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Molecular mechanisms underlying intraspecific variation in snake venom

Diana R. Amazonas[a], José A. Portes-Junior[a], Milton Y. Nishiyama-Jr[b], Carolina A. Nicolau[c], Hipócrates M. Chalkidis[d], Rosa H.V. Mourão[e], Felipe G. Grazziotin[h], Darin R. Rokyta[f], H. Lisle Gibbs[g], Richard H. Valente[c], Inácio L.M. Junqueira-de-Azevedo[b,*], Ana M. Moura-da-Silva[a,*]

[a] Laboratório de Imunopatologia, Instituto Butantan, Av. Vital Brazil, 1500, 05503-900 São Paulo, SP, Brazil
[b] Laboratório Especial de Toxinologia Aplicada, Instituto Butantan, Av. Vital Brazil, 1500, 05503-900 São Paulo, SP, Brazil
[c] Laboratório de Toxinologia, Instituto Oswaldo Cruz – IOC/FIOCRUZ, Av. Brasil, 4365, 21040-900 Rio de Janeiro, RJ, Brazil
[d] Programa de Pós-Graduação em Recursos Naturais da Amazônia, Laboratório de Bioprospecção e Biologia Experimental, Universidade Federal do Oeste do Pará - UFOPA, Rua Vera Paz, s/n, 68035-110 Santarém, PA, Brazil
[e] Faculdades Integradas do Tapajós/Faculdade da Amazônia – FIT/UNAMA, Rua Rosa Vermelha, 335, 68010-200 Santarém, PA, Brazil
[f] Department of Biological Science, Florida State University, Tallahassee, FL 32306, USA
[g] Department of Evolution, Ecology and Organismal Biology Ohio State University, Columbus, OH 43210, USA
[h] Laboratório Especial de Coleções Zoológicas, Instituto Butantan, Av. Vital Brazil, 1500, 05503-900 São Paulo, SP, Brazil

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ABSTRACT

Elucidating the molecular mechanisms underlying snake venom variability provides important clues for understanding how the biological functions of this powerful toxic arsenal evolve. We analyzed in detail individual transcripts and venom protein isoforms produced by five specimens of a venomous snake (*Bothrops atrox*) from two nearby but genetically distinct populations from the Brazilian Amazon rainforest which show functional similarities in venom properties. Individual variation was observed among the venoms of these specimens, but the overall abundance of each general toxin family was conserved both in transcript and in venom protein levels. However, when expression of independent paralogues was analyzed, remarkable differences were observed within and among each toxin group, both between individuals and between populations. Transcripts for functionally essential venom proteins ("core function" proteins) were highly expressed in all specimens and showed similar transcription/translation rates. In contrast, other paralogues ("adaptive" proteins) showed lower expression levels and the toxins they coded for varied among different individuals. These results provide support for the inferences that (a) expression and translational differences play a greater role in defining adaptive variation in venom phenotypes than does sequence variation in protein coding genes and (b) convergent adaptive venom phenotypes can be generated through different molecular mechanisms.

Significance: Analysis of individual transcripts and venom protein isoforms produced by specimens of a venomous snake (*Bothrops atrox*), from the Brazilian Amazon rainforest, revealed that transcriptional and translational mechanisms contribute to venom phenotypic variation. Our finding of evidence for high expression of toxin proteins with conserved function supports the hypothesis that the venom phenotype consists of two kinds of proteins: conserved "core function" proteins that provide essential functional activities with broader relevance and less conserved "adaptive" proteins that vary in expression and may permit customization of protein function. These observations allowed us to suggest that genetic mechanisms controlling venom variability are not restricted to selection of gene copies or mutations in structural genes but also to selection of the mechanisms controlling gene expression, contributing to the plasticity of this important phenotype for venomous snakes.

1. Introduction

Snake venoms show widespread variation in composition not only within and between species but also at higher taxonomic levels [1–3]. This variation is widely interpreted to represent adaptive variation that allows subduing and digesting prey or for defense against predators [4–6]. Venom heterogeneity also has important medical consequences by causing significant differences in venom toxicity and pathogenicity

* Corresponding authors.
E-mail addresses: inacio.azevedo@butantan.gov.br (I.L.M. Junqueira-de-Azevedo), ana.moura@butantan.gov.br (A.M. Moura-da-Silva).

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[7–9]. For example, broad differences in venom composition have been observed at multiple levels in rattlesnakes. In general, rattlesnake venoms can be classified as either type I or II according to the predominant expression of SVMP or PLA_2 phenotypes, respectively [10]. The occurrence of type I or II phenotypes has been reported in closely related species [11], within the same species (depending on their geographical distribution) [12,13] or during their ontogeny [14]. This phenotypic variation results in functional variation: the phospholipases in type II venoms exhibit neurotoxic activity leading to high levels of venom lethality [10] whereas type I venoms are considered coagulotoxic due to their high abundance in SVMPs, characterized as tissue-damaging digestive enzymes that affect the hemostasis of prey [15]. Consequently, these shifts in rattlesnake venom phenotypes alter the mechanisms by which prey are subdued (e.g. venoms with neurotoxic versus hemostatic effects) with clear consequences for the pathology of human envenomations.

