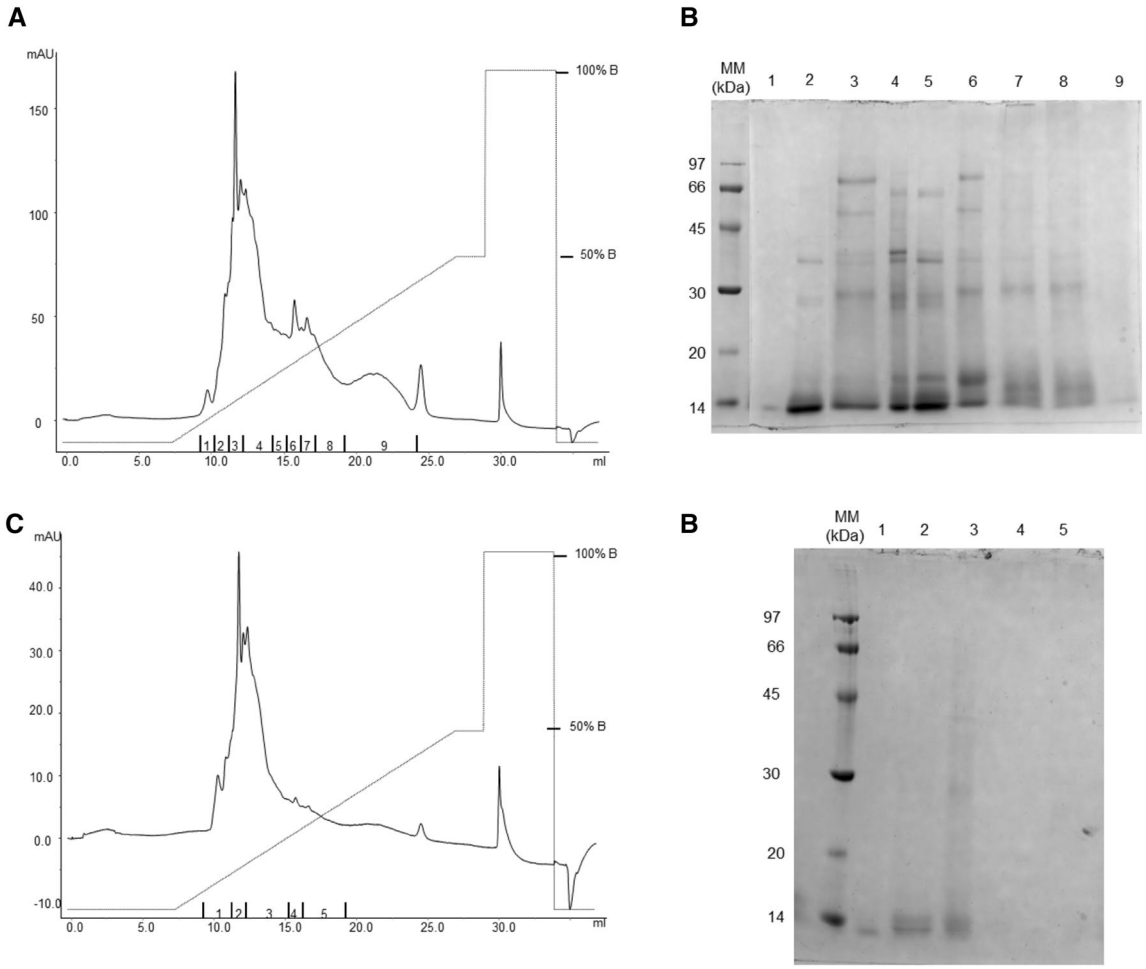

Fig. 3 **a** SDF (salt-displaced fraction) Anion exchange chromatography, after IEX batch processing, according to "Materials and Methods" section, under a linear gradient of NaCl (dotted line), UV monitoring at 280 nm. 1 mL fractions were automatically collected (X-axis numbering). *1–9* indicate fractions selected for SDS-PAGE analyses. **b** 12% SDS-PAGE profile of SDF samples *1–9* (*1* 11 min; *2* 12 min; *3* 13 min; *4* 14–15 min; *5* 16 min; *6* 17 min; *7* 18 min; *8* 19–20 min; *9* 21–25 min). **c** Acid-displaced fraction (ADF) anion exchange chromatography, after IEX batch processing, according to "Materials and Methods" section, under a linear gradient of NaCl (dotted line), UV monitoring at 280 nm. 1 mL fractions were automatically collected (X-axis numbering). *1–5* indicate fractions selected for SDS-PAGE analyses. **d** 12% SDS-PAGE profile of ADF samples *1–5* (*1* 11–12 min; *2* 13 min; *3* 14–16 min; *4* 17 min; *5* 18–20 min). Gel images were digitally composed for better visualization

