Venoms of Colubrids

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Abstract

Advancements in high-throughput technologies in the field of venomics, coupled with the increasing emphasis on a combination of proteomic, transcriptomic, and genomic approaches, have resulted in the ability to generate comprehensive venom profiles for many species of snakes. Rear-fanged snake venom research has slowly progressed due to the difficulties obtaining crude venom and a lack of interest in snakes that only rarely are responsible for human morbidity and mortality. However, current research into rear-fanged snake venoms has demon-

the evolution and origin of snake venom toxins within advanced snakes. These venoms still remain largely unexplored, and there exists within these venoms the potential to discover proteins of therapeutic significance or with unique characteristics. The majority of research conducted on these venoms has focused on protein chemistry and proteomic techniques (electrophoresis, enzymatic assays, liquid chromatography, and mass spectrometry), with fewer explorations of venom gland transcriptomes from expressed sequence tags (ESTs). Published research on rear-fanged snake genomes is not yet available, but such studies will provide insights into the evolutionary history of snake venom proteins and the regulation of toxin expression. Venom is a trophic adaptation, and as such, the evolution and abundance of venom proteins relates directly to prey capture success and organism natural history. Without this biologically relevant perspective, which considers the presence and evolution of rear-fanged venom proteins in terms of their biological significance to the organism, proteomic and genomic approaches could produce simply a list of proteins, peptides, transcripts, and genes.

Introduction

Snake venoms represent a critical innovation allowing advanced snakes (Caenophidian) to transition from a mechanical (constriction, as seen in

evolutionary relationships of venomous snakes as well as identifying compounds

sp. and for

(e.g., Pawlak et al. 2006, 2009). For example, NSA mice showed no adverse effects from the 3FTx irditoxin (from *B. irregularis* venom) at doses of at least 25 μg/g, whereas house geckos (*Hemidactylus frenatus*) and domestic chickens (*Gallus domesticus*) exhibited rapid flaccid paralysis, dyspnea, and increased respiratory rates at all doses tested, with an $LD_{50} < 0.55$ μg/g (Pawlak et al. 2009). This correlates closely with the diet of *B. irregularis*, which frequently feeds on birds and lizards, and demonstrates the importance of venom as a tropic adaptation and the need to acknowledge snake natural history when elucidating venom protein biological roles. LD_{50} values from mice alone (crude venom – 18–31 μg/g; Mackessy et al. 2006) would not have revealed the complexities of *B. irregularis* venom and would not have detected the presence of a prey-specific toxin.

Analyses of rear-fanged venoms by HPLC (high-performance liquid chromatography) size exclusion chromatography has revealed the presence of larger mass proteins, with acetycholinesterase and metalloproteinase activities limited to the first peaks, CRiSPs (cysteine-rich secretory proteins) found in the second peaks, followed by PLA_2s, and then 3FTxs when present (Peichoto et al. 2012). Ion exchange chromatography, especially cation exchange, has been shown to be an effective purification first step for 3FTxs present in rear-fanged snake venoms and

RP-HPLC has also been utilized as a first step for descriptive venomics because this technique provides a clear image of crude venom complexity by separating protein isoforms and exhibiting relative abundance of venom protein superfamilies when combined with SDS-PAGE or MS (Calvete et al. 2009; Fry et al. 2003c; Pawlak et al. 2006). A combination of liquid chromatography and soft ionization mass spectrometry (LC/MS) has been used to analyze crude rear-fanged snake venoms, including species from Colubrinae, Homalopsinae, Natricinae, Psammophiinae, Pseudoxyrhophiinae, and Xenodontinae (Fry et al. 2003c). An advantage to this technique is that it can be performed with limited amounts of material. MS molecular masses and LC retention information can also provide an idea of represented venom protein superfamilies in a crude venom (Fry et al. 2003c). However, ion suppression with coeluting proteins is a problem with electrospray mass spectrometry (ESI-MS), and proteins of lower abundance can be overlooked.

For identifications of purified venom proteins, N-terminal sequencing (Edman degradation) has been frequently used. N-terminal sequencing and tandem MS for de novo sequencing can provide reliable amino acid sequences, and automated de novo sequencing tools are increasingly becoming more robust. However, identification of proteins from rear-fanged snake venoms can still be problematic given the limited amount of database information currently available for rear-fanged venom protein sequences. A postsynaptic neurotoxin was isolated in the rear-fanged Rufous beaked snake (*Rhamphiophis oxyrhynchus*) but lacked sequence homology to any previously identified snake venom toxin in the databases, making it difficult to determine what venom protein family this neurotoxin represented (Lumsden et al. 2007).

Venoms are composed of both enzymatic and nonenzymatic proteins, as well as small peptides and other organics (Mackessy 2010), and numerous enzyme assays have been developed for the detection of the major snakemass

Weldon and Mackessy 2012). *Philodryas patagoniensis* venom has been reported to contain proteolytic activity greater than the venom of *Bothrops alternatus*, and the venom of *P. baroni* was reported to exhibit proteolytic activity 25 times greater than the activity reported for *B. jararaca* (Sanchez et al. 2014). Hemorrhagic SVMPs and serine proteinases are responsible for severe local inflammation and tissue necrosis in human envenomations, and significant bleeding has been reported from rear-fanged snake envenomations, likely due to the presence of these toxins (Weinstein et al. 2011). Assaying rear-fanged snake venoms for proteolytic activity, particularly SVMP activity, can be useful to predict the potential envenomation hazard these snakes could pose to humans.

Snake venom metalloproteinase classes differ in structure with regard to domain composition; P-Is have only the metalloproteinase domain, P-IIs have an additional disintegrin domain, and class P-IIIa-c have a metalloproteinase, disintegrin, and cysteine-rich domain, with P-IIId having an additional lectin domain (Fox and Serrano 2010). The only SVMPs to date that have been discovered in rear-fanged snake venoms have been of the P-III class, which have been characterized in several venoms, including *Dispholidus typus* and *Alsophis portoricensis* venoms, among others. Although full venomic analyses (protein digestion, followed by peptide mass fingerprinting) was not utilized to identify protein families in several of these studies, SVMP activity was detected using an azocasein substrate confirming the presence of SVMPs in these venoms. A combined proteomic and transcriptomic analysis of the venom of *Philodryas olfersii*, a rear-fanged venomous snake of South America with growing medical significance, revealed toxin similarities to those of snakes belonging to the family Viperidae, with the P-III class of SVMPs being the most abundant protein in the venom (Ching et al. 2006). P-III SVMPs are also the most abundant compounds in the venoms of *Thamnodynastes strigatus* (Ching et al. 2012) and of *Hypsiglena* sp. (McGivern et al. 2014). Both one- and two-dimensional gel electrophoresis further confirmed the presence of P-III SVMPs not only in *P. olfersii* but also in *P. patagoniensis*, *P. baroni*, and *Hypsiglena torquata texana* venoms (Peichoto et al. 2012). It is thought that during the evolution of a front-fanged venom system, the various domains observed in P-III SVMP were gradually lost. The P-I and P-II classes of SVMPs are currently only found in Elapidae and Viperidae venoms (Fox and Serrano 2010; Mackessy 2010).

Acetylcholinesterase activity has been reported in several rear-fanged snake venoms, with this activity being most prominent in venoms of *Boiga* species such as *B. irregularis* (Mackessy 2002; Mackessy et al. 2006). This acetylcholinesterase activity appears to be su547.40002441(002(in)18.5(en)25.100(ve)-7(n)13.5(i)900075(o)-323(be)

preparations, suggestive of a bird-specific postsynaptic affinity (Pawlak et al. 2006). Irditoxin, a lizard- and avian-specific 3FTx from *B. irregularis*, was identified shortly after denmotoxin (Pawlak et al. 2009), and recently another prey-specific 3FTx, fulgimotoxin, was discovered in a New World rear-fanged snake, *Oxybelis fulgidus*, indicating that this phenomenon is not limited to Old World species and is likely more common in rear-fanged snake venoms (Heyborne and Mackessy 2013). Based on 1D SDS-PAGE and other data, 3FTxs are present in numerous venoms from rear-fanged snakes (Saviola et al. 2014).

