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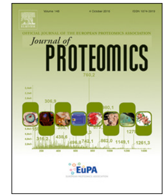

Transcriptome-facilitated proteomic characterization of rear-fanged snake venoms reveal abundant metalloproteinases with enhanced activity

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ABSTRACT

High-throughput technologies were used to identify venom gland toxin expression and to characterize the venom proteomes of two rear-fanged snakes, *Ahaetulla prasina* (Asian Green Vine Snake) and *Borikenophis portoricensis* (Puerto Rican Racer). Sixty-nine complete toxin-coding transcripts from 12 venom protein superfamilies (*A. prasina*) and 50 complete coding transcripts from 11 venom protein superfamilies (*B. portoricensis*) were identified in the venom glands. However, only 18% (*A. prasina*) and 32% (*B. portoricensis*) of the translated protein isoforms were detected in the proteome of these venoms. Both venom gland transcriptomes and venom proteomes were dominated by P-III metalloproteinases. Three-finger toxins, cysteine-rich secretory proteins, and C-type lectins were present in moderate amounts, but other protein superfamilies showed very low abundances. Venoms contained metalloproteinase activity comparable to viperid snake venom levels, but other common venom enzymes were absent or present at negligible levels. Western blot analysis showed metalloproteinase and cysteine-rich secretory protein epitopes shared with the highly venomous Boomslang (*Dispholidus typus*). The abundance of metalloproteinases emphasizes the important trophic role of these toxins. Comprehensive, transcriptome-informed definition of proteomes and functional characterization of venom proteins in rear-fanged snake families help to elucidate toxin evolution and provide models for protein structure-function analyses.

1. Introduction

Snake venoms contain a variety of proteins and peptides that function primarily in prey immobilization and digestion, and secondarily as a mechanism of defense [1]. Venoms have allowed advanced snakes (Caenophidia) to transition away from the use of constriction (commonly seen in more basal "henophidian" snakes), and instead to rely on a chemical means of prey capture [2]. Among the Caenophidia, rear-fanged venomous snakes represent diverse, unique evolutionary lineages where a mechanically less complex venom delivery system has evolved, paralleling the more complex injection system of front-fanged snakes. Rear-fanged venomous snakes have a relatively low-pressure venom delivery system and lack the large venom storage reservoir (gland lumen) seen in front-fanged venomous snakes. In the rear-fanged snake venom gland, venom proteins are produced and typically stored intracellularly, and upon deployment, they are released more slowly into a main duct conducting venom to the base of rear maxillary teeth, which may be grooved or modified, but never hollow (hollow fangs are only seen in front-fanged venomous snakes) [3].

Rear-fanged snake venoms have remained largely unexplored, and this dearth of knowledge contrasts strongly with the extensive research on front-fanged snake venoms. It has been estimated that fewer than 3% of rear-fanged snake venom proteomes have been described [4]. Venom research has focused on elapid (cobras, kraits, mambas, and relatives) and viperid (vipers and pit vipers) venoms because these snakes produce significantly larger venom yields and are responsible for the vast majority of snake envenomations of humans [1]. Most rear-fanged venomous snakes are unable to deliver sufficient quantities of venom to produce systemic envenomation effects in humans, but at least five species (*Dispholidus typus, Thelotornis capensis, Rhabdophis tigrinus, Philodryas olfersii*, and *Tachymenis peruviana*) are believed to have caused human fatalities [5–9].

In general, rear-fanged snake venoms show lower complexity than those of front-fanged snakes, commonly manifesting only 20–40 protein spots on 2D sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2D SDS-PAGE), while front-fanged snake venoms show considerably higher complexity, commonly displaying well over 100 protein spots [10]. Despite lower compositional complexity, rear-fanged venomous

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snakes sometimes exhibit front-fanged snake (elapid or viperid-like) venom phenotypes [11, 12]. Venom composition is likely closely linked to snake diet [13–17], with rear-fanged snake venoms producing examples of prey-specific neurotoxins [14, 18, 19], in addition to the examples of toxin taxon-specific receptor binding observed for elapids [20, 21]. In several cases, rear-fanged snake venoms have been documented to contain novel protein superfamilies with several distinct trajectories [14, 22–26].

The majority of venom protein superfamilies have representatives in both front-fanged and rear-fanged snake venoms [1, 6]. Some of the most prominent superfamilies include snake venom metalloproteinases (SVMPs), phospholipases A_2 (PLA_2s), serine proteinases, three-finger toxins (3FTxs), cysteine-rich secretory proteins (CRiSPs), proteinase inhibitors, and C-type lectins [1, 26]. Snake venom metalloproteinases are one of the most abundant components of viperid venoms [4] and are responsible for local and systemic hemorrhage often seen following viper envenomations [27, 28]. These metalloproteinases are zinc-dependent enzymes that consist of multiple domains thought to have evolved from early neofunctionalization of an ADAM-like (a disintegrin and metalloproteinase) ancestral sequence before the radiation of advanced snakes [29], and they may also serve a predigestive function during envenomation [30]. Myotoxic metalloproteinases have been observed in venoms of rear-fanged snakes, with proteolytic activity up to 25 times greater than that of some pitvipers [31–33]. There have been several SVMPs identified in rear-fanged snake venoms [10, 24, 34]. One of these metalloproteinases, alsophinase, was characterized from the venom of *Borikenophis* {formerly *Alsophis*} *portoricensis* (Puerto Rican Racer), a New World rear-fanged snake [33]. Bites from *B. portoricensis* have been reported to cause edema and ecchymosis, likely from the SVMPs present in this venom [35, 36].