3.2 Zymography

In order to assess whether the proteins present in the *D. melanostictus* skin secretion solution would retain their biological activities after the currently presented sample processing approaches, we performed zymograms (Fig. 4) by copolymerizing the 12% SDS-PAGE gel with three different substrates: (i) casein, (ii) gelatin and (iii) hyaluronic acid. Only samples obtained from SDF were tested due to the more evident protein yield. The SDF fraction displayed activity over casein, as well as enzymes present in fractions 4, 6 and 7 at approximate molecular masses of 100, 35 and 20 kDa, as observed by the negative staining of the gel. (Fig. 4a, c, boxed for better visualization). On the other hand, no gelatinolytic or hyaluronidasic activities were detected (Fig. 4b, c).

4 Discussion

Sample preparation can be a crucial step in Scientific analysis, and its importance increases in the same rate as the sensitivity of the following employed/desired analytical technique does. The need to analyze complex, viscous matrices is not new, and diverse approaches have been successfully employed. For instance, Barker et al. had introduced a technique called Matrix solid-phase dispersion (MSPD) [28]. This procedure is simple, not expensive and allows working

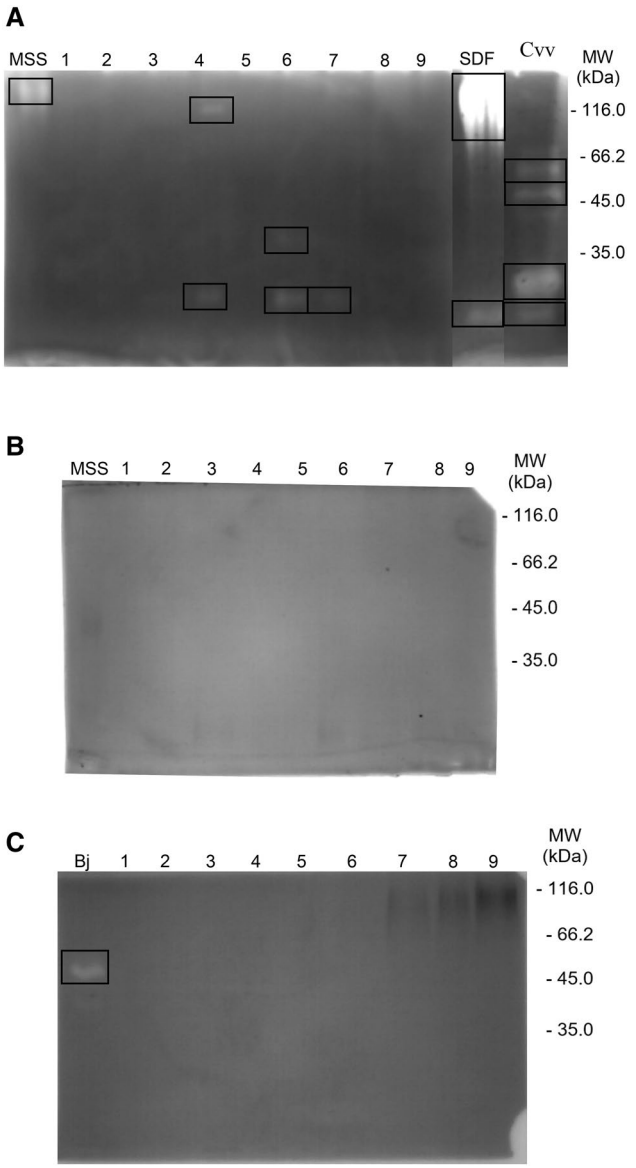

Fig. 4 Zymograms: biological activity retrieval assays of SRF chromatographic fractions. Samples *1–9* (Fig. 3a) were lyophilized, resuspended in water and submitted to a 12% SDS-PAGE gel copolymerized with **a** casein, **b** gelatin or **c** or hyaluronic acid. *D. melanostictus* skin secretion (MSS) and SDF aliquot fraction were lyophilized, resuspended in water and submitted to a 12% SDS-PAGE gel copolymerized with casein or gelatin. Positive controls were: *Crotalus viridis viridis* (10 ng) for casein and *Bothrops jararaca* (Bj) venom (40 µg) for hyaluronic acid assay. The boxes indicate the biological activity, for better visualization. Gel images were digitally composed for better visualization

with solid, semisolid or viscous samples. It consists, basically, into grinding the sample with a solid sorbent in a mortar using a pestle. Then, the blend is passed through a column or cartridge and the analytes are eluted with organic solvents [29, 30]. With this approach it is possible to identify pesticides from soil and antibiotics, insecticides or caffeine from animal or plant tissues [28, 30], among others. Other methods have been developed to study complex biological material, such as Dispersive liquid–liquid micro-extraction to determine polar or non-polar analytes from marine sediment extract; Stir-bar-sorptive extraction, meant to analyze molecules with medium to low polarity, and divergent volatility, employed in the study of aqueous samples (or extracts) [31].