More Recent Approaches

"First generation" venomics (e.g., Calvete et al. 2009) has been an exceptionally successful means to generate near-complete catalogs of venom proteins (Fig. 3), and this approach has also been applied to venoms of rear-fanged snakes. In recent years, the emergence of "omic" technologies has revolutionized venom research by integrating detailed high-throughput approaches to generate systematic venom studies involving whole genomes, transcriptomes, and proteomes (Calvete 2013). To date, a comprehensive approach, with (proteomics) MS/MS peptide sequencing of separated venom components (usually by RP-HPLC or 2D gel electrophoresis) combined with a species-specific venom gland transcriptome, has provided the most complete venom compositional coverage (Wagstaff et al. 2009; McGivern et al. 2014; Paiva et al. 2014; Goncalves-Machado et al. 2015). The change from 454 pyrosequencing to Illumina sequencing technology has also offered greater transcriptome coverage and depth (Rokyta et al. 2011; McGivern et al. 2014). MS/MS identification of peptide sequences relying on online protein sequence databases, such as the Mascot online server, can overlook unique isoform variations and can be unsuccessful at recognizing novel venom proteins if only small peptide fragments are used for protein identification. By generating a complementary transcriptome, MS/MS peptide sequences can be more precisely identified to the corresponding transcript, and translated transcripts will provide full protein sequences. Obtaining full sequences using only proteomic methodologies (such as N-terminal sequencing and MS/MS de novo sequence determinations from many peptide fragments) would otherwise be much more labor intensive and expensive.

Approaches to venom characterization have largely focused on mass spectrometry to generate complete venom profiles. The two primary MS methods for whole proteins include matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS and electrospray ionization MS (Kukhtina et al. 2000). These methods are frequently used to provide more accurate molecular masses for individual venom components and peptide fragments, and both allow for high-throughput analysis of complex samples. Mass spectrometric de novo sequence determination

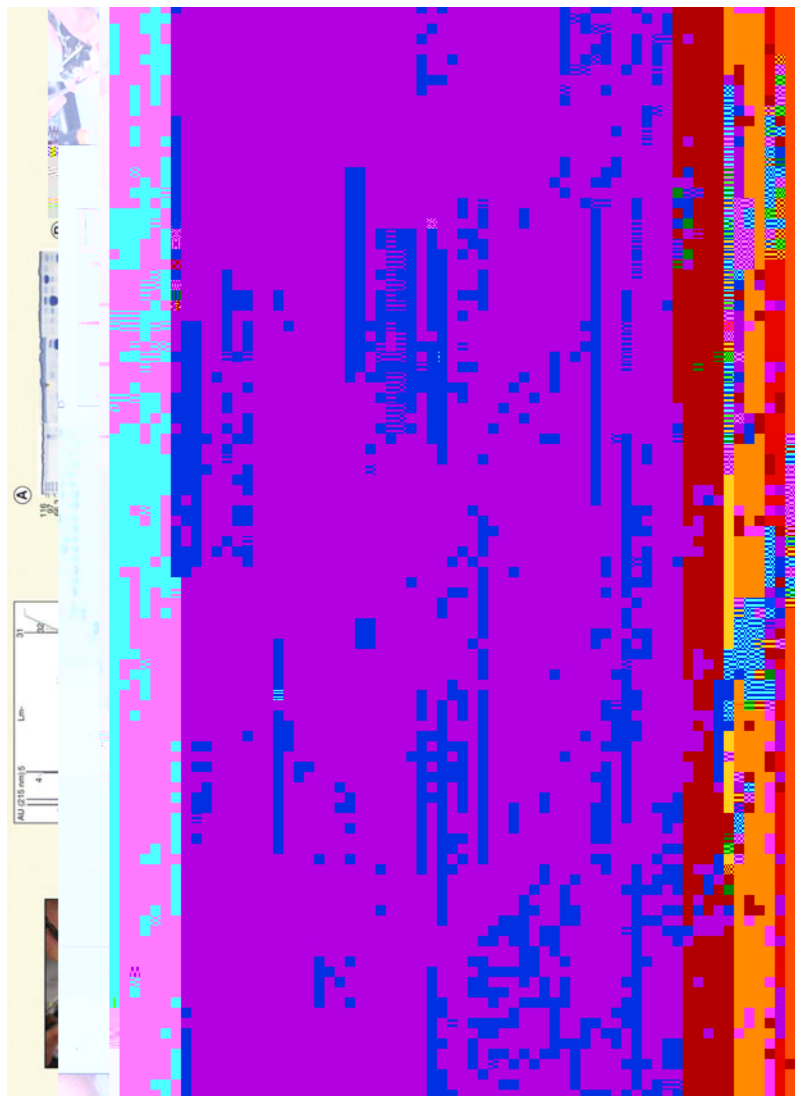

Fig. 3 An example of a venomics analysis of viper venom. Venom is fractionated using RP-HPLC and 1D SDS-PAGE; bands are digested and subjected to MS analysis (Reproduced from Calvete (2014))

pyroglutamate which must be removed prior to Edman sequencing (e.g., Pawlak et al. 2009; Heyborne and Mackessy 2013).

Top-down and bottom-up approaches are seen in proteomic literature regarding rear-fanged venomous snakes, where a top-down approach is done with intact venom proteins and a bottom-up approach is accomplished using proteolytic peptide mixtures. A top-down MALDI-TOF MS method using rear-fanged snake venom has revealed as many as 49 distinct protein masses (Peichoto et al. 2012). Top-down strategies allow for more complete characterization of protein isoforms and post-translational modifications (Han et al. 2008; Petras et al. 2015). Post-translational modifications found in rear-fanged snake venom proteins have yet to be studied in detail, and many opportunities exist for continued work using top-down MS methods.

(general venom description and biochemical activity) and transcriptomic data. For P. baroni, P. olfersii, and P. patagoniensis,

and have been observed to have novel protein superfamilies that would be missed using MS/MS peptide matching techniques alone (due to the lack of rear-fanged snake venom protein sequences in current databases). Genomic and transcriptomic data is becoming more readily available for venomous snakes (Rokyta et al. 2011, 2013; Vonk et al. 2013

levels of transcriptional and translational regulation of venom proteins that give rise to the variation that is seen in venom composition between species and even within individuals.

Venom gland transcriptome analyses are powerful for determining venom transcript expression, but genomic sequences can provide insight into venom gene transcriptional regulation (i.e., promoter sequences) and mechanisms resulting in venom protein diversity (i.e., alternative splicing events and/or gene dosage

biochemical and toxicological data, have allowed for a detailed examination of intraspecific, geographic, and ontogenetic venom variability primarily aimed at addressing the venom composition of dangerously toxic snakes of the families Elapidae and Viperidae (Calvete 2014; Calvete et al. 2009, 2012). Venomics also allows for identification of venom compounds that may be further examined for potential therapeutic value. Although the vast majority of venomic studies have included species that are of great medical significance, rear-fanged venomic studies are increasing and providing information on venom composition of these poorly known snakes.

Currently, only a few complete rear-fanged venomous snake proteomes are available, and most commonly, a bottom-up strategy is seen. There are primarily two bottom-up proteomic workflows. There is a "sort-then-break" approach, which includes performing protein fractionation and separation prior to protein digestion, followed by peptide analysis by peptide mass fingerprinting or de novo peptide sequence determination (Han et al. 2008). This workflow is seen in the venomic approach to venom profiling as mentioned above and was utilized with the venom of the rear-fanged snake *Thamnodynastes strigatus*. 2D gel electrophoresis was the method of separation before in-gel trypsin digestions and identification of individual protein spots using a MALDI Q-TOF (matrix-assisted laser desorption ionization quadrupole time-of-flight) Premier mass spectrometer (Ching et al. 2012). Also, several protein SDS-PAGE bands from the venoms of *Trimorphodon biscutatus lambda*, *Philodryas olfersii*, *Philodryas patagoniensis*, *Philodryas baroni*, and *Hypsiglena torquata texana* were also digested with trypsin and analyzed with MALDI-TOF/TOF (tandem matrix-assisted laser desorption ionization time of flight) to confirm the presence of PLA_2s, CRiSPs, and 3FTxs within

