Toxinology

P. Gopalakrishnakone *Editor-in-Chief*

Juan J. Calvete *Editor*

Venom Genomics and Proteomics

Toxinology

Editor-in-Chief
P. Gopalakrishnakone

In recent years, the field of toxinology has expanded substantially. On the one hand it studies venomous animals, plants and micro organisms in detail to understand their mode of action on targets. While on the other, it explores the biochemical composition, genomics and proteomics of toxins and venoms to understand their three interaction with life forms (especially humans), development of antidotes and exploring their pharmacological potential. Therefore, toxinology has deep linkages with biochemistry, molecular biology, anatomy and pharmacology. In addition, there is a fast-developing applied subfield, clinical toxinology, which deals with understanding and managing medical effects of toxins on human body. Given the huge impact of toxin-based deaths globally, and the potential of venom in generation of drugs for so-far incurable diseases (for example, diabetes, chronic pain), the continued research and growth of the field is imminent. This has led to the growth of research in the area and the consequent scholarly output by way of publications in journals and books. Despite this ever-growing body of literature within biomedical sciences, there is still no all-inclusive reference work available that collects all of the important biochemical, biomedical and clinical insights relating to toxinology.

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Venom Genomics and Proteomics

With 80 Figures and 14 Tables

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Series Preface

The term TOXIN is derived from the Greek word *Toeikov* and is defined as a substance derived from tissues of a plant, animal, or microorganism that has a deleterious effect on other living organisms. Studying their detailed structure, function, and mechanism of action as well as finding an antidote to these toxins is the field of TOXINOLOGY, and the scientists are called TOXINOLOGISTS.

In recent years, the field of toxinology has expanded substantially. On the one hand, it studies venomous animals, plants, and microorganisms in detail to understand their habitat, distribution, identification, as well as mode of action on targets, while on the other, it explores the biochemical composition, genomics, and proteomics of toxins and venoms to understand their interaction with life forms (especially humans), the development of antidotes, and their pharmacological potential for drug discovery. Therefore, toxinology has deep linkages with biochemistry, molecular biology, anatomy, pharmacology, etc. In addition, there is a fast developing applied subfield, clinical toxinology, which deals with understanding and managing medical effects of venoms and toxins on the human body following envenomations. Given the huge impact of envenomation-based deaths globally and the potential of venom in the generation of drugs for debilitating diseases (e.g., diabetes, chronic pain, and cancer), the continued research and growth of the field is imminent.

Springer has taken the bold initiative of producing this series, which is not an easy target of producing about 12 volumes, namely, biological toxins and bioterrorism, clinical toxinology, scorpion venoms, spider venoms, snake venoms, marine and freshwater toxins, toxins and drug discovery, venom genomics and proteomics, evolution of venomous animals and their toxins, plant toxins, and microbial toxins.

Singapore

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I would like to sincerely thank the section editor of this volume, Juan J. Calvete, for the invaluable contribution of his expertise and time and the authors who obliged with my request and provided a comprehensive review on the topics.

Springer provided substantial technical and administrative help by many individuals at varying levels, but special mention should go to the Springer editors for their tireless effort in bringing these volumes to reality.

Singapore

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Volume Preface: A Bright Future for Venomics

In any habitat of our planet there is competition for resources, and every ecosystem on Earth supporting life contains organisms that use venom as a predatory or defence strategy. Biological research has been continuously enhanced by advances in technology, and consequently, molecular toxinology has undergone a huge development with the implementation of omic approaches, particularly comparative genomics, venom gland transcriptomics, and mass spectrometry–based venom proteomics, to study venomous organisms and their toxins. Advances in instrumentation and methodologies have fueled an expansion of the scope of toxinological studies from simple biochemical analysis involving few molecules at a time to the systematic study of whole genomes, transcriptomes, and proteomes. Hypothesis- and omic-driven research of the composition of venoms and the principles governing the evolution of venomous systems lays the groundwork for unveiling the enormous potential of venoms as sources of chemical and pharmacological novelty. Basic and applied research on venoms and their toxins represents the Yin and Yang of venomics, two sides of the same coin. Thus, on the one hand, the functional parallelism between venom toxins that target with a high degree of specificity receptors involved in maintaining vital systems of the prey or victim, and disease conditions in which the activity of these receptors is dysregulated, suggests that the rational use of toxins or toxin mimetics represents an essentially unexplored strategy to cure pathologies which do not respond to currently available therapies. In this sense, venoms must thus be regarded as "oceans of opportunity" for the pharmaceutical industry and venomics as a discovery science. On the other hand, envenomation by snakebite or scorpion sting constitutes a highly relevant, but often neglected, public health issue in many tropical and subtropical regions of the world. A robust knowledge of venom composition and of the onset of ontogenetic, individual, and geographic venom variability may have an impact on the treatment of bite victims and in the selection of specimens for the optimized use or the generation of improved antidotes. In addition, never before has the study of venoms

attracted researchers from different scientific disciplines. This convergence toward ven(omics) is, in part, motivated by the fundamental implication of an understanding of the enormous capacity of nature to provide organisms of very different evolutionary histories, with both divergent and converged adaptive solutions, in fields such as ecology, evolution, structural and functional toxinology, and molecular taxonomy. This volume of the handbook series *Toxinology* includes 20 chapters that illustrate some directions being taken by the fields of molecular toxinology, the biotechnological applications of venom toxins, and antivenom production. Comparing the content of the chapters of *Venom Genomics and Proteomics* with state-of-the-art reviews published only 5–10 years ago, not only the enormous progress made since the implementation of omics technologies in the field of toxinology is apparent, but one also realizes the road still ahead to reach a holistic view of even a venomous system. Advancement in high-throughput technologies in the field of venomics has resulted in the ability to generate comprehensive venom profiles for many species. However, research on snake genomes is still in its infancy, although such studies are eagerly awaited to gain insights into the evolutionary history of snake venom proteins, including the mechanisms that originated venom and the regulation of toxin expression. Fortunately, this objective is also at the reach of current omic technologies. Therefore it sounds not crazy to predict a bright near future to the field of molecular toxinology.

January 2016

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His research studies include structure function studies, toxin detection, biosensors, antitoxins and neutralization factors, toxinogenomics and expression studies, antimicrobial peptides from venoms and toxins, and PLA2 inhibitors as potential drug candidates for inflammatory diseases. The techniques he employs include quantum dots to toxinology, computational biology, microarrays, and protein chips.

Prof. Gopalakrishnakone has more than 160 international publications, 4 books, about 350 conference presentations, and 10 patent applications.

He has been an active member of the International Society on Toxinology (IST) for 30 years and was president from 2008 to 2012. He is also the founder president of its Asia Pacific Section, a council member, as well as an editorial board member of *Toxicon*, the society's official journal.

His research awards include the Outstanding University Researcher Award from the National University of Singapore (1998); Ministerial Citation, NSTB Year 2000 Award in Singapore; and the Research Excellence Award from the Faculty of Medicine at NUS (2003).

His awards in teaching include Faculty Teaching Excellence Award 2003/4 and NUS Teaching Excellence Award 2003/4. Prof. Gopalakrishnakone also received the Annual Teaching Excellence Award in 2010 at both university and faculty levels.

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Dr. Juan J. Calvete (Valencia, Spain, 1957) is research professor of the Spanish Research Council (CSIC) and head of the Structural and Functional Venomics Laboratory at the Instituto de Biomedicina de Valencia (http://www3.ibv.csic.es/index.php/es/investigacion/genomica/upr).

He studied biology at the University of Valencia and earned his Ph.D. degree in biochemistry from Complutense University (Madrid, 1985). Dr. Calvete completed postdoctoral training in protein chemistry and structural biology at the Banting Institute (Toronto, Canada, 1987), the Max-Planck-Institute für Biochemie (Martinsried, Germany, 1988–1992), and the Institut für Reproductionsmedizin (Hannover, Germany, 1993–1998) prior to assuming his current position in the Instituto de Biomedicina de Valencia in 1998. Dr. Calvete has coauthored more than 380 scientific publications in peer-reviewed journals and monographic books, which have received over 13,600 citations according to the Google Scholar database (http://scholar.google.com/citations?user=3J6y5bkAAAAJ&hl=en).

His current research focuses on structural and functional proteomics of snake venoms, having developed proteomics-based tools ("venomics" and "antivenomics") for exploring the evolution, composition, interactions with antivenoms, and biotechnological applications of venoms and toxins.

Since 2011, Dr. Calvete serves as president of the European Section of the International Society on Toxinology (http://www.toxinology.org/European_Section.htm). Dr. Juan J. Calvete has served as first president of the Spanish Proteomics Society (SEProt; http://www.cbm.uam.es/seprot/seprot/sobrelaseprot.htm). Currently, he is an editorial board member of *Toxicon*, *Journal of Venom Research*, and *Toxins* and editor-in-chief of the *Journal of Proteomics* (http://www.journals.elsevier.com/journal-of-proteomics).

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Deadly Innovations: Unraveling the Molecular Evolution of Animal Venoms

1

Kartik Sunagar, Nicholas R. Casewell, Sandeep Varma, Rohan Kolla, Agostinho Antunes, and Yehu Moran

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Abstract
Understanding the nature and strength of natural selection that influences the evolution of genes is one of the major aspects of modern evolutionary biological studies. Animal venoms are complex cocktails of biologically active compounds that are secreted in a specialized gland and actively delivered to the target animal through the infliction of a wound. The injected molecules cause a disruption in the normal physiological and biochemical processes of the victim, typically in order to facilitate the feeding or defense of the venomous animal. Venom components have been theorized to have originated from the physiological protein-encoding genes. The molecular evolution of venom has been suggested to be influenced by numerous ecological and evolutionary factors, such as diet, prey distribution, predator pressure, ontogenetic shifts, and the gender of the animal. Because of the medical importance of venom proteins, many of which are amenable for therapeutic or diagnostic development as pharmaceutical compounds while others cause catastrophic pathology in envenomed humans, a comprehensive understanding of the origin of venom proteins and the evolutionary forces shaping their biodiversity is essential to unravel the complete biodiscovery potential of this nature's most biochemically complex cocktail. The current state of knowledge regarding venom evolution, as well as the potential relevance of this topic in the advancement of the biomedical field, has been reviewed here.

Introduction

Venomous animals have fascinated humans for millennia, and for good reasons: injection of even miniscule amounts of certain venom components can result in rapid paralysis and death of animals. The evolution of venom, one of nature's most complex biochemical concoctions, has underpinned the predatory success and diversification of numerous animal lineages. Such a cocktail of pharmacologically active peptides, proteins, salts, and organic molecules is often employed for both predation and defense by the secreting animal. While most cnidarians, centipedes, spiders, scorpions, and snakes primarily employ venom for securing a meal, others like certain insect orders, echinoderms, venomous fishes, and helodermatid lizards mainly employ venom for defensive purposes. Intriguing fossil evidence suggests that certain extinct animal lineages, such as the theropod dinosaur *Sinornithosaurus* (Gong et al. 2010) – a genus of feathered dromaeosaurid dinosaur – and the pantolestid mammal *Bisonalveus browni* (Fox and Scott 2005), also employed venom. Certain ancestral mammalian lineages, such as *Euchambersia*, a genus from the family Therocephalia, are also speculated to have had venom glands attached to their canine teeth. As a result, it has been theorized that this lineage could have used venom for predation in the Late Permian epoch, 250 million years ago. The extant venomous organisms belong to a vast variety of lineages, such as arthropods (bees, wasps, caterpillars of certain moths – *Lonomia,* scorpions, spiders, and centipedes), cnidarians (jellyfish, sea anemones, corals, and hydras),

mollusks (cone snails, squid, cuttlefish, octopus), fishes (sharks, stingrays, chimeras, teleost fishes like catfish, stonefish, scorpion fish, lionfish, surgeonfish, rabbitfish, onejaws, etc.), amphibians (certain species of salamanders with sharp ribs for injecting venom), mammals (vampire bats, certain species of shrews, male platypus, slender loris), and reptiles (venomous snakes and lizards: monitor lizards, helodermatid and Anguimorpha lizards). Although venom is known to be widely distributed among arthropods, with three out of four major lineages (Chelicerata, Myriapoda, and Hexapoda) being venomous, until recently there was no evidence to support the venomous nature of crustaceans. Lately, it was theorized that remipede crustaceans are likely to employ venom for predation, and therefore members of all four major groups of arthropods are, in fact, venomous (von Reumont et al. 2014).

Venom is defined as a secretion produced in the specialized glands/cells of an animal, delivered to the target animal through the infliction of wound, irrespective of the size of the wound. Upon introduction into the target animal, this complex cocktail disrupts the normal physiological and biochemical processes to facilitate feeding by or defense of the secreting animal (Fry et al. 2009a). Using this definition of venom, some authors have strongly argued that it is not necessary for an animal to cause rapid paralysis and death of the envenomed animals, or impose danger to humans, in order to be classified as “venomous.” Moreover, this definition of venom not only encompasses animals that are traditionally recognized as venomous (centipedes, scorpions, spiders, snakes, and venomous lizards) but also reinforces the venomous nature of hematophagous animals, such as vampire bats, leeches, and ticks, that employ a concoction of biochemical compounds to facilitate their feeding. By considering the aforementioned definition, it appears that venom has been independently recruited on at least 30 occasions in animals (Fry et al. 2009b).

Despite the fact that most venomous animals use sophisticated warning behavior (audible and visual) to avoid close encounters, thousands of people die every year worldwide from accidental envenoming by various animals. It is estimated that nearly four million people suffer envenoming every year and over 90,000 succumb to its fatal effects (Kasturiratne et al. 2008). The frequency of human bites/stings by venomous animals emphasizes the importance of venom research. Furthermore, bites from certain organisms (e.g., many species of viperid snakes and spiders) can induce hemolysis, myolysis, and necrosis and, in doing so, inflict permanent damages on the envenomed victim. Moreover, inter- and intraspecific variations in venom composition, driven by factors, such as geography, diet, phylogenetic history, and predator pressure, complicate the production of effective antivenom. Therefore, understanding the genetic basis of the diversification of venom-encoding genes in venomous animals can provide fundamental biological insights into their species evolution, ecological specialization, and genetic novelties, which may be of further importance for antivenom, pesticide development, and drug discovery research. Additionally, they provide unparalleled models for understanding molecular adaptations associated with predator–prey interactions, including perhaps coevolutionary arms races, and the convergence of biochemical functions.

Origin of the Venom Arsenal

It has been postulated that venom-encoding genes originate via the recruitment of body protein or physiological protein-encoding genes, especially those involved in key regulatory or biochemical processes, into the venom gland (Fry 2005). Many of these genes then undergo a "birth-and-death" model of evolution, where multiple copies of the gene are generated via repeated gene duplication events (Nei et al. 1997). Of these multiple copies, some degenerate or become pseudogenes, while others, which are now relieved of any preexisting negative selection pressures, gain novel biochemical functions via neofunctionalization. The tissue location from which venom toxins appear to have originated from remains largely unknown for almost every venomous animal lineage. For example, in snakes, venom toxins may have originated from genes expressed in a diversity of different tissue types (Fry 2005). Previously it was hypothesized that venom in snakes has evolved via the modification of the pancreatic system (Kochva 1978, 1987; Kochva et al. 1983). Although this hypothesis has since been discounted due to a lack of evidence from the tissue location of genes encoding toxin paralogs, the deep sequencing of small RNAs from the venom glands of the king cobra (*Ophiophagus hannah*) (Vonk et al. 2013) revealed that miR-375, a characteristic microRNA (miRNA) of the vertebrate pancreas, was transcribed in the venom glands as well. Thus, the authors provided strong support for the link between the pancreatic and venom gland secretory tissues, which was originally suggested decades ago (Kochva 1987). Therefore, although evidence for the entire snake venom system to have evolved from the pancreas is currently limited, aspects of it, such as some regulatory components and certain venom components [e.g., phospholipase A_2 (PLA_2) enzymes of vipers] may have a pancreatic origin.

Recently, an alternative hypothesis, known as the "restriction" hypothesis, was postulated to explain the origin of venom-encoding genes in snakes (Hargreaves et al. 2014). This hypothesis suggests that venom-encoding genes may have originated from genes that were ancestrally expressed in multiple tissues, including the venom gland. Where gene duplication was associated with this process, the duplicate copies are suggested to follow subfunctionalization, where one copy is restricted to the venom gland, while the other continues to serve the original physiological role. Over the course of evolutionary time, the latter is lost from the venom gland. The authors support this hypothesis by examining transcriptomes of physiological tissues and "salivary glands" of nonvenomous snakes. Although this hypothesis highlights the uncertainty regarding whether duplication events precede or follow recruitment events, the proposed restriction hypothesis does not starkly contradict the recruitment hypothesis, and further evidence is required to elucidate the timing of different processes relating to the co-option of genes for venom functions.

Recently, an alternative model of venom gene evolution was proposed. Based on observations of the location of expression of venom toxin homologues in the Burmese python (*Python bivittatus*), snake venom homologues were noted to be co-expressed in multiple tissue types, although not all tissue types, and at lower

levels overall than all snake genes in general (Reyes-Velasco et al. 2014). Based on these results, the authors proposed the “stepwise intermediate nearly neutral evolutionary recruitment” (SINNER) model of venom toxin gene evolution. The model was defined as having three main steps, which may or may not involve the process of gene duplication. Firstly, the expression of proto-venom genes is thought to be expressed in oral secretory glands at low levels (essentially constitutive expression); secondly, a switching to higher levels of tissue-specific expression in the oral/venom glands occurs; before lastly, a reduction in expression levels in nonvenom-related tissues occurs, driven largely by the degree of nonvenom tissue toxicity (Reyes-Velasco et al. 2014). Notably, this model implies that a nearly neutral phase of evolution occurs during venom gene recruitment, which balances the evolution of venom gene toxicity with its location and abundance of expression.

Diversification of the Venom Arsenal

Many venom-encoding genes, particularly those that may be involved in potential predator–prey arms races, have been suggested to diversify rapidly via the “birth-and-death” model of evolution (Fry 2005) under the influence of positive Darwinian selection – a phenomenon that involves a greater accumulation of non-synonymous to synonymous mutations (Yang et al. 2000). Thus, besides gene duplication events, mutations play an equally important role in generating the diversity of venom-encoding gene families in the animal kingdom. To explain the rapid evolution of conotoxins and certain snake venom components, some authors suggested gene duplication and accelerated evolution to be the underlying reasons (Duda and Palumbi 1999; Ohno et al. 1998). The recombination process, which is known to influence the evolution of immunoglobulin genes, has also been speculated to play a major role in generating toxin diversity (Olivera et al. 1999). It has also been suggested that all these molecular and evolutionary mechanisms could act in synergy to govern the rapid evolution of venom-encoding genes (Conticello et al. 2001). Based on previous speculations that DNA polymerases may act as “mutases” under certain conditions or in certain chromosomal regions (Radman 1999), Conticello et al. (2001) examined the role of DNA polymerases in inducing variation in the toxin-encoding genes of cone snails (Conticello et al. 2001). They reported a bias for transversions over transitions in these genes, and since DNA polymerase V has been shown to exhibit such a bias for transversions over transitions (Maor-Shoshani et al. 2000), the authors speculated that such a molecular mechanism could facilitate the diversification of toxin-encoding genes. Position-/codon-specific conservation has also been put forward to explain the molecular evolution of venom-encoding genes (Conticello et al. 2000). Since cysteine residues are encoded by two codons (TGC and TGT), transition or substitution of C by T or vice versa in the 3rd codon position is expected to be evolutionarily neutral. Interestingly, when cysteine codon usage was examined in conopeptide genes, extreme codon-specific conservation was observed, even in rapidly evolving regions (Conticello et al. 2000). The authors suggested that certain

"protecting molecules" may bind to such codons during replication in order to prevent them from accumulating mutations like their adjacent hypervariable chromosomal regions. They further speculated that these "hyperprotected" codons might act as signals for the "mutator complex," which would introduce hypervariations in adjacent regions.

The mechanism of "accelerated segment switch in exons to alter targeting" (ASSET: Doley et al. 2009) was proposed to explain the evolution of three-finger toxins (3FTx), one of the most potent venom components in certain snakes. Since the general organization of the 3FTx gene is highly conserved and ordered, it was proposed that segments in exonic regions have been exchanged with distinctly different ones. This "switching" of segments was suggested to be responsible for the resultant functional diversity in 3FTx. It was speculated that point mutations by themselves cannot account for the functional diversity of this toxin superfamily, and instead they only participate in fine-tuning the receptor binding capabilities that originate via ASSET. This study further suggested that molecular mechanisms, such as genetic recombination, splicing variation, and independent recruitments, contribute to this segmental switching of venom-encoding genes. Thus, ASSET was put forward to explain the evolution of the 3FTx superfamily and, perhaps, many other toxin families. However, a later study suggested that recombination events were unlikely to account for the tremendous functional and sequence variation of 3FTx genes (Sunagar et al. 2013a). Hypermutational sites were found to be concentrated in extremely short segments. The authors argued that the process of recombination would not have discriminated between structurally/functionally important and unimportant amino acid residues, and it would have been difficult to conserve important residues while segments recombined at such high rates. A diversity of 3FTx members examined revealed that 3FTxs are characterized by well-conserved residues (e.g., eight cysteine residues), and none of the hypermutational sites coincide with such structurally/functionally important sites (Sunagar et al. 2013a). The fact that 3FTx genes have been recruited at the base of the snake lineage rules out independent recruitment events contributing to sequence variation.

Consequently, an alternate mechanism called "rapid accumulation of variation in exposed residues" (RAVER) of toxins was instead proposed to explain the sequence and functional diversity of most toxins involved in predation, including 3FTx (Sunagar et al. 2013a). It was hypothesized that most venom components involved in predation adopt focal mutagenesis, in which a large proportion of mutations in toxins accumulate under the influence of positive Darwinian selection in structurally and functionally insignificant regions. While deleterious mutations in structurally/functionally important regions are most likely filtered out of the population by purifying selection, rare mutations in these regions that increase the potency of toxins may be selected for and may further lead to the origination of novel toxin families. Moreover, it was suggested that the accumulation of mutations in certain regions of toxins, such as the molecular surface, confers an adaptive advantage. Indeed, a diversity of venom proteins in numerous animals have been demonstrated to evolve via RAVER (Sunagar et al. 2013a; Brust et al. 2013; Ruder et al. 2013; Low et al. 2013; Kozminsky-Atias and Zilberberg 2012; Fig. 1),

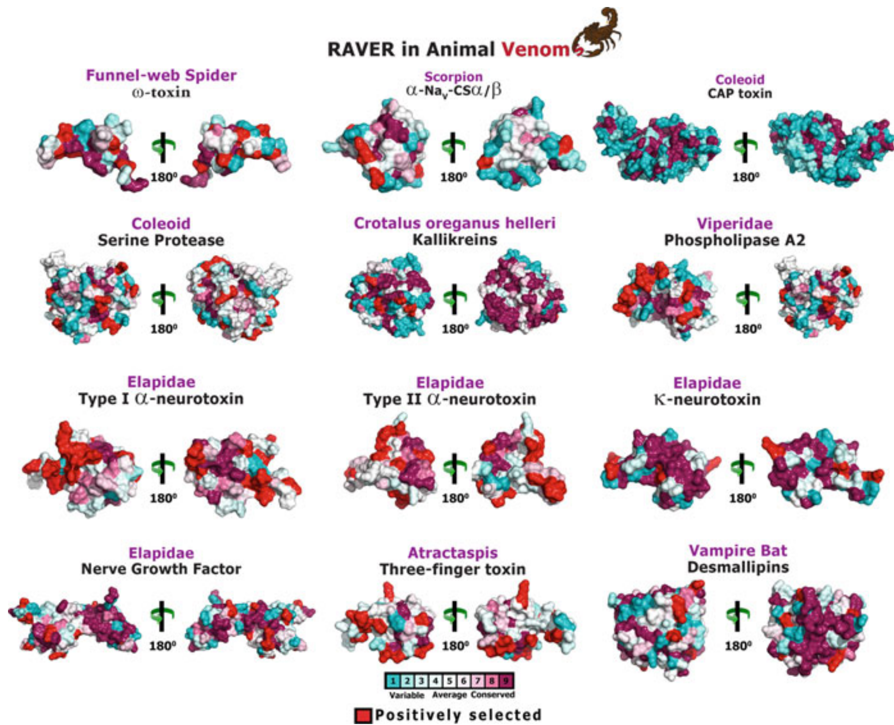

Fig. 1 RAVER in animal venoms. Three-dimensional homology models of various animal venom components depicting their molecular surface variability are presented. A color code is provided to depict sites experiencing evolutionary hypervariability and conservation. Positively selected sites are indicated in *red*

which introduces novel amino acid residues on the molecular surface of the toxin capable of nonspecifically interacting with the novel targets in prey animals and causing a myriad of pharmacological effects (Sunagar et al. 2013a; Kini and Chan 1999). Since the synthesis and secretion of venom proteins is an energetically expensive process (Nisani et al. 2012), RAVER ensures the structural integrity of proteins important for predation, while enabling a diversity of surface motifs to expand the range of vulnerable prey species. Another study, which reported RAVER in the common vampire bat, *Desmodus rotundus*, theorized that this mechanism could be extremely beneficial to hematophagous animals, as it is likely to delay and/or prevent the development of immunological resistance in the prey by introducing extremely variable protein surface chemistry (Low et al. 2013). Unsurprisingly, it has previously been demonstrated that prolonged feeding by vampire bats results in the development of immunological resistance in prey animals (Delpietro and Russo 2009).

Type I and type II toxins, which are sodium channel modulators, are commonly found in sea anemone venoms (Moran et al. 2009a). Genome sequence data analysis has shown that these cnidarians harbor multiple copies of genes encoding these

toxins, and that each copy is more similar to one another than to homologous genes in related species (Moran et al. 2008a). Sequence conservation at the nucleotide level is not limited to the coding regions but can also be detected in untranslated regions (introns) of toxin-encoding genes. As a result, this unusual pattern of sequence conservation cannot be explained by purifying selection. This rare phenomenon, known as concerted evolution, was initially documented in rDNA genes (genes encoding ribosomal RNA). Concerted evolution was shown to occur via unequal crossover events (Eickbush and Eickbush 2007). It was theorized that concerted evolution in sea anemone type I and type II neurotoxin-encoding gene likely aids in maintaining several copies of the same gene in order to enable the secretion of these toxins in large amounts. Moreover, multiple copies of the same gene may also facilitate the production of large amounts in a relatively short time. Indeed, it was later reported that the mode of delivery of these toxins occurs via the secretion of peptides to the tentacle surface by the ectodermal gland cells, indicating a demand for quicker and increased production of these toxins (Moran et al. 2012).

Alternative splicing, which results in the production of multiple protein isoforms using the same genetic template (Black 2003), is another process that has been proposed to aid in toxin diversification. It has been suggested that multiple toxin forms generated via this pathway could target different ligands in prey animals (Servent et al. 1997) or could lead to the origination of novel toxins altogether. For example, acetylcholinesterase has been suggested to be encoded via alternative splicing mechanisms, where both the toxin and the physiological body protein are encoded by the same gene (Cousin et al. 1998). Alternative splicing was also shown to play a role in the control of expression of type I neurotoxins in the sea anemone *Nematostella vectensis* (Moran et al. 2008b). Additionally, gene deletions and insertions are also known to play an important role in generating novel toxins. For example, denmotoxin found in the venom of the mangrove cat snake (*Boiga dendrophila*) has been suggested to have gained an exon via insertion events (Pawlak et al. 2006).

Identifying Molecular Evolutionary Regimes

The principal goal of modern molecular evolutionary biology is to identify and understand the forces that shape the genetic diversity of life. Mutation, which is considered as a raw material for genetic variation, is the weakest among evolutionary forces that govern the evolution of genes, since it cannot rapidly alter the frequency of mutants in a population. Advantageous and deleterious mutations increase and decrease, respectively, the fitness of an individual relative to the wild type (individuals with the plesiotypic sequence), while neutral mutations are generally expected to be neither detrimental nor beneficial. Without the influence of natural selection, reverse mutations could nullify the effect of these primary mutations, and the chance of fixation would solely rely on random genetic drift. Thus, the observed differences in genes are unlikely to result from mutations alone. However, mutations are important for generating multiple genetic variants or polymorphism in a population upon which various forces of natural selection can

act. Over time, depending on various factors (e.g., fitness and the size of the population), each variant could increase and eventually get fixed or alternatively get eliminated from the population. In smaller populations random genetic drift plays a major role, whereas selection becomes a stronger force in larger populations. When evolutionary selection pressure increases the prevalence of a beneficial mutation/trait, it is referred to as positive selection, while evolutionary forces that decrease the frequency of a deleterious mutation, to the point that it gets eliminated from the population, are termed as negative or purifying selection. Selection pressure that does not affect the frequency of mutations is known as neutral selection. Genetic drift influences the frequencies of all types of mutations and causes them to fluctuate randomly through time, with no net tendency toward increase or decrease, until the mutations become either fixed or eliminated.

"Summary statistics" offer the simplest methods of identifying the mode of natural selection. Summary statistics estimate the relative frequency of polymorphic sites from the sequence alignments of the gene under study. It should be noted that the sequences should either belong to alleles of the same gene or homologous genes and are either retrieved from multiple individuals of different populations/ species or from different individuals of the same population. These estimations result in the observed data (alternate model), which is then statistically compared to the results that would be expected under neutral evolutionary processes (null model). Significant deviation of the alternate model from the null model results in rejection of the null hypothesis, which in turn indicates the influence of natural selection on the evolution of the gene under study. It is noteworthy that the standard null hypothesis makes several important assumptions, such as absence of migration, random mating populations, infinite sites (absence of reverse mutations), a constant effective population size, constant mutation rates, absence of recombination, and, finally, the assumption that all mutations are either neutral or lethal. As a result, the rejection of the null hypothesis could arise from the violation of any of these assumptions and thus not necessarily point to the influence of natural selection.

Genes accumulate two main types of substitutions: synonymous (dS) substitutions that do not cause a change in the amino acid being encoded (hence silent substitutions) and non-synonymous (dN) substitutions that result in the replacement of the original amino acid (Eyre-Walker 2006). The relative abundance of these substitutions in genes (dN/dS ratio), which is denoted by the letter ω, indicates the nature of selection influencing the evolution of the gene under examination. Neutral evolution is characterized by a dN/dS ratio of 1, as the rate of non-synonymous substitutions equals that of synonymous substitutions (Yang et al. 2000). As a result, the significant statistical deviation of ω from 1 indicates the role of natural selection. When synonymous substitutions accumulate more rapidly than non-synonymous substitutions (dN/dS < 1), it indicates the role of negative selection, while the greater accumulation of non-synonymous to synonymous mutations (dN/dS > 1) is a hallmark of positive Darwinian selection (Yang et al. 2000). While ω is an indicator of the nature of selection, it is not informative regarding the "strength" of selection. Thus, omega values should not be compared to gauge the strength of selection pressures on different genes or homologues of the same gene.

Maximum-likelihood models (Goldman and Yang 1994; Yang 1998) implemented in the codeml of the PAML package of programs (Yang 2007) are often employed to understand the molecular evolution of venom-encoding genes. Site, branch, and branch–site models are available in PAML to assess the relative accumulation of dN and dS across sites, branches, and sites and branches, respectively (Yang and Nielsen 2002; Zhang et al. 2005). The Bayes empirical Bayes (BEB) approach implemented in site model 8 identifies amino acid sites under selection (Yang et al. 2005) by calculating the posterior probability that a particular amino acid belongs to a given selection class (neutral, conserved, or highly variable). Sites that have a greater posterior probability ($PP \geq 95\ \%$) of belonging to the "$\omega > 1$ class" are identified as positively selected. Several models in the HyPhy package (Pond et al. 2005) are also available for identifying the regime of natural selection. Here, the choice of the model depends on the size of the dataset, as well as the scientific question itself. For instance, single likelihood ancestor counting (SLAC) is designed for large datasets (40 or more sequences), random effects likelihood (REL) for intermediate-sized datasets (20–40 sequences), and fixed effects likelihood (FEL) for intermediate to large datasets (over 50 sequences) (Murrell et al. 2013). Fast, Unconstrained Bayesian AppRoximation (FUBAR) can be used for analyses of very large datasets (Murrell et al. 2013). It has been suggested that a strong influence of negative selection on a majority of lineages in the phylogeny can mask the signal of positive selection (Murrell et al. 2012). To identify sites that are influenced by both episodic and pervasive influences of positive selection, the mixed effects model of evolution (MEME) was proposed (Murrell et al. 2012). Thus, several tools are available to identify the nature of natural selection on venom-encoding genes.

Characteristics of Animal Venom Proteins

Recruited from Physiological Body Protein-Encoding Genes

As outlined in the sections above, venom components are recruited from physiological protein-encoding genes, either via modifications and gene hijacking or through repeated gene duplications. Additionally, mechanisms like alternative splicing may aid in the secretion of toxins in the venom gland by utilizing the same genetic framework that codes for the physiological protein in other tissues. Often, certain domains are deleted from physiological proteins in order to recruit them into an envenoming role. For example, tolloid, a highly conserved astacin metallopeptidase, was duplicated in *N. vectensis* several times, and the novel copies have been recruited for expression within the stinging cells (nematocytes) of the sea anemone (Moran et al. 2013). These paralogs have lost CUB (complement C1r/C1s, Uegf, Bmp1) domains, which are responsible for high specificity of tolloid toward chordin, a chief participant in the BMP pathway and a pivotal component in animal morphogenesis (Marques et al. 1997). The loss of this domain has transformed these novel proteins into potent toxins, as they now lack the selectivity of the plesiotypic (basal) tolloid

proteins (Moran et al. 2013). Recruitment of a gene in the venom gland of an animal may not always rely on gene duplication events. The physiological proteins may be overexpressed in the venom gland, which when injected into the prey animal may interfere with the homeostasis. Nerve growth factors (NGF) in advanced snake venoms have been hypothesized to have evolved in this manner (Sunagar et al. 2013b). Although NGF is not overexpressed in the venom gland by all of the advanced snakes, they may still serve a toxic function (Sunagar et al. 2013b), since they are capable of causing apoptosis of cells that lack tyrosine kinase (TrkA) receptors (Frade and Barde 1999).

