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Combined proteomic and functional analysis reveals rich sources of protein diversity in skin mucus and venom from the *Scorpaena plumieri* fish

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ABSTRACT

The biological activities observed upon envenomation by *Scorpaena plumieri* could be linked to both the venom and the skin mucus. Through a proteomic/functional approach we analyzed protein composition and biological activities of the venom and skin mucus. We identified 885 proteins: 722 in the Venomous Apparatus extracts (Sp-VAe) and 391 in the Skin Mucus extract (Sp-SMe), with 494 found exclusively in Sp-VAe, being named *S. plumieri* Venom Proteins (Sp-VP), while 228 were found in both extracts. The majority of the many proteins identified were not directly related to the biological activities reported here. Nevertheless, some were classified as toxins/potentially interesting molecules: lectins, proteases and protease inhibitors were detected in both extracts, while the pore-forming toxin and hyaluronidase were associated with Sp-VP. Proteolytic and anti-microbial activities were linked to both extracts, while the main toxic activities – cardiovascular, inflammatory, hemolytic and nociceptive – were elicited only by Sp-VAe. Our study provided a clear picture on the composition of the skin mucus and the venom. We also show that the classic effects observed upon envenomation are produced by molecules from the venomous gland. Our results add to the growing catalogue of scorpaeniform fish venoms and their skin mucus proteins.

Significance: In this study a large number of proteins – including classical and non-classical toxins – were identified in the venomous apparatus and the skin mucus extracts of the *Scorpaena plumieri* fish through shotgun proteomic approach. It was shown that the toxic effects observed upon envenomation are elicited by molecules originated from the venomous gland. These results add to the growing catalogue of scorpaeniform fish venoms and their skin mucus proteins – so scarcely explored when compared to the venoms and bioactive components of terrestrial animals.

Data are available via ProteomeXchange with identifier PXD009983

1. Introduction

The spotted scorpionfish *Scorpaena plumieri* is one of the most venomous fish in the Atlantic Ocean, being responsible for many accidents in the Brazilian coast. The representatives of this species use their venom to protect themselves against potential predators. They possess a primitive venom apparatus formed by 13 dorsal, 3 anal and 2 pelvic fin spines associated with venomous glandular tissue – which is no more than a grouping of secretory cells – within/along grooves located in the anterior portion of these spines, which are covered by a mucous-rich integumentary sheath [1].

Envenomation occurs through mechanical pressure on the spines, which tears the integumentary sheath encasing them, causing the venom to enter the wound. Clinical manifestations of accidents include intense pain, edema, erythema, occasional skin necrosis, adenopathy, nausea, vomiting, agitation, malaise, sweating, diarrhea, tachycardia and arrhythmias [2]. Thus, although not lethal, envenomation by *S. plumieri* may lead to potentially severe outcomes.

In this fish species, the ill-defined structure of the venom gland makes the collection of venom for scientific research technically difficult. Nevertheless, for more than ten years our research team has been conducting classic biochemical and physiopharmacological studies on

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the venom of *S. plumieri*, using the extract from its venomous apparatus. We have described lethal, hemolytic, cardiovascular, inflammatory, pore formation in cell membranes, inhibition of integrin-binding, hemagglutination and enzymatic activities related to this venom extract [3–10].

However, considering the anatomical features of the glandular tissue and the mucus that covers the spines, it is quite possible that during envenomation not only the venom but also the mucus enters the wound [11]. While fish venoms usually have an exclusive protective function, fish epidermal mucus exhibits many roles. Besides working as a mechanical and biochemical barrier between the fish and the surrounding water, other functions like lubrication, osmoregulation and locomotion (for a detailed review, see [12]), formation of shoals, recognition of individuals and homing of migratory populations have been attributed to fish mucus [13].

Furthermore, it was also demonstrated that skin mucus plays a major role in preventing the colonization by parasites, bacteria and fungi [14, 15]. In fact, antibacterial activity against a broad range of infectious pathogens has been described in epidermal fish mucus [16, 17]. Therefore, mucous secretion has been considered a key component of fish innate immunity mechanisms and the first line of defense against pathogens [18].

These multiple roles have been related to several bioactive substances present in the mucus, which are secreted by the epidermal goblet and epithelial cells or derived from the circulatory system [19]. An array of humoral defense molecules such as immunoglobulin M (IgM), complement, agglutinins (lectins), factors of the acquired immune system [20, 21], C-reactive protein, carbonic anhydrase, calmodulin, hemolysin, glycopeptides, antimicrobial peptides, lysozyme, proteases and glycolipids [18, 20, 22–29] were identified or purified from this epidermal secretion.

Due to the difficulties involved in dissecting/isolating the glandular tissue from surrounding tissues during venom extraction, it stands to reason that the biological activities reported in experimental conditions could be linked to both the venom and skin mucus secretion. Thus, in an attempt to dissociate the activities of *S. plumieri* venom from those caused by the skin mucus alone, we employed a high-throughput tandem mass spectrometry proteomic approach combined with pharmacological and biochemical assays in order to assess their protein composition and bioactive properties.

In this study we aim to reveal the protein profile of the venomous extract and the skin mucus from *S. plumieri*, potentially contributing with fundamental information for future basic research, and even – through a better understanding of venomous fish toxinology – aiding in the search for new clinical strategies to treat the envenomation. Finally, seeing as fish venoms remain considerably less studied when compared to terrestrial venoms, the exploration of their full potential opens a myriad of possibilities for the development of new drugs and for a deeper understanding of complex physiological processes.