However, such techniques are not suitable for biological samples, especially for proteins (enzymes), due to the frequent use of organic solvents that typically hinder the biological activity from the protein. The chemical integrity of the molecule is preserved allowing, for instance, accurate molecular mass determination, proteomic identification, Edman degradation and other analytical approaches that would not rely on—or assess—the native protein conformation, such as circular dichroism, infrared spectroscopy or kinetic assays.

Pineda Guerra et al. compared four protein extraction methods when performing proteomic analyses of the Africanized bee venom [32]. Authors assessed different combinations of sample processing steps, namely: re-suspension (7 M urea, lysis buffer or water); precipitation (acetone or trichloroacetic acid) and solubilization (7 M urea and 4% CHAPS or lysis buffer). But authors' analyses were limited to electrophoresis, thus protein conformation and/or activity were not considered in their study.

Amphibian skin secretions are a rich source of biological molecules, most of them being secondary metabolites, amino acid derivatives, peptides and proteins [5–10, 14]. Normally, such molecules are 'easily' prospected and purified through standard chromatographic approaches [8, 14], not requiring special sample preparation steps. Our group has been successfully isolating and biochemically characterizing peptides [33], alkaloids [15], steroids [7], low molecular mass molecules [8], and describing proteins through proteomic approaches [27] from the amphibian skin for many years. However, when it comes to the purification of such proteins, some complications arise.

The Bufonidae family is made up of comparatively large animals, capable of providing grams of crude skin secretion just by mechanical stimulation, which are rich in proteins and low molecular mass components [7], but lack bioactive peptides [34]. Although ideal candidates for providing a 'good' biological sample, all previous studies have reported issues in *Rhinella* sample preparation and manipulation [7, 16, 22]. Right after collection, attempts to re-suspend the material fail, due to its high viscosity, with the need to employ mechanical filters and/or successive centrifugation steps to remove particulate or insoluble matters (e.g., 'lost' molecules). Alternately, one can employ liquid–liquid partition using immiscible solvents (such as dichloromethane:water); however, this approach is mainly

indicated to retrieve low molecular mass molecules [15]. Yet, even after the above-mentioned sample preparation techniques, the chromatographic analyses sometimes are impaired because of over pressuring, clogging or UV signal saturation. All these problems were experienced by these authors during the initial analyzes of *D. melanostictus* skin secretion solution.

There were major proteins present in this skin secretion solution (Fig. 1b, lane MSS) that were spread through a broad molecular mass range (14–66 kDa), making it 'easy' to analyze (by SDS-PAGE) whether molecules were efficiently separated or not, i.e., no overlapping of similar molecular masses would raise doubts on the separation.

'Classical' sample preparation (proteins) typically starts with an ion exchange chromatography [35], a coarse technique that allows the grouping of molecules that will be further separated based on other molecular features. Figure 1 presents both the cation and anion exchange chromatographic profiles and the corresponding SDS-PAGE analyses. The majority of the proteins present in the crude skin secretion (MSS lanes) were lost during the chromatographic separation (lanes 1–3 Fig. 1b, lanes 1–4 Fig. 1d). Even though the SDS-PAGE of the anion exchange chromatography was poorer in term of detected protein bands (Fig. 1d vs. 1b), the chromatographic profile itself was better in term of adsorved molecules (Fig. 1c vs. 1a). Therefore, anion exchange chromatography was selected as the first step for protein purification.

Some sample preparation was attempted for the anion exchange chromatography, and such modifications yielded the profile presented in Fig. 2, with the corresponding SDS-PAGE analyses. In spite of the profile resolution improvement, a significantly large unbound fraction was detected. Such fraction saturated the UV detection and delayed the isocratic elution for two more minutes prior to gradient separation. Still, no efficient protein separation was obtained, as analyzed by the SDS-PAGE depicted in Fig. 2b.

Based on information gathered so far, authors were convinced that anion exchange was indeed the best first-step for the amphibian protein purification, but chromatography saturation was still an issue. It was then that batch, instead of column, chromatography was proposed. It was our understanding that, by performing the anion exchange in a batch, the unbound fraction would not be a chromatographic issue anymore and we would be able to focus on the adsorbed fraction. Displacements based either on pH or ionic strength were performed, the ionic strength displacement being more efficient, as presented in Fig. 3b versus 3d (pH displacement).

Yet, the definitive 'proof of concept' was still missing: have proteins lost their spatial folding/conformation and, therefore, their biological activity as a consequence of the sample preparation procedures?