Most Toxins Are Secretory Proteins

All known animal toxins are characterized by an N-terminal signal peptide domain consisting of a short stretch of amino acids (5–30 residues), which are important for localization (e.g., translocation outside the nucleus). This sequence is cleaved during the maturation process of the toxin. Since these amino acids are not found in the mature toxin, they are generally excluded from the adaptive process experienced by most mature toxins and their biochemical receptors in prey animals. Toxins may also be secreted in a precursor form that undergoes the maturation step involving the removal of the propeptide region. Indeed, several toxins retrieved from sea anemone, spiders, scorpions, and cone snails are secreted as propeptides that undergo several posttranslational modifications to attain the mature form (Sollod et al. 2005). Similar to the signal peptide region, the propeptides also appear to be excluded from the evolutionary arms race. However, propeptides of certain toxins, such as the snake venom metalloproteases (SVMP), can be an exception to this rule, as discussed in sections outlined below.

The Majority of Toxins Are Cysteine Rich

Covalent disulfide bonds, which are formed by the oxidation of two cysteine residues, are known to be responsible for the structural stability of several proteins, including extracellular toxins (Betz 1993; Norton and Pallaghy 1998). They facilitate the formation of intramolecular bridges responsible for the proper folding of proteins, as well as intermolecular bridges that enable the formation of complex proteinaceous structures. In animal venoms, disulfide bond formation may further facilitate the highly efficient targeting of biological receptors in prey animals, by creating unique folding patterns. Site-directed mutagenesis studies have revealed that modifications of most cysteine residues involved in the formation of such disulfide bonds result in partial to complete loss of protein function, exemplifying the importance of these residues in venom components (Welker et al. 2011). Unsurprisingly, certain venom components, such as the cysteine-rich secretory proteins (CRISP), have as many as 16 cysteine residues that remain evolutionarily constrained across several animal lineages (Sunagar et al. 2012). A strict cysteine-

encoding codon conservation was revealed by the examination of codon usage in various toxin-encoding genes (Conticello et al. 2000).

Many Toxins Undergo Posttranslational Modifications

Venom proteins are also known to undergo a range of posttranslational modifications, such as proteolysis, disulfide bridging, glycosylation, C-terminal amidation, N-terminal pyroglutamine formation, palmitoylation, and isomerization. These processes are not only important for acquiring structural variations, but may also be vital for gaining novel biological activities. Toxin complexes, which are often retrieved from animal venoms, result from covalent and non-covalent interactions between different and the same venom proteins and can be more efficient pharmacologically in comparison to individual venom components. The formation of quaternary structures can expose amino acid residues that were previously hidden and result in the recognition of novel biological targets in prey animals (Doley and Kini 2009). Such synergistic activity of venom proteins has been theorized to increase the potencies of animal venoms (Doley and Kini 2009). Protein glycosylation is a process of attaching a carbohydrate moiety to the hydroxyl or other functional groups in proteins in order to improve their stability. Glycosylation has also been theorized to enable proper folding of proteins and, consequently, also have an effect on their biological activities (Elbein 1991). This posttranslational modification has been noted in several venom components, including NGF (Earl et al. 2006), SVMPs (Takeya et al. 1989), factor X-activating proteins (Gowda et al. 1994), and serine proteases (Zelanis et al. 2012) of snakes and several toxic proteins in spiders (Veiga et al. 1999). Glycosylation of NGF in snake venoms has been theorized to increase the circulation time of this toxin type. Due to the importance of this PTM, glycosylation sites in these toxins are known to be evolutionarily constrained (Sunagar et al. 2013b).

Several toxins in spiders and scorpions are known to undergo C-terminal amidation (Benkhadir et al. 2004). Nearly 12 % of all known spider toxins have been reported to be modified via this posttranslational modification (Herzig et al. 2011). C-terminal amidation of alpha scorpion toxin, which inhibits the inactivation of voltage-gated sodium channels by binding at the receptor site 3, was shown to be vital for the biological activity of these toxins in the common European scorpion (*Buthus occitanus tunetanus*) (Benkhadir et al. 2004). Similarly, artificial amidation of ShK toxins from the Caribbean carpet anemone (*Stichodactyla helianthus*), which are potent blockers of the potassium ion channel, was shown to potentiate an enhanced ion channel blocking ability (Pennington et al. 2012). Thus, amidation appears to be an important posttranslational modification of toxin proteins and has been speculated to protect these toxins from proteolytic cleavage. Conversion of proline to hydroxyproline has also been documented in various sea anemone species (Honma et al. 2005; Phelan and Blanquet 1985); however, the underlying biological advantages of this posttranslational modification remain unclear.

Variability in the Evolutionary Rates of Toxins

It is often assumed that venom components rapidly evolve under the influence of positive selection. However, it should be noted that the evolutionary rate depends on a number of factors. The mechanism of action in particular governs the rate of toxin evolution. For instance, cytotoxic 3FTx, a unique member of the 3FTx family found in cobra venoms, exhibits a diversity of biological activities via nonspecific mechanisms. Although the precise molecular mechanism through which cytotoxic 3FTx exerts its toxic effects remains to be understood, an extremely well-conserved hydrophobic patch that covers nearly 40 % of the molecular surface has been theorized to interact with hydrophobic regions of the cell's phospholipid bilayer (Condrea 1976). Thus, in contrast to α-neurotoxic 3FTx that specifically bind to prey receptor sites, cytotoxic 3FTxs have been theorized to escape the classic predator–prey arms race (Sunagar et al. 2013a). In contrast to earlier studies which suggested that cytotoxic 3FTxs evolve under the influence of positive selection (Gong et al. 2000), state-of-the-art selection assessments revealed extreme negative selection governing their evolution (Sunagar et al. 2013a).

Similarly, a study examining intraspecific variation of venom in the Southern Pacific rattlesnake (*Crotalus oreganus helleri*) reported unusual conservation of sequences in the highly secreted crotamine toxins (myotoxic β-defensin peptides) (Sunagar et al. 2014). All the hypermutational sites that were detected in these toxins were concentrated in nonsecreted regions and, as a result, did not participate in the envenoming process. In contrast, the amino acid sequence of the mature protein was extremely well conserved (~76 %) and under the influence of negative selection. The authors postulated that the unusual sequence conservation observed may be reflective of the unique mode of action of crotamines. These toxins are known to bind nonspecifically to their negatively charged cell membrane targets via cationic molecular surface, resulting in membrane destabilization. Hence, evolutionary forces of selection act in favor of the accumulation of positively charged residues on the surface-exposed regions of the molecule. It is perhaps not surprising that 29 % of the surface moieties of these toxins are constituted by positively charged amino acids. Similarly, the sea anemone type I and type II toxins represent other examples of toxins where sequence conservation has a mechanistic explanation. The concerted evolution of these neurotoxin-encoding genes in sea anemones results in homogenization and extreme sequence conservation within each species (Moran et al. 2008a, 2009b).

Differential Evolution of Domains

Venom components exhibit many of the typical evolutionary characteristics of proteins, including variability in the evolutionary rates of different domains. It appears that regions that are involved in toxicity evolve at rapid rates, whereas regions involved in structural stability remain highly conserved. Differential evolution of domains was suggested for cone snail toxins, where the mature peptide

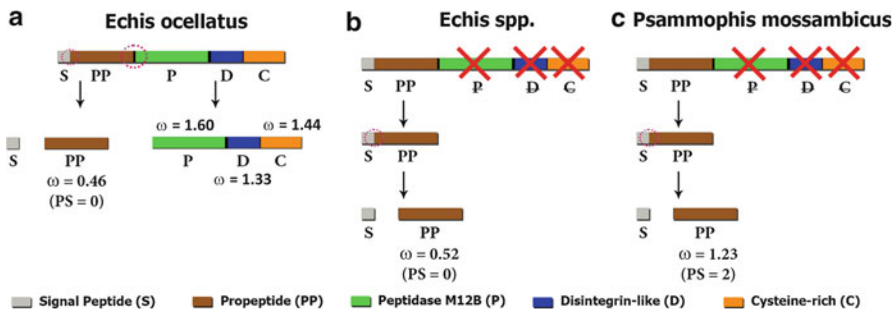

Fig. 2 Differential evolution of domains. This figure depicts the differential evolution of propeptide domains in (**a**) *Echis coloratus* multidomain, (**b**) *Echis* spp. monodomain, and (**c**) *Psammophis mossambicus* monodomain SVMPs. Each domain (*C* cysteine-rich, *D* disintegrin, *P* protease, *PP* proprotein, and *S* signal peptide) is differently color coded for comparisons. Model 8 computed omega values and the number of positively selected sites detected ($PP \geq 0.95$, Bayes empirical Bayes approach) are also indicated (Adapted with permission from Brust et al. 2013)

exhibited the highest mutation rate, in comparison with the propeptides and signal peptide domains (Conticello et al. 2001). The authors of this study noted that the rate of evolution in regions that code for the mature peptide was one order of magnitude higher than the regions encoding the remaining regions. Differential evolution of domains can also be noted in group III SVMPs, which are composed of signal peptide, propeptide, metalloproteinase, disintegrin, and cysteine-rich domains (Brust et al. 2013; Fig. 2). The mature secreted toxins are typically combinatorial variations of the latter three domains. The SVMP-encoding genes of the olive whip snake (*Psammophis mossambicus*) are unique in containing only the signal and propeptide domains. Research on these fascinating venom components has shown that in order to compensate for the absence of the typical toxic domains, the *Psammophis* SVMP propeptide has evolved rapidly (Brust et al. 2013). Bioassays revealed surprising neofunctionalization of this typically redundant domain to cause specific inhibition of mammalian alpha7 neuronal nicotinic acetylcholine receptors (Brust et al. 2013). Unlike *Psammophis*, some species of *Echis* express both the unusual monodomain (propeptide only) SVMP and the typical multidomain potent toxin. As a result, diversifying selection only acts upon the multidomain form that interacts with prey receptors, while the propeptide region evolves under evolutionary constraints. This data demonstrates that regions in toxins that experience a predator–prey arms race adopt an elevated rate of evolution. This phenomenon was also noted in CRISP glycoproteins found in the salivary secretions of various animals and the venoms of the members of Toxicofera (snakes and venomous lizards). It was reported that the cysteine-rich domain (CRD) of CRISPs, which has been theorized to be responsible for the ion channel targeting activity of these proteins, experiences relatively increased influence of positive selection, while the structurally important regions, such as the 12 cysteine residues, remain extremely well conserved (Sunagar et al. 2012).

Venom Evolution is Influenced by Species Ecology

The characterization of animal venoms is not only essential for the biodiscovery process, where novel bioactive compounds with useful functions for therapeutics are discovered, but also for treating animal envenoming. Various environmental and ecological factors, such as diet, prey size, predatory pressures, geographical distribution, gender, and temperature, may play an important role in shaping the venom composition of animals. Venom varies not only across species, but may vary within this species as well (Sunagar et al. 2014; Daltry et al. 1996; Mackessy 2010; Fry et al. 2002; Boldrini-Franca et al. 2010), with different populations relying on different biochemical concoctions for prey capture and defense. Ontogenic (developmental stage dependent) variation of venom, where various developmental stages employ different cocktails of toxins, has also been documented in numerous animals (Durban et al. 2011, 2013; Gibbs et al. 2013). Interestingly, venom variation has also been reported between the different venom glands of the same individual snake (Johnson et al. 1987).

Since venom components mostly target certain physiological receptors that remain evolutionarily conserved in various animals, venomous organisms are often assumed to employ the same biochemical concoction for predation and defense (Dutertre et al. 2014). Fascinatingly, it was recently documented that some cone snails can switch between different concoctions of venom, as a response to predatory or defensive stimuli (Dutertre et al. 2014). Cone snails were shown to employ a paralytic toxin arsenal for defense, while employing a highly prey-specific concoction for predation. The defensive venom arsenal of cone snails, which is known to be responsible for fatal human envenoming, was shown to be highly potent in blocking neuromuscular targets. Interestingly, these defensive and predatory concoctions appear to be synthesized in completely different parts of the cone snail venom gland, with the predatory cocktail originating in the distal region, while the defensive venom being secreted in the proximal region of the venom gland (Dutertre et al. 2014). A similar phenomenon was documented in the Transvaal thick-tailed scorpion (*Parabuthus transvaalicus*) which was reported to employ a "prevenom" for defense (Inceoglu et al. 2003). This transparent venom is secreted in small amounts and is rich in K^+ salts and peptides. The authors speculated that *P. transvaalicus* only employs this venom to deter predators or when preying upon small-sized prey animals. *P. transvaalicus* was suggested to employ the actual venom – a dense and cloudy secretion – only when certain stimuli, such as deterring predators or predating upon large prey animals, are reached (Inceoglu et al. 2003). Salt-rich prevenom was theorized to participate in envenoming by depolarizing cells and decreasing local electrochemical gradients and was found to be at least twofold more efficient than the actual venom in depolarizing myotubes at equivalent high doses. The actual venom on the other hand could bring about the same effect even at very low concentrations, indicating its greater potency in comparison with the prevenom (Inceoglu et al. 2003). This process of employing two different types of venom was suggested to aid in the preservation of the metabolically expensive venom.

The influence of diet on venom variation has been documented in various animals. The Malayan pit viper (*Calloselasma rhodostoma*), which feeds on varying amounts of amphibians, reptiles, and endotherms across its distribution range, was shown to have compositionally different venoms across this range, which correlated with different prey types predated upon in different geographical regions (Daltry et al. 1996). Variation in prey species has also been shown to influence the venom arsenal over the lifetime of a snake. A study on *Bothrops jararaca* and the Urutu viper (*B. alternatus*) reported venom variation due to dietary shifts across developmental stages. Venoms of juvenile *B. jararaca* exhibited greater toxicity toward anurans than to endothermic prey, such as rodents. As adult *B. jararaca* shifted their diet from small lizards and amphibians to endothermic prey, their venom was found to be more toxic to endothermic animals. In contrast, the venoms of both juvenile and adult *B. alternatus* remained highly toxic to endothermic prey animals, which they predate upon throughout their life (Andrade and Abe 1999). Another study examining the stomach and hindgut contents of saw-scaled vipers revealed that venom of species largely specialized in feeding on arthropods (*E. carinatus sochureki* and *E. pyramidum leakeyi*) was significantly more toxic to scorpions than those (*E. ocellatus*) that only fed on arthropods occasionally. Similarly, the toxicity of venom from saw-scaled vipers that specialize in feeding on vertebrates (*E. coloratus*) was significantly lower toward scorpions (Barlow et al. 2009). Finally, in contrast to land snakes, sea snake venoms are known to be extremely streamlined due to their fish-specific diet. As a likely result of convergent evolutionary processes, only relatively few key fish-specific toxins remain in the venom of these snakes. Furthermore, because of this diet-related adaptive process, a single sea snake antivenom can efficiently neutralize envenoming associated with several other sea snake lineages, as well as the venoms of sea krait, which originated independently from sea snakes.

Venom composition has been reported to vary in relation to ontogeny or the developmental stage (Durban et al. 2013; Mackessy 1988; Guercio et al. 2006; Mackessy et al. 2006). Such differences in venom composition were theorized to be a result of prey specificity. However, examination of juvenile dusky pigmy rattlesnakes (*Sistrurus miliarius barbouri*), which were raised on different diet, revealed little difference in the relative abundance of each of the major toxin types at the end of 26 months (Gibbs et al. 2011), indicating that variability in diet may not impact the overall venom composition. However, females older than 26 months raised on different diets exhibited differences in expression levels of several venom components (Gibbs et al. 2011). This may suggest a combined effect of development, gender, and diet on the venom composition. Examination of venom composition of the Central American rattlesnake (*Crotalus s. simus*) revealed a putative molecular mechanism that might be responsible for sculpting venom composition in snakes (Durban et al. 2013). Proteomic examinations revealed ontogenetic shifts in the venom composition of this snake, which involves a gradual reduction in the expression of certain toxins, such as serine proteinases and PLA_2s (e.g., crotoxin), followed by an increased secretion of PI and PIII metalloproteinases, when these snakes reach an age of 9–18 months. Many RNA transcripts have been theorized to be regulated by

microRNAs (miRNA) (Ambros 2004). Deep sequencing of small RNAs from the venom glands of various developmental stages of *C. s. simus* retrieved several miRNAs that were complementary to transcripts of various venom components. Nearly 1,000 copies of miRNAs that were complementary to PIII-SVMP transcripts were retrieved from the venoms of newborn snakes, while 590 copies complementary to the β-subunit of crotoxin were retrieved from adults. Interestingly, the neurotoxic venom of newborn snakes lacks hemolytic SVMPs, while the hemolytic venom of adults lacks neurotoxic components like PLA_2s. Hence, this study suggested that miRNAs might be involved in the regulation of venom gland transcriptomes of *C. s. simus*, resulting in ontogenetic shifts in venom composition. Research on honeybees (*Apis mellifera*) has revealed season- and age-dependent variation in venom components like 5-hydroxytryptamine or 5-HT (serotonin) (Owen and Sloley 1988). Differences in concentrations of 5-HT were identified via liquid chromatography. Similar to other components (e.g., melittin, histamine, and catecholamines), the amount of 5-HT varied in relation to the age of the animal, with the younger working bees having lower concentrations of 5-HT than the older bees. The amount of 5-HT increased with the age of the working bees.

Different populations of the same venomous species can also employ different concoctions of toxins. For example, geographical variation in venom composition was observed in the bark scorpion (*Centruroides edwardsii*) – a widespread species of scorpion from Colombia. Venoms collected from scorpion populations in two different locations of the Magdalena River Valley were reported to exhibit different biochemical properties. Significant differences were observed not only in the molecular masses of phospholipase enzymes from the Tolima and Antioquia regions, but also in the minimum hemolytic dose (MHeD), which is the smallest amount of a reagent required to induce the complete lysis of a defined amount of red blood cells. The venom of *C. edwardsii* from Antioquia was reported to have higher MHeD levels, which is probably reflective of its higher phospholipase concentration (Estrada-Gomez et al. 2014). Similarly, examination of the venom of the common lancehead viper (*Bothrops atrox*) from Colombia revealed that it is comprised of 26 different proteins belonging to nine toxin families, with PI metalloproteinase and K49-PLA_2 molecules accommodating the majority of the secreted toxins, while the species from Brazil, Peru, and Ecuador predominantly contained PIII metalloproteinases (Nunez et al. 2009). Examination of the venom gland proteomes and transcriptomes of various populations of *Crotalus oreganus helleri* (the Southern Pacific rattlesnake), one of the most medically significant snakes in all of North America, revealed that despite these populations being separated from each other by relatively short geographical distances, their venom composition was remarkably different (Sunagar et al. 2014). Snake venom metalloproteinases (SVMPs), which are hemorrhagic and tissue-destroying toxins, were abundantly secreted by the Catalina Island and Phelan rattlesnake populations, secreted in only moderate amounts by the Loma Linda population and at trace levels by the Idyllwild population. The Idyllwild population of the San Jacinto Mountains was also unique in secreting a potent presynaptic neurotoxic PLA_2 complex, previously only identified in the Mohave rattlesnake (*Crotalus scutulatus*) and the Neotropical rattlesnake

(*Crotalus durissus terrificus*). This study also identified variations in molecular regimes adopted by toxin families in different populations and thus revealed why the venom of this species has been a clinician's nightmare.

The composition and secretion of venom may also vary between different sexes (gender-specific variation). Gender-specific variation of venom in the hobo spider (*Tegenaria agrestis*), the most commonly found species in Europe and the United States, has been thoroughly studied. Studies on different populations of this species in Marysville and Tungstead Quarry have revealed that females yield more venom than males (Binford 2001). Differences in venom potencies were also observed, with that from the female *T. agrestis* being more potent toward insects than the venom from males (Binford 2001). Another study reported that the male *T. agrestis* venoms are actually more potent toward mammals than those of females (Vest 1987). Peptide toxins, which exhibit insect and mammal specificity, from the venoms of these spiders were identified by a later study (Johnson et al. 1998). Similarly, venom from the female lesser Asian scorpion (*Buthus eupeus*) was found to be twice as potent toward mice and guinea pigs, in comparison to the venoms of males (Kashnikova 1979).

Environmental conditions have also been implicated in inducing variation in the venom composition. However, direct evidence correlating venom variation with the changes in environmental conditions is still missing. For example, a study carried out isoelectric focusing of venom proteins to understand their seasonal variation in rattlesnakes revealed little change (Gregory-Dwyer et al. 1986). Seventeen individuals from three different species (*Crotalus viridis helleri*, *Crotalus molossus molossus*, and *Crotalus atrox*) were housed under controlled lighting and environmental conditions to depict seasonal variations. The isoelectric focusing test, which aims at separating biological molecules according to their isoelectric point (pI), failed to identify differences in bands during the course of the study. The authors thus concluded that venom composition in these snakes remained unchanged, regardless of the external cues the animals are subjected to.

Although venom has been a subject of great interest for decades, mechanisms through which environmental and ecological factors exert a potential influence on venom composition are not well understood. The availability of high-throughput sequencing data due to the recent advancements in "omics" technologies has revolutionized research in venom biology. Advancements in bioinformatic methodologies to understand that the molecular evolutionary regimes of venom-encoding genes evolve will be crucial for addressing such current shortcomings in venom biology.

Conclusion and Future Directions

Characterization of venom and understanding how venom components evolve can have substantial implications in medicine and agronomics. In medicine, there are two particularly important avenues that may benefit: (i) the drug development process, where novel biochemical compounds serve as templates for developing novel drugs – also known as biodiscovery – and (ii) the development of efficient "next-generation" antivenoms for treating bites from various venomous organisms.

Drug development is an expensive and time-consuming process, and it can take many years before a novel medicine is approved for use as a therapeutic agent. The attrition rate of molecules that enter the drug discovery pipeline is very high, with nearly 10,000 molecules being rejected after years of testing, tweaking, and screening, before yielding a single molecule with approval for therapeutic use (Robuck and Wurzelmann 2005). Target identification and lead optimization are the two early stages in the drug development process that can greatly benefit from liaising with evolutionary venomics. While "target" is the physiological protein to which the experimental drug would bind to result in a desired physiological change, "lead" is a compound that can bind to the target but needs tinkering to attain ideal pharmacokinetic and pharmacodynamic properties (Hughes et al. 2011). Various components of venom, which have evolved over millions of years to specifically bind to certain ligands in prey animals, serve as efficient probes for identifying important physiological receptors. For example, α-bungarotoxin, a three-finger toxin from the banded krait (*Bungarus multicinctus*), enabled the isolation and characterization of nicotinic acetylcholine receptor (nAChR) in humans (Nirthanan and Gwee 2004). This further led to our understanding of myasthenia gravis, an autoimmune neuromuscular disease (Chu 2005).

Venom components can also serve as templates for designing successful lead compounds with greater affinities toward a specific target ligand. One example where this has been validated is the identification of renin–angiotensin–aldosterone axis as a target for the reduction of blood pressure. It was observed that venom of the Brazilian viper, *Bothrops jararaca*, caused a sudden catastrophic drop in blood pressure. It was found that the venom of this snake contained a substance that inhibited the angiotensin converting enzyme (ACE). This enzyme plays a key role in producing angiotensin II, a hypertensive peptide, from angiotensin I. Further studies on this molecule yielded the first synthetic ACE inhibitor, captopril, which became a multimillion dollar antihypertension drug. This is also one of the first examples of structure-based drug design, where a naturally occurring compound is modified to produce a novel drug. Captopril further paved the way for the discovery of drugs like angiotensin receptor blockers and direct renin inhibitors, all of which target the same process (Patlak 2004). The number of molecules that are experimentally screened for use in drug development can be drastically reduced through bioinformatic approaches, such as molecular docking experiments that examine the binding efficiencies of bioactive compounds to target ligands (Meng et al. 2011). By using this technique, it may be possible to identify extremely efficient molecules from a large pool of bioactive compounds. Phylogenetic clustering of novel sequences with toxins that have been pharmacologically evaluated might therefore enable the rapid identification of candidate compounds with potential as pharmacological leads. In comparison with the traditional experimental high-throughput screening, virtual screening using bioinformatics can be advantageous, as it is direct, economically efficient, and less time consumptive. Molecular evolutionary studies on venom components and their physiological homologues may also be beneficial, as they unravel the evolutionary mechanisms that permit changes in genes from nontoxic to toxic functions. Moreover, they provide an insight into the

molecular evolution of these proteins by revealing hypervariable and extremely conserved regions, which would be beneficial from a pharmacological point of view. For example, evaluation of the molecular evolutionary regimes of CRISPs in toxicoferans and their nontoxic homologues in mammalian lineages led to the identification of extremely well-conserved regions in these proteins (Sunagar et al. 2012). The physiological homologues of CRISPs are known to play important roles in the mammalian reproductive pathway. Hence, by designing lead compounds that can specifically target such regions in these proteins, highly efficient contraceptive pills could be developed. To date, studies incorporating an evolutionary perspective to identify lead compounds from toxins have been very limited.

Animal venoms, particularly the toxic secretions of spiders and sea anemone, have been suggested to be extremely useful for the development of bioinsecticides [for a review, see (King and Hardy 2013)]. Nearly 14 % of the world's annual crop production, which includes ~20 % of stored grains, is estimated to be destroyed by numerous species of pest insects (Oerke and Dehne 2004), resulting in a loss of over 100 billion (USD) every year (Carlini and Grossi-de-Sa 2002). Numerous arthropod insect species have been implicated in contributing to this significant agronomical loss, and to date, chemical pesticides are heavily used in order to manage them. Although these agrochemical pesticides are extremely efficient in managing agricultural pests, the lack of phyletic specificity has resulted in tremendous collateral damage, both to the environment and human health (Dawkar et al. 2013). The lack of diversity in the biological activities of these compounds has also been suggested to result in substantial numbers of pesticide-resistant insects. A novel class of insecticides known as bioinsecticides, which are developed from organisms and their derivatives (e.g., animal venoms, recombinant viruses, peptidomimetics, and transgenic plants), have been envisioned to be both environment friendly and highly efficient in managing arthropod pests. They have a very short shelf life, and hence, the harmful effects of pesticides to the environment, which are invariably associated with the use of agrochemicals, are greatly reduced. Venoms from sea anemones and spiders are complex cocktails of biologically active compounds that have evolved over millions of years to target ion channels and various other physiological receptors of prey animals – invariably arthropod insects – to bring about rapid paralyses and death. Certain sea anemone toxins, such as Av3 from *Anemonia viridis*, have been shown to be extremely potent against insects. Although these potent neurotoxins can target arthropod voltage-gated sodium channels (Navs), they have no measurable effect on vertebrates (Moran et al. 2007). Understanding the molecular evolution of such toxins and the potential coevolution of these toxins with their natural targets may provide invaluable insights for the development of highly efficient bioinsecticides. These studies will not only reveal regions in toxins that are likely to be involved in the arms race (as suggested by their rapid rate of evolution), but may also unravel putative target sites in insect prey (e.g., highly conserved regions in proteins). Furthermore, molecular docking experiments could enable the rapid screening of a large pool of biologically active insect-specific toxins in order to select efficient candidates for testing.

Understanding the molecular evolution of venom components can also be beneficial for designing efficient antivenoms. The global burden of envenomations from various organisms, particularly snakes, and the morbidity and mortality rates associated with them are quite staggering. The figure for snakebites alone according to one study ranges between 1.2 and 5.5 million worldwide, resulting in 20,000–90,000 deaths every year (Kasturiratne et al. 2008). Many of these victims either do not receive treatment or receive inadequate treatment. The treatment of envenomation currently rests on the administration of antivenom, which is essentially a mixture of immunoglobulins developed by injecting small quantities of venom into animals and then purifying the resulting hyperimmune sera. Administering large quantities of such animal immunoglobulins – the majority of which are nonspecific to venom and, in some cases, also contain non-immunoglobulin impurities – can lead to allergic reactions ranging from a simple rash, serum sickness, and/or pyrogen reaction to life-threatening anaphylactic shock. In addition, less immunogenic venom components and low molecular weight toxins, such as 3FTxs, are particularly difficult to neutralize. Moreover, the production of antivenom is inherently dependent on the genetic variability of venoms derived from different individual specimens. However, in reality, antivenoms are usually developed from venoms pooled from locally available populations of venomous organisms. Such antivenoms may not be efficient in treating bites from different populations of the same species, where extensive venom variation exists. Theoretically, it would be difficult to target and effectively neutralize certain predatory venom components that rapidly accumulate variations under the process of adaptive evolution. Therefore, understanding the regime of molecular evolution and identification of hypermutational and conserved sites is crucial for the production of next-generation antivenoms that are designed to target extremely conserved domains of such rapidly evolving toxins (Wagstaff et al. 2006). Such antivenoms could be advantageous as they are likely to be cross-specific and hence effective even against widely distributed venomous species with varying venom compositions.

Overall, the incredible molecular complexity of venom is driven by a myriad of forces, such as morphological refinement of the venom delivery system, shifts in feeding ecology, potential coevolutionary chemical arms races, and evolution of toxin complexes with multiple subunits. This complexity is both a curse and a blessing: on one hand it can manifest as numerous distinct pathological sequelae that significantly complicate the clinical treatment of envenoming, while also offering extraordinary drug discovery potential due to the generation of a vast pool of venom peptides that have been evolutionarily designed to interact with a range of specificities toward receptors that play crucial roles in human physiology.

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Cross-References

▶ Applications of Snake Toxins in Biomedicine
▶ Industrial Production and Quality Control of Snake Antivenoms
▶ Scorpion Venom Gland Transcriptomics and Proteomics: An Overview
▶ Snake Venom Peptidomics
▶ Squamate Reptile Genomics and Evolution

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Squamate Reptile Genomics and Evolution

2

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Abstract

Squamates exhibit some of the most extreme and fascinating biological adaptations among vertebrates, including the production of a wide diversity of venom toxins. The rapid accumulation of genomic information from squamate reptiles is generating important new context and insights into the biology, the regulation and diversity of venom toxins, and the evolutionary processes that have generated this diversity. It is an exciting time as we discover what the unique aspects of the squamate genome can tell us about the molecular basis of such interesting and diverse phenotypes and explain how the extreme adaptations of

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squamate biology arose. This chapter reviews what is known about major patterns and evolutionary trends in squamate genomes and discusses how some of these features may relate to the evolution and development of unique features of squamate biology and physiology on the whole, including the evolution and regulation of venom toxins. It also discusses current challenges and obstacles in understanding squamate genome size, diversity, and evolution, and specific issues related to assembling and studying regions of squamate genomes that contain the genes and regulatory regions for venom toxins. Evidence is presented for a relatively constant genome size across squamates even though there have been major shifts in genomic structure and evolutionary processes. Some genomic structural features seem relatively unique to squamates and may have played roles in the evolution of venom toxins.

Introduction

Overview of Squamate Reptiles

Squamates, including lizards and snakes, are a diverse lineage of reptiles that are unique from other branches on the reptilian tree of life. This radiation represents a particularly interesting history in the evolution of vertebrates. Beginning with a limited number of ancestral reptiles in the mid-Triassic, present-day Squamata comprise over 9,000 species (Reptile Database; www.reptile-database.org) inhabiting a large diversity of habitats globally, making it one of the most important and speciose vertebrate radiations. Novel adaptations to a wide spectrum of habitats and ecological roles have led to an even wider array of behaviors, phenotypes, and life history traits that are unique to squamates. The current influx of information about squamate genome structure, content, and diversity holds great potential to enlighten us about the unique and diverse adaptations possessed by these species, including the evolution and regulation of diverse venom toxins.

The Squamata diversification began nearly 250 million years ago (MYA), and divergence times among amniotes and within squamates have been estimated previously (Hedges et al. 2006; Castoe et al. 2009b). The order Squamata is comprised of the suborders Iguania (comprised of exclusively lizards) and Scleroglossa (which includes the remaining lizards, amphisbaenians, and snakes). The Squamata diverged from its sister group, the Sphenodontia (tuataras) ~240 MYA. These lineages split from other reptilian groups (birds, crocodiles, and turtles) ~275 MYA. Within the Squamata, the suborder Scleroglossa is further subdivided into infraorders Amphisbaenia, Anguimorpha, Gekkota, Scincomorpha, and Serpentes. The Gekkota diverged other Scleroglossan lineages ~200 MYA. The Scincomorpha diverged from the clade containing Amphisbaenia, Anguimorpha, and Serpentes greater than 180 MYA. The divergence time for the split between Amphisbaenia and the Anguimorpha-Serpentes clade is estimated at ~179 MYA. Finally, the divergence time between Anguimorpha and Serpentes is ~175 MYA.

The intrinsic medical relevance of venom has made it a focus of fascination and study for hundreds of years. Recently, additional interest in squamates and their toxins has motivated whole new fields of inquiry: the potential for a role for venom toxins in medicine, understanding the evolutionary origins of these toxins from presumably nontoxic ancestral proteins, understanding how these toxins are regulated at multiple levels and through ontogeny, and their variation among species, populations, and individuals (Calvete 2010; Casewell et al. 2012). The precise definition of a "venom" or "toxin" has not, however, been clear or consistent in the literature. For example, some practical definitions of "venoms" appear to include all proteins expressed in venom or other oral glands in squamates, regardless of their biological activity or toxicity. A greater understanding of the evolutionary relationships among putative venom toxins and their origins will provide important clues to guide this debate. Furthermore, the relationship between changes in sequence, structure, and function is expected from a greater understanding of squamate genomics, and this will reshape our conceptual understanding of venoms and their origins.

