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N-glycome profiling of *Bothrops jararaca* newborn and adult venoms

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ABSTRACT

Glycosylation is an important post-translational modification of snake venom proteins and contributes to venom proteome complexity. Many snake venom components are known to be glycosylated, however, very little is known about the carbohydrate structures present in venom glycoproteins. Previous studies showed that the ontogenetic shift in diet, from ectothermic prey in early life to endothermic prey in adulthood, and shift in animal size are associated with changes in the venom proteome of the snake *Bothrops jararaca*. In this study we explored the composition of the *N*-glycome released from newborn and adult *B. jararaca* venom proteins. We used an ion trap mass spectrometer (IT-MS) to disassemble glycan structures based on the use of several pathways of MS (MSn) and demonstrate the presence of some structural isomers in both newborn and adult venom *B. jararaca N*-glycans. The main *N*-glycans identified in both venoms are of the hybrid/complex type however some mannose-rich type structures were also detected. The *N*-glycan composition of newborn and adult venoms did not vary indicating that differences in the utilization of the *N*-glycosylation motif could be the explanation for the differences in the glycosylation levels indicated by the differential electrophoretic profiles previously reported for *B. jararaca* newborn and adult venoms.

1. Introduction

Snake venoms are complex mixtures containing many different biologically active proteins and peptides. An increasing number of proteomics studies have been carried out in order to understand the complexity of snake venoms and their subsets of toxin families [1–3]. Variability in composition is a ubiquitous feature among species and recently it has taken on an in-depth qualitative and quantitative focus [1,3,8–10]. Venom proteomes can vary according to several factors, such as age, gender, habitat, and, in this context, the snake diet is often considered the variable driving factor. A number of examples of snake venom variability have been reported from differential expression of certain toxin families during ontogenetic development to identification of distinct toxin isoforms in a single specimen, to distinct processing sites in toxin precursors [4–9]. Complementary mass spectral analyses have increased exponentially the number of venom proteomes explored, a term usually referred to as *venomics* [10]. However, the lack of a broad and comprehensive database for snake proteins, especially venom proteins, frequently leads to manual interpretation of spectra via *de novo* sequencing. Alternatively, Expressed Sequence Tags (ESTs) databases can be utilized, a feature that has improved the knowledge of the proteins in the venom gland [3,10]. However, the vast majority of snake venom proteins are post-translationally

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modified and, even though the modifications can be predicted by conceptual translation of cDNA sequences present in such databases, the extent and complexity of a given post-translational modification (PTM), such as glycosylation, is virtually unpredictable based solely on *in silico* analysis.

Glycosylation is a key protein post-translational modification (PTM) process and has an array of implications in the structure and function relationship of proteins as well as in the role displayed by glycosylated proteins in biological pathways [11]. Aberrant glycosylation has also been reported and, in some cases, is associated to the degree of tumor malignancy [12]. In this respect, the elucidation of glycan structures plays a major role in glycobiology, and has been the main goal of numerous laboratories. Several studies have focused on the determination of glycan structures and composition and mass spectrometry has proven to be a reproducible and robust approach [13–17]. Like most PTMs, glycosylation provides a means of expanding genome expression. Thus, considering that variability, we decided to analyze the *N*-linked glycan composition, frequently referred to as the N-glycome, of a South American Viperid species, *Bothrops jararaca*. This species is one of the most abundant in Brazil, inhabiting the rain forest as well as more open vegetation areas [18]. Although primarily nocturnal and generalist, it exhibits a notable ontogenetic shift in diet, feeding on ectothermic prey (mainly arthropods, lizards, and amphibians) through its juvenile phase and on endothermic animals (mainly small mammals) during adult life [19,20]. Due to its broad geographical distribution, this species is responsible for the majority of the accidental envenomation cases by *Bothrops* genus in Brazil. Recently we have reported the variability in the venom proteome and peptidome of newborn and adult *B. jararaca* [9]. The glycoproteomic analysis showed that N-glycosylation seems to be the most prominent post-translational modification in *B. jararaca* toxins and the N-glycosylation profiles differed for newborn and adult venom toxins [9]. Moreover, we showed that the subproteome of glycoproteins with affinity for Concanavalin A lectin is composed mainly by Snake Venom Serine Proteinases (SVSPs) and Snake Venom Metalloproteinases (SVMPs), which are the main toxin families verified in this species by transcriptomic analysis [21].