In *Bothrops* pit vipers, PLA_2 molecules do not exhibit neurotoxic activity; instead, they show a tissue-damaging myotoxic activity [16]. Perhaps as a consequence, the majority of *Bothrops* species show a rattlesnake-like type I venom phenotype with high SVMP content [17], with only a few examples of type II venoms, such as *B. jararacussu* venom [18] which is usually less toxic (higher LD_{50}), at least in laboratory mouse models. Nevertheless, interspecific and intraspecific variability have also been reported for venoms from *Bothrops* species that show an SVMP-rich type I phenotype. Such variation arises through differences in the abundance of specific venom proteins in each toxin group, leading to a quantitative variability in venom activities. The studies on venom composition for *Bothrops* species report that this type of variation can be influenced by factors such as ontogeny [19,20], gender [21], and broad scale geographical variation [22,23]. However, to our knowledge, no previous studies in *Bothrops* have attempted to characterize venom variation among specimens belonging to the same ontogenetic stage, gender, and geographical location. The use of quali-quantitative transcriptomics and proteomics approaches have been used for addressing questions about the nature of links between genotypes and phenotypes for adaptive traits like venom [24,25] and for devising strategies used for antivenom development [26].

Here, we used transcriptomic and proteomic approaches to characterize in detail venom variation for five individuals of the common lancehead (*B. atrox*) sampled from two relatively close populations located on the North and South banks of the Amazon River in the west of Pará State, Brazil. Previous analyses of pooled venom from individuals from these populations showed broad similarity in HPLC profiles but also differences in the sizes of specific venom peaks. Despite these differences, functional analyses of pooled venoms from these populations showed that they are indistinguishable in terms of their functional properties [27]. Other work has shown that these populations are genetically distinct and diverged from each other < 0.5 MYR bp but remained connected through low levels of gene flow [28]. Thus, these populations exhibit similar venom phenotype likely due to adaptation due to similar prey communities in similar habitats and therefore offer the opportunity to use our ability to dissect in detail the molecular variation in venom to assess whether common adaptive phenotypes have the same or different molecular bases [29].

In the present work, we have also, we quantified venom variation in unprecedented detail, both at individual and population levels, and then understood its molecular basis in terms of differences in expression and translation of different toxin proteins. We used transcriptomic and proteomic analyses to quantify patterns of paralogue/isoform expression in each venom followed by linking the observed paralogues with protein sequences, for which function had been previously determined, to understand the role of each isoform in determining the observed venom phenotype. We then used this information to assess three broad questions about the molecular basis of adaptive phenotypic variation using venom as model: 1) What role does expression and translation play in generating phenotypic variation in a complex molecular adaptation? 2) Is there a difference in patterns of variation among different functional categories of venom proteins? 3) Do functionally similar venom phenotypes have the same or different molecular bases?

2. Material and methods

2.1. Snakes and venoms

Individual *B. atrox* adult female snakes were collected from two locations in the western part of the State of Pará, Brazil. Two specimens were collected from a recently cleared pasture area, which was previously an upland forest in the municipality of Oriximiná, on the North shore of the Amazon River (S 01°46′03.39″; W 55°43′53.31″) and three specimens were collected in Floresta Nacional do Tapajós, a National Forest located in the municipality of Belterra, next to the Tapajós River, about 80 km south of the Amazon River (S 03°03′59.03″; W 54°58′8.94″), under ICMBio/SISBio license 32098-1. After capture, the snakes were immediately transferred to the Herpetarium of Faculdades Integradas do Tapajós, in Santarém, and anesthetized using CO_2 for venom extraction. Venom samples were collected using manual extraction techniques and individually freeze-dried for proteomic studies. For transcriptomic analysis, total RNA was extracted from venom gland tissue collected four days after venom extraction. The snakes were anesthetized with sodium pentobarbital (30 $mg{\cdot}kg^{-1}$, s.c.) and decapitated. Venom glands were then dissected and immediately frozen in liquid nitrogen followed by storage at −80 °C for further mRNA isolation. Animal care and procedures used in the handling of snakes were in accordance with the guidelines of the Ethical Committee for Animal Research of Instituto Butantan (1244/14).

2.2. Venom fractionation by reverse phase chromatography

Individual *B. atrox* venoms were fractionated using reversed-phase high-performance liquid chromatography (RP-HPLC) following previously described methods [17]. Briefly, 5 mg of crude lyophilized venom was dissolved in 250 µL of 0.1% trifluoroacetic acid (TFA) and injected onto a Vydac C18 column (250 mm × 4.6 mm, 10 µm particle size) coupled to a Shimadzu LC 20 - AT HPLC system. Proteins were eluted at 2 mL/min with a gradient of 0.1% TFA in water (solution A) and 0.1% TFA in acetonitrile (solution B) (5% B for 5 min, 5–15% B over 10 min, 15–45% B over 60 min, 45–70% B over 10 min, 70–100% over 5 min, and 100% B for 10 min). The fractionation was monitored at 214 nm.

2.3. Proteomic characterization by shotgun mass spectrometry

Replicates of each individual venom sample (50 µg of protein) were reduced and alkylated before treatment with trypsin solution (0.2 µg/µL), as previously described [30]. The tryptic digests were desalted using in-house made columns packed with Poros R2 resin (Life Technologies, USA) and subjected to reversed-phase nanochromatography coupled to nanoelectrospray high resolution mass spectrometry for peptide analysis. Each digest was analyzed in triplicate in the mass spectrometer, as previously described for proteome analysis of several bothropic venoms [27].

Tandem mass spectra were processed and searched against an in-house database using the search tools Mascot (Matrix Science, London, UK; version 2.4.1) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). The database used to identify the MS/MS spectra is composed of the full-length precursor proteins predicted from the transcriptomes of the same five specimens of *B. atrox* described in this manuscript. Protein identification was based on the presence of proteotypic peptides relating to each venom protein isoform; search parameters were as described in the literature [27].

Scaffold (version Scaffold_4.6.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein