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Characterising the enzymatic profile of crude tentacle extracts from the South Atlantic jellyfish *Olindias sambaquiensis* (Cnidaria: Hydrozoa)

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ABSTRACT

Jellyfish venoms are of medical and biotechnological importance, with toxins displaying antimicrobial, analgesic and anti-tumor activities. Although proteolytic enzymes have also been described, detailed characterisation of these proteins is scant in *Olindias* spp. High throughput mass spectrometry profiling of cnidarian venoms has become increasingly popular since the first description of the proteomic profile of putative toxins isolated from nematocysts of the hydrozoan jellyfish *Olindias sambaquiensis* describing the presence of orthologous enzymes as presented in venoms of advanced species as snakes. Rigorous bioinformatics analyses can aid functional annotation, but biochemical assays are prerequisite to unambiguously assign toxic function to a peptide or protein. Here we present results that experimentally confirm previously predicted proteomic analysis that crude venom extracts from tentacles of *O. sambaquiensis* are composed of polypeptides with metalloproteinase, serine proteinase and phospholipases A_2 activities. Surprisingly, levels of serine proteinase and phospholipase A_2 activities were comparable to those observed in venoms of *Bothrops* snakes which were used as positive controls in this study. Hence, these data offer new opportunities to explore serine proteinase and phospholipase A_2 activities in the clinical sequelae following *O. sambaquiensis* envenomation, with future possible biopharmaceutical applications.

1. Introduction

Human encounters with jellyfishes are common, although envenomation vary in toxicity ranging from mild symptoms to sometimes lethal consequences depending on several factors including the species or geographical distribution (Haddad Junior et al., 2002; Lumley et al., 1988). Different toxic effects have been reported in jellyfish extracts such as neurotoxic (Carneiro et al., 2011; Lassen et al., 2012), myotoxic (Endean, 1987; Yanagihara and Shohet, 2012) and pore forming cytolytic biological activities (Haddad Junior et al., 2014; Li et al., 2013; Long and Burnett, 1989; Wang et al., 2013). Enzymes such as proteases (Calton and Burnett, 1982, 1983; Gusmani et al., 1997; Lee et al., 2011) and phospholipases (Carneiro et al., 2011; Gusmani et al., 1997; Stillway and Lane, 1971) have also been reported in venoms of some jellyfish species, as have toxins with potential biotechnological interest including antimicrobial peptides from *Aurelia aurita* (Shenkarev et al., 2012), toxins with antineoplastic and analgesics effects from *Pelagia noctiluca* (Ayed et al., 2012a, 2012b).

Olindias sambaquiensis is a hydrozoan jellyfish species endemic to the southwestern Atlantic, and is fairly common from the north coast of the State of São Paulo (Brazil) to the province of Buenos

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Aires (Argentina) (Oliveira et al., 2016; Vannucci, 1951). The species has already been associated with accidents with swimmers and fishermen, in which the victim developed an intense painful condition and a skin patter of the lesions of short round marks, or sometimes with short welt lines, the lesions may develop into blisters or necrosis (Haddad Junior et al., 2010; Mosovich and Young, 2012; Resgalla Junior et al., 2011). Several reports are available on *O. sambaquiensis* envenomings. However, biochemical characterisation of the toxic components of *O. sambaquiensis* venom is scarce.

The first use of high throughput mass spectrometry to characterise a jellyfish venom described the putative toxins isolated from nematocysts of *O. sambaquiensis* (Weston et al., 2013). This study notably also revealed that amino acid sequences of these putative toxins were orthologs to many representatives of peptide toxins found in higher animals, strongly implicating that venoms may have arisen from basal metazoan origins. The putative toxins of *O. sambaquiensis* were dominated by proteases and phospholipases A_2 mostly from snake venoms. Proteases are abundant toxins especially in the venom of Viperidae snakes, with important biological roles leading to hemostatic disturbances, cytotoxicity and tissue disruption (Markland, 1998; Moura-da-Silva et al., 2007). Phospholipases A_2 are also a major components of snake venoms with potentially life-threatening neurotoxic and myotoxic action (Gutiérrez and Lomonte, 2013). Modern molecular biology techniques such as genomics, transcriptomics and proteomics can generate huge amounts of sequence data relatively quickly. This has certainly resulted in an explosion of new toxins being described in known and newly described venomous animals. Advanced bioinformatics methods can be applied to functional annotation (Gacesa et al., 2015; Starcevic et al., 2015), but biological assays are required to unambiguously assign toxin function. In this study, we present the results of an analysis that aimed to experimentally confirm the presence of enzyme activities previously identified by mass spectrometry proteomics of venom isolated from nematocysts of *O. sambaquiensis*, and to determine function by comparison with the activities of similar enzymes from snake venoms.