The aim of this study was to explore the composition of the *N*-glycome released from newborn and adult *B. jararaca* venom proteins. Since a number of toxins in this venom are glycosylated it was reasonable to assume that they could display a role in human envenomation cases. We have used an ion trap mass spectrometer (IT-MS) to disassemble glycan structures based on the use of several pathways of MS (MSn). To the best of our knowledge this is the first report on the application of this approach to analyze snake venom glycans. Here we demonstrate the presence of some structural isomers in both newborn and adult *B. jararaca* *N*-glycans.

2. Materials and methods

2.1. Venom samples

Pooled venom from 694 two-week old newborns (359 male and 335 female specimens) and 110 adults (49 male and 61 female specimens older than 3 years) from São Paulo State (Brazil) was used in this study. The venom was milked, 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 [22] (Coomassie Plus Assay Kit, Thermo Scientific, USA) and bovine serum albumin as a standard.

2.2. Release of N-linked glycans

Enzymatic *N*-deglycosylation was carried out with the glycosidase PNGaseF (New England Biolabs Inc, USA) under denaturing conditions, according to manufacturer's protocol. Briefly, 1 mg of venom proteins was dissolved in 300 μL of 0.04 M ammonium bicarbonate, followed by the addition of 15 μL glycoprotein denaturing solution (5% SDS, 0.4 M DTT). The mixture was heated at 90 °C for 15 min. After cooling, 15 μL of reaction buffer was added (0.5 M sodium phosphate, pH 7.5), followed by the addition of 15 μL of 10% NP-40 and 2 μL of PNGase F (1000 U). The reaction mixture was incubated for 18 h at 37 °C.

2.3. Reduction and permethylation of released N-glycans

Enzymatic released *N*-glycans were applied onto C-18 Solid Phase Extraction (SPE) cartridges (Grace Davidson Discovery Sciences, USA) and eluted with 5 mL of 5% acetonitrile in water containing 0.1% TFA. Eluted *N*-glycans were dried in SpeedVac, dissolved in HPLC grade water and desalted using Porous Graphitized Columns (PGC, Grace Deerfield, IL, USA). Eluted *N*-glycans were dried in SpeedVac and dissolved in 200 μL 1 M $NaBH_4$ in 0.01 M NaOH solution [14]. The samples were left overnight at room temperature. The reduction solution was chilled, terminated by the addition of glacial acetic acid, and diluted with 2 mL of absolute ethanol before drying under a nitrogen stream. Borate esters were removed by repeated addition and drying of a 1% acetic acid in methanol solution. After reduction procedure, the enzymatic released *N*-glycans were submitted to desalting using PGC as described above. Micropermethylation procedure was carried out according to the method of Ciucanu and Kerek [23]. Briefly, sodium hydroxide beads were packed in a spin column (Harvard Apparatus, Holliston, MA, USA) and suspended in 500 μL of acetonitrile to prevent moisture absorption. Prior to sample application, acetonitrile was removed by centrifugation and the column was washed three times with 300 μL DMSO, spinning the column at 1000 rpm for 2 min. Glycan samples were dissolved in 90 μL DMSO, followed by the addition of 0.9 μL of HPLC grade water and 16.8 μL of iodomethane. The samples were applied onto the spin columns, centrifuged at 1000 rpm for 2 min and the eluates were repetitively applied onto the spin columns (10 times). The collection tube was placed in ice bath and 300 μL of HPLC grade water was added, followed by addition of the same volume of chloroform. The organic layer was washed ten times to remove any sodium hydroxide, iodomethane or impurities that might be present. Finally, permethylated samples were dried in SpeedVac and dissolved in 80% methanol prior to mass spectrometry analysis.

2.4. Mass spectrometry and data analysis

Mass spectra were obtained using an LTQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with