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Characterization of Brown spider (*Loxosceles intermedia*) hemolymph: Cellular and biochemical analyses

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ABSTRACT

This is the first study on the hemolymph from a spider of the *Loxosceles* genus. These animals are responsible for a great number of envenomation cases worldwide. Several studies on *Loxosceles* venoms have been published, and the knowledge about the venom and its toxins is considerable, not only regarding the biological and biochemical characterization, but also regarding structural, genetic and phylogenetic approaches. However, the literature on *Loxosceles* hemolymph is nonexistent. The main goal of the present study was to characterize biochemically the hemolymph content, and especially, to identify its different hemocytes. Moreover, many papers have already shown molecules whose source is the hemolymph and their very interesting activities and biomedical applications, for example, antifungal and antibacterial activities. A 2D-SDS-PAGE of brown spider hemolymph showed approximately 111 spots for pH 3–10 and 150 spots for pH 4–7. A lectin-blotting assay showed that hemolymph carbohydrate residues were similar to those found in venom. Several types of TAG and DAG phospholipids were found in the hemolymph and characterized by HPTLC and mass spectrometry. Four different hemocytes were characterized in *Loxosceles intermedia* hemolymph: prohemocyte, plasmatocyte, granulocyte and adipohemocyte. This paper opens new possibilities on toxinology, studying an unknown biological material, and it characterizes a source of molecules with putative biotechnological applications.

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1. Introduction

Loxosceles venoms are well-studied topics in toxinology, with a broad range of studies concerning their composition and toxic effects (Gremski et al., 2014). Spiders from the *Loxosceles* genus, also known as brown spiders, are responsible for a great number of envenomations, which generally trigger cutaneous effects and development of a dermonecrotic lesion. Occasionally, victims can present systemic symptoms (fever, nausea, hemolysis, and kidney commitment), and in rare cases, the victims can die (Paludo et al., 2006; Chaim et al., 2011). Considering the medical relevance of *Loxosceles* genus, its venom has been well characterized, and several toxins have been identified and studied. Phospholipases-D, metalloproteases, hyaluronidases, and insecticidal peptides, among others, have already been described and characterized as components of *Loxosceles* venoms, whose effects are due to the synergistic effect of the several toxins. Transcriptome analysis has also been performed, showing the percentages of each toxin in the total mRNA of the venom gland (Gremski et al., 2010; Fernandes-Pedrosa et al., 2008). Concerning proteomic approaches, there are 3 studies on the venom of *Loxosceles* spiders (Machado et al., 2005; dos Santos et al., 2009; Zobel-Thropp et al., 2014). In contrast, the hemolymph from *Loxosceles* has been neglected by the scientific

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community. Arachnid hemolymph and immune system are generally not well investigated. Hemolymph is a transparent fluid that transports nutrients, hormones, oxygen and cells (hemocytes) through the open circulatory system of arthropods. Hemolymph nourishes spider tissues, regulates homeostasis, triggers healing and regeneration and protects them from external infections (Cunningham et al., 1994; Zachariah and Mitchell, 2009; Jalal et al., 2010; Hernández-Martínez et al., 2003). However, the few studies performed had already pointed to its rich composition and putative biotechnological applications (Vilcinskas and Wedde, 2002; Kanost, 1999; Trabalon et al., 2010). Hemolymph is in direct contact with the venom gland, which suggests that its composition is worthy of investigation. Herein, *Loxosceles intermedia* hemolymph was biochemically characterized, and its hemocytes were identified.

2. Materials and methods

2.1. Hemolymph collection from Brown spider

Adult *L. intermedia* spiders were individually anesthetized with chloroform for approximately one minute. Then, for the puncture method, each spider was immobilized with an adhesive tape and the hemolymph was collected by a puncture on the dorsal vase using a needle and ultrafine syringe (BD Micro-Fine ™ + Demi 8 mm, Diameter 0.30 mm) (Fig. 1) and then solubilized in PBS (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 2 mM KH_2PO_4) containing 5% Protease Inhibitor Cocktail for mammalian tissues – PIC (Sigma, St. Louis, MI, USA). For the leg method, legs were cut off at the patellar region and the hemolymph was collected using a micropipette with a regular tip. The procedure following the collection was the same as the one already described for the puncture method. All the assays were conducted using a hemolymph pool from male and female adult spiders (at least 5–10 animals).

2.2. Gel electrophoresis (SDS-PAGE)

The protein concentrations of hemolymph samples were determined by the Coomassie Blue method, using bovine serum albumin (BSA) for the standard curve (Bradford, 1976). *L. intermedia* hemolymph was analyzed by denaturant electrophoresis with continuous gradient 5–15% (w/v) polyacrylamide gels under reducing and non-reducing conditions following Laemmli (1970). The gels were staining with Coomassie Brilliant Blue R-250 (30 μg of hemolymph) (Bio-Rad, Botafogo, RJ, Brazil) or monochromatic silver (3 μg of hemolymph) (Wray et al., 1981). In these analyses a hemolymph pool from 30 spiders was used. The molecular mass markers were acquired from Sigma (Sigma–Aldrich, St. Louis, MO, USA).