homologues are expressed in other tissues, suggestive that these “toxins” are either coexpressed in many tissues or are “reverse recruited” from the venom gland for other physiological roles in other tissues (Casewell et al. 2012; Reyes-Velasco et al. 2015;

identify compounds that constitute an entire venom for a species but will also provide a detailed description of venom composition of rear-fanged snakes that may be of medical significance with regard to snakebite. Further, rear-fanged venomics may provide insights into the utilization of these proteins for therapeutic drug development.

Proteomics, especially the high-throughput venomic and shotgun proteomic methods, have increased the sensitivity and speed at which a whole venom can be characterized and the abundances of individual venom protein families determined. Although the vast majority of rear-fanged snakes may be considered as nonthreatening to humans, proteomics allows identification of venom protein families which can further assist with examining the evolutionary relationships among venomous snakes and their toxins. For example, proteomic screening of the venom of

and susceptibility). At the molecular level, there is the evolution of protein catalytic/ligand-binding sites and targeting. At the organismal level, there are selection pressures brought on by prey availability, preference, and susceptibility to specific toxin effects. Therefore, the biological roles of venom proteins should be incorporated into high-throughput proteomic, transcriptomic, and genomic results aimed at understanding venom evolution.

Rear-fanged venomous snakes encompass several families and subfamilies of the Colubroidea, and collectively they include the largest number and diversity of venomous snakes. To explore the biological roles of rear-fanged snake venoms, or individual toxins within these venoms, it is important to use adequate toxicity models for assays that match the biology of the snake being studied. Understanding the diversity of venom components and their differential effects toward specific prey will facilitate a greater understanding of the selective mechanisms driving snake venom evolution and adaptation (Mackessy et al. 2006; O'Donnell et al. 2007). Future studies should take into account the interactions between the snake's venom and its natural prey, since toxicity is best defined within the context that it is being used. There is a need for inbred nonmammalian vertebrate species to be used as models for LD_{50} assays, as well as for viable nonvertebrate models of whole organism toxicity. Such models would be ideal for toxinologists interested in receptor-ligand evolution and positive selection of venom proteins involved in coevolutionary predator/prey arms races (Mackessy 2002). Some of ar00tri9t448.29998(r-liga

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Recently, five venoms from rear-fanged snake species were evaluated for potential antileishmanial activity. Exposure to relatively high levels of these rear-fanged snake venoms resulted in cytotoxicity toward cultured promastigote states of *Leishmania major* and venom of one species, *T. b. lambda*, showed significant cytotoxicity even at lower doses (Peichoto et al. 2011). Because rear-fanged snake venoms contain many of the same venom protein families as front-fanged venomous snakes, and because structural motifs of venom proteins are conserved but possess activities and specificities that may be highly variable, exploration of rear-fanged snake venom proteins could uncover some highly useful compounds. Anticoagulants in rear-fanged snake venoms include SVMPs, serine proteases, and phospholipase A_2 enzymes (Saviola et al. 2014), and initial analyses indicate that at least some may show higher specificities than homologues from front-fanged snake venoms (Weldon and Mackessy 2012). These variants provide opportunities to decipher the subtleties in functional sites in order to understand the plasticity of venom protein structure and function. Venom proteins can serve as templates for biomedical engineering and provide insight into selective receptor binding (Kini and Doley 2010), as is exhibited by several 3FTxs from rear-fanged snake venoms. Without the selectivity of alpha-bungarotoxin, a 3FTx from venom of the Many-banded krait (

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