Importance of Genomic Resources for Squamates

Prior to discussing genomes and genomics, it is worthwhile to briefly discuss what a "complete genome" means in a practical sense, and why different "complete genomes" might differ tremendously in their information content. A "complete genome" represents a hypothetical reconstruction of the genome based on combining information from multiple types of sequence reads. Vertebrate genomes are normally sequenced from a single representative individual of the species of interest. Often the larger, more repetitive, and more heterozygous a genome is, the harder it is to reassemble in silico. Among "complete genomes," there may exist a large range of completeness and accuracy. In practice, "complete genomes" are comprised of thousands of "scaffolds" or "scaffolded contigs," which represent the largest genomic chunks that could be put together into single reconstructed pieces.

In addition, biologically motivated questions require genome annotation (identification of repeat elements, genes, and untranslated regions). Annotation can be based on predicted similarity to previously studied species or on empirical data from the same organism. Transcriptome analysis is a particularly important empirical method used to evaluate the total set of transcripts produced by a species and estimate transcript and splice forms produced by genes (for a given tissue surveyed). The number and diversity of tissue types surveyed for transcriptome studies may have a large impact on the quality of these empirical annotations. Ultimately, these considerations mean that different complete and annotated genomes will be of different quality and thus different utilities. It is therefore important that when available data is surveyed, these factors relating to information content and quality of genome assemblies and annotations are taken into account.

For several decades, the vast majority of what was known about squamate genes and proteins was focused on venom proteins and the transcripts that encode them.

The majority of snake gene sequences in Genbank, for example, are from venom gland cDNA sequencing. Studies of venom gland transcriptomes have, however, lacked context due to the lack of transcriptomes from other tissues and from other squamate reptiles. Similarly, studies of venom genes focused on inferring patterns of selection in squamate venom proteins, and on inferring the genetic and ontological origins of venom genes in squamates, have suffered from a lack of knowledge of the full complement of genes in squamate species and the expression patterns of these genes across many different tissue types. This lack of context with which to understand and interpret the origins, relationships, and patterns observed in venom toxins has limited comparative analyses and limited our understanding of the evolution of venom toxins. Dramatic increases in computer power and decreasing costs have improved the feasibility of large-scale genome projects, and numerous full-scale squamate genome projects have recently begun to emerge.

Available Squamate Genomes

In recent years, numerous squamate genome projects have been proposed and initiated. Several have now been completed, and many more are expected in the near future. The Carolina anole lizard (*Anolis carolinensis*) genome project provided the first complete and annotated squamate genome that is now freely available. Through comparisons with avian and mammalian genomes, the *Anolis* genome yielded insights into the evolution of amniotes and into differences between the genome birds and mammals and that of *Anolis*, and likely other squamates (Alfoldi et al. 2011). Major findings include a high degree of similarity between *Anolis* and avian microchromosome structure, but with the caveat that the *Anolis* microchromosomes show a greater degree of repeat content than was found in mammalian and avian genomes (Alfoldi et al. 2011). Another peculiarity was the finding that *Anolis* lacked GC-biased isochores (or long segments of similar GC-content that differ across the genome), in contrast to mammals and birds.

In addition to the *Anolis* lizard genome, several snake genome projects have been completed. A high-quality draft genome of the Burmese python (*Python molurus bivittatus*), complete with annotations, has recently been assembled and released (Castoe et al. 2013). Transcriptomic resources have also been made available and are being developed further (Castoe et al. 2011c). This genome project was largely motivated by the importance of this species in studies of the molecular basis of extreme physiological and phenotypic traits, including the ability for some snakes (such as the python) to undergo tremendous fluctuations in metabolism after eating massive prey items. This nonvenomous snake genome is also expected to aid in understanding the evolutionary origins of venom toxins in other snake lineages. The genome for the venomous king cobra (*Ophiophagus hannah*) is also currently available and provides insights into the molecular basis for the evolution of the sophisticated snake venom system (Vonk et al. 2013). Numerous other squamates have been targeted for genome sequencing through the efforts of the Genome 10 K community (G10KCOS) and the Beijing Genomics

Table 1 Status of current squamate reptile genome projects as of July 2013 (Data were gathered and adapted from GenBank (NCBI) the Genome 10 K public lists (G10K), literature, and personal communications)

Species	Family	Common name	Status	Source
Lizards				
Podarcis muralis	*Lacertidae*	Wall lizard	In progress	G10K
Shinisaurus crocodilurus	*Shinisauridae*	Chinese crocodile lizard	Completed	G10K
Pogona vitticeps	*Agamidae*	Bearded dragon	Completed	G10K
Ophisaurus harti	*Anguidae*	Chinese glass lizard	In progress	G10K
Eublepharis macularius	*Geckonidae*	Leopard gecko	In progress	G10K
Heloderma suspectum	*Helodermatidae*	Gila monster	Proposed	G10K
Anolis apletophallus	*Polychrotidae*	Slender anole	In progress	G10K
Anolis carolinensis	*Polychrotidae*	Green anole	Published	NCBI
Aspidoscelis tigris	*Teiidae*	Western whiptail	Proposed	G10K
Varanus komodoensis	*Varanidae*	Komodo dragon	Proposed	G10K
Snakes				
Boa constrictor	*Boidae*	Boa constrictor	Completed	G10K
Thamnophis sirtalis	*Colubridae*	Garter snake	In progress	Castoe et al. (2011b)
Ophiophagus hannah	*Elapidae*	King cobra	Completed	Vonk et al. (2013)
Leptotyphlops dulcis	*Leptotyphlopidae*	Texas blind snake	In progress	Castoe, personal communication
Python molurus	*Pythonidae*	Burmese python	Completed	Castoe et al. (2013)
Crotalus horridus	*Viperidae*	Timber rattlesnake	In progress	Sanders, personal communication
Crotalus viridis	*Viperidae*	Prairie rattlesnake	In progress	Castoe, personal communication

Institute (BGI) and by various consortia or individual laboratories. Currently available information about many of these target species is available via the Genome 10 K website (http://genome10k.soe.ucsc.edu), and a summary of all known projects is provided in Table 1.

Genomes for the nonvenomous garter snake (*Thamnophis sirtalis*; Castoe et al. 2011b), the Texas blind snake (*Leptotyphlops dulcis*), the venomous prairie rattlesnake (*Crotalus viridis*), and the timber rattlesnake (*Crotalus horridus*)

are currently being sequenced. The genome of the Boa constrictor (*Boa constrictor*) is complete and available but lacks any annotation (Bradnam et al. 2013; www.assemblathon.org). The addition of multiple snake genomes to the *Anolis* genome is expected to yield new and valuable insight into the evolution of amniote and squamate genomes and provide much needed "omic" context to existing information on venom proteins, genes, and transcriptomes. In addition to new snake genomes, there are multiple lizard genomes, including individuals of *Heloderma*, *Pogona*, and *Varanus* – all lizard members of the clade "Toxicofera," which also includes snakes and is proposed to have evolved venoms on its ancestral lineage (Fry et al. 2006). These and other lizard genomes are expected to provide tremendous and much needed evolutionary and comparative context for understanding the origins of venoms in squamates, the number of times venoms may have evolved, and from what genetic and ontological sources.

Squamate Genome Size

Genome size is an important metric for inferring large-scale changes across genomes, for estimating the effort required to sequence and assemble a genome, and for identifying what characteristics of interest might be related to changes in genome size. Indeed, repetitive element content, organism longevity, metabolic rate, and development rate have all been proposed to correlate with genome size (Gregory 2001). Though there does not appear to be a correlation between genome size and organism complexity, genome size does have an impact on cellular physiology, nuclear volume, and overall cell size (Gregory 2005). Squamate genome sizes have been estimated using three main methods: Feulgen density (FD), static cytometry (SFC), and flow cytometry (FCM). Estimates of genome size based on the full collection available from all these methods suggest that squamate genome size is relatively variable. The current method of choice, flow cytometry, is likely the most accurate technique to estimate genome size (Leutwiler et al. 1984; Hedley et al. 1985), although all previous summaries (and analyses) of genome size have incorporated all three estimates despite their differences in accuracy and precision. Genome size estimates using each of the three techniques for all squamates with data currently available in the Animal Genome Database (Gregory 2013) are summarized in Fig. 1, separated by technique. These estimates (Fig. 1a) show multiple forms of bias across methods. Squamate genome size estimates from Feulgen density and static cytometry are bigger and have a much higher variance than other measurements. These two techniques are thus less precise and possibly less accurate than flow cytometry (Fig. 1a). Thus, previous perspectives of high variance in genome size among squamate reptiles based on these estimates may be artifactual, due to the methodological inconsistency of the techniques used. There is a strong argument for careful interpretation of genome size estimates made by methods other than flow cytometry methods.

The average squamate haploid genome size estimate based on flow cytometry is 1.9 Gbp ($n = 90$, range = 1.3–3.0 Gbp; Fig. 2b). This average is intermediate in size

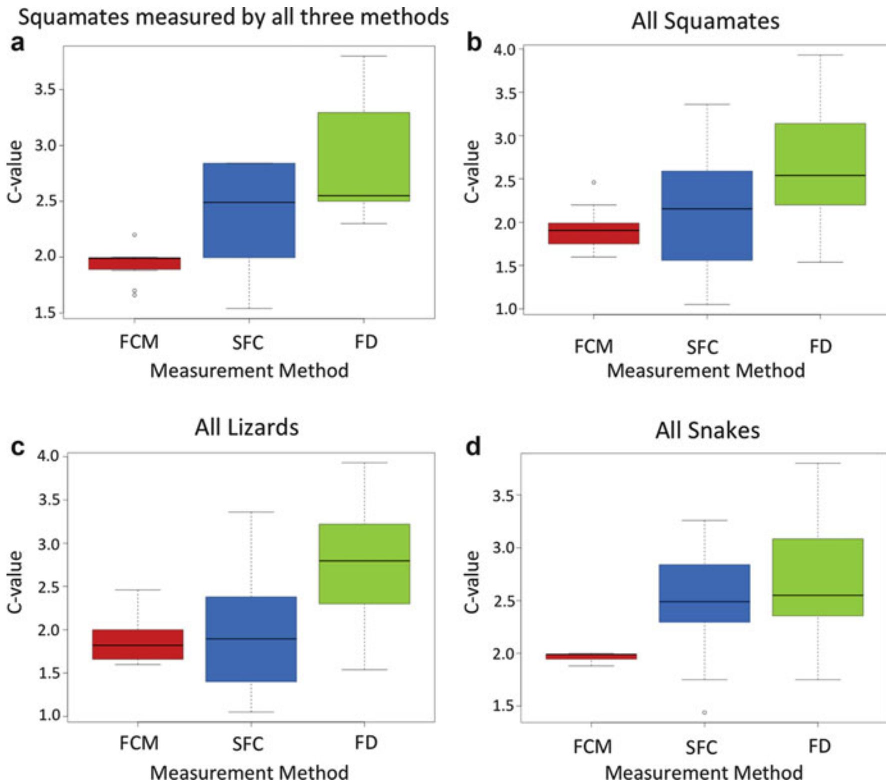

Fig. 1 Box-plot comparisons of genome size estimates based on three different methods. For all panels, methods are abbreviated: *FCM* flow cytometry, *SFC* static flow cytometry, *FD* Feulgen density. (**a**) Genome size estimates for all lizard and snake species that have been measured by all three methods. (**b**) Genome size estimates for all snake and lizard species that have been measured by at least one of the three methods. (**c**) Genome size estimates for lizard species that have been measured by at least one of the three methods. (**d**) Genome size estimates for snake species that have been measured by at least one of the three methods (Data based on the Animal Genome Size Database (Gregory 2013))

between birds (1.4 Gbp) and mammals (3.5 Gbp) and is also smaller than other non-avian reptiles (3.2 Gbp in Testudines and Crocodilia and 5.0 Gbp in *Sphenodon* (Janes et al. 2010b)). The average lizard genome size based on flow cytometry is also 1.9 Gbp ($n = 58$, range = 1.3–2.8 Gbp; Fig. 2c). The average snake genome size based on flow cytometry is also 1.9 Gbp ($n = 32$, range = 1.5–3.0 Gbp; Fig. 2d). Previous work on the pattern of genome size evolution found that the Reptilia have experienced continuous gradual evolutionary change in genome size with no rapid shifts in genome size since the early reptile radiation (Organ et al. 2008). Other research, however, has found that larger genomes evolve in size at faster rates than smaller genomes in reptiles (Oliver et al. 2007). These previous studies all have used data from all three methods of genome size estimation above, and it is unclear if or how this may have impacted their conclusions.

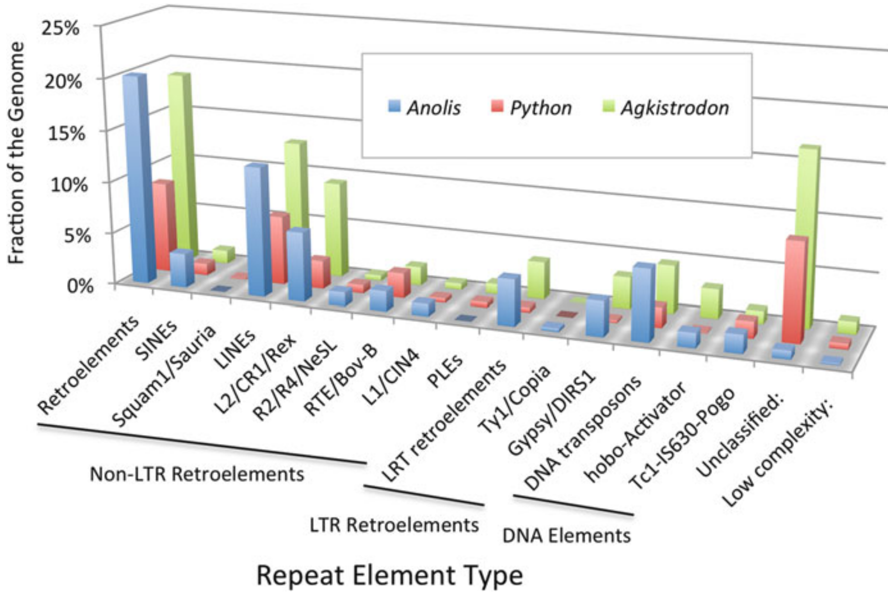

Fig. 2 Comparison of genome repeat content between *Anolis carolinensis*, *Python molurus bivittatus*, and *Agkistrodon contortrix*. Various repeat element families and their overall classification are shown on the *horizontal axis*. The *vertical axis* indicates the proportion of the total genome constituted by a repetitive element (Data based on Castoe et al. (2011a) and analysis of the repeat-masked complete *Anolis* genome from the UCSC Genome Browser)

Squamate Genome Structure

The Mitochondrial Genome

Genomics typically invokes reference to the nuclear genome, although the mitochondrial genomes of squamate reptiles have been studied most thoroughly to date. This smaller organellar genome typically contains 13 protein-coding genes central to mitochondrial oxidative metabolism function, the ribosomal and tRNAs to accomplish translation of these proteins, and a control region that functions in mitochondrial genome replication and transcription. Snake mitochondrial genomes have been of particular interest because they evolved a number of characteristics that are unlike most vertebrates. With the exception of the scolecophidian snakes (blind snakes and their relatives), which are the most ancestral extant group of snakes, all snakes appear to have a duplicated mitochondrial control region, and both control region sequences are maintained at nearly identical sequences by an unknown mechanism of concerted evolution (Kumazawa et al. 1996; Jiang et al. 2007). Molecular evolutionary evidence suggests these control regions are both likely to act as origins of genome replication (and probably also as promoters

for RNA synthesis) (Jiang et al. 2007; Castoe et al. 2009b). It has been hypothesized that these duplicate control regions might function in the rapid metabolic upregulation in some snakes, which is associated with feeding (Jiang et al. 2007; Castoe et al. 2009b). In addition to large-scale genome structure, studies have shown that snake mitochondrial proteins have experienced an extreme adaptive event that included unprecedented coevolutionary change along with a great excess of radical amino acid replacements. These findings imply that snake oxidative metabolism might function uniquely among vertebrates, due to the large number of unique and radical changes observed in these snake proteins (Castoe et al. 2008). Further evidence for the largest known episode of convergent molecular evolution having occurred between the proteins of snakes and acrodont lizards implies strong convergent patterns of selection (Castoe et al. 2009a). The uniqueness of squamate mitochondrial genome structure and protein evolution raises many questions about the scope of adaptation in the nuclear genomes of squamates and if there might have been evolutionary interactions between extreme metabolic adaptation and the evolution of venom systems in squamate reptiles.

Nuclear Chromosomal Structure

Chromosomal variation is far greater in reptiles than in mammals, mainly due to the presence of microchromosomes (Olmo 2005). Microchromosomes are structurally and functionally similar to macrochromosomes but are roughly half the size of macrochromosomes on average (Rodionov 1996). They are two to three times more gene dense than macrochromosomes (Smith et al. 2000), and avian microchromosomes appear to have a higher recombination rate than macrochromosomes (Rodionov et al. 1992). Compared with macrochromosomes, nucleotide content in microchromosomes tends to be GC rich and contains higher frequencies of CpG dimers, and these microchromosomes are also relatively depauperate in repetitive elements (Hillier et al. 2004).

On average, squamates have 36.6 chromosomes (range = 27–51 chromosomes) divided roughly into one half macrochromosomes (average = 18, range = 12–35) and one half microchromosomes (average = 18.9, range = 2.1–24) (Olmo and Signorino 2013). Snakes appear to have relatively highly conserved karyotypes, with the most common diploid number being $2n = 36$. Karyotypes of snakes typically consist of eight pairs of macrochromosomes and 10 pairs of microchromosomes (Matsubara et al. 2006; Srikulnath et al. 2009). Lizards, in contrast, have large variations in chromosome number and morphology. In lizards, one of two main karyotypes tends to be observed in a given species: either a mixture of macrochromosomes and microchromosomes or few or no microchromosomes (Srikulnath et al. 2009). No phylogenetically controlled correlation exists between haploid genome size and the number of microchromosomes, macrochromosomes, and total chromosomes (Organ et al. 2008), so it is difficult to make any inferences about relationships between chromosome number and genome size.

Sex Chromosomes

Sex determination in squamates results from one of two mechanisms, both of which are scattered across various squamate lineages. One mechanism, which is more common in non-squamate reptiles (e.g., turtles and crocodilians), is temperature-dependent sex determination (TSD), in which the sex of offspring is governed by incubation temperature. The more common sex-determination mechanism in squamates is genetic sex determination (GSD), where chromosomal inheritance dictates sex. In most squamates GSD follows a Z/W sex chromosome system. Among squamates, snakes are straightforward in this respect, and all exhibit a sex chromosome system with female heterogamety (ZW), which is the general trend across the squamate tree of life (Janes et al. 2009). Analyses of snake sex chromosomes have revealed increased differentiation in a phylogenetic gradient from pythons to colubroid snakes (Matsubara et al. 2006). In contrast, lizards can have either heterogametic males or females, possess X/Y or Z/W sex chromosome systems, and sometimes exhibit TSD. This diversity of sex-determining mechanisms makes squamates an ideal system for understanding sex determination (Ezaz et al. 2005).

The Z/W sex-determination system parallels the better-known X/Y sex-determination system in that the W sex chromosome is often a degenerated copy of the Z sex chromosome just as the Y sex chromosome is often a degenerated copy of the X sex chromosome. Sex-determining genes have been resolved for mammals (*Sry*) and birds (*Dmrt1*), but not for squamates. *Dmrt1* has been mapped to autosomal chromosomes in four snake species (Matsubara et al. 2006) and is therefore not the sex-determining factor for snakes. Additionally, *Dmrt1* from the chicken Z chromosome has been mapped to both Z and W sex chromosomes in *Gekko hokouensis*, and analyses of the *Anolis* genome further indicate that *Dmrt1* is unlikely to be the sex-determination gene (Alfoldi et al. 2011). Complicating the search for a sex-determination locus in squamates is the finding that sex chromosomes are not homologous between reptile groups, which are consistent with sex chromosomes evolving many times independently in reptiles (Ezaz et al. 2009).

Genomic GC-Isochore Structure

GC isochores are large tracts of genomic DNA with internally relatively homogeneous base composition that varies over large chromosomal scales. GC-rich isochores positively correlate with many important genomic features, including recombination rate, gene density, epigenetic modifications, intron length, and replication timing, implying their importance as functional genomic elements (Janes et al. 2010b). The *Anolis carolinensis* genome was found to lack GC-rich isochores, which was an unexpected result (Alfoldi et al. 2011). Recent analyses of the Burmese python and king cobra genomes indicate a higher degree of GC-isochore structure than *Anolis* (Castoe et al. 2013). These findings may suggest that snakes have re-evolution GC isochore since their divergence from *Anolis* or that GC isochore was lost in an ancestor of *Anolis*.

Ultraconserved Regions

Ultraconserved elements (UCEs), or small stretches of the genome that are conserved across distantly related vertebrates, have become popular for inferring the phylogenetic relationships among vertebrate organisms (McCormack et al. 2012); Crawford et al. 2012 discovered a dramatically increased substitution rate in UCEs in the squamate lineage, and particularly in snakes. Squamates, therefore, appear to show a shift in conserved regulatory genomic regions that have otherwise remained relatively static in other amniote lineages. Other research on long, conserved noncoding sequences (LCNSs), another class of highly conserved genomic elements, found that a higher percentage of these sequences is conserved in reptiles, which may reflect differing roles and constraints in gene regulation in the reptile lineage (Janes et al. 2009, 2010a). Future studies on squamate genomes may provide additional insights into the evolutionary patterns of conserved genomic elements and the functional consequences of changes in such conserved genomic regions in squamates.

Transposable Element Diversity

Although our current knowledge of vertebrate genome structure and diversity is strongly slanted towards mammals, new sequence-based information on reptilian genome structure and content is just beginning to emerge (Shedlock et al. 2007; Kordis 2009; Novick et al. 2009; Piskurek et al. 2009; Castoe et al. 2011d, 2013). Like most vertebrates, large portions of squamate genomes are comprised of repeat elements, and based on the small numbers of examples known, squamate genomes appear to contain a highly diverse repertoire of repeat element types (Shedlock et al. 2007; Castoe et al. 2011d, 2013). In contrast to the genomes of mammals and birds, most (non-avian) reptile genomes are comprised of a particularly diverse repertoire of different types of transposable elements (TEs) and multiple apparently active TE types, subtypes, and families (Fig. 2). Whereas mammal and bird genomes often have undergone recent expansion of one or a small number of TEs, such as L1 LINEs and Alu SINEs in humans, reptilian genomes examined have experienced recent (and presumably ongoing) activity and expansion of multiple TE types; this is particularly true of the squamate reptiles studied to date (Castoe et al. 2013). Based on preliminary genomic analyses of the lizard *Anolis*, trends in the squamate lineage include an increase in simple sequence repeat (SSR) content, the dominance of CR1 LINE retroelements, and a high overall diversity of retroelements (Shedlock et al. 2007; Novick et al. 2009; Piskurek et al. 2009).

Genomic sample sequencing and analysis of unassembled random genomic sequences from two snake species (*Python molurus bivittatus* and *Agkistrodon contortrix*) determined that among the snakes, the relative abundance of different repeat elements varies widely, while genome size and repeat element diversity do not (Fig. 2). Sample sequencing from ten total snake genomes indicates that repeat content varies widely, while the diversity of repeat elements stays fairly consistent

(Castoe et al. 2013). It is also notable that major differences in repeat element content between snakes is based on the difference in abundance of most repeat element classes rather than expansion or contraction of one or a few repeat element groups (Castoe et al. 2011d, 2013). Two groups of non-LTR retrotransposons, CR1 LINEs and Bov-B LINEs, appear to be particularly abundant and active in snake genomes (Castoe et al. 2013). There are also probably several classes of abundant SINEs in snakes, but they have not been identified and are either novel or too divergent to be recognized by RepBase libraries and therefore are likely included in the set of "unclassified" repeats (Fig. 2). It is notable that previous studies have overestimated the abundance of Bov-B LINEs in snakes and lizards (Walsh et al. 2013) due to an incorrect annotation of a hybrid Bov-B/CR1 LINE (as a Bov-B LINE) reference sequence in RepBase (Castoe et al. 2011d). Current information on the transposable element landscapes of squamates suggests that there appears to be major shifts in abundance and presumably activity of multiple transposable element families, and a greater sampling of species is necessary to understand at what temporal scale and at which nodes in the squamate tree such shifts may have occurred.

Horizontal Transfer of Transposable Elements

Knowledge of the presence and absence of transposable element types across vertebrate lineages remains fragmentary due to the limited sampling of vertebrate genomes; this is especially the case for squamate reptiles. Despite this, different types of elements in squamate genomes, including LINEs (Kordis and Gubensek 1997, 1998, 1999), SINEs (Piskurek and Okada 2007; Piskurek et al. 2009), and DNA transposons (Gilbert et al. 2008; Pace et al. 2008), may owe their origins to horizontal transfer. Multiple studies have inferred horizontal transfer of Bov-B LINE retrotransposons between mammals and snakes or squamate reptiles to explain the enigmatic distribution of these elements across amniote vertebrates (Kordis and Gubensek 1997, 1998). Based on phylogenetic analysis of Bov-B sequences from available vertebrate genomes and the sampled genomes of the python and copperhead, multiple episodes of horizontal transfer of Bov-B LINEs to or from squamate reptiles appear to have also occurred (Castoe et al. 2011d; Fig. 3). In Fig. 3, horizontal transfer is implicated because sequences of Bov-B from squamates are extremely closely related to similar sequences from mammals. Multiple transfers are indicated by the result that two clades of snakes do not form a clade exclusive of lizards, implying multiple independent transfers to ancestral lineages of snakes and/or squamates. Similarly, space invader (SPIN) elements, a type of hAT DNA transposon, are also inferred as having been independently horizontally transferred into the genomes of multiple tetrapod lineages within the last 15–46 million years (My), including into multiple lineages of squamates (Gilbert et al. 2008; Pace et al. 2008; Novick et al. 2009; Castoe et al. 2011d). Gilbert et al. (2012) determined that at least 13 independent episodes of SPIN element horizontal transfer events took place within Squamata within the last 50 My on at least three different continents.

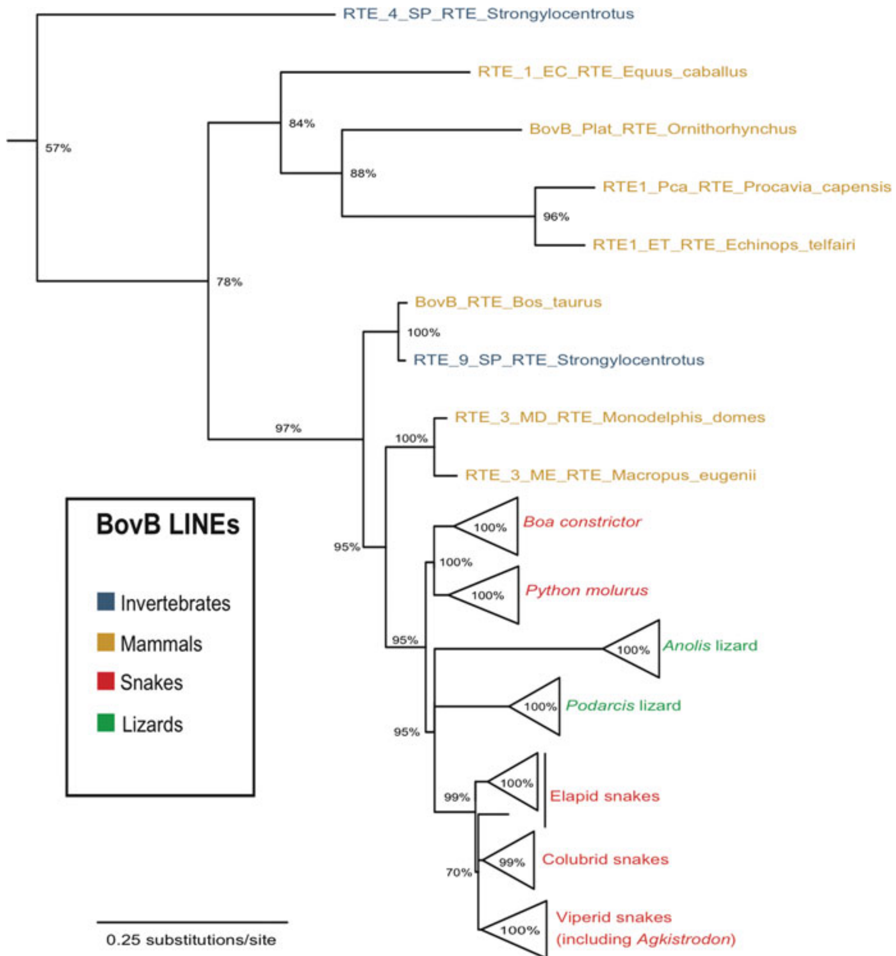

Fig. 3 Bayesian phylogenetic tree comparing the relationships of Bov-B LINEs in a variety of vertebrate and invertebrate lineages. Lineage names are color coded based on taxonomy (see key), and posterior probabilities for nodal support are indicated. The topology suggests multiple horizontal transfers between deeply diverged vertebrate and invertebrate lineages (Figure adapted from Castoe et al. (2011d), supplemental figure S8)

Evidence suggests that these transfers may have been mediated by parasites (Gilbert et al. 2010; Walsh et al. 2013).

Microsatellite Seeding by Transposable Elements

It has been shown that transposable elements may occasionally contain microsatellite or simple sequence repeats (SSRs) on their tails and are therefore capable of seeding

novel microsatellite loci on large scales throughout the genome. Snake genomes are the most extreme example of this in vertebrates (Castoe et al. 2011d). Analysis of two snake genome samples indicated a conspicuous increase in the genomic SSR and low complexity content, apparently indicating a secondary increase in SSR evolution and turnover in snakes (Castoe et al. 2011d). It is notable that this change must have occurred subsequent to the slowdown in SSR evolution and turnover earlier in the reptilian lineage (Shedlock et al. 2007). Snake1 (L3) CR1 LINEs appear to increase in frequency in snakes (Fig. 2), and also seed microsatellites, because the 3-prime tail of these elements contains a microsatellite repeat (Castoe et al. 2011d, 2013). These LINEs tend to contain one of two SSR repeat sequences, both of which are related in sequence (Fig. 3). The impact of such SSR seeding is extreme and obvious in the genome of the copperhead (*Agkistrodon*), in which Snake1 CR1 LINEs have become relatively abundant compared to python (Fig. 3). Specifically, a majority of all SSRs in the copperhead are one of three closely related sequences (AGA, AGAT, or AGATA; Fig. 3). Sequence sampling of ten total snake genomes indicates that these microsatellite-seeding Snake1 CR1 LINEs have expanded extensively in colubrid snakes, providing further details supporting a trend seen in the comparison between python and copperhead (Castoe et al. 2013).

Microsatellites may alter genome recombination structure and rates and, together with other repeat elements (e.g., CR1 LINEs), may facilitate unequal crossing over events that lead to tandem duplication of segments of the genome. From what is currently known about snake genome structure, it appears that most venom genes (Casewell et al. 2012) are derived from other nontoxic gene families that experienced gene duplication. Interestingly, the current model for the evolution of venom toxins (at least in snakes) includes the tandem duplication of genes (Ikeda et al. 2010). Snake1 CR1 LINEs are also notable because, from what is currently known of snake genomes, they occur at high frequency throughout phospholipase venom genes in viperid snakes (Ikeda et al. 2010; Fig. 4c), in numerous other venom genes in viperids and elapids (Castoe et al. 2011d), and in Hox gene clusters of colubrid snakes (Di-Poi et al. 2010). Therefore, transposable elements and seeding of microsatellites may have contributed to the genomic context that facilitated the evolution and radiation of venom loci in snakes.