Since this is among the first biochemical characterization studies for the *D. melanosticus* skin secretion, no hint on which protein-based biological activity to be assayed was available. As a consequence, we chose to evaluate the presence of hydrolases; namely, peptidases and glycosidases which have already been described before for other amphibians [36, 37]. Figure 4 presents the three zymograms performed in this work. The peptidasic activity could be detected on the casein-based zymogram, suggesting the presence of serine peptidases, due to the substrate specificity and lower molecular mass (~ 20–25 kDa, lanes 4, 6 and 7). Interestingly, the crude skin secretion (lane MSS) showed only peptidase activity at higher molecular mass (~ 100 kDa), which was detected in lane 4. These results were observed, prominently, in SDF fraction. The lack of detection for the lower molecular mass peptidase activity in the crude skin secretion could be due the fact that these enzymes would be much less (relatively) concentrated than high molecular one in the crude skin secretion.

High molecular masses protein displaying peptidase activity are normally related to serine peptidases belonging to the subtilisin family, whereas low molecular mass enzymes are typical trypsin-like peptidases [38]. However, further studies are needed to properly classify the detected peptidase activity.

The lack of activity over collagen (gelatin, Fig. 4b) indicates the absence of metallopeptidases, as well as no hyaluronidase activity was detected (Fig. 4c). Both metallopeptidases and hyaluronidases are typically found in snake venoms and their presence is normally related to venom spread and initial prey digestion [39, 40]. Such enzymatic activities were assayed here from a herpetological perspective, i.e., concerning the zoological relationship between amphibians and reptiles.

Although promising from a biotechnological perspective (poor temperature, pH, ionic strength and/or osmotic pressure control), enzymes present in the amphibian skin secretion have rarely been studied. According to our experience, one major reason would be the difficulties in processing and handling such biological material. Usually, researchers have been characterizing proteins from anuran skin aqueous extracts. Kawasaki et al. isolated a histone H2B with antimicrobial activity from *Rhacophorus schlegelii* [41]. Zhao et al. found a trypsin inhibitor in *Bufo andrewsi* [42]. *D. melanostictus* skin extracts were prospected before and albumin and BMP1 could be identified [43, 44]. However, few studies succeeded in identifying proteins from amphibian skin secretion. Zhao et al. purified baserpin and lysozyme from *B. andrewsi* [45, 46] and Zhang et al. isolated KPHTI, a trypsin inhibitor, from *Kaloula pulchra hainana* [47]. It is our understanding that such shortage relates exclusively to sample preparation procedures.

The sticky and adhesive characteristics of some amphibian skin secretions can bring methodology difficulties; however, such biological features are likely to play important roles for the animal. Evans and Brodie studied these properties in the skin secretion of different amphibians [23]. The authors state that *Dyscophus antongilii* skin secretion is five times stronger than rubber cement and suggest that the high adhesive capacity could work as an anti-predator defense. Accordingly, the identification of a trypsin inhibitor in *Dyscophus guineti* skin secretion by Konig et al. may be related to the secretion storage in the cutaneous glands [20]. Once the glandular content is released, the inhibitor would quickly dissociate allowing the fast agglutination of the molecules present in the secretion leading to its adhesive characteristic.

The aim of this work was to develop a method that allows studying the sticky and adhesive amphibian skin secretion. This new approach in sample preparation is likely to permit that new studies isolate, identify and characterize novel proteins from amphibian skin secretions. As a consequence, a better understanding of the role of these proteins in the physiology, defense strategies and antimicrobial actions happening in the skin of these animals would be possible. Naturally, other viscous samples would directly benefit from the currently described methodology.

Although we have focused on bioactive protein retrieval approaches, the sample processing we present here works both ways. Supplemental material 5, Fig. 5 shows that the low molecular mass content significantly improves in the unbound batch fraction. This is extremely important for those interested in these molecules, for the enriched fraction can be further processed (C18-RP-HPLC, for example) without risking injecting high molecular mass proteins in the column which might clog and/or precipitate, obstructing the column and impairing the separation process.

5 Concluding Remarks

In this study we describe, for the first time, a straightforward and reproductive method to process the viscous, glue-like, adhesive amphibian skin secretion. By using a batch ion-exchange chromatography sample preparation strategy, followed by traditional, well-established column chromatography, we were able to obtain soluble samples that smoothly underwent different biochemical processing steps. Furthermore, the unbound fraction retained the majority of low molecular mass molecules (alkaloids and steroids) that interfere in the chromatographic steps, as well as in the colorimetric protein concentration estimation procedures, due to their high UV absorbance. Removal of such molecules allowed proper protein sample processing.

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Compliance with Ethical Standards

Conflict of interest Authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with animals performed by any of the authors.

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