Ahaetulla prasina (family Colubridae; Asian Green Vinesnake), is native to large areas of southeast Asia. It is an arboreal snake with a diet of small, nestling birds, lizards, and frogs [37]. *Borikenophis portoricensis* is a rear-fanged “colubrid” snake (family Dipsadidae) native to numerous islands in the Caribbean. This is a ground-dwelling, diurnal snake with a diet consisting primarily of lizards (*Anolis* sp.) and *Eleutherodactylus* frogs [36, 38]. These snakes both have similar dietary preferences, but occupy rather different ecological niches: *Ahaetulla* is an elongate arboreal species found in broad regions of southeast Asia, while *Borikenophis* is a terrestrial predator ranging from dry scrub forests to lowland tropical forests in the Caribbean. They represent diverse model species in which to explore the adaptive significance of these two (potentially important) factors affecting venom composition. The present work explores venom gene expression in *A. prasina* and *B. portoricensis* venom glands and compares gene expression to venom proteome composition. By characterizing the venom gland transcripts, the venom proteome, and venom enzyme activity, a better understanding of toxin gene expression, venom composition, and the biological roles of rear-fanged snake venom proteins can be obtained, as well as identifying any potential human health hazards these snakes could pose [9, 39].

2. Materials and methods

2.1. Reagents

TRIzol reagent was purchased from Life Technologies (San Diego, CA, U.S.A.). Stranded mRNA-Seq kit and Library Quantification Kit (Illumina® platforms) were purchased from KAPA Biosystems (Boston, MA, U.S.A.). Agencourt AMPure XP reagent was from Beckman Coulter, Inc. (Brea, CA, U.S.A). Novex Mark 12 unstained molecular mass standards, MES running buffer, LDS sample buffer, nitrocellulose membranes and precast 12% Bis-Tris NuPAGE electrophoretic gels were obtained from Life Technologies (San Diego, CA, U.S.A.). Pierce BCA protein assay kit was purchased from Thermo Fisher Scientific (Rockford, IL, U.S.A). Phospholipase A_2 assay kit was purchased from Cayman Chemical Co (Ann Arbor, MI, U.S.A.). SAIMR Boomslang antivenoms (South African Vaccine Producers, LTD.; batch Y00651, expiration March 2013) was a gift from the Sedgwick County Zoo, Wichita, KS, USA. All other reagents (analytical grade or better) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). All reagents and supplies used for molecular work were certified nuclease-free.

2.2. Venom glands and venom collection

Ahaetulla prasina (n = 4) imported from Indonesia (Bushmaster Reptiles) and *Borikenophis portoricensis* (n = 3) originating from Guana Island, British Virgin Islands were maintained in the University of Northern Colorado Animal Resource Facility in accordance with UNC-IACUC protocol #9204. One snake of each species was used for venom gland collection. Both snakes were adults, with *A. prasina* measuring 1000 mm snout-to-vent and weighing 150 g, and *B. portoricensis* measuring 580 mm snout-to-vent and weighing 75 g. Venom was manually extracted from rear-fanged snakes using the method of Hill and Mackessy (1997) with subcutaneous injections of ketamine-HCl (20–30 mg/kg) followed by pilocarpine-HCl (6 mg/kg) [40]. Adult *Crotalus viridis viridis* venom was obtained by manual extraction from a wild-caught specimen (Weld Co., Colorado, USA). All venoms were centrifuged at 9000 x g for 5 min, frozen at −80 °C, lyophilized, and stored at −20 °C until use. Four days post-extraction, when mRNA levels are highest [41], rear-fanged snakes were heavily anesthetized with isoflurane and euthanized *via* skull-cervical severing, and venom gland tissue was then collected. Tissue from each of the venom glands (right and left glands) from *A. prasina* was placed directly into TRIzol reagent for immediate RNA isolation. Gland tissue from *B. portoricensis* had been collected five years previously and had been stored in RNAlater at −80 °C before RNA was isolated for the current study. All procedures were approved by the UNC Institutional Animal Care and Use Committee (IACUC protocol 9204.1).

2.3. RNA isolation, library preparation and next-generation sequencing

RNA isolation was performed following the TRIzol reagent manufacturer's protocol with an additional overnight −20 °C incubation in 300 μL 100% ethanol with 40 μL 3 M sodium acetate. Total RNA from each species was resuspended in nuclease-free H_2O and poly-A+ RNA was selected from 4 μg of total RNA using KAPA Stranded mRNA-Seq kit oligo-dT beads. KAPA Stranded mRNA-Seq kit manufacturer's protocol for library preparation was followed for Illumina® sequencing. Products of 200–400 bp were selected by solid phase reversible immobilization using Agencourt AMPure XP reagent. PCR library amplification consisted of 14 cycles. Libraries were then checked for proper fragment size selection and quality using an Agilent 2100 Bioanalyzer. Library concentration was determined following KAPA Library Quantification Kit manufacturer's protocol, and each venom gland library was equally pooled and sequenced on an Illumina® HiSeq 2000 platform lane at the UC Denver Genomics core to obtain 100-bp paired-end reads.

2.4. Transcriptomics: Assembly, annotation and quantification

The quality of the sequenced reads was assessed using the Java program FastQC (Babraham Institute Bioinformatics, UK), and low-quality reads (Phred+33 score < 30) and contaminating adaptor sequences were removed using Trimmomatic with a sliding window of 4 bps [42]. To obtain the best venom gland transcriptome assembly, two assembly approaches were used in combination with different k-mer sizes and assembly algorithms. A Trinity (release v2014-07-17) *de novo* assembly of paired-end reads was completed with default parameters (k-mer size 25) [43]. A second *de novo* assembly was completed with the program Extender (k-mer size 100) [44]. For Extender, reads were first merged with PEAR (Paired-End read mergeR v0.9.6; default parameters) if their 3′ ends overlapped to create longer contiguous sequences [45]. The Extender assembly was performed specifying the

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