Integrated bottom-up and top-down analysis reveals new proteoforms in the venom of an individual Bothrops pauloensis specimen

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Introduction: *Bothrops pauloensis*, popularly known as the painted jararaca, is a medium-sized venomous snake found in Brazil, Paraguay, and Bolivia. Reports of envenoming cases caused by *B. pauloensis* are scarce, but the accidents are characterized by hemorrhage, inflammation, local myonecrosis and blood clotting perturbations, typically associated to accidents involving the Bothrops genus. The venom proteome of *B. pauloensis* as well as its ontogenetic variations have been previously characterized. However, these studies were carried out using venom pools and did not capture individual variation of toxin proteoforms. In this work we collected the venom of a single individual, fractionated and analyzed the venom proteoforms of main toxin families using a combined strategy of top-down and bottom-up analysis. **Material and methods:** *B. pauloensis* venoms were obtained from the Herpetology Laboratory of Instituto Butantan (São Paulo, Brazil), from the extraction of a single adult male specimen. Venoms were quantified using the Bradford Reagent (Sigma-Aldrich, B6916), according to the manufacturer's recommendations. Subsequently, 100 ?g of crude venom was loaded in C18 stage tips and fractionated with different percentages of ACN in 0.1% TFA: 30%, 35%, 45% and 80%. The venom eluates were splitted in six aliquots, dried in a vacuum concentrator (Concentrator Plus, Eppendorf) and stored at -20 °C until digestion. Venom fraction aliquots were digested with five different enzymes: trypsin, Asp-N, chymotrypsin, thermolysin and Glu-C, according to manufacturer's recommendations. Digestion samples and the remained intact aliquots of each fraction were desalted using C18 stage tips and submitted to LC-MS/MS analysis. Top-down analyzes of intact venoms fractions were performed on the nanoAcquity UPLC chromatographic system (Waters) coupled to a Synapt G2 HDMS mass spectrometer (Waters) operated in the data-independent acquisition MS^E mode. Bottom-up analyzes of digested aliquots were performed on a nLC Easy 1200 system (Proxeon Biosystem) coupled to an Orbitrap Exploris 240 (Thermo Scientific) mass spectrometer operated in the data-dependent acquisition mode. Multiply charged mass spectra of intact toxins were acquired on MassLynx 4.1 and manually deconvoluted. MS/MS raw data were analyzed in PEAKS Studio X+ (Bioinformatics Solution Inc.) using de novo analysis and database search with the Serpentes database. Mutations were mapped using the Spider search module. Experimental intact masses were matched with the theoretical masses of proteoforms. **Results and discussion:** The main toxins PLA_2, LAAO, metalloproteases and bradykinin potentiating peptides were identified, and also new proteoforms. Most of the toxins were identified in the 35% and 45% fractions, consistent with the results reported in venomics studies. The new proteoforms were identified through the integrated analysis of de novo sequencing and bottom-up and top-down proteomics. **Conclusion:** Integrated bottom-up and top-down analysis of Bothrops pauloensis venom revealed a hidden complexity in the venom and identified new proteoforms of main toxins at the individual level.

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