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Proteomic identification of gender molecular markers in *Bothrops jararaca* venom

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ABSTRACT

Variation in the snake venom proteome is a well-documented phenomenon; however, sex-based variation in the venom proteome/peptidome is poorly understood. *Bothrops jararaca* shows significant sexual size dimorphism and here we report a comparative proteomic/peptidomic analysis of venoms from male and female specimens and correlate it with the evaluation of important venom features. We demonstrate that adult male and female venoms have distinct profiles of proteolytic activity upon fibrinogen and gelatin. These differences were clearly reflected in their different profiles of SDS-PAGE, two-dimensional electrophoresis and glycosylated proteins. Identification of differential protein bands and spots between male or female venoms revealed gender-specific molecular markers. However, the proteome comparison by *in-solution* trypsin digestion and label-free quantification analysis showed that the overall profiles of male and female venoms are similar at the polypeptide chain level but show striking variation regarding their attached carbohydrate moieties. The analysis of the peptidomes of male and female venoms revealed different contents of peptides, while the bradykinin potentiating peptides (BPPs) showed rather similar profiles. Furthermore we confirmed the ubiquitous presence of four BPPs that lack the C-terminal Q–I–P–P sequence only in the female venom as gender molecular markers. As a result of these studies we demonstrate that the sexual size dimorphism is associated with differences in the venom proteome/peptidome in *B. jararaca* species. Moreover, gender-based variations contributed by different glycosylation levels in toxins impact venom complexity. *Biological significance: Bothrops jararaca* is primarily a nocturnal and generalist snake species, however, it exhibits a notable ontogenetic shift in diet and in venom proteome upon neonate to adult transition. As is common in the *Bothrops* genus, *B. jararaca* shows significant sexual dimorphism in snout–vent length and weight, with females being larger than males. This sexual size dimorphism suggests the tendency for female specimens to feed on larger prey, and for male specimens to go on a diet similar to that of juveniles. Variation in the snake venom proteome is a ubiquitous phenomenon occurring at all taxonomic levels. At the intraspecific variation level, the individual contribution to the venom proteome is important but effects contributed by age and feeding habits may also affect the proteome phenotype. Whether sex-based factors play a role in venom variation of a species that shows sexual size dimorphism is poorly known. The use of proteomic strategies supported by transcriptomic data allows a more comprehensive assessment of venom proteomes uncovering components that are gender-specific.

1. Introduction

The variability found in venom proteomes is an intriguing aspect of the evolution of venomous snakes. The venom proteome determined by a given snake genome can vary depending on various endogenous or exogenous factors [1–6]. As the process of protein synthesis is a major connection in the relationship between genome and phenotype of cells and tissues, post-translational modifications (PTMs) are a significant source of protein variation. At the level of intraspecific venom variation, the individual contribution to the proteome/peptidome is important, however, the effects contributed by environmental conditions, age, gender, and feeding habits may also influence the proteome picture exhibited by each specimen [7–11]. Dietary divergence among male and female snakes has important ecological implications as well as might influence venom composition. Although sexual size dimorphism may be the most

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general reason for the sexes to diverge in diets, it is not the only one, and in several taxa, males and females eat different types or sizes of prey even at the same body sizes [12]. In predators that swallow their prey whole, such as snakes, gape size sets the limit to the ingestible prey size or the swallowing capacity [13,14,15]. Moreover, the degree of sexual dimorphism in body size or relative head size is related to the spectrum of ingestible prey selection for each sex [13,16]. Adult male and female snakes may diverge strongly in dietary composition with males feeding on smaller prey and females usually foraging on larger mammals [17,18].

In Brazil, *Bothrops jararaca* is one of the most abundant venomous snake species [19] and is responsible for the majority of envenomation cases involving humans in South and Southeastern Brazil (SINAN/SVS, Brazilian Ministry of Health, 2015 [20]). *B. jararaca* exhibits adult size dimorphism in which female specimens are larger, and, interestingly, in a clinical study that analyzed cases of bites caused by *B. jararaca* and the snake was brought by the victim to the Vital Brazil Hospital of the Butantan Institute, São Paulo, Brazil, it was reported that the majority of the cases were caused by female and juvenile snakes [21]. In another study on the sexual dimorphism of *B. jararaca*, it was reported that female specimens produce larger amounts of venom with higher protein content compared to male specimens. Moreover, venom from female individuals showed higher hyaluronidase, hemorrhagic, and lethal activities [22]. As hypothesized by the authors, these data indicated that sexual dimorphism in *B. jararaca* is associated with niche partitioning with adult males and females diverging strongly in feeding biology.

A number of reports have been published on the composition of *B. jararaca* venom gland transcripts as well as venom proteome diversity, taking into account distinct biological/ecological features such as ontogenetic variability [23–27], intraspecific and geographical variability [28,29] and sexual dimorphism [30,31]. These studies revealed that *B. jararaca* venom undergoes proteome rearrangement upon neonate to adult transition [25,26], and that individual venom proteome variability is present from a very early animal age and is not a result of ontogenetic and diet changes [28]. Moreover, significant geographic variation in the composition of *B. jararaca* venom from Southeastern and Southern populations within the Atlantic rainforest areas in Brazil has been demonstrated [29].