Genomics of Squamate Venom Toxins

Genetic and Genomic Structure of Squamate Venom Toxins

The ability to leverage emerging high-throughput technologies for genomic, transcriptomic, and proteomic analysis continues to improve our understanding of the squamate lineage and the evolution of squamate venoms. Developing a deeper knowledge of toxin gene structure, and the genomic context in which toxin genes exist and in which they have evolved, is central for understanding the evolutionary origins and regulation of these genes. Most of what is currently known about squamate venom genes, however, provides little genomic context because it is

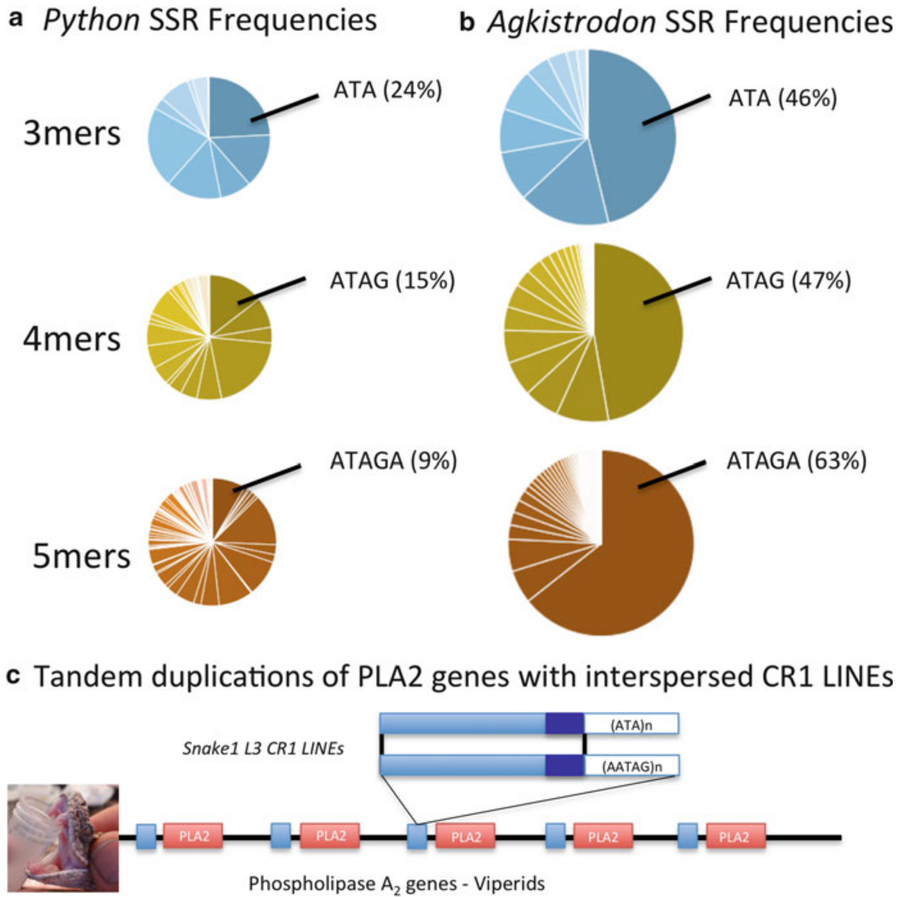

Fig. 4 The estimated proportions of simple sequence repeat (SSR) content within *Python molurus bivittatus* and *Agkistrodon contortrix* genomes based on random unassembled genomic sequence data and a plausible connection between the taxonomic bias in SSR content and venom evolution. (**a**) 3, 4, and 5mer SSR estimates for *Python* only. (**b**) 3, 4, and 5mer SSR estimates for *Agkistrodon* only. (**c**) Hypothetical representation of phospholipase A2 (PLA2) venom genes in viperids, which are interspaced by Snake1 L3 CR1 LINEs whose associated SSR tails have putatively led to altered recombination and thus the tandem duplication motif that is common in venom genes (Figures based on data from Castoe et al. (2011a), and PLA2 gene cluster sequence reported by Ikeda et al. (2010))

based on cDNAs of venom gland transcripts, thus providing information only about the transcribed exons and UTRs. Because there are multiple opportunities for regulation of gene expression and protein activity after transcription (e.g., siRNA, miRNA, translation efficiency, posttranslational modification, etc.), there remain many gaps in our knowledge in relating mRNA transcript levels directly to levels of functional toxins in venoms. We expect that with the availability of venomous snake reference genomes like that of the king cobra (Vonk et al. 2013), we will be better equipped to fill these gaps in our understanding.

Venom genes have been shown to often occur in duplicated tandem arrays (Ikeda et al. 2010), and the evolution of venoms is thought to involve the duplication of nontoxic physiological protein-coding genes that are subfunctionalized or neofunctionalized to become venom toxins (Casewell et al. 2012; Vonk et al. 2013). Additionally, alternative splicing may provide further variation in functional venom proteins, increasing the number of protein products per locus, as has been shown in *Vipera lebetina* (Siigur et al. 2001). This typical structure of a venom gene locus can make it difficult to accurately translate information from transcriptome data. Based on transcriptome sequences, for example, it might be difficult to discern the difference between different alleles at the same locus, alternative splice forms from the same locus, or different recently duplicated loci. Therefore, in the absence of reference genomes for squamate reptiles, there is some ambiguity in translating venom protein diversity to transcript diversity and ultimately to inferences of venom locus diversity in the genome. Such inferences are made more difficult in studies where the aim is to use transcriptome or proteome data to analyze genetic variation in venom loci across individuals and populations because allelic variation among individuals may further complicate this mapping to genomic loci. The recent release of a draft genome sequence for the king cobra will help to fill this void, although this draft genome estimate was not able to completely assemble venom gene regions. This resource has, however, already provided important support for the tandem duplication model of the gene duplication and neofunctionalization for venom locus evolution and indicates ontological or developmental links between the venom gland and the pancreas based on similarities in small RNA expression (Vonk et al. 2013).

Despite substantial progress in forging connections between the genome, venom genes, their transcripts, and venom proteins and their effects, there are still substantial advances to be made with the availability of genomic resources for squamates. One critical and fundamental step forward would be the availability of well-assembled and annotated genomes for multiple venomous squamates to provide multiple complete genome references in which venom genes, along with their genomic context, can be directly linked to venom gene transcripts and venom proteins. Additionally, many other important questions regarding the genetic and ontological origins of venom toxins require additional genomic and transcriptomic resources for squamates to fully address, including the following: (1) are venom loci exclusively expressed in the venom glands or some forms expressed elsewhere in the organism? (2) Are there specific sequences that are identifiable that target venom genes for transcription only in the venom glands? (3) What were the expression patterns and biological functions of ancestral venom genes prior to their recruitment as venoms? (4) Is there evidence that certain sequences, such as simple sequence repeats or transposable elements, have played a central role in facilitating duplication and diversification of venom gene loci?

Challenges Facing Genome Assembly of Squamate Genomes and Venom Gene Regions

Our ability to confidently study the genomic context of venom genes is limited by our ability to not only collect genomic information, but further by the ability to accurately reconstruct the regions of the genome in which venom genes occur. If the prevailing view that most venom genes in squamates have undergone duplication is correct, assembling these regions of the genome is difficult. Moreover, in cases where this duplication occurred via tandem duplication (Ikeda et al. 2010), de novo genome assembly of these regions of the genome will be particularly difficult. Venom genes are also known to contain relatively high allelic variation, increasing the likelihood for heterozygosity at venom loci, which is known to make genome assembly more difficult. The evidence that these tandem duplicate copies may also be interspersed with highly repetitive transposable elements and other repeats further complicates genome assembly. These factors collectively make venom-related regions of the genome difficult to confidently reconstruct in de novo genome assemblies, particularly with current sequencing strategies that employ short sequence reads. As a result, it is expected that some of the most difficult regions of squamate genomes to assemble will be those that are of the greatest interest and value for studying venom, and even "complete genomes" may provide limited and fragmentary information about the genetic structure and genomic context of venom genes. As sequencing technologies continue to evolve, there is hope that hybrid sequencing approaches that combine multiple different types of reads (including perhaps low quality but very long sequences) may help in accurately assembling these critical regions of squamate genomes.

Conclusion and Future Directions

Squamates represent an extensive and ancient component of vertebrate evolutionary history and biodiversity, yet their genomic diversity has been remarkably poorly studied in comparison to mammals and birds. Multiple aspects of their extreme biology, including the evolution of a great diversity of toxic venoms, argue strongly for the importance of establishing genomic resources for squamates to illuminate key connections between genotypes and key phenotypes of interest. Emerging evidence implies that squamates have a relatively consistent genome size across species, yet may have marked difference in genomic repetitive content, making them excellent models for understanding relationships between genome size and repeat content. Squamates also are of biological interest because they represent an ideal comparative system for studying mechanisms of sex determination in vertebrates.

The sequencing and annotation of complete vertebrate genomes are increasingly feasible and affordable. An emerging central goal of toxinological research is to develop a seamless understanding of the connection between the genome and venom

toxins, incorporating gene regulation and the forces that act to modulate transcription and translation of venom genes. This is, of course, complicated by difficulties discussed above, including problems assembling tandem venom gene arrays in the genome, differentiating alleles, isoforms, and loci from transcriptomic data. Recent evidence also strongly implicates a role for small RNA in the modulation of ontogenetic and other shifts in venom composition (Calvete 2010). Furthermore, although no studies to date have identified such effects, it is reasonable that there may be epigenetic regulatory effects that additionally modulate expression of venoms. Among vertebrates, snakes in particular possess a tremendous number of unique or extreme phenotypes. A greater understanding of the molecular and genomic basis of these phenotypes holds exciting potential to increase broad understanding of the function and functional flexibility of the vertebrate genome and to illuminate the mechanisms by which such unique phenotypes can be evolutionary created from the raw material of the common vertebrate genome plan.

As more squamate genome and gene expression data become available, the toxinological community might consider a careful reevaluation of the precise language used for putative toxins upon discovery and acceptable criteria to be used to identify genes as "venom toxins." This also requires better organism-wide context of where else various genes are expressed and what genomic content is associated with those genes. Such studies would also have good potential for forging new links between the structure and evolutionary processes of squamate genomes, and how these might have shaped the evolution of venoms and other extreme phenotypes of squamates. Furthermore, understanding the ancestral state of venom gene orthologs in nonvenomous and venomous squamate species would provide novel insight into what processes and genomic features, at what times during squamate evolution, initiated and fostered the evolution of squamate venoms.

It is motivating that, although we know relatively little about squamate genomes currently, the details about squamate genomes that we do know tell a compelling story about the uniqueness and relatively extreme features of squamate genomes compared to other lineages of vertebrates and suggest an exciting future of discovery as more squamate genome information becomes available. This chapter has outlined multiple arguments motivating additional squamate genome sequence information as central to exposing the details of some of the most intriguing biological features known in vertebrates, including the evolution and function of deadly venom toxins and other extreme aspects of squamate biology. As a new generation of genome sequencing technology becomes more established and inexpensive, this data will likely begin to become available, making the coming years exciting for squamate biologists, toxinologists, vertebrate evolutionary biologists, and genome scientists.

Cross-References

► Shotgun Approaches for Venom Analysis
► Snake Venom Peptidomics
► Snake Venom Phospholipase A_2: Evolution and Diversity

▶ Snake Venom Proteopeptidomics: What Lies Behind the Curtain
▶ Venoms of Colubrids

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Venoms of Colubrids

3

Cassandra M. Modahl, Anthony J. Saviola, and Stephen P. Mackessy

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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 demonstrated the existence of novel venom proteins and has provided insight into

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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 Henophidians) to a chemical (venom) means of subduing prey (Kardong et al. 1997). The complex mixture of proteins and peptides which constitute a snake's venom contribute to multiple biological functions, including immobilizing, dispatching, and digesting prey (Mackessy 2010). However, rear-fanged venomous snakes are particularly interesting due to the fact that some species of these "colubrid" clades utilize constriction in addition to venom for facilitating prey capture. For example, venom from the brown tree snake (*Boiga irregularis*) contains a prominent heterodimeric three-finger toxin (3FTx) that is specifically toxic toward lizard and avian prey (Pawlak et al. 2009), and because this 3FTx is nontoxic toward mammalian prey, these snakes will instead constrict mammals (but not lizards; Mackessy et al. 2006). The specific receptor binding exhibited by 3FTxs originates from the accelerated accumulation of nucleotide substitutions within exons and the resulting changes to protein amino acid sequence and structure (Kini and Doley 2010; Sunagar et al. 2013), and in this case, a taxon-specific toxin has evolved. Venomic techniques combined with transcriptomics, genomics, natural history, behavior, and the recognition of venom as a trophic adaptation offer a powerful approach to unraveling the complex evolutionary history of venoms. This chapter considers the application of combined approaches toward the study of rear-fanged snake venoms.

Research centered on venom composition and individual protein characterization provides insight into the biological roles of venom compounds and

evolutionary relationships of venomous snakes as well as identifying compounds which evoke toxic symptoms resulting from snakebite and directly contributes toward production of more efficient antivenoms. Because front-fanged venomous snakes belonging to the families Elapidae and Viperidae produce significantly larger venom yields, and are responsible for the vast majority of human envenomations, venom research has primarily focused on species within these two families of snakes (Mackessy 2010). Rear-fanged venomous snakes, on the other hand, appear to exhibit a less derived venom delivery apparatus, produce significantly lower venom yields, and are generally perceived as nonthreatening to humans; as a result, they are generally understudied relative to the front-fanged snakes (Saviola et al. 2014). The large majority of rear-fanged venomous snakes are unable to deliver lethal quantities of these toxins or even enough toxins to result in systemic envenomations, but at least five species (*Dispholidus typus, Thelotornis capensis, Rhabdophis tigrinus, Philodryas olfersii,* and *Tachymenis peruviana*) are believed to have caused human fatalities (Kuch and Mebs 2002; Mackessy 2002; Prado-Franceschi and Hyslop 2002; but see Weinstein et al. 2013). Increasing awareness of severe, at times fatal, envenomations from rear-fanged snakes has led to a slowly growing interest in their venoms. In addition, advances in research techniques have resulted in a modest increase of data on individual toxins and on the composition and complexity of rear-fanged snake venoms.

Even though a single species may produce a venom with more than 100 protein components, snake venom proteins belong to a small number of enzymatic and nonenzymatic superfamilies. Some of these well-recognized venom protein families include phospholipases A_2 (PLA_2s), serine proteinases, snake venom metalloproteinases (SVMPs), three-finger toxins (3FTxs), proteinase inhibitors, and lectins (Mackessy 2010). These major venom protein families are found in almost all snake venoms, including many rear-fanged snake venoms (Mackessy 2002). In general, rear-fanged snake venoms show lower complexity than the venoms of front-fanged snakes, with upward of 40 expressed proteins visible following 2D SDS-PAGE (two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis), while front-fanged snake venoms show considerably higher complexity. In several cases, rear-fanged snake venoms have also been documented to contain novel protein superfamilies, thus providing a more comprehensive view of venom evolution (Ching et al. 2006; OmPraba et al. 2010; Pawlak et al. 2009). With the introduction of high-throughput proteomic and nucleic acid sequencing methods, detailed venom descriptive work encompassing the proteome, transcriptome, and genome is now possible with the relatively small amount of starting material obtained from rear-fanged snakes. These techniques, combined with protein biochemical characterizations and snake natural histories, will continue to elucidate the evolution and biological roles of rear-fanged snake venom components. The goal of this chapter is to provide a review of previous work, current research and methods, and future applications involving rear-fanged snake venoms.

Previous and Current Research on Rear-Fanged Snake Venoms

Classical Approaches

Since the mid-twentieth century, the study of snake venom toxinology has developed into a formalized scientific discipline. Originally, rear-fanged (opisthoglyphic) snakes were regarded as nonthreatening to humans, but the tragic deaths of herpetologists Karl Schmidt (due to envenomation from *D. typus*) and Robert Mertens (*Thelotornis capensis*) brought attention to the venomous potential of bites from rear-fanged snakes. These events initiated an increase in studies on these venoms (Weinstein et al. 2011), and the discovery that many rear-fanged venom secretions exhibit complex immunoidentity with numerous medically important viperid and elapid species (Minton and Weinstein 1987) further stimulated research endeavors into rear-fanged snake venoms. Some lethal venom components, such as 3FTxs, PLA_2 enzymes, and snake venom metalloproteinases (SVMPs), that were once thought to be found exclusively in elapid or viperid venoms now appear to be significantly abundant compounds in numerous species of rear-fanged snakes (Fry et al. 2003a; Mackessy 2002; Huang and Mackessy 2004; Peichoto et al. 2011). However, due to limited accessibility of specimens and low venom yields obtained during extractions, studies involving rear-fanged snake venoms have progressed slowly compared to the extensive work examining front-fanged snake venoms (Mackessy 2002). The now common utilization of anesthetics, such as ketamine hydrochloride, followed by a subcutaneous injection of the parasympathomimetic pilocarpine hydrochloride to stimulate venom secretion, has not only improved snake handling and safety for both the animal and handler but has also resulted in greatly increased venom yields (Rosenberg 1992; Hill and Mackessy 1997; Mackessy 2002). Since it is now possible to obtain sufficient quantities of venom, coupled with the continuing advancements in biochemical characterization and high-throughput proteomic, transcriptomic, and genomic techniques, it is now feasible to develop a much better understanding of the composition and complexity of rear-fanged snake venoms.

Traditional methods, such as the use of one- and two-dimensional gel electrophoresis, provide a quick and basic approach for identifying venom compounds present in crude venoms. For 1D SDS-PAGE (one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis), as little as 10–30 μg of crude venom separated in 12 % acrylamide precast gels provides a clear molecular fingerprint of potential venom compounds (Fig. 1), allowing for inter- and intraspecific comparisons of venom variation (e.g., Mackessy et al. 2006; Peichoto et al. 2012). Rear-fanged venoms that have been studied typically demonstrate a greater complexity in the higher molecular mass regions following SDS-PAGE (Peichoto et al. 2012). Two-dimensional gel electrophoresis provided additional venom compositional information in an analysis of South and North American opisthoglyphous snake species, allowing for the detection of multiple protein isoforms that shared similar molecular masses but differed in isoelectric points. Acidic proteins in the 30–40 kDa range, which would have been difficult to distinguish using only one-dimensional gel electrophoresis, showed differential expression in *Philodryas*

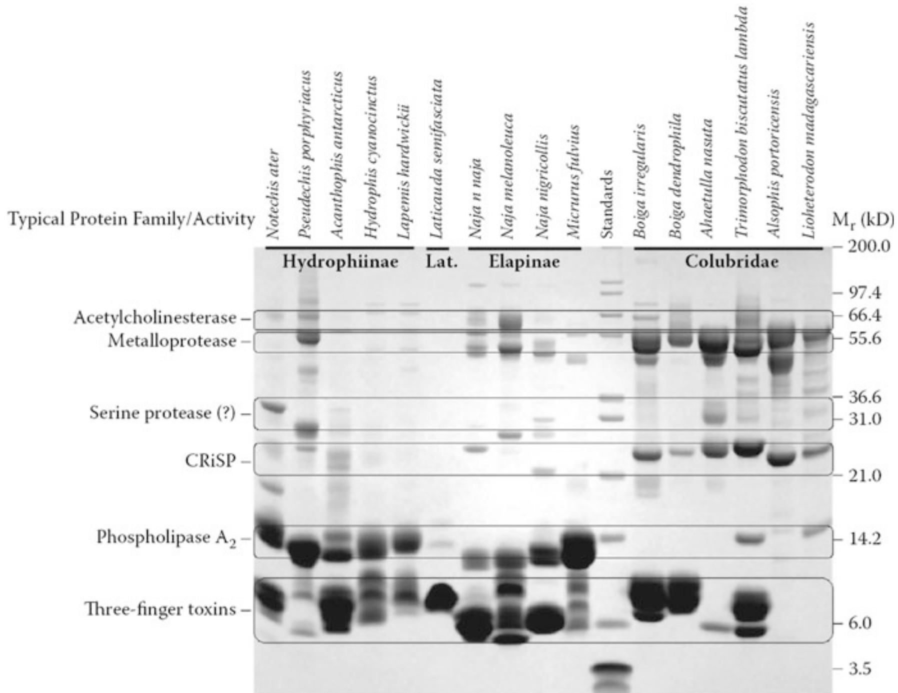

Fig. 1 One-dimensional SDS-PAGE of elapid and rear-fanged (Colubridae) snake venoms; 3–20 protein bands are visible. Note that proteins of the size of 3FTxs are shared among both clades and that SVMPs are commonly found as a major component of rear-fanged snake venoms but are less abundant in most elapid venoms (Reproduced from Mackessy (2010))

sp. and for *Trimorphodon biscutatus lambda* (Peichoto et al. 2012). The greater resolution provided by 2D gel electrophoresis is also an ideal fractionation method for in-gel trypsin digestion and mass spectrometry (MS) analysis. This technique allows for the identification of multiple protein isoforms that can exist in a venom protein superfamily (Fig. 2), such as the multiple matrix metalloproteinase isoforms found in the rear-fanged snake *Thamnodynastes strigatus* (Ching et al. 2012).

Whole-organism toxicity, another traditional venom characterization method, allows for identification of lethal doses (LD_{50}); this dosage reflects the amount of a substance required to kill half of the injected organisms within a 24-h period. Low LD_{50} values indicate the presence of potent (often neurotoxic) venom components, but it can be difficult to obtain enough material, especially purified individual venom components, from some rear-fanged snakes (Mackessy 2002). LD_{50} values have classically been determined using a mouse model because mice are easy to maintain, can be obtained in an array of essentially "reagent grade" strains, and are included in the diet of many venomous snakes (da Silva and Aird 2001). Because the diets of rear-fanged venomous snakes often encompass a broader range of prey taxa, species that regularly feed on nonmammalian prey may produce venom toxicity and other physiological data in mice that are not biologically relevant

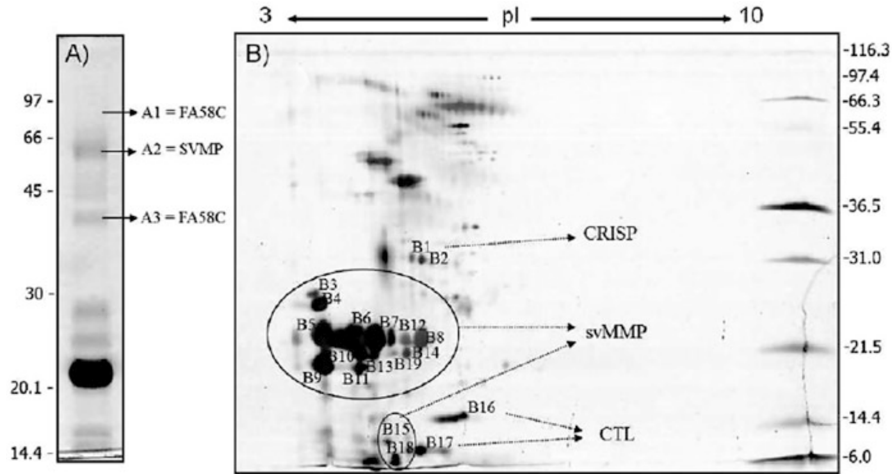

Fig. 2 1D SDS-PAGE (**a**) and 2D SDS-PAGE (**b**) of *Thamnodynastes strigatus* venom. Note the greater resolution of proteins in B – most proteins of this venom have an acidic pI (Reproduced from Ching et al. (2012))

(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 was used successfully for the isolation of the 3FTxs fulgimotoxin and denmotoxin (Heyborne and Mackessy 2013; Pawlak et al. 2006). Anion exchange columns have been successful for purifying SVMPs from rear-fanged snake venoms (Weldon and Mackessy 2012). Reversed-phase (RP) HPLC is a common final polishing step for the removal of salts from size exclusion or ion exchange chromatography, but this method can result in denaturation of some venom proteins, in particular metalloproteinases and other enzymes.

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 snake venom enzyme superfamilies. These assays include substrates that can indicate the presence of proteases (SVMPs and serine proteinases), acetylcholinesterases, PLA_{2s}, L-amino acid oxidases, hyaluronidases, and phosphodiesterases in rear-fanged snake venoms (Mackessy 2002). Proteolytic activity has been assayed for using several substrates, including casein yellow, azocasein, collagen, and fibrinogen (Sanchez et al. 2014). Zymogram gels, which are copolymerized with gelatin, have also been used to characterize rear-fanged snake venom proteins with proteolytic activity (general endoproteinase activity) (Hill and Mackessy 2000; Weldon and Mackessy 2010).

Using azocasein substrate, SVMP activity has been identified in many rear-fanged snake venoms, including the venoms of *Dispholidus typus*, *Philodryas* sp., *Hydrodynastes gigas, Hypsiglena torquata,* and *Alsophis portoricensis* (Hill and Mackessy 2000; Mackessy 2002; Peichoto et al. 2007; Weldon and Mackessy 2012). This list of species includes both New World and Old World rear-fanged snakes and is suggestive of potential local tissue damage and hemorrhage if bitten by these species (Peichoto et al. 2012; Sanchez et al. 2014). Currently, there are several rear-fanged SVMPs that have been further characterized, such as patagonfibrase from *Philodryas patagoniensis* and alsophinase from *Alsophis portoricensis*, both of which demonstrate alpha-fibrinogenolytic and hemorrhagic activities (Peichoto et al. 2007;

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 substrate specific as it lacks activity toward a butyrylcholine substrate (Mackessy 2002). Acetylcholinesterase activity is commonly detected in venoms with the use of the substrate acetylthiocholine that reacts with dithiobisnitrobenzoate to produce a colorimetric determination of activity. For the detection of PLA_2 activity, 4-nitro-3-(octanoyloxy) benzoic acid and egg yolk phosphatidycholine Type IV substrates have been used to assay activity in venoms of *Boiga dendrophila, Diadophis punctatus regalis, Dispholidus typus, Leptodeira annulata, Malpolon monspessulanus, Rhabdophis subminiata, Thelotornis capensis, Rhamphiophis*

oxyrhynchus, and *Trimophodon biscutatus lambda* (Hill and Mackessy 2000; Huang and Mackessy 2004). The PLA_2 trimorphin has been purified and characterized from *Trimorphodon biscutatus lambda* venom.

CRiSPs are also widespread in reptile venoms and exhibit a remarkable degree of sequence conservation (Heyborne and Mackessy 2010; Peichoto et al. 2009), and several members of this superfamily have been found to interact with different target proteins, such as cyclic nucleotide-gated ion channels as well as L-type Ca^{2+} and K^{+} channels (Yamazaki and Morita 2004). The biological functions of many CRiSPs remain relatively unknown. A CRiSP isolated from the rear-fanged snake *Helicops angulatus* has been shown to exhibit robust neurotoxic activity that results in immediate respiratory paralysis in mice (Estrella et al. 2011), while patagonin, a CRiSP characterized from the venom of *P. patagoniensis,* was found to cause muscular damage (Peichoto et al. 2009). Rear-fanged venom CRiSPs appear to show the same conservation of structure and diversification of function as seen for 3FTxs.

In the absence of biochemical and biological assays, it can be difficult to predict the activity of venom proteins. Large venom protein superfamilies such as 3FTxs and PLA_2s can exhibit a diversity of activities, ranging from neurotoxicity as a result of specific receptor binding to general cytotoxicity resulting in tissue necrosis. Researchers should be careful assigning protein activity based solely upon sequence similarity to other proteins or if only limited biochemical assays have been conducted. In the case of PLA_2s, pharmacological effects may be dependent or independent of enzymatic activity; therefore, a biochemical assay focused only on PLA_2 enzymatic activity could overlook other pharmacological activities, such as neurotoxicity (Mackessy 2010). Many high-throughput venom descriptive techniques, such as those based on MS/MS (tandem mass spectrometry) data, are limited when it comes to evaluating structure-function variation within venom protein families. Venom proteins can share similar amino acid sequences and have different structural arrangements as a result of post-translational modifications or interactions between other venom proteins or substrates. It is also possible for toxins to have similar structural appearances but exhibit vastly different receptor targets or activities. An example of this is the diversity of biological activities exhibited by venom 3FTxs, which include neurotoxicity, enzyme inhibition, cardiotoxicity, cytotoxicity, ion channel blockage, and anticoagulation effects (Kini and Doley 2010).

Venoms of several rear-fanged snakes in the family Colubridae (*sensu stricto*) contain 3FTxs that maintain the same conserved three β-sheet stabilized loops (from which the name "three-finger toxin" originated) commonly seen in 3FTxs from elapid venoms. Several 3FTxs from rear-fanged snakes have taxon-specific receptor binding affinities which has not been observed for elapid 3FTxs. Denmotoxin, from venom of the rear-fanged mangrove cat snake (*Boiga dendrophila*), was the first prey-specific 3FTx identified and displayed potent postsynaptic neuromuscular activity by irreversibly inhibiting chick biventer cervicis nerve-muscle preparation twitches, but it induced much smaller and reversible inhibition of twitches in mouse hemidiaphragm nerve-muscle

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 is especially of interest for protein sequences that have a blocked N-terminus, making it more difficult to determine the amino acid sequence from Edman degradation. Rear-fanged snake venom 3FTxs commonly have an N terminal

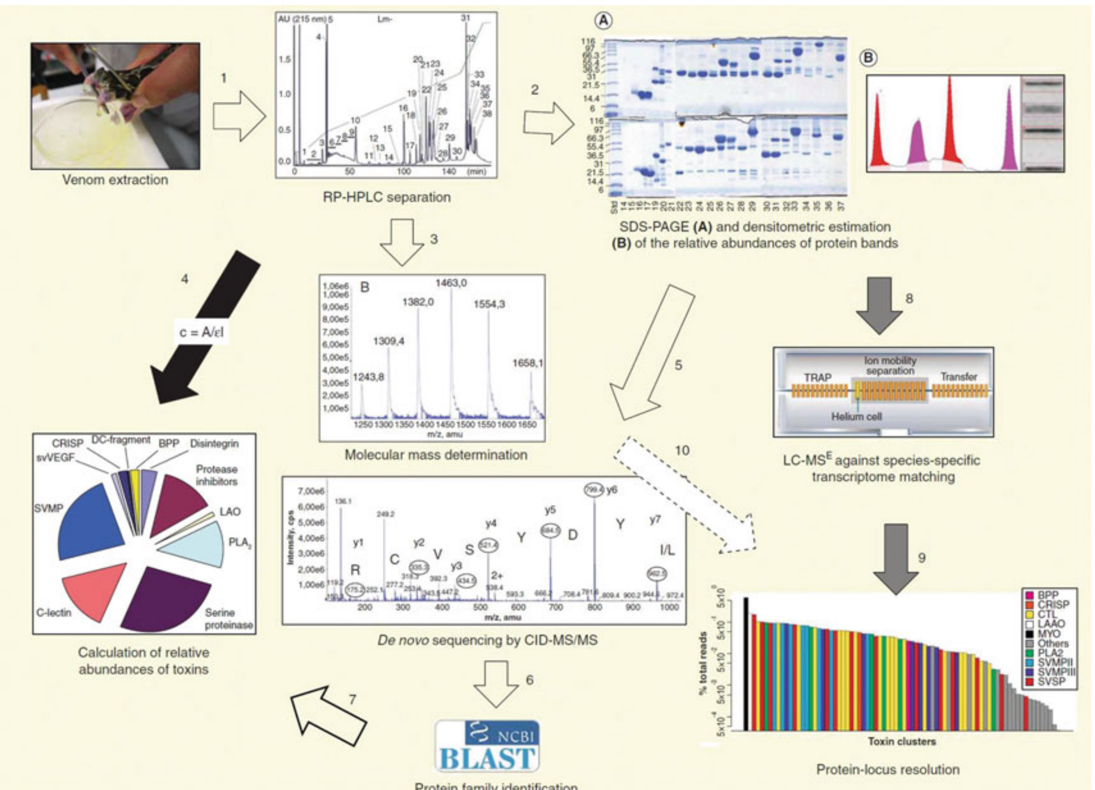

Fig. 3 An example of a venomics analysis of viper venom. Venom is fractionated using RP-HPLC and 1D SDS-PAGE; bands are digested and subjected to MS analysis (Reproduced from Calvete (2014))

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.

A bottom-up approach, such as tandem MS performed on proteins digested with proteases such as trypsin (most commonly used), chymotrypsin, or Glu-C, generates a spectrum of fragmented singly charged peptide ions that can be matched to databases for protein identification (peptide mass fingerprinting) or can be used for de novo sequence determination (i.e., Chapeaurouge et al. 2015). Collision-induced dissociation (CID) is the most widely used MS/MS technique for this type of venom analysis. This technique creates a series of backbone fragmentations at the peptide bond, resulting in b- and y-fragment ions. MASCOT, SEQUEST, or other databases are searched using algorithmic comparisons of proteins derived from genomic sequencing or known protein amino acid sequences to identify unknown proteins based on their peptide fragment spectra.

However, post-translational modifications of venom proteins are not detectable when examining the venom genome or transcriptome, and discrepancies between the proteome and the transcriptome of a single species have been noted (Pahari et al. 2007; Sunagar et al. 2014). Translation blockages (Wang et al. 2010) are also not detectable based on transcriptome data alone, and therefore genomic and transcriptomic data do not fully represent the compounds that may constitute a species' venom. In addition, the increased sensitivity of transcriptomics results in all venom gland mRNA (messenger ribonucleic acid) being sequenced, and therefore it can be difficult to discern which transcript sequences are translated and secreted as venom proteins and which are simply endogenous cellular proteins. Adopting a combination of "omic" approaches allows for a species-specific transcriptome database of all potential venom protein components in a venom to be matched with MS/MS-generated peptide fragments from the crude venom proteome. Proteomics can also be used to check the accuracy of transcriptome assembly and translation. If peptide MS/MS sequences do not match a single species-specific transcriptome, it could be suggestive of erroneous contig (contiguous sequence from overlapping DNA reads representing a transcript) assembly, sequencing errors that resulted in a reading frame shift, or incorrect reading frame selection (Calvete 2014). Therefore, a combination of genomic, transcriptomic, and proteomic data is necessary to fully understand venom composition and evolution.