2. Material and methods

2.1. Collection of S. plumieri specimens and preparation of the extracts from the venomous apparatus and skin

The extracts were obtained from wild specimens of *S. plumieri*, collected on shallow water beaches on the coast of Espírito Santo state – Brazil. The capture was authorized by the *Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis* – IBAMA (the Brazilian Public Agency for Environmental Affairs). The extract from the venomous apparatus (Sp-VAe) was obtained according to [30] adapted by [3]. Fish skin mucus was collected from the dorsolateral area – far from the spines – by carefully scraping the surface with a plastic spatula and was homogenized in ultrapure water at 4 °C. The homogenate was centrifuged for 30 min at 4 °C/14.000 *g* and the supernatant was collected and named *Scorpaena plumieri* Skin Mucus extract – Sp-SMe. Protein concentration of both aqueous extracts was determined by Lowry Method [31], using bovine serum albumin as standard. Freshly extracted Sp-VAe and Sp-SMe were used in biological assays or stored at −80 °C for further analysis.

2.2. Biochemical characterization

2.2.1. Proteomic analysis

2.2.1.1. Sample preparation. Lyophilized samples (50 µg) were dissolved in 75 µL of water and precipitated with trichloroacetic acid (TCA) 6.1 N to a final concentration of 25% (w/v) and held overnight at 4 °C. After a centrifugation step (14.000 *g*, 30 min at 4 °C) the pellet was washed twice with 500 µL of ice-cold acetone, air-dried and resuspended in 100 µL of 8 M urea in 100 mM Tris-HCl, pH 8.5. Protein samples were reduced, alkylated and digested with trypsin (Promega, MS grade) as previously described by [32]. Briefly, reduction was performed by adding 1 µL of 10 mM TCEP (tris (2-carboxyethyl) phosphine) to the samples and mixing gently. Samples were incubated at 56 °C for 15 min. Next, 5 µL of 100 mM iodoacetamide (IAA) were added to each tube. Samples were incubated at room temperature for 15 min in the dark. Afterwards, a solution of 125 µL of water, 15 µL of 1 M ammonium bicarbonate containing 10% of 100 mM $CaCl_2$ was added to the tubes and vortexed gently. Finally, a trypsin (Promega) solution (1 µg enzyme:25 µg protein) was added to each tube, and trypsinolysis proceeded overnight at 37 °C. The reaction was stopped by the addition of 10 µL of acetic acid. Peptide samples were submitted to solid-phase extraction on C_{18} cartridges (SEP PAK – Waters) according to manufacturer's instructions. Desalted tryptic fragments were kept at −20 °C until analysis by mass spectrometry.

2.2.1.2. Liquid chromatography - mass spectrometry - nano-LC-MS/MS. Two micrograms of trypsinized samples, solubilized in 0.1% trifluoracetic acid solution (TFA, Fluka®) were injected into the nanoUHPLC UltiMate® 3000 (Dionex®) system. Peptides were initially trapped on a Nano-Trap Acclaim PepMap100 C_{18} column (100 µm i.d. × 2 cm, 5 µm, 100 Å; Thermo Scientific®) and washed for 3 min using 2% acetonitrile (ACN)/water/0.05% TFA solution, at a flow rate of 7 µL/min. Next, reverse phase chromatography was performed using the capillary column Acclaim PepMap100 C_{18} RSLC (75 µm i.d. × 15 cm, 2 µm, 100 Å; Thermo Scientific®) conditioned at 40 °C, at a flow rate of 300 nL/min. A linear gradient of solvents (A) 0.1% formic acid in water and (B) 80% ACN in water with 0.1% formic acid was applied as follows: 3.8 to 50% B over 120 min, followed by a sharp ramp to 99% B. Spectral data of eluted ions were generated in the positive mode using a Q Exactive mass spectrometer operating a data-dependent acquisition method (Thermo Scientific®, Bremen, Germany). The ion source was 2.1 kV and capillary temperature set to 250 °C. Survey scans were acquired at a resolving power of 70,000 at *m/z* 200, mass range 300 to 2000 *m/z* and AGC target of 1e6 ions in up to 250 ms. Data dependent tandem mass spectra were acquired for up to 10 most intense precursor ions that reached 1e5 ions in up to 120 ms. Selected parent ions were dissociated by Higher Energy Collision Induced Dissociation (HCD) with stepped Normalized Collisional Energy (NCE) of 28–30 and product ion spectra were acquired at a resolution of 35,000. Ions with charge state > 5 and singly charged were not selected for fragmentation. A dynamic exclusion time of 30 s was set to prevent repetitive analysis of ions.

2.2.1.3. Data analysis. Obtained spectra were submitted to database search, de novo sequencing, and quantification using PEAKS Studio 8 software and PEAKS Q module (Bioinformatics Solutions Inc.). Search was performed against 89,475 NCBI protein sequences (29,188,211 residues) from Scorpaeniformes order. Parameters considered: (i) Enzymatic specificity of trypsin (K/R not before P), allowing up to two missed cleavage sites; (ii) Error tolerances of up to 10 ppm for precursors and 0.02 Da for product ions; (iii) Cysteine