2. Experimental

2.1. Collection of animals and preparation of crude tentacle extracts

An expedition to collect specimens of hydromedusae *O. sambaquiensis* was held on September 23th 2014 in the São Sebastião Channel, northern coast of the State of São Paulo, Brazil, along the Enseada and Cigarras beaches (23°41′28.5″S 45°21′25.6″W) at an average depth of 10 m. Animals were caught using trawling nets with a 25 mm mesh. Each trawl lasted approximately 20 min. Specimens of *O. sambaquiensis* were morphologically identified based on the presence of four radial canals of orange color with pleated gonads along these canals, long marginal tentacles present in large numbers and bell diameter not exceeding 10 cm (Vannucci, 1951). Animals were transported alive in buckets with local sea water to the Centro de Biologia Marinha (CEBIMar), Universidade de São Paulo in São Sebastião, where tentacles were excised and stored in microtubes with seawater on dry ice for transport to the Laboratório de Imunopatologia, Instituto Butantan where the samples were stored at −80 °C until required. The tentacles were centrifuged for 3 min at 3000 × g at 4 °C to remove sea water and the tentacles then gently homogenized in a tissue homogeniser in 2 mL 10 mM sodium acetate, pH 5.0. The homogenate was then centrifuged for 5 min at 15,000 × g at 4 °C and the supernatant recovered. The protein concentration of the crude tentacle extract was determined using a Bradford assay (Bradford, 1976) and the electrophoretic profile was analysed by SDS-PAGE (Laemmli, 1970).

2.2. Proteolytic activity assays

Metalloproteinase activity of *O. sambaquiensis* tentacle extracts was assessed by Fluorescence Resonance Energy Transfer (FRET) using the following substrates: Abz-AGLA-EDDnp (GenOne, Rio de Janeiro, RJ, BR), with broad specificity; Mca-PLAQAV-Dpa-RSSSR-NH_2 (R&D Systems, Minneapolis, MN, USA), specific for ADAMs (A Disintegrin And Metalloproteinases) and Mca-PLGL-Dpa-AR-NH_2 (R&D Systems, Minneapolis, MN, USA), specific for MMPs (Matrix Metalloproteinases) and the results compared to the metalloproteinase activity of *Bothrops jararaca* crude venom, run at the same experiments, as a control of an animal venom in which metalloproteinase activity is relevant. For the assays, Abz-AGLA-EDDnp was made up to a concentration of 200 μM in 10 mM $CaCl_2$, 150 mM NaCl and 0.05% (v/v) Brij 35 (dissolved in 50 mM sodium acetate pH 5.0 or 50 mM Tris to give two solutions at pH 7.5 or 9.0), as described by Kuniyoshi et al. (2012), with modifications. The reaction mixtures (50 μL containing 10 μg tentacle extract or 1 μg *B. jararaca* venom and 50 μL of substrate) were incubated at 4 °C, 25 °C or 37 °C for 10 min. Jararhagin (5 μg/mL), a *B. jararaca* venom metalloproteinase (Paine et al., 1992), was used as positive control. The reactions were monitored every minute over the 10 min reaction using a SpectraMax M2 fluorimeter (Molecular Devices, Sunnyvale, CA, USA) at λ_{EM} 420 nm and λ_{EX} 320 nm in kinetic mode. The specific activity of three independent experiments was calculated in relative fluorescence units (RFU/min/μg). Mca-PLAQAV-Dpa-RSSSR-NH_2 was dissolved to 10 μM in 25 mM Tris containing 2.5 mM of $ZnCl_2$ and 0.005% Brij 35, pH 9.0. The reaction mixture contained 50 μL substrate and 50 μL containing 10 μg tentacle extract or 1 μg *B. jararaca* venom. Recombinant enzyme ADAM-17 (R&D Systems, Minneapolis, MN, USA) was used as a positive control at a concentration of 0.2 μg/mL. Mca-PLGL-Dpa-AR-NH_2 was used at a concentration of 10 μM in buffer 50 mM Tris containing 10 mM $CaCl_2$, 150 mM NaCl and 0.05% Brij 35, pH 7.4. Again, the reaction mixture contained 50 μL substrate and 50 μL containing 10 μg tentacle extract or 10 μg *B. jararaca* venom. Recombinant enzymes MMP-2 and MMP-9 also used at 0.2 μg/mL concentrations as positive controls. All reaction mixtures were monitored every minute using a SpectraMax M2 fluorimeter (Molecular Devices, Sunnyvale, CA, USA) at λ_{EM} 405 nm λ_{EX} 320 nm in kinetic mode. The specific activities of MMPs and ADAMs were calculated from three independent experiments in U/mg using the conversion factor (pmol/RFU) derived from the standard curve of 7-amino-4-Methylcoumarin (Sigma-Aldrich, St. Louis, MO, USA).

Serine proteinase activity of *O. sambaquiensis* tentacle extracts was assessed by the method of Zhu et al. (2005) with modifications and the results also compared to the serine proteinase activity of *B. jararaca* crude venom, run at the same experiments, as a control of an animal venom in which serine proteinase activity is relevant. The assays were performed using 20 μL of 500 μM of the chromogenic substrate L-benzoyl-arginyl-p-nitroanilide (L-BAPNA) (Sigma-Aldrich, St. Louis, MO, USA). The substrate was incubated at 4 °C, 25 °C or 37 °C for 40 min with 20 μL containing 5 μg tentacle extract or 5 μg *B. jararaca* venom and 160 μL 50 mM sodium acetate buffer, pH 5.0 or 50 mM Tris-HCl pH 7.5 or 9.0. As a positive control, porcine pancreas trypsin (Sigma-Aldrich, St. Louis, MO, USA) was used at a concentration of 25 μg/mL. Proteolysis of the substrate was measured at 405 nm.

2.3. Zymography

In-gel zymography (Heussen and Dowdle, 1980; Snoek-van Beurden and Von den Hoff, 2005; Vandooren et al., 2013) was used to estimate the molecular mass of proteases in the tentacle extract using casein and gelatin as indicator substrates. Samples