Currently, several rear-fanged genera, including *Boiga*, *Hypsiglena,* and *Philodryas*, are the best characterized venoms using a combination of proteomic

(general venom description and biochemical activity) and transcriptomic data. For *P. baroni, P. olfersii,* and *P. patagoniensis,* it has been shown that the majority of venom proteolytic activity is from SVMPs, with low levels of activity toward substrates for serine proteinases (Sanchez et al. 2014). The venom of *Boiga irregularis* was found to be dominated by 3FTxs (Fig. 4a), while that of *Hypsiglena* was dominated by SVMPs (Fig. 4b); the venom of *Boiga* appeared more "elapid like," while the venom of *Hypsiglena* was more "viper like" (McGivern et al. 2014). A combination of proteomics and transcriptomics is ideal for rear-fanged venomous snakes, particularly considering that these venoms are not well characterized

Fig. 4 (continued)

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) and is enhancing the understanding of evolutionary relationships between venom compounds, and the snakes which produce them. As these databases grow, they will allow for the investigation of the multiple

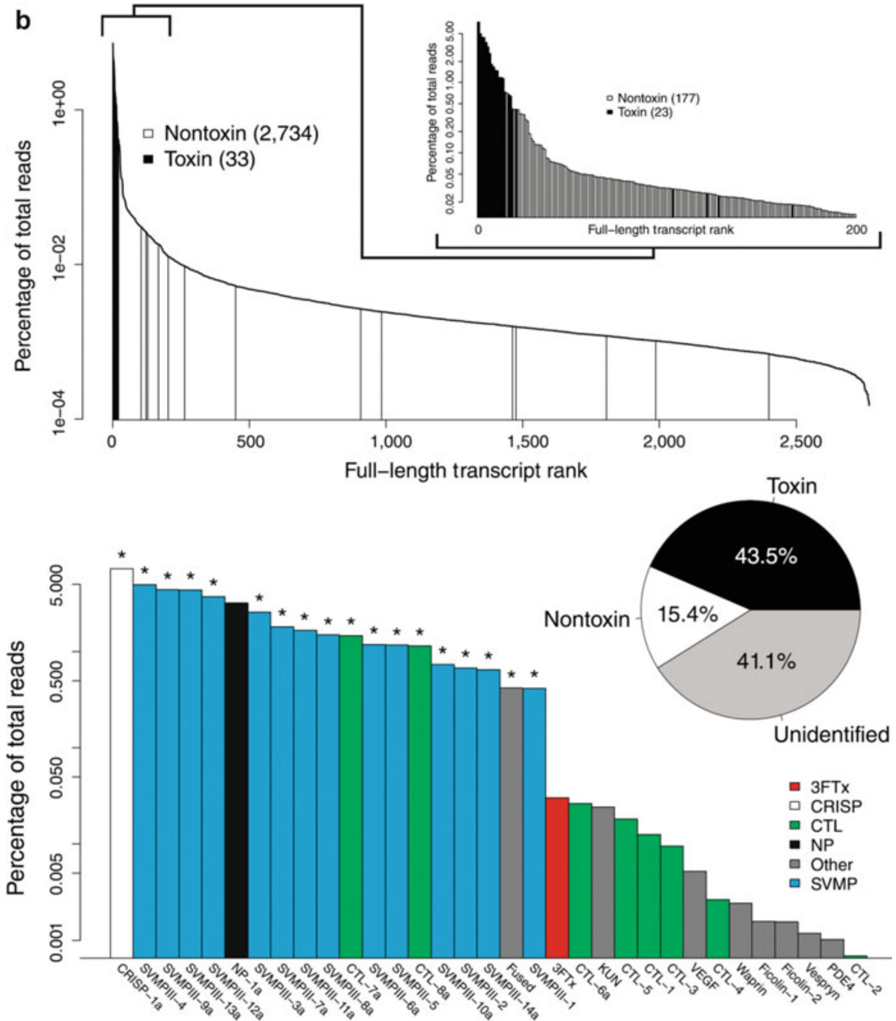

Fig. 4 Transcriptome of the venom gland of the brown tree snake (**a**) and of the desert night snake (**b**). The tree snake venom is rich in 3FTx transcripts, while the night snake transcriptome is dominated by (P-III) SVMP transcripts (Reproduced from McGivern et al. (2014))

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 effects). A combined genomic and transcriptomic approach will also allow for splicing variations to be identified. Splicing variations allow for different functional proteins to be transcribed using the same exons, and this may help lead to binding to different receptors. New exons may also be inserted into the gene. Three-finger toxins in rear-fanged snakes have been found to have extended N-terminal segments compared to elapid and viperid 3FTxs, and for denmotoxin, this is the result of a newly inserted exon two. Currently, the function of this longer N-terminal region is unclear, but this is currently the only full 3FTx gene sequence for a rear-fanged snake and provides insight into additional mechanisms of evolution of these toxins (Pawlak and Kini 2008).

High-Throughput Proteomic Approaches to the Study of Snake Venoms

Protein chemistry methodologies have been utilized to examine snake venoms very early in the history of modern venom biochemical research. However, recent advances in proteomic techniques and the utilization of mass spectrometry have greatly expanded the understanding of venom composition, allowing for the field of venom proteomics (venomics) to flourish. The term “snake venomics” (see Fig. 3) was developed by Calvete and coworkers and has been important as a standardized venom characterization protocol and a semiquantitative estimation of venom protein relative abundances (see Calvete 2013 for a review). By using absorbance at 215 nm during the primary RP-HPLC separation (which roughly correlates with the abundance of peptide bonds), a percentage value can be assigned to each chromatographic peak, and in combination with a densitometric lane scan of SDS-PAGE run using individual chromatographic peaks, the relative percentage of different venom components that make up the overall crude venom can be determined (Calvete et al. 2009). Standard venomic protocols involve chromatographic (usually RP-HPLC) and electrophoretic techniques to separate crude venom proteins which are then digested into peptides with proteolytic enzymes (most commonly trypsin). Individual peptide ions can also be fragmented by collision-induced dissociation, with the resulting daughter fragment ions identified by manual inspection. This approach identifies venom proteins based upon multiple lines of evidence, including molecular mass, peptide mass matching, several peptide sequence identifications, and determination of the number of cysteine residues present. These data typically allow for the identification of most toxin classes found in snake venoms (Calvete 2014). These methodologies, incorporated with

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 some of these venoms (Peichoto et al. 2012).

An alternative is the "break-then-sort" approach, where protein digestion is performed without any prefractionation/separation and peptides are separated by multidimensional chromatography followed by tandem MS analysis (Han et al. 2008). This technique is referred to as "shotgun proteomics." Both methods are heavily reliant on the high-throughput advances in mass spectrometry, allowing for the identification of multiple peptide fragments to assemble an overall complete venom profile.

"Shotgun" methods involve the production of small sequence fragments of a greater whole that are identified and then assembled into a larger picture. In the case of shotgun venom proteomics, overall venom composition is determined from the identity of the fragmented peptide ions after a whole venom protein digestion. This approach was used to resolve the proteome of the rear-fanged dog-faced water snake (*Cerberus rynchops*) and resulted in the identification of a novel snake venom protein family (veficolins) that is speculated to induce platelet aggregation and/or initiate complement activation (OmPraba et al. 2010). Shotgun proteomics offers an alternative to venomics and can be particularly useful for rear-fanged snake venoms because they are typically much less complex than Elapidae or Viperidae venom proteomes. A shotgun technique can also be used on individual venom proteins; once a venom protein is purified, it can be digested with several different proteases,

and the resulting peptide fragments from different digestion libraries can be assembled to resolve the complete amino acid sequence (Bandeira et al. 2007).

Shotgun proteomics and venomics both offer insight into the complete composition of rear-fanged snake venoms and are high-throughput and sensitive techniques that can be done using relatively little starting material. These methods can also allow for rapid de novo elucidation of primary structure (amino acid sequence and post-translational modifications) of single peptides in a complex mixture or peptides derived by in-solution or in-gel proteolysis of larger proteins. Relative abundances of venom protein families can be estimated using these techniques; however, it is more difficult to determine relative abundances with shotgun venom proteomics. Shotgun proteomics results can be strongly biased, with portions of abundant proteins being overrepresented in many spectra and low-abundance protein spectra not being seen at all (Bandeira et al. 2007; Calvete 2014). The increased complexity of the generated peptide mixture requires highly sensitive and efficient separation. On the other hand, with venomics, it is also possible that during RP-HPLC separation, before electrophoresis and MS/MS sequencing, highly hydrophobic and/or large proteins may elute poorly and be absent or underrepresented in abundance (based upon chromatographic peaks and electrophoretic results).

Both of the above methods rely on the identification of peptide masses/ionization patterns. Peptide mass fingerprinting and de novo MS/MS sequence determination methods are high throughput, less labor intensive, and more cost effective than N-terminal sequencing, but a limitation to the reliance on peptide masses is that certain combinations of amino acids can have indistinguishable masses, therefore creating ambiguity. An example of this is the assignment of isobaric (Ile/Leu) or quasi-isobaric residues (Lys/Gln or Phe/Met-ox), although methods such as high-resolution Fourier transform ion cyclotron resonance or Orbitrap mass analyzers can be used to discriminate between quasi-isobaric residues (Calvete 2013). It can also be difficult to identify correctly all peptide sequences with peptide mass fingerprinting, especially those with unexpected modifications or from proteins that are absent from databases. Identification based on shared peptide sequences in databases often does not allow differentiation between isoforms, and snake venoms can have multiple different isoforms present, each with potentially different pharmacological activities (Calvete 2014; Kini and Doley 2010; Mackessy 2010). Complete amino acid sequences for large, unknown proteins from bottom-up methods is also not possible due to the incomplete recovery of a full tryptic peptide set. Although tryptic digestion followed by LC-CID-MS/MS (liquid chromatography collision-induced dissociation tandem mass spectrometry) is ideal for the identification of toxin classes, it does not provide information about the quaternary structure of individual toxins or toxin activities. After individual venom proteins are cleaved into peptide fragments, they can no longer be used for follow-up biochemical or pharmacological assays. In recent years, venomics and shotgun venom proteomics have become highly sensitive techniques to provide information obtaining to overall venom composition for a snake species, but biochemical and pharmacological assays are needed for complete venom protein characterization.

High-Throughput Transcriptomic and Genomic Approaches to the Study of Snake Venoms

The majority of colubrid venom studies have focused on the protein composition and enzymatic properties of these venoms, with relatively few published venom gland transcriptomes or venom protein transcripts. Although venom compositional and biochemical studies can help to infer clinical symptoms of envenomation and the biological roles of these proteins, venom protein transcripts can also be used to derive venom composition and predict protein activity. Transcriptomic studies can provide a starting point for proteomic methods when crude venom material is lacking or of low venom yield, commonly an issue with rear-fanged venomous snakes. A venom protein transcript can be translated to acquire an entire protein amino acid sequence, and this allows for identification of protein superfamilies and functional protein domains. Obtaining toxin transcripts can also assist in the assembly and completion of protein sequences where trypsin digests or N-terminal sequencing provides only partial sequence. Transcripts also provide information about the evolutionary history of venom protein superfamilies and can be used for the reconstruction of ancestral sequences. One can then explore questions such as the origin of venom and the mechanisms responsible for venom evolution and adaptation (Casewell et al. 2012, 2013). Transcript sequences are needed for positive selection analysis within protein superfamilies, because to establish protein amino acid sites under positive selection, protein transcripts must be used to determine where single-nucleotide polymorphisms are occurring and are resulting in nonsynonymous mutations (Sunagar et al. 2013).

Transcriptomic and genomic methods offer many exciting opportunities for future studies, and the cost of next-generation DNA sequencing is becoming more affordable. With rear-fanged snakes comprising several Colubroidea families and subfamilies, many novel venom transcripts likely exist and remain unexplored. Colubrid transcriptomes and genomes offer the opportunity to identify novel venom protein families and scaffolds and provide insight into the evolutionary histories of ubiquitous venom protein families (Ching et al. 2006; Fry et al. 2012; OmPraba et al. 2010).

Currently, complete venom gland transcriptomes have only been published for *Philodryas olfersii* (Ching et al. 2006), *Cerberus rynchops* (OmPraba et al. 2010), *Thamnodynastes strigatus* (Ching et al. 2012), and *Boiga irregularis* and *Hypsiglena* sp. (McGivern et al. 2014), and some venom transcript sequences from *Dispholidus typus, Telescopus dhara, Trimorphodon biscutatus, Liophis miliaris, Liophis poecilogyrus, Leioheterodon madagascarensis, Psammophis mossambicus,* and *Rhabdophis tigrinus* are also available (Fry et al. 2012). The venom gland transcriptome from *Cerberus rynchops* revealed a novel venom protein family, ryncolin, that was the first discovered venom protein to exhibit sequence similarity to ficolin (a mammalian protein with collagen-like and fibrinogen-like domains) (OmPraba et al. 2010). The venom gland transcriptome from *Thamnodynastes strigatus* was found to be largely composed of matrix metalloproteinases, unrelated to the metalloproteinases found in other Colubroidea

snake families (Ching et al. 2012). This was the first time that matrix metalloproteinases were identified as a prominent venom component and were discovered to make up the majority of *T. strigatus* venom (both in abundance of transcripts and proteins) (Ching et al. 2012). A combined RNA-seq (ribonucleic acid sequencing, completed with the generation of complementary deoxyribonucleic acid libraries [cDNA]) and mass spectrometry analysis of venom glands and venoms from two species indicated that there are very different venom compositional "strategies" present among rear-fanged snakes, reminiscent of differences seen between elapid and viperid species (McGivern et al. 2014). Other protein families identified in an analysis of several rear-fanged snake venom glands include lipocalin, phospholipase A_2 (type IIE), vitelline membrane outer layer protein, and ribonucleases (Fry et al. 2012). Besides identifying novel venom protein families, rear-fanged snake venom gland transcriptomes have provided sequences that have helped resolved venom protein evolutionary histories, such as the evolution of C-type natriuretic peptides throughout Colubroidae (Ching et al. 2006).

The number of rear-fanged snake venom transcripts and genes will certainly increase as sequencing technologies have become more available and affordable. Next-generation sequencing (NGS) allows for multiple venom gland transcriptomes to be sequenced in parallel and removes the need for tedious *E. coli* cloning procedures (Durban et al. 2011). The majority of current rear-fanged snake venom gland transcriptomes have been constructed by first selecting mRNA from gland tissue (usually from a gland removed 3–4 days after venom extraction), generating cDNA libraries by reverse transcription, cloning these sequences with the use of plasmid vectors and transformed *E. coli*, and then randomly picking clones to be sequenced with chain-terminating Sanger sequencing technology. This methodology can introduce bias into a study since smaller cDNA fragments have higher transformation efficiency, or transcripts could be partially expressed in *E. coli* with lethal effects (Durban et al. 2011). There is also the chance of missing transcripts that occur in low abundance if not enough colonies are selected. NGS techniques remove these biases and create larger sequence assemblies.

Given that there is still extensive proteomic work to be done to determine protein families that compose rear-fanged snake venoms, care must be taken when identifying "venom protein" transcripts from a rear-fanged snake venom gland transcriptome without proteomic evidence. It is probable that some transcripts for endogenous cellular proteins that are not secreted from the gland, and do not serve as functional venom components, will be encountered. Several published rear-fanged snake transcriptomes have provided proteomic evidence to support the translation and secretion of identified venom protein transcripts with the use of two-dimensional gel electrophoresis and/or HPLC separation and mass spectrometric analysis (Ching et al. 2006, 2012; McGivern et al. 2014; OmPraba et al. 2010), and until there is a better understanding of the venom protein families occurring in rear-fanged snake venoms, this approach should be a standard practice.

Ancestral venom proteins had diverse activities and performed physiological roles in a variety of tissues (Fry 2005). Recent analyses have determined that toxin

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; Hargreaves et al. 2014). It has been suggested that these genes are coexpressed in a variety of tissues and then following gene duplications are restricted to expression in the venom gland after transcriptional regulation changes within other tissues. Multiple tissue transcriptomes are critical in order to understand the evolutionary history of venom gene superfamilies and the events following venom gene duplication, subfunctionalization, and neofunctionalization within these gene families, as well as how these processes influence venom protein adaptability. Future tissue transcriptomes will also help to provide insight into the origin and evolution of venom proteins, because nontoxin homologues are needed to construct venom gene trees (Casewell et al. 2012). There is a need to look at more tissues to explore differential expression of venom gene homologues in other tissues and the possibility of "reverse recruitment"; current studies are limited in that only a handful of venom gene families, tissues, and snake species have been analyzed (Casewell et al. 2012; Hargreaves et al. 2014; Reyes-Velasco et al. 2015; Junqueira-de-Azevedo et al. 2015). Venom evolution is a very complex and dynamic system, and research in this area has applications for studies involving other proteins that experience accelerated evolution and novel functionality gain.

It is still largely unknown what mechanisms are responsible for the regulation of venom genes. Promoter regions, transcription factors, methylation, as well as other mechanisms of gene regulation remain largely unexplored in venomous snake genomes. Techniques such as ChIP-Seq (chromatin immunoprecipitation sequencing) and RIP-seq (RNA immunoprecipitation sequencing) are possible future approaches to determine the regulatory proteins binding to DNA and RNA involved in the transcription and translation of venom genes and transcripts. Multiple tissue transcriptomes provide the ability to observe these differential expression patterns.

Changes to DNA sequence can directly affect gene products and influence the evolution of a protein. Although protein sequences can provide information regarding potential structure and function, the coding gene sequence (CDS) can reveal hidden single-nucleotide polymorphisms (SNPs) or elucidate molecular evolutionary patterns. Venom gene nucleotide polymorphisms can have significant impacts on venom proteins; a dinucleotide deletion in a 3FTx gene resulted in the loss of neurotoxic activity in the marbled sea snake (*Aipysurus eydouxii*) (Li et al. 2005).

Venom genes experience increased nucleotide substitution rates, especially within exon regions, as compared to other protein-coding genes (Doley et al. 2009). Venom protein genes have the flexibility to accumulate mutations at an increased rate due to the presence of multiple gene copies resulting from gene duplications and subfunctionalization. If one sequence develops a detrimental mutation, other copies of the gene remain present and functional. This mechanism also allows for venom gene neofunctionalization. Venom multigene families have been identified as evolving by this "birth-and-death" gene model (Fry et al. 2003b; Vonk et al. 2013). Venom proteins typically possess a stable structural core maintained by multiple disulfide bonds, and nonsynonymous nucleotide

substitutions that alter nonstructural, surface-exposed residues can change protein-targeting interactions, such as targeting new receptor types (Doley et al. 2009). This allows for a venom protein family to develop multiple activities, and having multiple gene products provides a selective advantage over the optimization of a single gene product by allowing for evolutionary "experimentation."

There have been several mechanisms proposed to explain accelerated gene neofunctionalization rates within venom gene superfamilies, including accelerated segment switching in exons to alter targeting (Doley et al. 2009) and rapid accumulations of point mutations in exposed residues (Sunagar et al. 2013). Transposable elements have also been shown to produce protein diversity. The python genome was found to have a large abundance of retroelements in comparison to its size (Castoe et al. 2013), especially LINEs (long interspersed elements). LINEs have been associated with creating protein diversity by carrying along genetic material from transposition events and therefore resulting in additional exon segments. Transposable elements can contribute to nonhomologous recombination, one of the mechanisms responsible for generating gene duplications.

Recently, the first snake genomes, the Burmese python (*Python molurus bivittatus*) and king cobra (*Ophiophagus hannah*), were completed (Castoe et al. 2013; Vonk et al. 2013). These snake genomes provide genomic scaffolds that will facilitate assemblies and annotation for other snake genomes. Genomic sequencing also provides potential full venom protein sequences. Databases with genomic sequences can be translated into all possible reading frames and matched to resulting peptide fragments obtained by tandem mass spectrometry. Many proteomic techniques will therefore be aided by the addition of complete snake genomes and in combination with venom gland transcriptomes, these genomes will provide overall insight into venom protein expression and evolution. A complete genome of a rear-fanged venomous snake is currently unavailable, but once finished, this genomic information will be accessible to compare venom multigene families within different snake families. For example, in the case of the rear-fanged snake *Boiga dendrophila*, the full gene sequence of the venom 3FTx denmotoxin was found to exhibit unique gene organization compared to 3FTx gene sequences found in Elapidae. Three-finger toxins are usually composed of three exons and two introns; however, denmotoxin was found to have an additional exon. Exon shuffling is a general mechanism for the creation of new genes (Pawlak and Kini 2008), and in the case of SVMPs, loss of exon segments has contributed to the evolution of the P-II and P-I classes.

Conclusion and Future Directions

The Promise of Venomics, Transcriptomics, and Genomics

With the advancements in venom extraction methods for rear-fanged venomous snakes and in proteomic and molecular techniques, it is now possible to complete full analyses on rear-fanged snake venoms. This work will not only allow us to

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 *Thamnodynases strigatus* indicated that the most abundant protein family consisted of a new kind of matrix metalloproteinase unrelated to the traditional SVMPs documented in all families of venomous snakes (Ching et al. 2012). This same study further identified the presence of a lactadherin-like factor V/VIII C-terminal domain as a part of the proteome, in addition to well-known venom protein families such as SVMPs, C-type lectins, and CRiSPs. Rear-fanged snake venoms contain many of the same venom protein families that are found in medically significant venomous snakes, such as Viperidae and Elapidae, and as more transcript sequences are acquired and matched to corresponding purified proteins, in combination with biochemical and pharmacological assays, recurring venom protein domains can begin to be better defined and associated more directly with specific activities and molecular mechanisms of action (Fig. 5).

Transcriptomics in combination with proteomics offers the ability to specifically identify the abundance of each venom protein, even between isoforms, from MS/MS peptide spectra and provides greater sequence coverage than what can be accomplished using only de novo sequence determination methods. Comparisons between proteomes and transcriptomes also provide insight into the translational regulation of venom proteins. This "omic" approach was used to discover that microRNAs could potentially explain ontogenetic translational regulation. MicroRNAs produced in rear-fanged venom glands remain unexplored, though microRNAs have begun to be examined in Elapidae and Viperidae (Durban et al. 2013; Vonk et al. 2013). Transcriptomes assembled from a variety of snake body tissues as well as venom gland tissue of rear-fanged snake species will provide venom protein and nonvenom homologue transcript sequences. These sequences can then be used to construct gene trees in order to reveal complete venom protein evolutionary histories and identify amino acid sites under positive selection.

Genomics in combination with transcriptomics allows for the exploration of transcriptional regulation seen within venom protein families and the unique evolution of venom protein genes. Genomics has revealed the dynamic evolution and adaptation of the venom system, such as the massive and rapid expansion of venom gene families that correlates with their functional importance in prey capture (Vonk et al. 2013). The expansion of venom protein gene families may occur in response to an evolutionary arms race between venomous snakes and their prey.

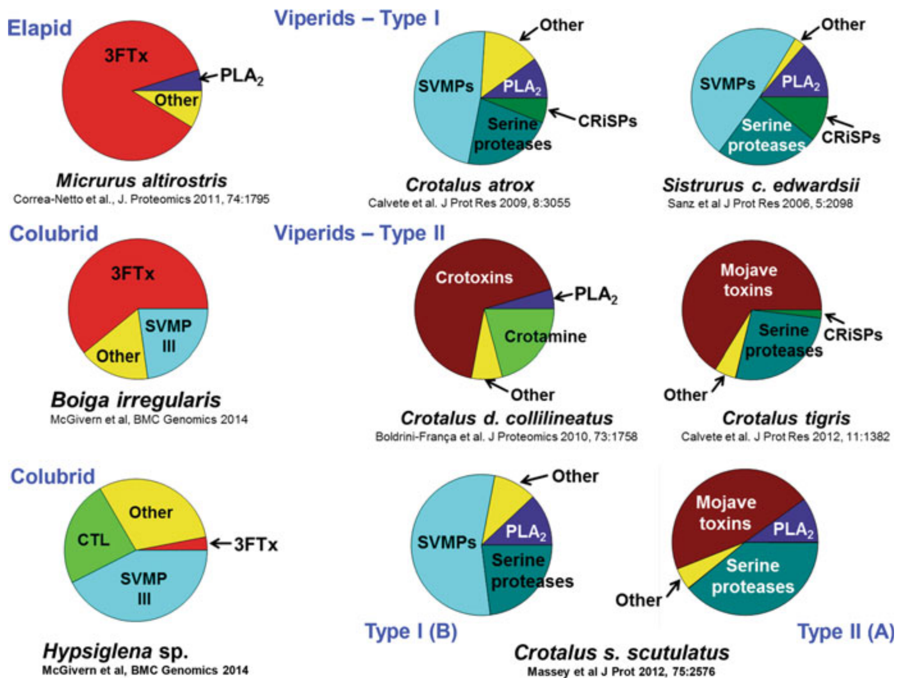

Fig. 5 Comparative proteomes and transcriptomes of elapid, colubrid, and viperid snakes. Note that potent toxins (3FTxs, crotoxins, Mojave toxins) dominate the venom profiles of the elapid, one colubrid (*Boiga*), and type II viperid venoms, while SVMPs dominate in venoms from the other colubrid (*Hypsiglena*) and type I viperid venoms. Pie charts were based on data in respective papers cited

Venom gene sequences are therefore ideal for studying accelerated patterns of evolution and the association between genotype and adaptive phenotypes.

The combination of proteomics, transcriptomics, and genomics in the study of rear-fanged snake venoms can provide a holistic approach to understanding venom protein evolution and regulation, which in turn impacts overall crude venom composition. However, it is also important to view the evolution and expression of rear-fanged venom proteins in terms of biological significance. Venom is a trophic adaptation, and as such, the presence and abundance of these proteins relates directly to prey capture and organism natural history. Without this biological perspective, proteomic, transcriptomic, and genomic approaches could simply generate a list of proteins, peptides, transcripts, and genes.

Forgotten Aspects of Understanding Venom Evolution

Snake venom prey-specific toxins provide an ideal model to study genotype-phenotype fitness interactions because the function of phenotypic variation (venom composition) can be related to the nature of the adaptation (prey preference

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 these toxins may prove useful for understanding diversification and evolution of important ion channels, such as the nicotinic acetylcholine receptor; based on selective toxicity of *B. irregularis* venom and irditoxin, there appears to have been some form of coevolutionary adjustment between predator armaments (venom) and prey susceptibilities (receptor binding) which imparts particular effectiveness against specific prey types (Mackessy et al. 2006; Pawlak et al. 2009). The differential activities of these and other toxins could be exploited for the development as molecular tools for dissecting receptor-ligand binding interactions, and they may provide clues for their exploitation as therapeutics.

Potential for Drug Development from Venom Proteins and Peptides

A number of toxins have proved to be excellent research tools to decipher the molecular details of physiological processes, and several have led to the development of novel therapeutic agents (Lewis 2009; Takacs and Nathan 2014). Captopril, the first successful venom-based drug, was developed from bradykinin-potentiating peptides from the venom of the lancehead viper (*Bothrops jararaca*) and is still on the market as an antihypertensive drug. Other venom-based drugs include tirofiban (aggrastat) and integrilin (eptifabatide) that were both designed from the structure of snake venom disintegrins (Saviola et al. 2014; Vonk et al. 2011; Calvete et al. 2010). There are many rear-fanged venomous snake species that have venoms yet to be studied, providing an untapped source of proteins with novel activities for therapeutic development (Saviola et al. 2014).

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 (*Bungarus multicinctus*), the knowledge of the distribution of nicotinic acetylcholine receptors and neurotransmitter communication wouldn't have advanced to its current state. Because venom proteins originated from ancestral proteins that served cellular physiological (housekeeping) roles and often have exceptionally high binding specificities, they are ideal candidates for unraveling cellular signaling pathways and the resulting disruption of these pathways. Rear-fanged snake venoms provide a largely unexplored source of toxins, and with recent advances in proteomic, transcriptomic, and genomic approaches, there should be an increase in future research focused on these venoms.

Cross-References

► Applications of Snake Toxins in Biomedicine
► Shotgun Approaches for Venom Analysis
► Squamate Reptile Genomics and Evolution

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Phylogenetics of Scorpions of Medical Importance

4

Adolfo Borges and Matthew R. Graham

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Abstract

This chapter assesses the phylogenetic relationships between scorpions and sodium channel-active scorpion toxins (NaScTx) of medical significance, almost entirely contained within the family Buthidae, with the exception of *Hemiscorpius lepturus* (Hemiscorpiidae). Within Buthidae, venom capable of severe and lethal scorpionism appears to have evolved multiple times among and within major morphological groups. Published mitochondrial sequence data from two markers (*COI* & 16S) were used to construct a partial maximum likelihood phylogeny for Buthidae. The resulting topology is largely congruent

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with results from comparative analysis of morphological data. Old World and New World buthids appear to be split, suggesting that some of the higher-level patterns in Buthidae can be explained by the breakup of Pangea. Provided that the venom composition should be more similar among closely related than distant species, the phylogeny can be used to predict which of the less dangerous species could also produce potent venoms. Clinical, phylogenetic, and toxinological evidence were also used to interpret the evolution and biogeography of these medically significant venomous taxa and the evolution of their toxic molecules. The existence of species-specific NaScTx repertoires in scorpions is probably the consequence of coevolution and arms races at the molecular and biochemical levels to overcome the ever-evolving structure of receptor sites (including sodium channels) in their predators and preys.

Introduction

Scorpions are an ancient and widespread group, mostly known for the extreme toxicity of some species to humans. The approximately 2,140 extant (Recent) scorpion species are contained within 14–18 families (see "Phylogeny of Buthidae" section), although Buthidae is the largest and most widely distributed family, with ~1,023 spp. (Fig. 1a). Scorpions of medical importance are considered hereby as those with a sting that produces severe or lethal envenomation in humans. Table 1 presents an updated list of the world's scorpion species positively identified in the literature as responsible for severe/lethal scorpionism (based only on reports including taxonomical verification), together with their range of distribution and clinical manifestations. The majority of medically relevant species belong to family Buthidae, the only exception being the Indo-Arabian *Hemiscorpius lepturus* (Hemiscorpiidae). Envenomations by the latter are generally characterized by coagulation disorders and local necrosis, which markedly differ from the neurotoxic and cardiotoxic symptoms characteristic of buthids. The 45 buthid taxa identified in Table 1 responsible for severe/lethal scorpionism comprise less than 8% of recognized species in this family and represent groups with both Old World and New World origins (see section "Toxinological Diversity in Buthidae: Old World Versus New World"). The broad range of genera containing these noxious species, however, explains the distribution of areas with the highest incidence of scorpionism in the world. In particular, many regions in Central and South America, the Middle East, Asia, and northern and southern Africa harbor species that are responsible for significant morbidity and pediatric mortality (Fig. 1b and Table 1).

