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Short communication

Potamotrygon cf. *henlei* stingray mucus: Biochemical features of a novel antimicrobial protein

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ABSTRACT

Antimicrobial molecules are important components of the innate immune system in vertebrates. They have been studied widely in several fishes, but little is known about these defence factors in stingrays, which are thought to have less sophisticated adaptive immune systems when compared to other teleosts. Stingrays from the specie *Potamotrygon* cf. *henlei* are distributed throughout the rivers of central-west Brazil, being the cause of numerous envenomations occurring in the dry seasons.

In a previous study, we reported that the mucus of the stingray *P.* cf. *henlei* shows antimicrobial effects. Here, to analyze the antimicrobial compounds from the mucus of *P.* cf. *henlei*, we employed solid-phase extraction, chromatographic separation followed by ESI-MS, and Edman degradation. A protein similar to the β-chain of hemoglobin was identified, isolated and partially sequenced by Edman degradation. This protein has a molecular weight of 16072.8 Da, and was shown to be active against bacteria (*Micrococcus luteus* and *Escherichiacoli*) and yeast (*Candida tropicalis*) without hemolytic activity. Effects of this new protein in the microcirculation environment were also evaluated. The results obtained provide fundamental information for future basic research, clinical diagnosis and development of new therapies to accident treatment. To the best of our knowledge, this is the first description of a bioactive polypeptide from the mucus of a stingray.

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1. Introduction

The skin of fish constitutes a pivotal immunological protection against the external environment. The layer of mucus on the fish surface, considered the first line of defence, participates in a number of functions including disease resistance, respiration, ionic and osmotic regulation, locomotion, reproduction, communication, feeding and nest building (Negus, 1963; Ingram, 1980; Shephard, 1994; Zhao et al., 2008). The mucus, such as that produced by the skin of the stingrays, has a complex set of components, which may include amino acid residues, peptides, complex carbohydrates, glycopeptides, glycolipids and other chemicals (Klesius et al., 2008; Alexander and Ingram, 1992; Birkemo et al., 2003).

Fish epidermal mucus was found to display antimicrobial activity against broad range of infectious pathogens (Mozumder, 2005; Hellio et al., 2002). We recently described the antimicrobial activity of catfish *Cathorops spixii* mucus (Ramos et al., 2012). Moreover, histone H2B and two ribosomal proteins are examples of proteins with antimicrobial activity that have been isolated from

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epidermal mucus of Atlantic cod (Bergsson et al., 2005). Members of some families of antimicrobial peptides (AMPs) were also found to be important innate defence components in the epidermal mucosal layer of Moses sole fish (*Pardachirus marmoratus*) (Oren and Shai, 1996), winter flounder (*Pleuronectes americanus*) (Cole et al., 2000), Atlantic halibut (*Hippoglossus hippoglossus*) (Birkemo et al., 2003), Hagfish (*Myxine glutinosa L.*) (Subramanian et al., 2009) and catfish (*Pelteobagrus fulvidraco*) (Su, 2011). These observations suggest that mucus is a good source of novel molecules for fish and human health-related applications.

We also recently reported that the mucus of the stingray *Potamotrygon.* cf. *henlei* shows antimicrobial effects and a pro-inflammatory response (Monteiro-Dos-Santos et al., 2011). The aim of the present study was therefore to identify and characterize the major component(s) with antimicrobial activity in the mucus of *P.* cf. *henlei*, which is a very common stingray found in northern and central-western rivers from Brazil (Carvalho et al., 2003). Their spines are hard, sharp, bilaterally retroserrated and covered by an integumentary sheath with a ventrolateral glandular groove containing venom glands along both edges (Halstead, 1970) and the mucus of biological importance that covers the entire body of these animals. This study employed a screening approach on mucus components that were purified by RP-HPLC, and characterized by ESI-MS and Edman degradation. By this approach, several compounds including peptides were obtained and a protein similar to Hemoglobin β-chain was identified, isolated and characterized. Following antimicrobial and hemolytic assays, intravital microscopy was used to image the effects of the protein on the microcirculation.