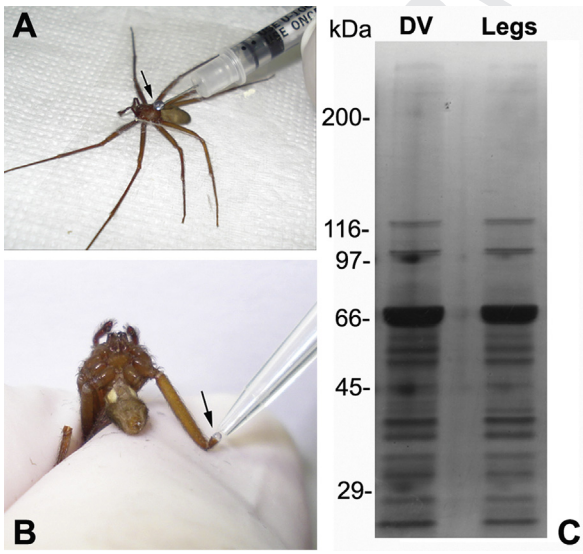

Fig. 1. Hemolymph collection of *L. intermedia* spider. A: Hemolymph collection by puncture of the dorsal vase using a syringe and needle; B: Hemolymph collection by cutting the legs at the patellar region; C: SDS-PAGE protein profile of hemolymph collected by both methods (DV – dorsal vase; Legs), 3 μg of total protein stained with silver.

2.3. Two-dimensional gel electrophoresis (2DE)

Prior to isoelectric focusing (IEF), hemolymph samples (from 50 spiders) were solubilized in 2DE rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer, pH 3–10, 0.002% bromophenol blue and 100 mM DTT). The protein concentration was determined using a 2-D Quant Kit®, and 100–150 μg of hemolymph was diluted in 200 μL of 2DE rehydration buffer. To rehydrate the strips, hemolymph samples were applied on 13 cm immobilized pH gradient (IPG) gel strips at a linear range of pH 3–10 or pH 4–7 (GE Healthcare, Piscataway, NJ, USA) and incubated for 16 h at room temperature. The IEF was performed at 20 °C on an IPGphor unit (IPGphor 3 Isoelectric Focusing System – GE Healthcare, Pittsburgh, PA, USA) with a total voltage accumulation of 15,500 V for 5 h at 50 μA/strip. After focusing, the strips were incubated for 15 min in reducing solution (6 M urea, 30% glycerol, 2% SDS and 65 mM DTT) and followed by a 15-min incubation in alkylation solution (6 M urea, 30% glycerol, 2% SDS and 135 mM iodoacetamide). The SDS-PAGE step was performed using 10% polyacrylamide gels at the constant current of 45 mA per gel at 15 °C. For the colloidal Coomassie Brilliant Blue staining, the gel was incubated in fixation solution (1.3% TCA and 20% methanol for 1 h) and followed by an overnight incubation in staining solution (5% acetic acid and 50% ethanol for 1 h). The gel was subsequently incubated for 3 min in neutralization solution (0.1 M Tris pH = 6,5), washed in 20% methanol for less than a minute and stabilized in 5% ammonium sulfate for 1 h. The gel was maintained in a 1% acetic acid solution and scanned using an ImageScanner III LabScan 6.0 (GE Healthcare). The detection of the gel spot and calculation of the isoelectric point (pI) and molecular weight (MW) were obtained using Image Master 2D Platinum software (GE Healthcare). The gels were repeated tree times.

2.4. Lectin-blotting

For the evaluation of protein glycosylation we used the DIG Glycan Differentiation Kit (Roche Applied Science, Indianapolis, IN, USA). First of all, hemolymph samples were submitted to SDS-PAGE (5–15% gradient acrylamide), then the proteins were transferred onto nitrocellulose membranes that were then blocked with fat free casein buffer and incubated for 1 h at room temperature with distinct digoxigenin-labeled lectin.

s: *Sambucus nigra* agglutinin (SNA), *Peanut agglutinin* (PNA), *Datura stramonium agglutinin* (DSA) and *Galanthus nivalis agglutinin* (GNA). To recognize the lectins, the nitrocellulose sheets were incubated with anti-digoxigenin antibodies conjugated with alkaline phosphatase for 1 h at room temperature. The reaction was developed by NBT and X-phosphate from the kit. The reactions were performed following the manufacturer's recommendations.

2.5. Lipid analyses

For lipid analysis, the hemolymph was collected and solubilized

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