The number of noxious genera ($n = 6$) is higher in the Old World (Asia, Africa, Europe), whereas the number of toxic species ($n = 30$) is higher in the New World (the Americas) (Table 1), a trend that applies to the distribution of scorpion diversity in general (Nenilin and Fet 1992). In the Old World, buthid genera containing medically relevant species include *Androctonus* (northern Africa, Middle East), *Leiurus* (northern Africa up to Algeria, Middle East), *Buthus* (Mediterranean basin including northern Africa and southern Europe, in Africa

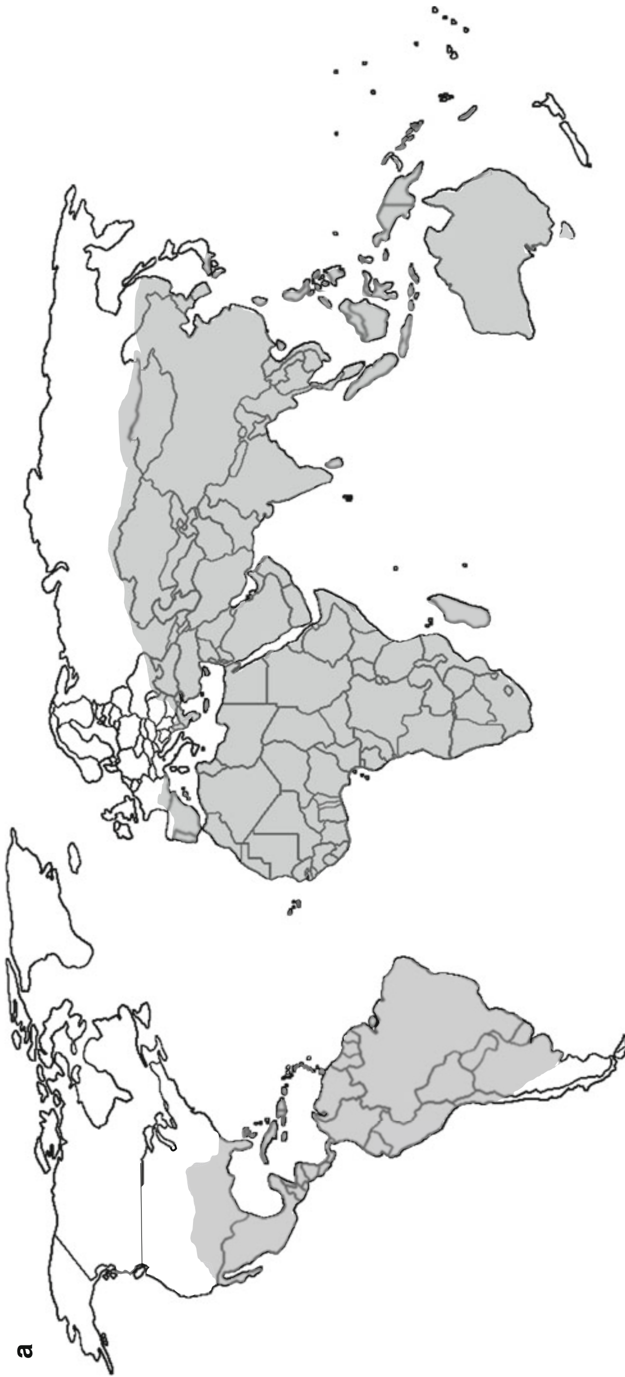

Fig. 1 (continued)

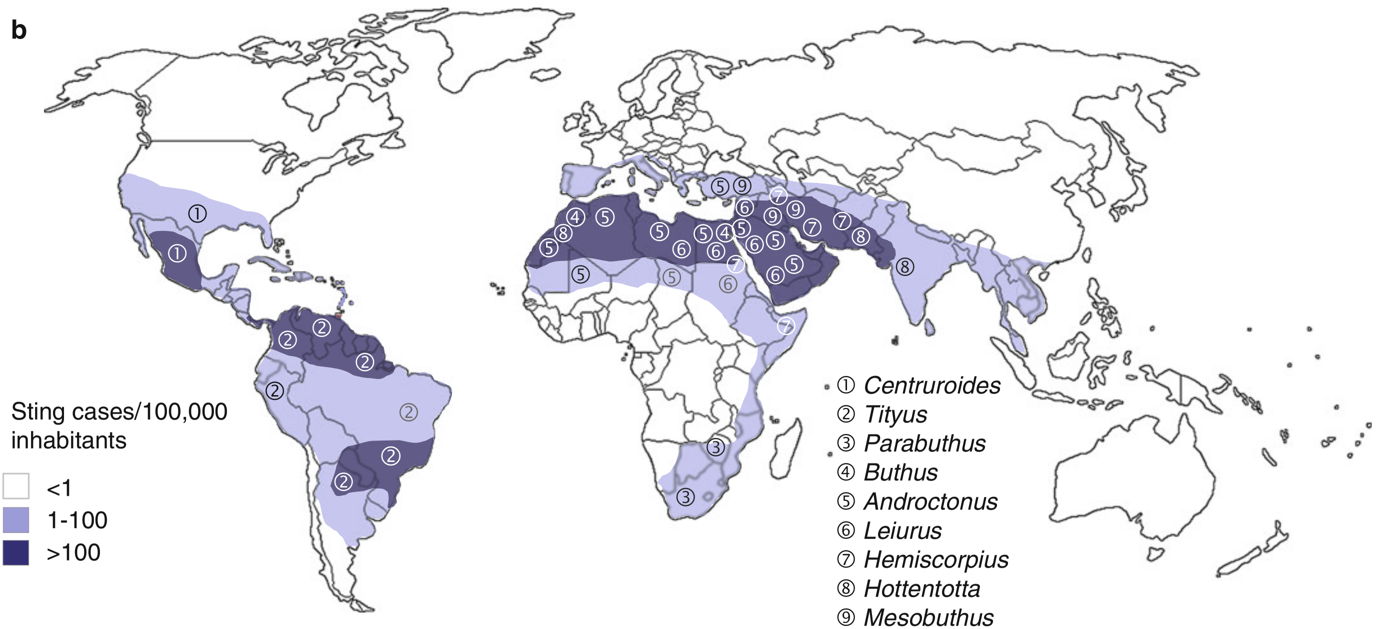

Fig. 1 (**a**) Worldwide distribution of the family Buthidae. (**b**) Areas of incidence of scorpionism in the world (based on Chippaux and Goyffon 2008), together with the distribution of medically significant buthid genera and including genus *Hemiscorpius* (family Hemiscorpiidae)

Table 1 List of scorpion species documented as medically significant

Species	Distribution	Manifestations in severe scorpionism	References
Androctonus australis	Chad, Egypt, Libya, Mauritania, Somalia, Sudan, Tunisia, India, Israel, Pakistan, Saudi Arabia, Yemen	Encephalopathy and cardiovascular collapse	Amitai (2005)
Androctonus bicolor	Algeria, Egypt, Eritrea, Libya, Morocco, Tunisia, Israel, Jordan, Syria	Tachycardia and respiratory distress	Amitai (2005)
Androctonus crassicauda	Armenia, Azerbaijan, Bahrain, Egypt, Iran, Iraq, Israel, Jordan, Kuwait, Oman, Saudi Arabia, Syria, Turkey, United Arab Emirates, Yemen	Parasympathetic and sympathetic manifestations, characterized by pain, hyperemia and edema, sinus tachycardia, pallor and cold extremities, nausea, vomiting, and restlessness	Amitai (2005)
Androctonus mauritanicus	Mauritania, Morocco	Not described but fatalities reported from Morocco	Amitai (2005)
Buthus occitanus complex	Algeria, Burkina Faso, Djibouti, Egypt, Ethiopia, Gambia, Gabon, Guinea-Bissau, Libya, Mauritania, Morocco, Nigeria, Senegal, Somalia, Sudan, Tunisia, Cyprus, Iraq, Israel, Jordan, Lebanon, France (southern), Greece, Portugal, Spain	Priapism, restlessness, vomiting, siderosis, abdominal pain and/or hyperdistention, tachycardia, polypnea/dyspnea, cyanosis, arterial hypotension, bradycardia	Amitai (2005)
Centruroides infamatus	Mexico (States of Aguascalientes, Colima, Durango, Guanajuato, Jalisco, Michoacán, Nayarit, Oaxaca, Puebla, Sinaloa, Veracruz, and Zacatecas)	Heart failure, pulmonary edema, circulatory shock, convulsions	Chávez-Haro and Ortiz (2014)
Centruroides limpidus	Mexico (States of Guerrero, México, Michoacán, Morelos, Querétaro, Puebla)	*Globus pharyngeus*, blurry vision, temporary blindness, nystagmus, dysarthria, muscle ataxia, abdominal distension, opisthotonos, convulsions, and priapism	Chávez-Haro and Ortiz (2014)
Centruroides noxius	Mexico (Nayarit state)	*Globus pharyngeus*, blurry vision, temporary blindness, nystagmus, dysarthria, muscle ataxia, abdominal distension, opisthotonos, convulsions, and priapism	Chávez-Haro and Ortiz (2014)
Centruroides sculpturatus[a]	USA (States of Arizona, California, Nevada, New Mexico); Mexico (Sonora)	Neurotoxic, predominantly cholinergic manifestations. Cranial nerve abnormalities, neuromuscular hyperactivity, and dysautonomias	Skolnik and Ewald (2013)

(*continued*)

Table 1 (continued)

Species	Distribution	Manifestations in severe scorpionism	References
Centruroides suffusus	Mexico (Durango state)	*Globus pharyngeus*, blurry vision, temporary blindness, nystagmus, dysarthria, muscle ataxia, abdominal distension, opisthotonos, convulsions, and priapism	Chávez-Haro and Ortiz (2014)
Centruroides tecomanus	Mexico (States of Colima, Michoacán)	*Globus pharyngeus*, blurry vision, temporary blindness, nystagmus, dysarthria, muscle ataxia, abdominal distension, opisthotonos, convulsions, and priapism	Chávez-Haro and Ortiz (2014)
Centruroides villegasi	Mexico (Guerrero state)	Not described	Chávez-Haro and Ortiz (2014)
Grosphus palpator[b]	Madagascar (Tananarive province)	Neurotoxic (predominantly cholinergic), *globus pharyngeus*, apnea	Bergman (1997)
Hemiscorpius lepturus	Iran (Khuzestan province), Iraq, Pakistan, Yemen	Severe and fatal hemolysis, secondary renal failure, deep and necrotic ulcers, ankylosis of the joints, thrombosis, cardiovascular failure	Jalali et al. (2010)
Hottentotta franzwerneri	Morocco	Not described but fatalities reported from Morocco	Amitai (2005)
Hottentotta tamulus	India, Pakistan	Myocarditis, pulmonary edema, but no raised circulating catecholamines	Strong et al. (2014)
Leiurus hebraeus	Israel, Jordan, Lebanon, Syria, Saudi Arabia, Yemen	Sympathetic manifestations including tachycardia, hypertension, ventricular arrhythmia as well as parasympathetic manifestations, including priapism, hypersalivation, and muscular twitching	Amitai (2005)
Leiurus quinquestriatus	Algeria, Chad, Egypt, Ethiopia, Libya, Mali, Niger, Somalia, Sudan, Tunisia	Encephalopathy, pulmonary edema, seizures, and clinical features suggestive of myocarditis, e.g., heart failure, cyanosis, cardiogenic shock, and dysrhythmia	Amitai (2005)

Mesobuthus eupeus	Afghanistan, Turkey, Armenia, Azerbaijan, China, Georgia, Iran, Iraq, Kazakstan, Kyrgyzstan, Mongolia, Pakistan, Syria, Tajikistan, Turkmenistan, Uzbekistan, Russia (Astrakhan region)	Autonomic manifestations including hyperemia and edema	Amitai (2005)
Mesobuthus gibbosus	Cyprus, Syria, Turkey, Albania, Greece, Macedonia, Montenegro	Abdominal pain, muscle contractions, nausea, hypertension, hypotension, bradycardia, dyspnea, pulmonary edema, convulsion, and shock	Amitai (2005)
Parabuthus granulatus	Angola, Botswana, Namibia, South Africa	Predominantly sympathetic manifestations, respiratory distress hypertension, and pulmonary edema	Bergman (1997), Müller et al. (2011)
Parabuthus transvaalicus	Botswana, Mozambique, South Africa, Zimbabwe	Predominantly parasympathetic manifestations, with neuromuscular and cardiac alterations; profuse sialorrhea	Bergman (1997), Müller et al. (2011)
Tityus asthenes	Northern Peru, Ecuador (*cis*- and *trans*-Andean distribution), Pacific coast of Colombia, and Panama	Cardiopulmonary complications, respiratory distress (including tachypnea), vomits, edematous, or hemorrhagic acute pancreatitis; fatalities reported from Panama and Colombia, also probably from Ecuador	Borges et al. (2012), Otero et al. (2004)
Tityus bahiensis	South-eastern Brazil, Argentina (Misiones, Corrientes, and Santa Fé provinces)	Predominantly autonomic manifestations, including vomiting, agitation, sweating, dyspnea, bradycardia, tachycardia, tachypnea, somnolence/lethargy, cutaneous paleness, hypothermia, and hypotension. Manifestations less severe than in the case of *T. serrulatus*	Pucca et al. (2014)
Tityus breweri	Venezuela (Bolívar state)	Sympathetic and parasympathetic manifestations comprising tachycardia, arrhythmia, respiratory complications including tachypnea, and pancreatic alterations; muscle fasciculations	Borges et al. (2010b)
Tityus caripitensis	Venezuela (Monagas state)	Sympathetic and parasympathetic manifestations comprising tachycardia, arrhythmia, respiratory complications including tachypnea, and pancreatic alterations	Borges and De Sousa (2006)

(*continued*)

Table 1 (continued)

Species	Distribution	Manifestations in severe scorpionism	References
Tityus cerroazul	Panama (Coclé and Panamá provinces), Costa Rica (Limón province)	Not described but at least one death reported from Panama	Borges et al. (2012)
Tityus championi	Costa Rica (Puntarenas province), Panama (Chiriquí province)	Not described but at least one death reported from Costa Rica	Borges et al. (2012)
Tityus confluens	Argentina (Corrientes, Santa Fé, La Rioja, Córdoba, Tucumán, Salta, and Jujuy provinces)	Not described but several deaths reported from Argentina	de Roodt et al. (2009)
Tityus discrepans	Venezuela (Capital District, Miranda and Aragua states)	Neurotoxic, mainly cholinergic, including gastrointestinal manifestations, with scarce cardiopulmonary alterations	Borges and De Sousa (2006)
Tityus falconensis	Venezuela (Falcón and Lara states)	Diaphoresis, vomits, arterial hypertension, irritability, tachycardia, myocarditis, but no alterations of blood glucose or amylase levels	Borges and De Sousa (2006)
Tityus festae	Eastern Panama, Northern Colombia	Not described but at least one death reported from Panama	Borges et al. (2012)
Tityus isabelceciliae	Venezuela (Capital District)	Not described but fatalities reported	Borges and De Sousa (2006)
Tityus metuendus	Amazonian regions of Peru, Brazil, Bolivia	Not described but at least one death reported from Peru	Lourenço (1997)
Tityus neoespartanus	Venezuela (Margarita Island)	Adrenergic and cholinergic manifestations comprising tachycardia, arrhythmia, respiratory complications including tachypnea, and pancreatic alterations	De Sousa et al. (2007)
Tityus nororientalis	Venezuela (Sucre, Monagas and Anzoátegui states)	Adrenergic and cholinergic manifestations comprising tachycardia, arrhythmia, respiratory complications including tachypnea, and pancreatic alterations	Borges and De Sousa (2006)
Tityus obscurus	Brazil (Amapá, Pará, and Amazonas states), French Guiana, Suriname	Mainly central neurotoxicity, including general paresthesia, ataxia, dysarthria, myoclonus, and dysmetria	Pardal et al. (2014)

Tityus pachyurus	Costa Rica (Limón Province), central and western Panama, Colombia (Antioquia, Boyacá, Caldas, Cundinamarca, Huila, and Tolima departments)	Pulmonary edema, hypertension, heart arrest associated with ventricular tachycardia	Borges et al. (2012), Otero et al. (2004)
Tityus perijanensis	Western Venezuela (Zulia State), Northeastern Colombia	Cardiopulmonary manifestations and abdominal distress; central neurotoxicity	Borges and De Sousa (2006)
Tityus sabineae	Colombia (Caldas, Boyacá, and Cundinamarca departments)	Not described but at least one death reported	Lourenço (2000)
Tityus serrulatus	Southeastern Brazil	Predominantly autonomic manifestations, including vomiting, agitation, sweating, dyspnea, bradycardia, tachycardia, tachypnea, somnolence/lethargy, cutaneous paleness, hypothermia and hypotension, pulmonary edema. Manifestations like severe hypertension, seizures, priapism, and coma are less prevalent	Pucca et al. (2014)
Tityus stigmurus	Northeastern Brazil	Vomiting, nausea, abdominal pain, sialorrhea, neurological disorders (tremor, agitation, dizziness, difficulty in walking, contracture, blurred vision, pallor, and somnolence), cardiovascular disorders (tachycardia, hypertension, and hypotension), and breathing disorders (dyspnea). Manifestations less severe that in the case of *T. serrulatus*	Pucca et al. (2014)
Tityus trinitatis	Trinidad and Tobago	Neurotoxic, mainly cholinergic, including acute hemorrhagic pancreatitis and myocarditis	Borges (2014)
Tityus trivittatus	Argentina (Tucumán, Santa Fé, Catamarca, Santiago del Estero, San Juan, Mendoza, Formosa, Chaco, Entre Ríos, Buenos Aires, Corrientes, Córdoba, Misiones, La Rioja provinces)	Adrenergic hyperstimulation. In severe cases: shock, congestive cardiac insufficiency and arrhythmia, pulmonary edema, bradycardia, bradypnea, apnea, and coma	de Roodt (2014)
Tityus zulianus	Western Venezuela (south of Lake Maracaibo)	Cardiopulmonary manifestations as a result of massive adrenergic stimulation, pulmonary edema	Borges and De Sousa (2006)

[a] *C. sculpturatus* (range southern US and northern Mexico), synonymized with *C. exilicauda* (range Baja California), differs from the latter genetically and toxinologically (Fet et al. 2005)

[b] The *Grosphus* species associated with the accident has not been verified

from Senegal to Ethiopia), *Mesobuthus* (in Asia, from Lebanon to Korea; in the eastern Mediterranean basin), *Hottentotta* (throughout Africa; in Asia, from Lebanon to China, including the Arabian peninsula), and *Parabuthus* (southern and eastern Africa, the Arabian peninsula). In the New World, all reported scorpionism cases are due to envenomation by *Centruroides* (North, Central and South America, and the Caribbean) and *Tityus* (from northern Costa Rica to northern Argentina, including the Caribbean area) species (Fig. 1b). Clinical, phylogenetic, and toxinological evidence continue to elucidate the evolution and biogeography of these medically significant venomous taxa and the evolution of their toxic molecules.

Phylogeny of Buthidae

Higher-level scorpion systematics has recently experienced extensive revisions and heated controversy, with different authors proposing taxonomies with between 14 and 18 families (Prendini and Wheeler 2005; Soleglad and Fet 2003). Despite the conflicting taxonomic viewpoints, family Buthidae has remained relatively stable and contains all medically significant species except *Hemiscorpius lepturus* (family Hemiscorpiidae). A cladistic analysis of Buthidae, based on the relative position of the d_3 trichobothrium and DM_c carina on the pedipalp patella, suggests six main groups of buthid scorpions: *Buthus* group, *Ananteris* group, *Isometrus* group, *Charmus* group, *Uroplectes* group, and the *Tityus* group (Fet et al. 2005). Two main evolutionary lineages are represented by this approach: (1) the *Buthus* group, containing 39 predominantly arid-adapted Palearctic genera (especially from North Africa and Middle East), including all Old World noxious taxa (*Androctonus*, *Buthus*, *Leiurus*, *Hottentotta*), and (2) a second clade encompassing 43 predominantly Afrotropical genera, separated into the five remaining groups, which includes a few Oriental and Australian genus-level endemics (*Lychas*, *Isometrus*) and a separate Neotropical clade of nine genera, including all noxious taxa in the New World (*Centruroides*, *Tityus*) (Fet et al. 2005). The monophyly of the New World buthid clade has been confirmed by several lines of evidence, including the anatomy of book lungs and ovary uterine structure (Kamenz and Prendini 2008; Volschenk et al. 2008), molecular phylogenetics (Soleglad and Fet 2003), and toxinological data (Rodríguez de la Vega and Possani 2005). The latter have indicated that toxic peptides produced by New World scorpions, at least within the group of toxins altering voltage-gated sodium channel activation (β-NaScTx, see section "Physiopathology and Molecular Diversity in Scorpion Toxins"), belong to a different evolutionary lineage than Old World scorpions, indicating that toxinological divergence parallels results from the cladistic analysis. Thus, within Buthidae, severe/lethal scorpionism is not restricted to a single group but appears to have evolved multiple times among and within major buthid groups.

To examine the relationships among medically significant buthids, published mitochondrial sequence data from two markers (*COI* & 16S) gleaned from GenBank were used to construct a partial maximum likelihood (ML) phylogeny for Buthidae (Fig. 2). Data were available for 114 buthid taxa and the phylogeny

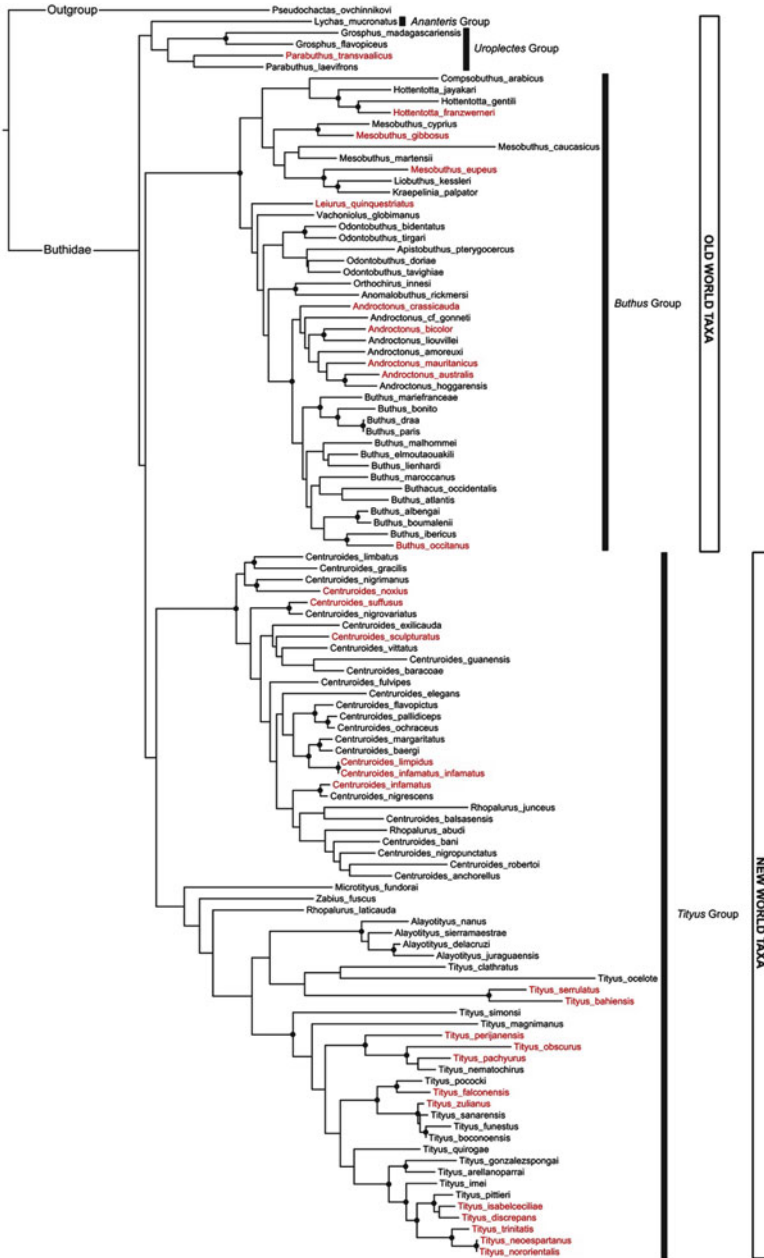

Fig. 2 Partial maximum likelihood (ML) phylogeny of Buthidae based on concatenated mitochondrial (*COI* & 16S) sequence data. Species considered to be medically significant are in *red*. *Black* dots indicate strongly supported nodes (bootstrap ≥ 70). *Black* bars represent morphological groupings (Fet et al. 2005). The ML phylogeny was generated using RAxML (Stamatakis 2006)

was rooted with *Pseudochactas ovchinnikovi*, a unique taxon currently considered to represent the sister group of Buthidae (Prendini et al. 2006). Although many nodes across the phylogeny are not strongly supported, especially at higher levels likely due to genetic saturation, the ML topology is congruent with results from comparative analysis of morphological data (Fet et al. 2005); the *Ananteris*, *Buthus*, *Tityus*, and *Uroplectes* groups are all monophyletic, although with varying degrees of support. Furthermore, Old World and New World buthids appear to be split, suggesting that some of the higher-level patterns in Buthidae can be explained by the breakup of Pangea.

Of the Old World taxa, all of the medically significant taxa except *Parabuthus transvaalicus* are members of the *Buthus* group. Within the group, species responsible for severe/lethal scorpionism are not monophyletic and are instead scattered across the phylogeny. Interestingly, medically significant species do not form monophyletic groups within *Mesobuthus* and *Androctonus* and are instead polyphyletic with respect to less dangerous congenerics. A similar trend occurs among New World taxa, of which only *Centruroides* and *Tityus* contain medically significant species. However, assuming that there is some phylogenetic signal to venom toxicity in scorpions, the Buthidae phylogeny can be used to make some important predictions regarding buthids not considered medically significant. In other words, if the composition of venoms is more similar among closely related than distantly related species, then the phylogeny can be used to predict which of the less dangerous species could also potentially possess potent venoms. Using this reasoning, envenomation by Old World *Mesobuthus cyprius* and *Buthus ibericus* should be taken seriously as both are strongly supported as sister to medically significant species. Of the New World taxa, *Centruroides nigrovariatus*, *C. nigrescens*, *Tityus nematochirus*, *T. pococki*, *T. sanarensis*, *T. funestus*, *T. boconoensis*, and *T. pittieri* are potentially dangerous as they are closely related to medically significant sister taxa.

Although many buthid genera are not represented in the above phylogeny, medically significant species are clearly the result of many independent evolutionary events that lead to venom capable of severe/lethal scorpionism. Sampling of additional taxa, as well as new genomics tools, will undoubtedly resolve some of the higher-level relationships among buthid taxa and further precipitate the understanding of relationships among medically important species.

Physiopathology and Molecular Diversity in Scorpion Toxins

Exacerbation of presynaptic neurotransmission is the main mechanism employed by buthid scorpions to subdue prey or deter predators by using toxins that affect voltage-gated sodium (Nav) and potassium channels (NaScTx and KScTx, respectively). These low-molecular-mass (3–8 kDa) proteins, collectively known as the CSα/β superfamily (cysteine-stabilized α/β scaffold, containing 3–8 cysteine residues forming three or four intramolecular disulfide bridges), act either by altering the channel's gating mechanism or blocking its selectivity filter (Rodríguez de la Vega et al. 2013). Considering that the Nav-active toxins (NaScTx) are responsible

for the most dangerous neurotoxic effects observed during human envenomation (Guerrero-Vargas et al. 2012), those scorpions producing the highest abundance and/or more active NaScTx variants toward skeletal/cardiac muscle and neuronal Nav isoforms are expected to produce the most toxic venoms against mammals. In fact, less toxic species within the Neotropical genus *Centruroides*, such as *C. margaritatus*, appear to produce venoms richer in potassium channel-active toxins (KScTx) and pore-forming (antimicrobial) peptides than their toxic congenerics from the Mexican Pacific versant (reviewed in Borges 2015). In the case of the Old World genus *Parabuthus*, it was originally assumed that all 22 species endemic to southern Africa were equally noxious, but a differential toxicity is observed both experimentally and clinically, with *P. granulatus* being the most toxic, followed by *P. transvaalicus* and more distantly by *P. capensis*, *P. raudus*, *P. villosus*, *P. kalaharicus*, and *P. schlechteri* (Müller et al. 2011). Significantly, mass spectral evidence indicates that *P. granulatus* venom only contains peptides in the NaScTx molecular mass range, whereas other *Parabuthus* species are richer in KScTx peptides (Dyason et al. 2002). A similar situation probably occurs in other genera such as the speciose genus *Tityus*, where envenomation by *T.* (*Atreus*) *brazilae*, *T.* (*Tityus*) *neglectus* (northern Brazil), *T.* (*Tityus*) *obtusus* (Puerto Rico), *T.* (*Archaeotityus*) *mattogrossensis*, and *T.* (*Tityus*) *adrianoi* only produces local symptomatology (Borges 2014; Pucca et al. 2014), whereas syntopic *Tityus* spp. from northern and southern South America can be life-threatening (see Table 1). Thus, mammalian toxicity of scorpion venoms is directly related to NaScTxs content.

Notwithstanding the fact that scorpion venom toxicity is the result of several variables including the age of the victim and the amount and delivery route of injected venom, differences in the toxin repertoires expressed by individual species are evident from the differential symptomatology elicited in envenomed humans. *Leiurus hebraeus*, which is the main problem species in Israel, exerts both neurotoxicity and cardiotoxicity. Myocarditis and pulmonary edema have been frequently reported from India following envenomation by *Hottentotta tamulus.* In Tunisia, severe envenomation has been caused by *Androctonus amoreuxi* and *Androctonus australis* resulting in encephalopathy and cardiovascular collapse. The Arizona bark scorpion, *Centruroides sculpturatus,* is mainly neurotoxic, of a predominantly cholinergic nature (Amitai 2005). As mentioned above, the clinical picture of envenomation by *Tityus*, the most diverse scorpion genus, is dependent on the species involved. *T. serrulatus*, from the Brazilian southeast, elicits autonomic manifestations, whereas envenomation by *T. obscurus*, in the Amazon basin including French Guiana, involves mainly central neurotoxicity, including general paresthesia, ataxia, dysarthria, myoclonus, and dysmetria (Pardal et al. 2014). In addition, envenomation by *T. discrepans* in northcentral Venezuela is characterized by gastrointestinal manifestations, contrasting with the predominantly cardiopulmonary symptomatology caused by *T. zulianus* in the Mérida Andes (Borges et al. 2010b). In the southern African genus *Parabuthus*, envenomation by *P. transvaalicus* elicits mainly cholinergic manifestations, whereas stings by *P. granosus* produces predominantly sympathetic manifestations (Bergman 1997; Table 1).

Underlying the generation of such different physiopathological effects is the fact that NaScTxs alter Nav gating either affecting the channel threshold of activation (β-toxins) or the inactivation process (α-toxins). Furthermore, β- and α-toxins, which differ in concentration in crude venoms among species and even among individuals, exhibit marked selectivity for different Nav isoforms (Gilles et al. 2000; Leipold et al. 2006). Despite the general similarity among vertebrate Nav subtypes (currently nine isoforms are known), these are expressed differentially depending on the tissue and cell type and their receptor-binding sites for NaScTxs vary (Gurevitz et al. 2014). There is evidence suggesting that minor changes in NaScTx primary structure promote dramatic changes in their pharmacological properties. For instance, the 64-residue-long NaScTx Tz1 from *Tityus zulianus* is 92% identical to toxin Tc48b from *T. obscurus* but for only five surface-located residues; however, Tz1 acts as a β-toxin and Tc48b as an α-toxin (Borges et al. 2004; Murgia et al. 2004). Since α- and β-toxins differ in their ability to promote in vivo release of different amounts and types of neurotransmitters (Vasconcelos et al. 2005), differences in their abundance in crude venoms can have physiopathological implications. Even toxins produced by phylogenetically related species can exhibit different profiles of Nav modification. Thus, toxin Ts1 from *Tityus serrulatus* produces both an excitatory (shifting of activation threshold) and depressant (Na^+ current blockade) effect on the Nav1.4 (skeletal muscle-specific) isoform (Leipold et al. 2007). In contrast, toxin Tt1g from *Tityus trivittatus* (95% identical to Ts1, differing in three surface-located residues) only produces a depressant effect on the same isoform, not affecting the channel's threshold potential (Coronas et al. 2014). Accordingly, predicted surface accessibility of hypermutable sites in α- and β-NaScTxs suggests that most point mutations, via positive selection, are located in the molecular surface and the loops connecting secondary structure elements, areas involved in receptor recognition and antibody binding. The rapid accumulation of non-synonymous replacements in exposed residues has therefore been suggested to play a significant role in the neofunctionalization of these toxins through variations in their surface chemistry (Sunagar et al. 2013).

As seen in other venomous taxa (Casewell et al. 2012), such species-specific toxin repertoires in scorpions are probably the consequence of coevolution and arms races at the molecular and biochemical levels to overcome the ever-evolving structure of receptor sites (including Navs) in predators and preys of scorpions. For example, alternative splicing in insects renders more Nav variants (from a single Nav background) in comparison with their vertebrate counterparts, producing a broader spectrum of both drastic and subtle differences in channel expression and gating properties (e.g., 69 Nav splice variants in *Blattella germanica* (Orthoptera) versus the nine vertebrate Nav isoforms) (Dong et al. 2014). In one such arms race, there are also examples of counter selection in predators to elude the scorpion venom effect: the Nav1.8 variant involved in nociception in the southern US desert mouse *Onychomys torridus* contains mutations that render this channel susceptible of inhibition by the NaScTxs of its prey, the North American scorpion *Centruroides sculpturatus*. While scorpion peptides that target Nav channels typically activate the channel (β-NaScTxs) or block inactivation (α-NaScTxs), prolonging channel

activity and increasing neuron excitability, *C. sculpturatus* NaScTxs inhibit *O. torridus* Nav1.8 Na^+ current and decrease neuron excitability, blocking neuronal signaling and inducing analgesia instead of pain (Rowe et al. 2013).

Vicariance and Scorpion Venom Evolution

The origins of higher-level diversity among scorpions are often attributed to vicariance due to the fragmentation of Pangea and subsequent continental drift (e.g., Soleglad and Fet 2003; Lourenço 1996), although new studies suggest that Recent scorpions diversified with the fragmentation of Gondwana ca. 180–165 Myr (Sharma and Wheeler 2014). Under either scenario, vicariance has likely been the predominant force driving venom evolution in scorpions through the emergence of different toxin repertoires, as suggested by recent work on the Chinese buthid *Lychas mucronatus* (Ruiming et al. 2010). The venom composition of *L. mucronatus* from Hainan and Yunnan provinces, 1,000 km apart, was evaluated at the transcriptome level. Although no significant difference was observed in the abundance of α- and β-NaScTx types, toxins from each population differed in their primary structures. Based on primary structures and cysteine pairing, KScTxs, which alter the gating and/or block the various K^+ channel selectivity filters, are classified into four subfamilies: α, β, γ, and κ (Bergeron and Bingham 2012). α-KScTxs in *L. mucronatus* from Yunnan were twofold more abundant than in the Hainan-sourced population and possessed more diverse primary structures: the most abundant α-type from Yunnan (GT028663) has no homologue in the Hainan population. Also, transcripts encoding antimicrobial (pore-forming) peptides accounted for 21% of all toxin-like peptides in the Yunnan population, whereas their abundance reached 40% in the Hainan population (Ruiming et al. 2010).

The forces driving these intraspecific toxinological differences in *L. mucronatus* are not known at present, nor for any other scorpion species. However, divergence of toxins from one species to the next, or even among populations of the same species, suggests that venoms are sensitive to environmental selection. The interrelations between natural selection and the genetic and molecular processes responsible for generating variation in toxins have been most extensively studied in snakes and gastropods, where diet is a crucial component, but have not yet been elucidated in scorpions (Casewell et al. 2012). Studies of the gastropod genus *Conus* suggest that venom peptide genes rapidly diversify because genes whose products act on other animals in the environment, termed exogenes (*sensu* Olivera 2006), will evolve quickly. For other diverse metazoan lineages, such as scorpions, similarly rapidly diversifying genes should be a major genetic foundation for generating biodiversity. RNA editing, gene duplication, and posttranslational modification have been presented as the mechanisms responsible for NaScTx diversity (Zhu et al. 2004; Zhu and Gao 2006a). In particular, RNA editing has been suggested as the mechanism in scorpions producing gene variants encoding different disulfide-bridge linking patterns ((Zhu and Gao 2006a) see below).

Phylogenetic Scale of Venom Variation in Scorpions

Of the 14–18 recognized scorpion families, those that have been investigated for toxin data using venom gland transcriptomes include Bothriuridae (genus *Cercophonius*), Buthidae (genera *Australobuthus*, *Centruroides*, *Isometroides*, *Isometrus*, *Hottentotta*, *Lychas*, and *Tityus*), Chaerilidae (genus *Chaerilus*), Iuridae (genus *Hadrurus*), Liochelidae (genera *Opisthacanthus*), Scorpionidae (genera *Heterometrus* and *Pandinus*), Scorpiopidae (genus *Scorpiops*), and Urodacidae (genus *Urodacus*). These studies have provided information to infer the phylogenetic scale of scorpion venom variation, including the evolution of vertebrate toxicity in Buthidae (Ma et al. 2012; Sunagar et al. 2013).

The toxin types currently found in one or several of these scorpion families may have been the result of individual recruitments. For instance, pore-forming peptides with antimicrobial and hemolytic activity are widely distributed in bothriurids, chaerilids, scorpionids, liochelids, and iurids, but lacking in buthids (Ma et al. 2012; Sunagar et al. 2013). Toxin sequences originally thought to be exclusive of Buthidae venoms include bacterial cell-wall-disrupting peptides, BPPs (bradykinin-potentiating peptides), and NaScTxs. Extensive sequencing of venom gland cDNA libraries in bothriurids, iurids, liochelids, scorpionids, and scorpiopids has not revealed thus far the existence of NaScTxs (Ma et al. 2012), but a distant homologue (Ctricontig80) has been found recently in the venom of the chaerilid *Chaerilus tricostatus* (He et al. 2013). Upon phylogenetic analyses, this 23-residue-long peptide, putatively packed by three disulfide bridges, clusters with birtoxin, an also three disulfide-bridged peptide from the southern African buthid *Parabuthus transvaalicus*; the latter is a potent anti-mammalian β-toxin (Inceoglu et al. 2001) but with nontoxic (insect-specific) homologues in *Androctonus* species (Abbas et al. 2011). All members in the birtoxin subfamily contain only three disulfides with a slightly smaller size (about 58 residues) relative to the NaScTxs with four disulfides. They share 40–60% sequence identity to β-toxins but display more diverse pharmacological activities, ranging from those with a characteristic β-toxin effect, altering sodium channel activation, to others serving as potassium channel blockers due to the development of a putative functional dyad in their α-helical region (Abbas et al. 2011). The *Chaerilus tricostatus* (Ctricontig80) sequence also clusters with phaiodotoxin, a NaScTx-like depressant insect toxin from *Anuroctonus phaiodactylus* (family Iuridae), with four disulfide bridges and an eight C-terminal cysteine that imposes a different disulfide-bridge pattern (Valdez-Cruz et al. 2004; Fig. 3).