2. Materials and methods

2.1. Collection of stingray mucus

Specimens of adult female and male ($n = 15$) *P.* cf. *henlei* fish were collected from the Manoel Alves River in the state of Tocantins, Brazil. Mucus dispersed all over the body was collected by scraping the skin with a glass slide, and immediately stored on ice, then diluted in 0.15 M phosphate-buffered sterile saline, pH 7.4, homogenized, and centrifuged (5000 × g for 20 min at 4 °C) for collection of the supernatant. The supernatant was collected and stored at −20 °C. Protein content was determined by the method of Bradford (1976) using bovine serum albumin (Sigma Chemical Co., St Louis, MO) as standard protein.

2.2. Purification of mucus

The supernatants were loaded onto solid phase extraction cartridges Sep-Pak, C_{18} (Waters Corporation, Taunton, MA, USA) equilibrated in acidified water (0.1% trifluoroacetic acid (TFA)). A single aliquot of 3 mg diluted in 3 mL of 0.1% TFA was loaded and the elution was performed sequentially with 40 and 80% acetonitrile. These fractions were further concentrated by a vacuum centrifugation. Aliquots of 1 mg of the samples were dissolved in 1 mL of deionized water in 0.1% TFA and centrifuged at 5000 × g for 20 min (10 °C). The supernatants were applied to a system of RP – HPLC (Äkta basic, Amersham Biosciences – Sweden) for the sample separation. The sample was loaded in a Jupiter C_{18} column (4.6 mm × 150 mm, 5 μm, Phenomenex, USA) in a two-solvent system: (A) TFA/H_2O (1:1000) and (B) TFA/Acetonitrile (ACN)/H_2O (1:900:100). The column was eluted at a flow rate of 1.0 mL/min with a 10–80% gradient of solvent B over 40 min. For protein purification, further additional steps of chromatography were necessary, using a Bio Basic C_8 column (4.6 mm × 250 mm, 5 μm, Thermo, USA) with optimized gradients. The HPLC column eluates were monitored by their absorbance at 214 nm.

2.3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to Laemmli (1970). Proteins (10 μg) from the mucus of *P.* cf. *henlei* were analyzed by SDS-PAGE 4–20% acrylamide gradient under reducing conditions. Prior to electrophoresis, the samples were mixed 1:1 (v/v) with sample buffer. The gels were stained with the Silver method.

2.4. Mass spectrometry (MS) and peptide mass fingerprint (PMF)

The fractions were analyzed by electrospray, with direct injection in an LC–MS Surveyor MSQ Plus (Thermo Electron, USA) under positive ionization mode. The needle and cone potential were set to 3.1 kV and 40 V, respectively. The aqueous sample solutions (10 μL) were directly injected at a 50 μL/min constant flow rate of acetonitrile H_2O/0.1% formic acid (1:1). External calibration was performed with NaI (Sigma) over m/z 100–2000.

Protein band was excised and in-gel trypsin digestion was performed according to Hellman et al. (1995). Nanospray MS/MS analysis was performed on tryptic digests of SDS-Page band of purified PcfHb using Q-Tof mass spectrometry (Q-TOF Ultima API Waters/Micromass, Manchester, United Kingdom). An aliquot (5 μL) of the resulting peptide mixture were injected into Symmetry C_{18} trapping column (5 μm particles, 180 μm i.d. × 20 mm, Waters, USA) to desalt the peptide mixture. The nano UPLC (Waters) conditions were 0.1% formic acid in water (solvent A) and acetonitrile with 0.1% formic acid as solvent B. The separations were performed at a flow rate of 0.6 μl/min using a 0–80% gradient of solvent B over 45 min. The LC system was coupled to a nano ESI source of the Q-ToF instrument using a BEH130C_{18} column (75 μm × 100 mm, 1.7 μm particles; Waters, MA, USA). Typical conditions were a capillary voltage of 3.1 kV, a cone voltage of 50 V, and source temperature of 70 °C. Data dependent acquisition (parent ions with 2, 3 and 4 charges) were automatically recognized by the charge state recognition software MassLynx 4.1 (Waters, USA). The peptide ions were detected by scanning from m/z 200 to m/z 2000 at a rate of 1 scan/s, and were subjected to collision-induced dissociation with argon in the 13–55 eV collision energy range. Product ions from MS/MS experiments were detected by scanning from m/z 50 to m/z 2000 at a rate of 1 scan/s. External calibration was performed using phosphoric acid (Merck, Darmstadt, Germany).

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