In this investigation we further explored the composition of male and female *B. jararaca* venoms in order to identify gender molecular markers. For this purpose, we analyzed venom samples consisting of pools from 49 male and 61 female specimens older than 3 years, using electrophoretic and mass spectrometric approaches for protein identification, evaluation of proteolytic activities and peptidomic analysis. As a result of these studies, we have highlighted some proteomic similarities and differences between male and female venoms in protein families that have been associated with envenomation pathogenesis. Assessing the proteome composition by a combination of SDS-PAGE and 2-DE and by shotgun proteomic analysis allowed the identification of both clear and subtle differences between male and female venoms and highlighted the role of glycosylation as a key PTM contributing to variability. We also confirmed the presence of four cleaved bradykinin potentiating peptides only in the female venom, which constitute gender-specific markers of the *B. jararaca* peptidome.

2. Material and methods

2.1. B. jararaca venom samples

Venom pools from *B. jararaca* adult specimens (49 male and 61 female specimens older than 3 years) from localities located within a radius of ~200 km from the city of São Paulo (Brazil), and kept in the Laboratory of Herpetology of Instituto Butantan (São Paulo, Brazil) under controlled conditions, were used in this study. The venom was extracted, pooled according to the gender, centrifuged for 30 min at 2000 ×*g*, 4 °C, to remove any scales or mucus, lyophilized and stored at −20 °C until use. Venom protein concentrations were determined using the Bradford reagent (Sigma, St. Louis, MO, USA) and bovine serum albumin (Sigma, St. Louis, MO, USA) as a standard.

2.2. Two dimensional electrophoresis (2-DE)

Prior to isoelectrofocusing (first dimension separation), venoms were solubilized in Destreak solution (GE Healthcare, Uppsala, Sweden) (1 mg/450 μL) containing 1% of immobilized pH gradient (IPG) buffer and sonicated for 10 min. First dimension IEF was carried out in an Ettan IPGphor Isoelectric Focusing System (GE Healthcare, Uppsala, Sweden) as described by the manufacturer. Precast IPG strips (24 cm, 3–10 linear or 4–7 linear) were employed for the first dimension separation, at 20 °C using a seven-phase electrophoresis program: 30 V for 6 h, 150 V for 2 h, 350 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 3000 V for 1 h and 5000 V for 13 h. Prior to running the second dimension separation, IPG strips were placed in the rehydration tray and the proteins in the strip were reduced and alkylated by sequential incubation in the following solutions: 0.05 M Tris–HCl, pH 8.4, 2% SDS; 30% glycerol, 6 M urea, 0.002% bromophenol blue (equilibration buffer—EB), 20 mg/mL DTT in EB, for 12 min; and then a solution of 30 mg/mL iodoacetamide in EB, for 10 min. They were directly applied to SDS-polyacrylamide gels (12%) (20 × 26 cm × 1.5 mm) for electrophoresis. The gels were fixed and then stained with silver.

2.3. Western blot analysis

After one dimensional gel electrophoresis (1-DE) [32], venom proteins (50 μg) were transferred to nitrocellulose sheets and Western blot analysis was carried out as described elsewhere [33] using (i) rabbit polyclonal antiserum containing antibodies against botrocetin, a C-type lectin from *B. jararaca* venom, kindly provided by Dr. Marcelo L. Santoro (Laboratório de Fisiopatologia, Instituto Butantan, Brazil) and (ii) rabbit polyclonal purified antibodies, raised against a nerve growth factor purified from mouse submaxillary glands (Sigma, St. Louis, MO, USA). Immunodetections were carried out with addition of secondary antibody (anti-rabbit IgG conjugated with horse radish peroxidase; Sigma, St. Louis, MO, USA).

2.4. In-gel protein digestion and mass spectrometric identification

Protein spots were excised and *in-gel* trypsin digestion was performed according to Hanna et al. [34]. An aliquot (50%) of the resulting peptide mixture was separated by C18 (8 cm × 75 μm) (Phenomenex, Torrance, CA, USA) RP-HPLC coupled with nano-electrospray tandem mass spectrometry on a LTQ-XL mass spectrometer (Thermo, San Jose, CA, USA) at a flow rate of 500 nL/min. The gradient was 0–80% acetonitrile in 0.1 M acetic acid over 30 min. The instrument was operated in the top ten mode, in which one MS spectrum is acquired followed by MS/MS of the top ten most-intense peaks detected, using 2.5 kV and 200 °C as source voltage and temperature, respectively. Full dynamic exclusion was used to enhance dynamic range — one spectrum before exclusion for 120 s. The resulting fragment spectra were searched using Mascot (version 2.4.1) search engine (Matrix Science, UK) against the UniProt database restricted to the taxa Serpentes (58,895 entries, downloaded on September 17th, 2015) with a parent tolerance of 1.5 Da and fragment tolerance of 1.0 Da. Iodoacetamide derivative of cysteine and oxidation of methionine were specified in Mascot as fixed and variable modifications, respectively. Mascot identifications required ion scores greater than the associated identity scores and 20, 30, 40 and 40 for singly, doubly, triply, and quadruply charged peptides, respectively.

Protein bands were excised from the SDS-polyacrylamide gel and subjected to *in-gel* trypsin digestion [34] and mass spectrometric analysis by LC–MS/MS. Peptide samples were automatically injected onto a trap column (5 cm, 100 μm I.D. × 360 μm O.D.) *in house* packed with Jupiter C-18 10 μm resin (Phenomenex, Torrance, CA, USA) in tandem with a C-18 analytical column (10 cm, 75 μm I.D. × 360 μm O.D.) *in*