A crucial feature in NaScTxs is that 3 (out of 4) disulfide bridges are buried in the structure, stabilizing the CSα/β scaffold, and are conserved across the family, whereas the fourth disulfide corresponds to an exposed wrapper disulfide bridge (WDB) which varies in position among different toxins (Rodríguez de la Vega and Possani 2005). Alteration of the WDB linkage pattern can lead to a functional switch of the NaScTxs via adjusting the conformation of key residues associated with toxin function. Whereas in most NaScTxs the fourth bridge links the N- and

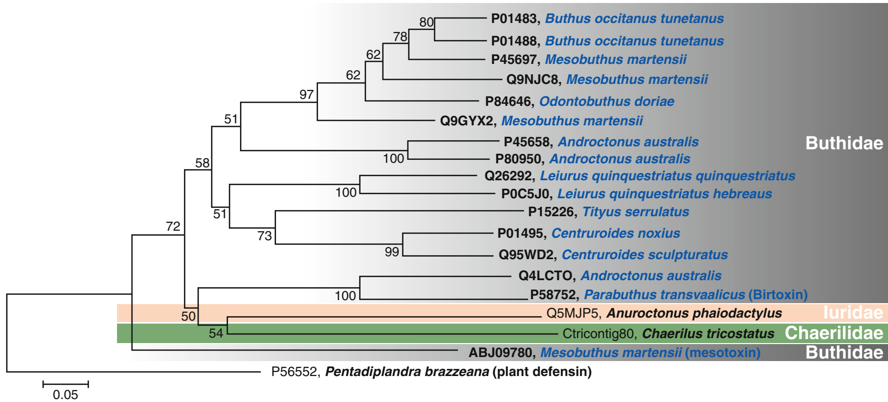

Fig. 3 Evolutionary analysis of NaScTxs sequences retrieved from scorpion venoms in the families Chaerilidae, Iuridae, and Buthidae (Adapted from He et al. 2013). Ctricontig80 is the NaScTx-like peptide sequence from *Chaerilus tricostatus* (Chaerilidae). P58752 is birtoxin from the scorpion *Parabuthus transvaalicus* (Buthidae), Q5MJPG is phaiodotoxin from the scorpion *Anuroctonus phaiodactylus* (Iuridae), and Q9GYX2 is toxin BmKα1 and ABJ09780 is mesotoxin, both from the scorpion *Mesobuthus martensii* (Buthidae). A plant defensin-like protein is used to root the phylogenetic tree

C-terminus, in the three disulfide-bridged members, the WDB is lacking. In phaiodotoxin the fourth bridge is located in the C-tail region (Zhu and Gao 2006a). In another group of NaScTx-like peptides (LVPs), which induce lipolytic responses in adipose cells and were originally thought to be exclusive of buthids, the evolutionary loss of the first cysteine removed the intramolecular WDB and allowed the assembly of an interchain WDB, resulting in a dimeric toxin with lipolytic instead of neurotoxic activity (Zhu and Gao 2006a). LVP-like sequences have been identified in *Cercophonius squama* (Bothriuridae) and *Urodacus manicatus* (Urodacidae) (Sunagar et al. 2013), rendering the buthid LVPs non-monophyletic. Thus, it has been suggested that an ancient peptide with three disulfide bridges might be the ancestor of NaScTx given that its scaffold can easily serve as a template for assembly of different types of toxins via evolution of the WDB in different positions (Zhu and Gao 2006b). NaScTx from buthids and non-buthids might have diverged from a common ancestor similar to mesotoxin, a three disulfide peptide from the buthid *Mesobuthus martensii* with a compatible size to typical NaScTx and considered evolutionary basal to α- and β-NaScTx (He et al. 2013). Considering the homology of the chaerilid Ctricontig80 peptide with mesotoxin and birtoxin-like peptides, and also taking into account the isolation of bothriurid and urodacid LVP-like sequences, NaScTx were probably recruited into the venom prior to the lineage split between buthids and non-buthids, which imply that toxins targeting Navs are among the most ancient components of scorpion venoms (He et al. 2013).

Toxinological Diversity in Buthidae: Old World Versus New World

Old World and New World buthids probably diverged during the Early Cretaceous (~140 Mya) as South America began to drift away from Africa, setting the stage for structural and functional divergent evolution of their venoms. NaScTxs of New and Old World buthids now differ in amino acid sequences, pharmacological action, and antigenic properties, although they still share characteristics suggestive of their common ancestry and conserved function, such as disulfide-bridge linkage pattern (Loret and Hammock 2001). Whereas α-NaScTxs, affecting Nav inactivation, have been found in scorpions throughout the world, anti-mammalian β-NaScTxs were originally assigned to New World scorpions in genera *Centruroides* and *Tityus*, and anti-insect selective β-toxins (depressant and excitatory) had been described only in the Old World. This suggested that diversification of β-NaScTxs into distinct pharmacological groups occurred after the splitting of Gondwana (Gordon et al. 2003). More recent research revealed, however, the existence of Old World toxins Lqhβ1 (from *Leiurus quinquestriatus*), BmK AS and BmK AS-1 (from *Mesobuthus martensii*), and AahIT4 (from *Androctonus australis*), all with a β-toxin fingerprint. The fact that Lqhβ1 displays both anti-mammalian (against Nav1.4) and anti-insect (against *para* channels from *Drosophila*) toxicity and that it is 41 to 50% identical to New World β-NaScTxs has suggested that the group of toxins represented by Lqhβ1 and AahIT4 evolved into the anti-insect selective depressant toxins in the Old World, and into β-toxins currently found in New World scorpions (Gordon et al. 2003).

Additional diversification of the β-NaScTx clade in the New World proceeded toward those with affinities to mammals (e.g., toxins Cn2, Css2, and Css4, from *Centruroides noxius* and *C. suffusus*), crustaceans (e.g., toxins Cn5, Cn11, and Cll1, from *C. noxius* and *C. limpidus*), and a group that acquired α-like activity while maintaining the structural features of β-NaScTxs (toxin CsE variants 1–3, from *C. sculpturatus*) (Gordon et al. 2003). *Tityus* β-NaScTxs from southern South America, such as Ts1, Tst1, and Tb1 (from *Tityus serrulatus* and *Tityus bahiensis*), are highly active on mammals and insects and thus seem to preserve ancient properties of Lqhβ1 in the New World (Rodríguez de la Vega and Possani 2005). Notwithstanding this effect of southern *Tityus* β-toxins, northern South American *Tityus* toxins might diverge from this scheme as toxin Bact-2 from *Tityus discrepans* (and also possibly Tz1 from *Tityus zulianus*, which is 98% identical to Bact-2) is a true anti-mammalian β-NaScTx that does not modify gating of the insect DmNav1 channel (Peigneur et al. 2012). The phylogenetic divergence existing among *Tityus* taxa across its distributional range in South America, illustrated by the split between *T. serrulatus* and *T. bahiensis* and northern *Tityus* species, is therefore paralleled by functional, structural, and immunological differences among their toxins (Borges et al. 2010a). Although not yet supported by a cladistic analysis, the abrupt split in the toxicity to humans that occurs within the spatial distribution of *Centruroides* (when noxious species, restricted to the

Mexican highlands, are compared with congenerics from other regions) also probably has a phylogenetic basis associated with the landscape history of Central America and the Caribbean (Borges 2015).

The *Isometrus* and *Uroplectes* groups make for interesting comparisons to the *Tityus* group from a toxinological standpoint. As mentioned above, *Parabuthus* species (in the *Uroplectes* group) produce β-NaScTxs, such as birtoxin, structurally and functionally related to North American *Centruroides* toxins (Inceoglu et al. 2001). The Eastern African *Babycurus centrurimorphus* (in the *Isometrus* group) produces toxin Babycurus-1 (Ben Khalifa et al. 1997), in which the 30-residue-long N-terminal sequence is most homologous (57–65%) to putative β-NaScTx RjAa14f from the Cuban *Rhopalurus junceus* (F2YLA3) and the North American Cex9 (*Centruroides exilicauda*; Q68PG6), CsEv5 (*Centruroides sculpturatus*; P58779), and CssIX (*Centruroides suffusus*; ADY17426), also in the β-NaScTx group. Homology of *Parabuthus* and *Babycurus* toxins to North American/Caribbean toxins suggests a common NaScTx ancestor to Caribbean/North American and southern/eastern African scorpions which is not shared by the genus *Tityus*. Importantly, upon the comparison of NaScTxs sequenced worldwide, β-NaScTxs from *Tityus* species cluster apart, together with several NaScTxs that affect inactivation (belonging to the α-class, Tf4 and Tc48a, from *Tityus fasciolatus* and *Tityus obscurus*), from clusters assigned to excitatory and depressant β-NaScTxs from Old World (Africa, Asia, and Europe) and the New World classical β-NaScTxs from scorpions of the genus *Centruroides* (Rodríguez de la Vega and Possani 2005). This indicates that toxins affecting Nav activation are not monophyletic and that New World buthid toxins represent multiple evolutionary lineages. Clearly, the analysis of more toxins from Afrotropical genera should shed light on the evolutionary relationships between South/North American and African buthid scorpions.

Fet et al. (2005) provide a biogeographical explanation for the generation of such toxin diversity between Old and New World scorpion peptides, as the result of the arms race between scorpions and predators. Mammal-specific NaScTxs, which constitute the most abundant components of venoms from extant, noxious Old World taxa, could have evolved during aridification of the Palearctic in the Tertiary period, when small burrowing mammals (mostly rodents) radiated into arid landscapes. In addition to being important nocturnal predators (Rowe et al. 2013), an increase in the number of rodents would have facilitated direct competition for space (burrows). Such a scenario explains the emergence of specific mammal-targeting toxins (used for defense, not for foraging) in predominantly burrow-living Palearctic buthids, as opposed to largely vegetation-inhabiting New World buthids such as *Tityus* and *Centruroides* species. The deserts of the New World are much younger, and competition with other arid-adapted scorpions – such as Bothriuridae, Caraboctonidae, and Vaejovidae – could have prevented New World taxa from diversifying as much as the Old World species. Venom, as it evolved, appears to have changed to serve a more of a defensive function in Old World buthids.

The Hemiscorpiidae

Hemiscorpius lepturus (family Hemiscorpiidae) is the sole member of order Scorpiones with venom that can cause severe and fatal hemolysis, secondary renal failure, deep and necrotic ulcers, ankylosis of the joints, thrombosis, and cardiovascular failure. These symptoms are clearly divergent from the typical neurotoxic/cardiotoxic buthid envenomation, resembling envenomations by spiders in genera *Loxosceles* and *Sicarius* (Araneae: Sicariidae) (Jalali et al. 2010). Biochemical characterization has indicated that neurotoxic components of *H. lepturus* venom are minor constituents, including Hemicalcin (a 33-mer peptide active on ryanodine-sensitive calcium channels representing 0.6% of the total protein content) and Hemitoxin (a 35-mer KScTx, representing 0.1% of the total protein). On the contrary, Heminecrolysin, a 33-kDa SMaseD (sphingomyelin D-degrading enzyme), is the main protein responsible for the pathological effects observed following *H. lepturus* envenomation. Although the SMaseD activity of Heminecrolysin is low compared to *Loxosceles* dermonecrotic enzymes, its lysophospholipase D catalytic efficiency is up to three orders of magnitude greater than spider SMaseDs, which explains the strong hemolytic capacity of *H. lepturus* venom (Borchani et al. 2013). Based on de novo sequencing, Heminecrolysin shares only limited sequence homology with *Loxosceles* SMaseDs, but its partial primary structure precludes any comparison with enzymes from other taxa, including ticks and bacteria (Borchani et al. 2011). Enzyme activities have been reported in venoms from several scorpion families: hyaluronidases and phospholipases (in families Buthidae, Scorpionidae, Caraboctonidae, Chactidae, and Liochelidae) and Zn^{+2}-dependent metalloproteases (e.g., antarease), only from buthids (Rodríguez de la Vega et al. 2013). Scorpion SMaseD activity, however, appears to be restricted to Hemiscorpiidae as it has only been reported from *H. lepturus*.

Scorpions in the family Hemiscorpiidae comprise a divergent group of species ranging from Somalia to Iran and Pakistan. Hemiscorpiids are a sister group to Hormuridae from which are thought to have diverged as a result of climatic changes in Gondwana during the Permian global warming (290–250 Myr) (Monod and Lourenço 2005). The genus comprises 14 species, but only *H. lepturus*, which is particularly abundant in southwestern Iran (Khuzestan province) is considered dangerous and potentially lethal. Iranian *Hemiscorpius* species are morphologically very similar and difficult to distinguish, so it is probable that *H. lepturus* is not the only species accountable for all severe envenomations. The fact that the African congenerics do not show the extreme sexual dimorphism and cytotoxic venom of *H. lepturus* indicates that Iranian species have probably been separated from the other hemiscorpiids for a considerable length of time (Monod and Lourenço 2005). The venom evolution (defensive?) strategy in *Hemiscorpius*, also an arid-adapted genus, clearly diverges from that of burrow-living Palearctic buthids, a probable result of a quite different arms race. Modern phylogenetic analyses will no doubt elucidate the enigmatic position of this genus from both evolutionary and toxinological standpoints in the near future.

Conclusion and Future Directions

In summary, venom capable of severe/lethal scorpionism is rare among scorpions, currently known from only about 2% of the 2,140 extant species. All but 1 of the 45 medically significant species are members of Buthidae, the largest and most widely distributed of the scorpion families, found in a variety of Old World and New World habitats. The presented phylogenetic analysis of Buthidae (Fig. 2), based on mitochondrial sequence data, is largely congruent with morphology-based cladistic analyses and suggests that venoms with the potential to produce severe/lethal scorpionism evolved multiple times in both hemispheres. Furthermore, if there is a phylogenetic signal to venom composition, then the following species, which are currently not considered to be medically significant, are predicted to also possess particularly dangerous venoms: *Mesobuthus cyprius*, *Buthus ibericus*, *Centruroides nigrovariatus*, *C. nigrescens*, *Tityus nematochirus*, *T. pococki*, *T. sanarensis*, *T. funestus*, *T. boconoensis*, and *T. pittieri*.

Structural and functional divergence of the toxins from one species to the next, or even among populations of the same species, suggest that venoms are sensitive to selective pressures among different environments. Multiple lines of evidence suggest that aridification-induced radiations of rodent predators could have been a primary selective pressure driving venom evolution in scorpions. In addition, rodent radiations could also be responsible for the emergence of noxious toxins, as mammalian toxicity of scorpion venoms is directly related to mammal-specific NaScTxs content. Although natural selection has been most extensively studied in snake and gastropod venoms, scorpions are also proposed as an ideal model system for venom research. Medically significant scorpion species are clearly the result of many independent evolutionary events that lead to venom capable of severe/lethal scorpionism. Additional sampling and novel tools from genomics could usher in a new era of discovery for scorpions of medical importance.

Cross-References

► Scorpion Venom Gland Transcriptomics and Proteomics: An Overview

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Scorpion Venom Gland Transcriptomics and Proteomics: An Overview

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Abstract

Recently, the OMIC technologies of proteomics and transcriptomics have been extensively applied to deeply understand the structure and molecular diversity of animal venoms from various species including scorpions. During the last decade, proteomics analysis of 27 different scorpion species has been conducted and revealed the complexity of scorpion venoms. Mass fingerprinting data clearly showed noticeable differences in the number of venom molecules (ranging from 60 to 665) between scorpion species. Also, as a complementary approach, the transcriptomics analysis of venom glands from 20 different scorpion species resulted in about 73,000 assigned ESTs which mainly correspond to neurotoxins, antimicrobial peptides, housekeeping proteins, hypothetical proteins, and a large number of unassigned types of scorpion venom peptides or proteins. The employment of transcriptomics in scorpion venomics is adding new insights

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about venom diversity and biological processes of venom gland as well as facilitating the identification of various novel biologically active peptides. In this chapter, scorpion gland transcriptomics will be mainly discussed with a brief introduction about scorpion venom and its proteomics analysis.

Introduction

Within the phylum of Arthropoda, scorpions are one of the most ancient living fossils (418 million years BP) on earth (Polis 1990; Dunlop et al. 2008). There are eighteen different families with more than 1,500 scorpion species. The family of Buthidae including about 40 % of scorpion species (81 genera and 570 species) is considered as the most medically important (Soleglad and Fet 2003). Systematically, scorpions are a basal pedigree of Arachnida (a large group of Chelicerata). Consequentially, scorpions play a critical role in the evolution of chelicerates and arthropods (Shultz 2007). Scorpions occupy a vast territory of the world from Africa to Asia, Australia, and America. They are particularly well adapted to survival in extreme habitats, and their ability to produce and deliver venoms is an important factor in this success (Rodriguez and Possani 2004). In addition to their venom system, scorpions have various morphological and behavioral features which make them amazing animals. Of these features, scorpions glow when exposed to certain wavelengths of UV light, and the tail plays a role in hunting and photosensation. Interestingly, the first complete genome sequence (32,016 protein-coding genes) of the Asian scorpion *Mesobuthus martensii* revealed new molecular pathways regulating these features. For example, the light-sensing gene Mmopsin3 plus other twenty visual signal transduction genes were transcribed in the scorpion tail of *M. martensii*. These findings confirm the contribution of scorpion tail in light sensation and in detecting the movement of preys under faint light (Cao et al. 2013). For these amazing features, a huge scorpion model (Battling for Survival) was constructed and installed in the Natural History Museum (London, UK) to simulate how scorpions attack and inject their venoms into victims (Fig. 1).

Intricacy and Pharmacological Significance of Scorpion Venoms

Scorpions naturally develop a highly specialized homologous venom apparatus (a pair of venom glands connected to the hypodermic aculeus) used for capturing prey and for defense against predators (Polis 1990). The mRNA sequences encoding for all venom peptides are transcribed in these glands and the produced venom stored until needed. Usually the prey is captured by scorpion pincers, and then the venom is injected through needlelike stinger (aculeus). Scorpion venom is a heterogeneous mixture of substances (100–700 different components) (Fig. 2) including inorganic salts, lipids, nucleotides, free amino acids, mucopolysaccharides, proteins (mainly enzymes), and peptides (Rodriguez de la Vega and Possani 2005;

Fig. 1 The *Battling for Survival* scorpion model (*Pandinus imperator*) in the arthropods section of the Natural History Museum in London, UK (Photo was taken by MA Abdel-Rahman, London 2014)

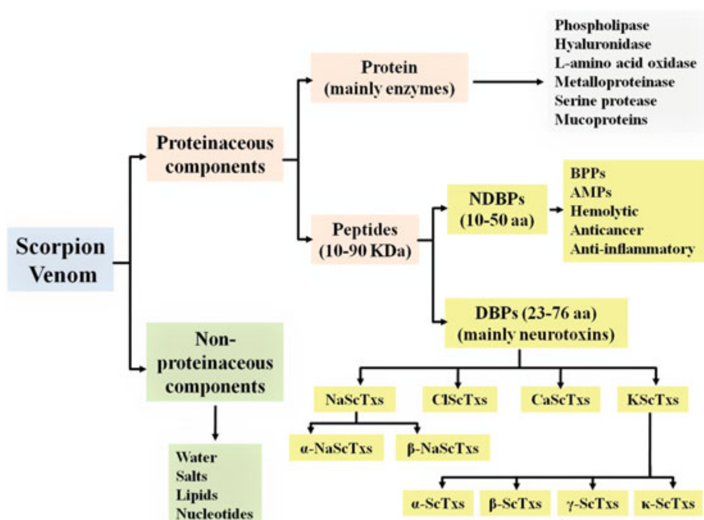

Fig. 2 A simplified chart showing the chemical structure of scorpion venom. Disulfide-bridged peptides (DBPs); non-disulfide-bridged peptides (NDBPs); bradykinin-potentiating peptides (BPPs); antimicrobial peptides (AMPs); sodium, potassium, calcium, and chloride ion channel toxins (NaScTxs, KScTxs, CaScTxs, and ClScTxs, respectively)

Abdel-Rahman et al. 2015). Scorpion venom peptides are the major constituents with various biological and pharmacological activities. According to their structure, venom peptides are classified into disulfide-bridged peptides (DBPs) and non-disulfide-bridged peptides (NDBPs) (Zeng et al. 2005). The majority of DBPs are short neurotoxins (23–76 amino acids stabilized by 3 or 4 disulfide bonds) which

exhibit conserved structure-function relationships. They specifically target various membrane ion channels (Na^+, Cl^-, K^+, and Ca^{+2}) and certain types of cellular receptors (e.g., ryanodine receptor). Clinically, the scorpion neurotoxin CTX or chlorotoxin (36 amino acids with four disulfide bridges) was purified from the venom of *Leiurus quinquestriatus* (DeBin et al. 1993) and has been applied in clinical trials to inhibit glioma cell migration/invasion. The antimetastatic activity of CTX appears to be based on two integrated pathways: (i) blocking chloride channels expressed only in glioma cells and (ii) inhibiting the enzymatic activity of matrix metalloproteinase-2 (MMP-2) overregulated in these cancer cells (Deshane et al. 2003). In an in vivo bio-imaging study, the labeled CTX with a far-infrared fluorescent imaging probe was used in surgical tumor restriction to define the borders of glioma cells (Stroud et al. 2011). The second important group of venom peptides is the NDBPs (10–50 amino acid residues) that display different activities such as anticancer, antimicrobial, anti-inflammatory, hemolytic, and bradykinin-potentiating peptides (BPPs) (reviewed in Zeng et al. 2005; Almaaytah and Albalas 2014). Excluding the phenomena of intraspecific diversity, it is estimated that there are more than 150,000 different peptides/proteins expressed in the venom glands of 1,500 different scorpion species (Rodriguez de la Vega et al. 2010). To date, only 871 peptides/proteins (ranging in length from 4 to 410 amino acid residues) have been derived from 72 scorpion species (retrieved from Tox-Prot database June 2014, http://www.uniprot.org/program/Toxins) (Fig. 3). Of these molecules, 638 peptides (73 %) have been characterized from 47 different scorpions belonging to the family of Buthidae (Fig. 4). Also, Tox-Prot data showed that the highest numbers of venom peptides (100 and 89) have been identified from the Asian scorpions *Mesobuthus martensii* (family Buthidae) and *Lychas mucronatus* (family Hormuridae), respectively.

OMIC Approaches in Scorpion Venom Research

Generally, studying venom composition using the cutting-edge OMIC technologies (transcriptomics and proteomics) is a critical step toward understanding biological roles of venoms and their potential application in research and medicine. Moreover, the study of animal toxinome adds valuable insights about some essential biological processes such as gene duplication in producing evolutionary novelties, generation of large multigene families, and molecular principles of ecological adaptations (King 2011; Brust et al. 2013). The application of OMIC approaches (proteomics and transcriptomics) in scorpion venom research is very advantageous for the following main reasons: (i) gaining better understanding of venom composition and evolution, (ii) revealing molecular mechanisms involved in venom toxicity, (iii) depicting an accurate image about interspecific and intraspecific venom diversity, (iv) discovery of novel pharmacological and diagnostic agents, (v) understanding biological processes taking place in scorpion venom glands, and (vi) developing effective antivenoms to circumvent the deleterious effects following scorpion envenomation. Recently, several proteomics and transcriptomics investigations have been performed to study

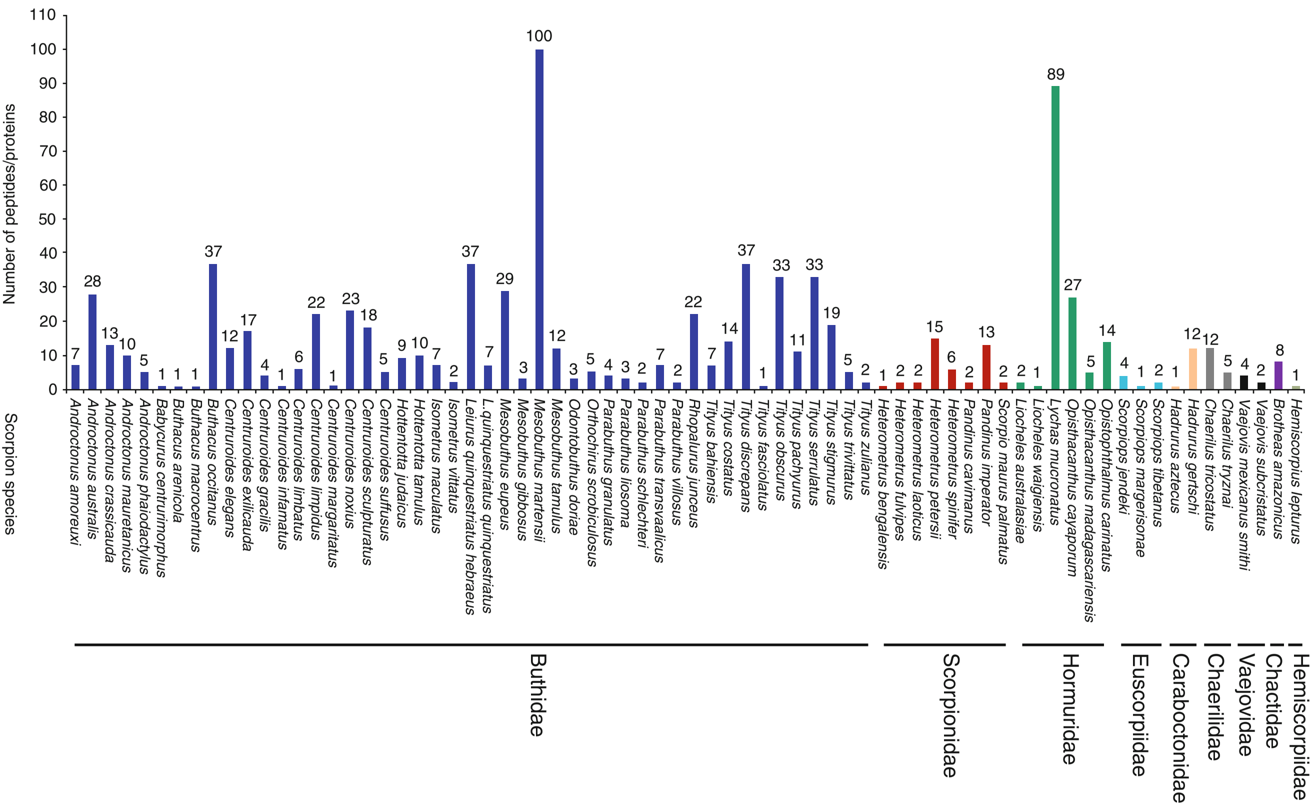

Fig. 3 Number of venom peptides/proteins (ranging in size from 4 to 410 amino acids) identified from 72 different scorpion species (Data retrieved from Tox-Prot project in 2014, http://www.uniprot.org/program/Toxins)

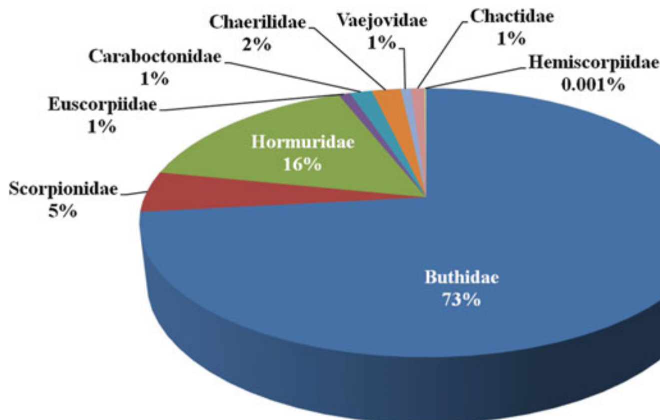

Fig. 4 Variation in the percentage of scorpion venom peptides/proteins characterized from various families

venomics of various scorpion species (reviewed in Abdel-Rahman et al. 2015; Table 1). In proteomics studies, methods of venom fractionation (e.g., SDS-PAGE and chromatography), mass spectrometry (MALDI-TOF/MS and electrospray ionization), and peptide sequencing (using MS/MS or Edman degradation) have been extensively applied to determine the total number and structure of molecules in a given scorpion venom. To date, using mass spectrometric analyses, 5,314 unique molecular masses have been identified in the venom of 27 different scorpions (Table 1). Of these molecules, 3,753 (70.6 %) are from the family Buthidae and the rest (1,561) from the families of Urodacidae (8.9 %), Scorpionidae (14.5 %), Hemiscorpiidae (4.2 %), and Vaejovidae (1.8 %). As shown in Table 1, the highest number of masses was identified in the venom of *Mesobuthus tamulus* (665 molecules, Newton et al. 2007), and the lowest numbers were recorded from the venoms of *Tityus cambridgei* and *Buthacus macrocentrus* (60 peptides each, Batista et al. 2004; Caliskan et al. 2012). A few of these molecules (5,314) were fully characterized. The main categories of these peptides are neurotoxins (sodium and potassium channel toxins), antimicrobial peptides, and BPPs.

In addition to venom proteomics, transcriptomics approaches have been applied to provide further insights about venom structure and diversity of various scorpion species (Table 1). Scorpion transcriptomics is a complementary approach to obtain venom peptide sequences through constructing venom gland cDNA libraries. It can be divided into two main types: (i) global profiling of the venom through random DNA sequencing of expressed sequence tags (ESTs) and (ii) fishing for transcript encoding a specific venom peptide/protein wherein its partial amino acid sequence has already been identified by proteomics analysis (Vetter et al. 2011). Until now (June, 2014), 73,234 expressed sequence tags (ESTs) have been obtained from 20 different scorpion species. The relative minor number of ESTs (35,944; 49.1 %) has been assigned from eleven scorpions of family Buthidae and the major number: 37,290 ESTs (50.9 %) from the families of Urodacidae, Scorpionidae, Euscorpiidae,

Table 1 List of scorpion venomics studies conducted through proteomics or transcriptomics approaches (2001–2014)

Family	Scorpion species	Transcriptomics (no. of ESTs)	Proteomics (no. of compounds)	References
Buthidae	*Androctonus crassicauda*		80	Caliskan et al. (2006)
	Androctonus mauretanicus		70–80	Oukkache et al. (2008)
	Buthacus macrocentrus		60	Caliskan et al. (2012)
	Buthus occitanus israelis	420		Kozminsky-Atias et al. (2008)
	Centruroides noxius	30,301		Rendon-Anaya et al. (2012)
	Centruroides tecomanus	130	104	Valdez-Velázquez et al. (2013)
	Hottentotta judaicus	537		Morgenstern et al. (2011)
	Isometrus maculatus	743		Ma et al. (2012)
	Leiurus quinquestriatus quinquestriatus		380	Nascimento et al. (2006)
	Leiurus quinquestriatus hebraeus		554	Nascimento et al. (2006)
	Lychas marmoreus obscurus		>100	Smith et al. (2012)
	Lychas mucronatus	1,289		Ruiming et al. (2010), Ma et al. (2012)
	Mesobuthus tumulus		665	Newton et al. (2007)
	Mesobuthus gibbosus	117		Diego-Garcia et al. (2014)
	Mesobuthus martensii	Whole genome (116 neurotoxin genes)	227	Cao et al. (2013), Xu et al. (2014)
	Parabuthus transvaalicus		100	Inceoglu et al. (2003)
	Parabuthus stridulus	111		Mille et al. (2014)
	Tityus serrulatus	1,629	380	Alvarenga et al. (2012), Pimenta et al. (2001)
	Tityus cambridgei		60	Batista et al. (2004)
	Tityus costatus		90	Diego-Garcia et al. (2005)

(*continued*)

Table 1 (continued)

Family	Scorpion species	Transcriptomics (no. of ESTs)	Proteomics (no. of compounds)	References
	Tityus stigmurus	540	>100	Almeida et al. (2012), Batista et al. (2007)
	Tityus bahiensis		464	Nascimento et al. (2006)
	Tityus discrepans	127	205	D'Suze et al. (2009), Batista et al. (2006)
	Tityus pachyurus		104	Barona et al. (2006)
Urodacidae	*Urodacus yaschenkoi*	172	274	Luna-Ramírez et al. (2013)
	Urodacus elongatus		>100	Smith et al., (2012)
	Urodacus armatus		>100	Smith et al. (2012)
Scorpionidae	*Heterometrus longimanus*		~78	Bringans et al. (2008)
	Heterometrus petersii	486	184	Ma et al. (2010)
	Opistophthalmus glabrifrons		>100	Smith et al. (2012)
	Pandinus imperator	34,481		Roeding et al. (2009)
	Pandinus cavimanus	184	339	Diego-Garcia et al. (2012)
	Scorpio maurus palmatus	101	65	Abdel-Rahman et al. (2013)
Euscorpiidae	*Scorpiops jendeki*	871		Ma et al. (2009)
	Scorpiops margerisonae	730		Ma et al. (2012)
Hemiscorpiidae	*Opisthacanthus cayaporum*	118	221	Silva et al. (2009), Schwartz et al. (2008)
Caraboctonidae	*Hadrurus gertschi*	147		Schwartz et al. (2007)
Vaejovidae	*Vaejovis spinigerus*		>100	Smith et al. (2012)
Total	***38 scorpion species***	**73,234 ESTs**	**5,314 molecules**	

Hemiscorpiidae, and Caraboctonidae (Table 1). However, although the number of ESTs has increased more than 10-fold during the last 2 years (2012–2014) (Abdel-Rahman et al. 2015), only 2,569 were assigned as scorpion toxin or toxin-like molecules (Diego-Garcia et al. 2014). For its significance as a powerful analytical strategy in scorpion venomics, scorpion venom gland transcriptomics will be discussed in the following sections.

Transcriptomics of Scorpion Venom Gland

Transcriptome of Active and Resting Venom Glands

Scorpion venom gland is a highly regulated organ, and many molecular factors are involved in its maintenance and regulation such as expressed genes. The venom gland-expressed genes are highly diverse with various physiological roles such as housekeeping functions, making venom precursors, and triggering venom ejection and delivery into prey (Ménez et al. 2006). In this context, is there a difference in the catalogue of gene expression between active and resting venom glands? As mentioned above, random screening of scorpion venom glands for nineteen species has been conducted using transcriptomics analysis. This approach is mainly based on generating cDNA libraries of venom glands and random DNA sequencing of high-quality ESTs (Fig. 5). In order to enrich venom-related transcripts, recently milked venom glands (after 2–5 days of electrical stimulation) were used to extract total RNA in all transcriptomics studies except *H. judaicus* and *T. stigmurus*. The main reason for using this method to construct cDNA library is that the transcriptional machinery of stimulated glands becomes active and involved in the process of venom regeneration. The transcriptomics profile of regenerating venom glands showed a high percentage of ESTs corresponding to toxin-like sequences (50–78 % in Buthidae and 30–44 % in non-Buthidae species) (Quintero-Hernández et al. 2011). In addition to neurotoxin-like peptides, various venom molecules were found such as antimicrobial peptides (AMPs) (reviewed in Harrison et al. 2014), phospholipase A2, serine proteases, metalloproteases, and lipolysis-activating peptides (LVPs). Recently, the team of Prof. Jan Tytgat identified a transcript that encodes a new putative chlorotoxin (Mgib88, 254 bp including the 3′-UTR) from the transcriptome of the Mediterranean scorpion *Mesobuthus gibbosus*. The results obtained in this work add new information and contribute to the classification of chlorotoxin genes into two groups for the genus Mesobuthus (Diego-Garcia et al. 2014).

On the other hand, there are two reports describing the transcriptome of replete venom glands (resting or full venom glands) of *H. judaicus* and *T. stigmurus* (Morgenstern et al. 2011; Almeida et al. 2012). The gene expression profiles of resting glands were significantly different from the profiles obtained from regenerating venom glands. For example, the replete venom glands comprised high abundance of transcripts encoding antimicrobial peptides as well as protease transcripts and low abundance of toxin transcripts. Moreover, three different pseudogenes

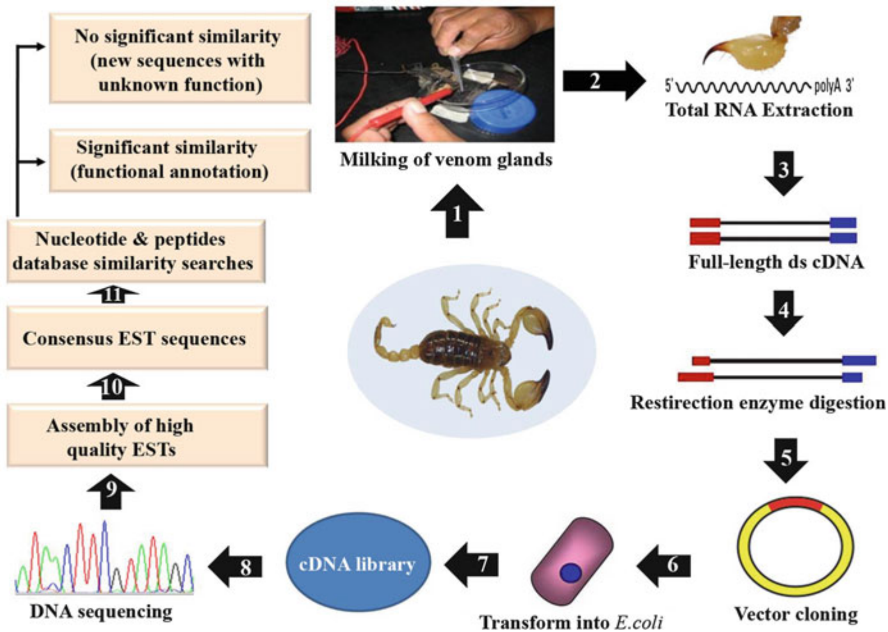

Fig. 5 A simplified flowchart showing steps of scorpion venom gland transcriptome analysis. Venom glands were electrically stimulated and milked 2–5 days ***1*** before total RNA extraction ***2*** which were used to prepare the full-length cDNA ***3***. The cDNA was digested ***4*** and the fractions ligated ***5*** into pDNR-LIB vector. Using electroporation, plasmids transformed ***6*** into DH5α and generated the cDNA library ***7***. Screening the transcriptome of venom gland through DNA sequencing ***8*** of colonies randomly selected and the high-quality ESTs used for bioinformatics analyses ***9–11***

or decommissioned toxins (U_{3m}-buthitoxin-Hj1α, skipped stop codon; U_{1m}-BUTX-Hj1c, accumulation of mutations; km-BUTX-Hj1c, loss of signal peptide coding sequence) were assigned in the transcriptome of *H. judaicus*. The characterization of pseudogenes and deactivated toxins may give an idea about the process of toxin decommissioning and death during the evolution of scorpion venoms (Morgenstern et al. 2011). The groups of Prof. Lourival Possani and Prof. Alfredo Herrera confirmed these findings through examining venom gland differential expression of the Mexican scorpion *Centruroides noxius*. The venom gland cDNA libraries of *C. noxius* were constructed before and after five days of venom milking. The statistical analysis of transcript abundance revealed that 3 % and 2 % of the isogroups were preferentially expressed during the active or resting states, respectively. Of these unique transcripts, sixteen toxin-like isogroups were abundant in the active venom glands against only eight isogroups that were represented in the replete glands (Rendon-Anaya et al. 2012). Accordingly, the transcriptomics studies of replete venom glands add new information about the dynamics of transcriptional changes in scorpion venom glands.

With the exception of *Pandinus imperator* (Roeding et al. 2009) and *C. noxius*, the classical protocol of constructing cDNA libraries and DNA sequencing (Fig. 5)

was applied in all transcriptomics studies of scorpion venom glands. The number of assigned transcripts ranges from 101 (*S. m. palmatus*) to 1,629 (*T. serrulatus*) (Table 1). The *P. imperator* transcriptome was the first transcriptomics analysis of a scorpion using a 454 pyrosequencing approach and obtained a total of 428,844 high-quality reads, which were assembled into 8,334 contigs and 26,147 singletons. In the case of *C. noxius*, Possani's and Herrera's group exploited the qualitative and quantitative capacities of the 454 pyrosequencing to conduct a global transcriptomics analysis of this scorpion. Using this platform, 30,301 ESTs have been characterized from both the milked and replete venom glands. The bioinformatics analysis revealed that these transcripts (30,301) correspond to 72 different toxin-like isogroups such as ion channel specific toxins (48), zinc metalloproteases (7), phospholipase (1), protease inhibitors (4), serine proteases (3), lipase (1), AMPs (1), and other venom components (7). Moreover, this approach revealed the presence of interesting components such as small RNA and the microRNA processing machinery (Rendon-Anaya et al. 2012).

Comparative Venom Gland Transcriptome Analysis

Using SDS-PAGE and mass spectrometry techniques, intraspecific venom variation has been studied in different scorpion spices such as *Tityus serrulatus* (Pimenta et al. 2003), *Mesobuthus tamulus* (Newton et al. 2007), *Leiurus quinquestriatus* (Omran and McVean 2000), *Scorpio maurus palmatus* (Abdel-Rahman et al. 2009), and *Rhopalurus junceus* (Rodríguez-Ravelo et al. 2013). The data of proteomics studies confirmed this phenomenon in scorpion venoms and revealed that environmental/biological factors (geographical distribution, climate, age, and sex) play a crucial role in the determination of venom components of a number of different scorpion species (e.g., Newton et al. 2007; Abdel-Rahman et al. 2009; Ma et al. 2009; Oliveira et al. 2013). Also, proteomics analysis has provided us with a precise image about the actual molecular diversity of scorpion venoms. However, we still have some major technological limitations to identify the unassigned masses, especially in comparative studies. In order to fill this gap and to study molecular diversity of scorpion venom within the same species, Ruiming and coworkers performed the first comprehensive comparative analysis of venom gland transcriptomes of the Asian scorpion *Lychas mucronatus* collected from two different and geographically isolated regions (Yunnan province and Hainan island) (Ruiming et al. 2010). Interestingly, the comparative analysis revealed noticeable differences in venom peptides and proteins between the two populations of *L. mucronatus* from different geographical regions (Fig. 6). As illustrated in Fig. 6a, there are clear differences in the relative proportion of venom categories (neurotoxins, AMPs, toxin-like peptides, gene products implicated in common cellular processes, sequences with no functional assessment, sequences with non-identified open reading frame, and atypical types of venom molecules) between the scorpion populations of Hainan and Yunnan provinces. For example, the proportion of ESTs corresponding to antimicrobial peptides is 24 % in Hainan

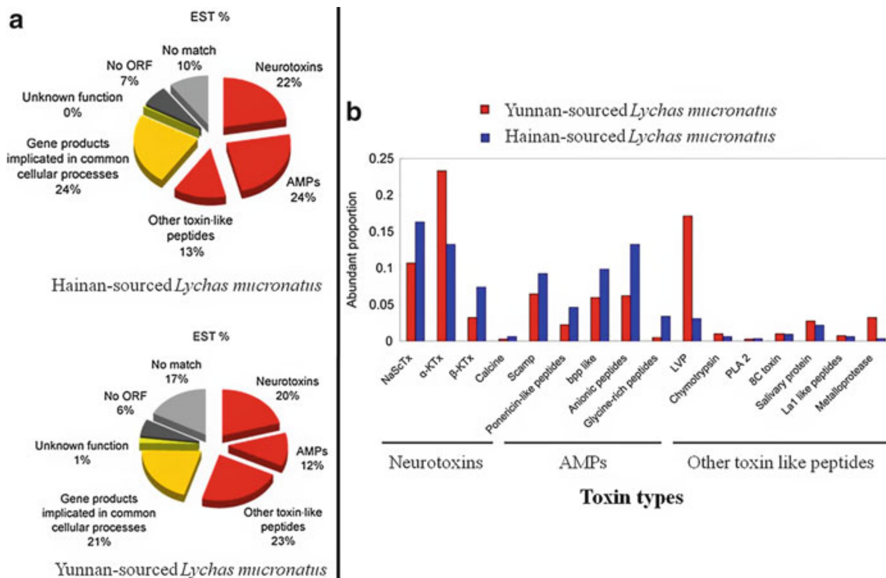

Fig. 6 Comparative venom gland transcriptome analysis of the *Lychas mucronatus* collected from two different isolated populations (Yunnan and Hainan provinces). **(a)** Relative proportion of each category of total transcripts from the venom gland library of the two scorpion populations. *Neurotoxins* include transcripts encoding putative toxins specific for Na^+, K^+, and Ca^{+2} channels. *AMPs* (antimicrobial peptides) include Scamp, ponericin-like peptides, bradykinin-potentiating-like peptides, glycine-rich peptides, and anionic peptides. *Other toxin-like peptides* include PLA2 and La1-like peptides. *Unknown function* includes ESTs similar with already described sequences with no functional assessment. *No ORF* includes sequences with non-identified open reading frame. *No match* includes ESTs having no homologous sequences in GenBank NCBI database. **(b)** Abundance of different toxin types (neurotoxins, AMPs, and other toxin-like peptides) between two populations of *L. mucronatus* (After permission from the authors, Ruiming et al. 2010)

province and 12 % in Yunnan-sourced *L. mucronatus*. Consequently, this comparison indicated that the population of Hainan can protect itself more effectively from pathogens. Figure 6b clearly demonstrates the dissimilarity of toxin-like peptides between the two scorpion populations. According to Zhao et al. (2007), the island of Hainan was separated from Chinese mainland about 2.5 million years ago. The long period of isolation doesn't compel the population of Hainan to dramatically change their toxin types. However, the primary structure and abundance of these toxin types significantly changed to enable the scorpion to survive in the new environment. Accordingly, transcriptomics analysis is a powerful complementary approach in this kind of studies for clarifying uniqueness in primary structure and exact abundance of venom toxins between different geographically isolated populations of the same scorpion species.

Scorpion Venom Gland Transcriptomics: A Gate to Drug Discovery

Using transcriptomics of scorpion venom glands, various new potentially active peptides have been characterized. These molecules include neurotoxins that modify permeability of different ion channels (reviewed in Quintero-Hernández et al. 2011; Rodríguez de la Vega et al. 2013), cytotoxins, LVPs, and AMPs. Because scorpion neurotoxins have been extensively reviewed, LVPs and AMPs identified in the transcriptome of venom glands will be discussed in this section. LVPs are long-chain toxins (~72 amino acid residues) that share high similarity in sequence to NaScTx, but LVPs contain seven cysteine residues instead of eight residues in NaScTx. This unique structure enables LVPs to form an interchain disulfide bridge and have potential biological activity on adipocyte lipolysis. Chai and coworkers (2012) concluded that bumarsin (72 amino acid LVP characterized from the scorpion venom of *M. martensii*) may provide a good model for developing novel drugs that can modulate cholesterol homeostasis through inhibiting the activity of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA). Soudani and colleagues (2005) have isolated and functionally characterized the lipolysis-activating peptide LVP1 from the venom gland of *Buthus occitanus tunetanus*. The chemical reduction/alkylation of LVP1 revealed a heterodimeric structure with two chains of LVP1α and LVP1β (8,877 and 8,807 Da, respectively). From nucleotide sequences of the corresponding cDNAs, the full amino acid sequences of both chains have been obtained. Each chain of LVP1α and LVP1β has a mature peptide (69 and 73 residues, respectively) containing seven cysteines and a signal peptide of 22 amino acids. LVP1 stimulated the lipolysis of rat adipocyte in a dose-dependent manner (EC_{50} ~ 1 μg/ml) and competes with radioligands for binding to adipocyte cell membrane. Moreover, LVPs have been characterized from the venom gland of other scorpions such as *B. martensii* (Zhu and Gao 2006) and *P. stridulus* (Mille et al. 2014). Zhu and Gao (2006) identified that two cDNA sequences are corresponding to α- and β-subunits of the lipolysis-activating peptide BotLVP1 from the venom gland of *B. martensii*. Interestingly, these results revealed the link between LVPs and NaScTxs and evidenced the presence of RNA editing mechanism in scorpion venom glands. This mechanism explained the functional switching of the BmLVP1 gene from adipocyte lipolysis to neurotoxicity through modifying the interchain pattern of wrapper disulfide bridge or WDB.

AMPs have been found in both vertebrates and invertebrates, existing in the skin, epithelial cells, blood, or hemolymph, as well as the venoms of various animals including scorpions (both Buthidae and non-Buthidae). They constitute a fundamental component of the innate defense system of scorpions. AMPS protect scorpions against the attack of a wide range of pathogens including bacteria, fungi, yeast, viruses, and protozoa via a multi-target mechanism of action. Until now, there are about 45 different AMPs that have been functionally characterized from scorpion venoms (Fig. 7, reviewed in Harrison et al. 2014). Structurally, the identified AMPs can be divided into two major categories: (i) cysteine-containing

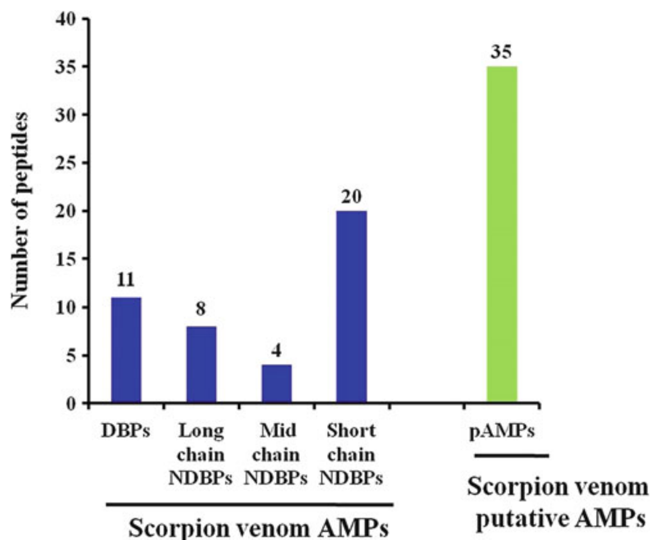

Fig. 7 Number of different types of antimicrobial peptides and putative antimicrobial peptides (*p*AMPs) identified from scorpion venoms. DBPs and NDBPs are disulfide-bridged peptides and non-disulfide-bridged peptides, respectively

peptides (26 % of the assigned molecules such as scorpine, opiscorpines, and bactridins) and (ii) non-cysteine-containing peptides with an amphipathic α-helix (74 %). Subsequently, Harrison and colleagues (2014) subdivided the second group into three types according to the length of their peptide chains: long-chain peptides (>35 amino acids such as pandinin-1), intermediate-chain peptides (20–35 amino acids such as meucin-24 and pandinin-2), and short-chain peptides (<20 amino acids such as IsCT and BmKb1) (Fig. 7). Recent transcriptomics studies revealed the high abundance of AMPS in the venom gland of various scorpions. For example, 24 % of all assigned ESTs of the venom gland of the Egyptian scorpion *S. m. palmatus* were corresponding to AMPs (Abdel-Rahman et al. 2013). Similar results were obtained from the venom gland of the scorpions *L. mucronatus* (Ruiming et al. 2010) and *T. stigmurus* (Almeida et al. 2012). Using a combination of transcriptomics and proteomics analysis, Luna Ramirez et al. (2013) characterized the first three short-chain α-helical AMPs from venom glands of the Australian scorpion *Urodacus yaschenkoi*. The three C-terminal amidated peptides UyCT1 (GFWGKLWEGVKNAI), UyCT3 (ILSAIWSGIKSLF), and UyCT5 (WSAIWSGIKGLL) are homologues (43–77 %) to the sequence of IsCT and showed a potent effect against Gram-positive and Gram-negative bacteria (MICs ranging from 1 to 25 mM). Also, the synergistic effect of these peptides was examined in order to increase their therapeutic index. Interestingly, these peptides have a greater affinity toward prokaryotic model membrane systems (K_d ranging

from 2 to 30 μM) than eukaryotic ones (K_d ranging from 150 to 200 μM) as confirmed through using dye release assays and isothermal titration calorimetry. These findings suggested that UyCT peptides could be good templates to develop new antibiotics against Gram-negative multiresistant bacteria, especially *A. baumannii* (Luna-Ramírez et al. 2014).

On the other hand, various putative AMPs assigned using the transcriptomics analysis of scorpion venom glands and their antimicrobial effects are still to be evaluated (Fig. 7). Based on sequence similarities, different cysteine-containing AMP-like peptides have been indentified such as OcyC7 (*O. cayaporum*, Silva et al. 2009), TcoScp1 (*T. costatus*, Diego-Garcia et al. 2005), HSP017C and HSP039C (*H. petersii*, Ma et al. 2010), SJE005C and SJE056C (*S. jendeki*, Ma et al. 2009), Pcav34 and Pcav23 (*P. cavimanus*, Diego-Garcia et al. 2012), Smp76 (*S. m. palmatus*, Abdel-Rahman et al. 2013), and MgibC6 (*M. gibbosus*, Diego-Garcia et al. 2014). Also, several non-cysteine-containing AMP-like peptides (similar to AMPs of IsCT, pandinin-1, pandinin-2, and BmKn2) have been found such as Hge027, Hge028, and Hge029 (*H. gertschi*, Schwartz et al. 2007); Ocy1, Ocy2, and Ocy3 (*O. cayaporum*, Silva et al. 2009); Pcav30 and Pcav7 (*P. cavimanus*, Diego-Garcia et al. 2012); Smp13, Smp24, and Smp43 (*S. m. palmatus*, Abdel-Rahman et al. 2013); HSP013C, HSP028C, and HSP049C (*H. petersii*, Ma et al. 2010); PsAMP5 (*P. stridulus*, Mille et al. 2014); and MgibC1, MgibC8, MgibC9, and Mgib253 (*M. gibbosus*, Diego-Garcia et al. 2014). It is worth mentioning that Smp13, Smp24, Smp43 (belonging to NDBP-5, NDBP-4, and NDBP-3 peptide sub-families, respectively), and Smp76 (scorpine-like peptide) are the first putative AMPs identified from the scorpion *S. m. palmatus* inhabiting the Sahara Desert of Egypt (Abdel-Rahman et al. 2013). All the precursors of Smp13, Smp24, and Smp43 contain a signal peptide, a mature peptide with no disulfide bridges (13, 24, and 43 amino acids, respectively), and a C-terminal pro-peptide. The mature peptide of Smp76 consists of 76 residues constrained by 3 disulfide bridges. Accordingly, the putative AMPs mentioned above may provide new templates for the development of new antimicrobial agents, and more effort should be done in this area.

Transcriptomics and Proteomics Data Consistency

The incomplete matching between transcriptomics and proteomics data has been reported in different scorpion species such as *H. petersii* (Ma et al. 2010), *U. yaschenkoi* (Luna-Ramírez et al. 2013), *C. tecomanus* (Valdez-Velázquez et al. 2013), and *S. m. palmatus* (Abdel-Rahman et al. 2013). For example, eleven venom-like peptides (Smp13, Smp22, Smp24, Smp27, Smp30, Smp33, Smp34, Smp43, Smp63, Smp73, and Smp76) have been characterized in the venom gland transcriptome of *S. m. palmatus*, with theoretical molecular weights 1,573.8, 2,344.7, 2,578, 3,046.4, 3,640.2, 3,842.5, 3,827.5, 4,654.3, 7,032.1, 7,870.1, and 8,396.8 Da. Of these molecules, only six components (Smp30, Smp33, Smp34,

Smp43, Smp73, and Smp76) were very similar to molecular masses (3,640, 3,843, 3,827, 4,656, 7,869, and 8,398 Da) identified through proteomics analysis of *S. m. palmatus* venom. Abdel-Rahman and colleagues (2013) concluded the main reasons that could be related to the incomplete consistency between proteomics and transcriptomics data:

1. The source materials, in most cases the crude venom, used in proteomics analysis are not milked from the same venom glands used to generate cDNA library.
2. Shortage in the availability of protein database, which is needed to structurally characterize putative peptides or proteins. Large numbers of putative sequences obtained by transcriptomics analysis are not fully characterized, and it is necessary to know the positions of signal peptide, pro-peptide, and mature peptide in order to precisely predict their molecular weights.
3. Some sequences can have posttranslational modification, which makes it difficult to calculate the exact molecular weight and correlate with the proteome.
4. The majority of high molecular weight proteins (especially housekeeping proteins) expressed in scorpion venom glands are not liberated in the milked venom.

Conclusion and Future Directions

The unique features of scorpions such as venom glands, fluorescence, and others attract investigators to apply various cutting-edge technologies (proteomics, transcriptomics, and high-throughput screening) to study biology and venomics of different scorpion species. The partial and global transcriptomics analysis has been applied to construct the venom gland cDNA libraries of 20 different scorpion species. Using this approach, several peptides with potential biological activities have been characterized, and they could be good templates to be developed into therapeutic agents. Moreover, transcriptome analysis revealed hundreds of new putative peptides (such as AMPs, neurotoxins, and LVPs), and the big challenge is to evaluate their biological effects. The next enormous challenge is to increase the number of transcriptomics studies on venom glands of other scorpions inhabiting different habitats such as desert environments. Taken together, venom transcriptomics is an efficient complementary approach in scorpion venomics and will attract more toxinologists to work on this exciting field of research and development.

Cross-References

► Shotgun Approaches for Venom Analysis

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Brown Spider Venom: The Identification and Biotechnological Potential of Venom Toxins

6

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Abstract

Brown spiders (*Loxosceles* genus) are venomous arthropods that use venom for predation and defense. These spiders have also been associated with human accidents, and the primary clinical manifestations are dermonecrosis with gravitational lesion spreading, hematological disturbances, and acute renal failure. *Loxosceles* venom comprises a complex mixture of toxins enriched in low molecular mass proteins (5–40 kDa). Characterization of this venom revealed three highly expressed protein classes: phospholipase-D family proteins, astacin-like proteases, and inhibitor cystine knot (ICK) peptides. A recent

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study also showed the presence of several other venom proteins, such as serine proteases, protease inhibitors, hyaluronidases, allergen-like toxins, and translationally controlled tumor protein (TCTP), expressed at low levels in *Loxosceles* venom. The Brown spider phospholipase-D family proteins have been well studied, and these toxins alone induce inflammatory responses, dermonecrosis, hemolysis, thrombocytopenia, and renal failure. In addition, the functional role of hyaluronidases as spreading factors in loxoscelism has been demonstrated. However, the biological characterization of other toxins has not been reported. Nevertheless, the mechanism by which *Loxosceles* toxins exert noxious effects is not fully elucidated. The aim of this chapter is to provide insights into Brown spider toxins, including the identification of novel toxins using molecular and proteomics approaches, and the biological characterization and structural description of toxins using X-ray crystallography. Putative biotechnological uses of *Loxosceles* toxins and future perspectives in this field will also be discussed.

Introduction

Spiders of the *Loxosceles* genus are one of the four groups of venom-producing spiders that cause significant clinical manifestations or human fatalities following envenomation (Palagi et al. 2013). This condition, referred to as loxoscelism, is commonly observed after accidents involving *Loxosceles* spiders and is characterized by several reactions. Although most bites are benign, local and systemic symptoms can emerge. Local reactions manifest as dark blue-violet necrotic wounds with gravitational spread that eventually become indurated, generating scar tissue (Chaim et al. 2011). In approximately half of cases, cutaneous lesions have been associated with nonspecific systemic symptoms, including fever, fatigue, headache, vomiting, pruritic reactions, and rash (Chaim et al. 2011; Isbister and Fan 2011). Systemic loxoscelism is a less frequent complication more often observed in children, with manifestations such as renal failure and hematological disturbances, i.e., disseminated intravascular coagulation and intravascular hemolysis (Isbister and Fan 2011).

Cutaneous loxoscelism has been described in several studies examining rabbit skin exposed to *Loxosceles* venom (Chatzaki et al. 2012; Silvestre et al. 2005). These studies describe the massive infiltration and aggregation of inflammatory cells, interstitial edema, increased vascular permeability, intravascular fibrin network deposition, thrombus formation inside the capillaries, and vessel degeneration. Longer exposure times lead to the degeneration and necrosis of the skin muscle and leukocyte infiltration into the skeletal muscle. The massive hemorrhage and destruction of epidermal integrity and necrosis of surrounding collagen near the epidermis have also been observed (Chatzaki et al. 2012; Gremski et al. 2014; Silvestre et al. 2005).

Viscerocutaneous loxoscelism is the systemic injury resulting from *Loxosceles* envenomation, and this condition occurs in up to 13 % of cases (Hogan et al. 2004).

Patients might develop acute kidney injury, which is responsible for most lethal cases of *Loxosceles* envenomation (Abdulkader et al. 2008). The analysis of mice renal tissue following the treatment of *Loxosceles intermedia* venom showed acute tubular necrosis in several nephrons, glomerular collapse, tubular epithelial cell cytotoxicity, eosinophilic material deposition in the tubular lumen, and basement membrane alterations (Luciano et al. 2004; Tambourgi et al. 1998). A direct effect of *L. intermedia* venom on kidney structures was also demonstrated in mice (Luciano et al. 2004). Using rats as models to examine *Loxosceles gaucho* venom nephrotoxicity, Lucato and coworkers (2011) revealed that this venom produces renal injury and rhabdomyolysis, independently from dermonecrotic injury or blood pressure changes. These authors attributed the observed renal injury to renal vasoconstriction and myoglobinuria because no direct effect of venom on isolated proximal tubules was demonstrated.

The severity of loxoscelism has been associated with the venom content. Several factors associated with the spider specimen, such as size, sex, species, nutritional state, and age, might influence the amount and content of venom. Evidence suggests that female venom exhibits the highest biological activity, positively correlated with higher sphingomyelinase activity (de Oliveira et al. 2005). *Loxosceles* venom primarily comprises a complex mixture of protein-based biologically active compounds with toxic and/or enzymatic activities (Gremski et al. 2014). Nonprotein compounds include sulfated guanosine derivatives, the major small-molecule components of the venom of *Loxosceles reclusa* (Schroeder et al. 2008). However, no correlation between loxoscelism and the presence of these molecules in the venom has been described. Additionally, the histamine content detected in *Loxosceles intermedia* venom is high enough to induce inflammatory responses (Paludo et al. 2009).

As previously mentioned, *Loxosceles* venom is enriched in protein-based molecules. The 1D and 2D electrophoresis profiles of the components from *L. intermedia* venom revealed two main groups of proteins at 20–40 kDa and 2–5 kDa (Fig. 1). The 2D map also showed that most spots were localized between pI 6 and 10, but the more intense spots were localized at neutral pI (Fig. 1). The 2D protein *Loxosceles* venom profile is similar among distinct species, such as *L. intermedia*, *L. adelaida*, *L. similis*, *L. gaucho*, and *L. laeta* (Barbaro et al. 2005; Luciano et al. 2004; Machado et al. 2005; Trevisan-Silva et al. 2013). Many protein and peptide toxins present in *Loxosceles* venom have been described, and the biological and biochemical properties of these compounds have been reported, providing insight into the pathophysiology of envenomation (Chaim et al. 2011; Hogan et al. 2004). Toxins, such as metalloproteases (da Silveira et al. 2007a; Feitosa et al. 1998), serine proteases (Veiga et al. 2000b), phospholipase-D family proteins (Chaim et al. 2006; da Silveira et al. 2007b; Tambourgi et al. 2002), hyaluronidases (Barbaro et al. 2005; da Silveira et al. 2007c), and insecticidal peptides (de Castro et al. 2004), have been detected in *Loxosceles* venom. Proteome analysis also revealed metalloproteases, serine proteases, phospholipases-D, and hyaluronidases in *L. intermedia* venom (dos Santos et al. 2009).

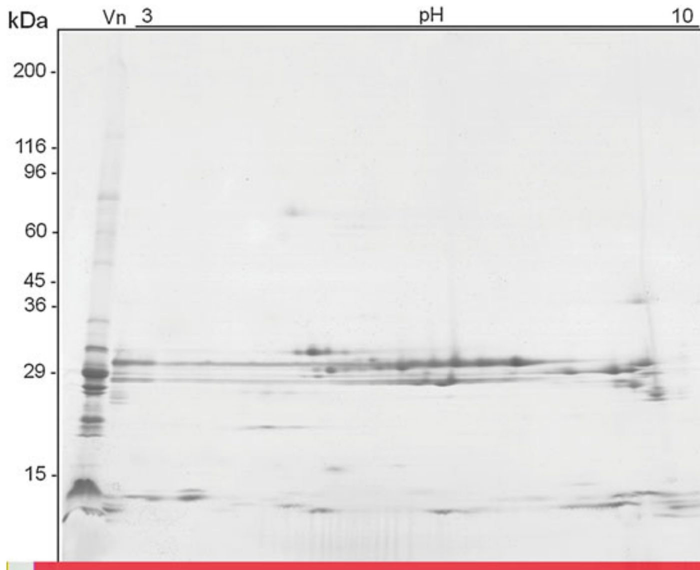

Fig. 1 Protein profiles from 1D and 2D gel electrophoresis analyses of *L. intermedia* crude venom. For 2D electrophoresis, samples of 150 μg of *L. intermedia* venom were subjected to isoelectric focusing (IEF) on 13-cm immobilized pH gradient gel strips with a pH linear range of 3–10. The second dimension analysis was performed on a linear SDS-PAGE gradient of 7.5–20 % under reducing conditions. The second dimension analysis was performed using isoelectric-focused venom and a sample of 20 μg of *L. intermedia* venom without IEF. The gels were stained with colloidal Coomassie Brilliant Blue. Vn indicates the 1D protein profile of *L. intermedia* venom. 3 pH 10 indicates the 2D protein profile of *L. intermedia* venom. Protein molecular mass markers are indicated on the left of the figure

More recently, transcriptome analyses were conducted to examine the protein content of *Loxosceles* venom obtained from the venom gland of two spider species: *L. laeta* and *L. intermedia* (Fernandes-Pedrosa et al. 2008; Gremski et al. 2010). The results showed the high expression of phospholipase-D and metalloprotease toxin families, and for *L. intermedia*, transcripts encoding ICK peptides (insecticidal peptides) were also highly expressed. Other toxin families, such as serine proteases, hyaluronidases, venom allergens, TCTP (translationally controlled tumor protein), enzymatic inhibitors, and C-type lectins, showed low levels of expression (Fernandes-Pedrosa et al. 2008; Gremski et al. 2010). These cDNA libraries provided an overview of the *Loxosceles* venom and facilitated the description of new molecules of biotechnological interest. Figure 2 displays the expression profile of the toxin families produced by *L. intermedia* venom gland, highlighting the main groups of venom components. Since then, several components, i.e., TCTP, hyaluronidase, *Loxosceles intermedia* recombinant dermonecrotic toxin 7 (LiRecDT7), and U2-SCRTX-Li1b, have been further explored and produced as recombinant molecules (Ferrer et al. 2013; Matsubara et al. 2013; Sade et al. 2011; Vuitika et al. 2013). Together with recombinant phospholipase-D and

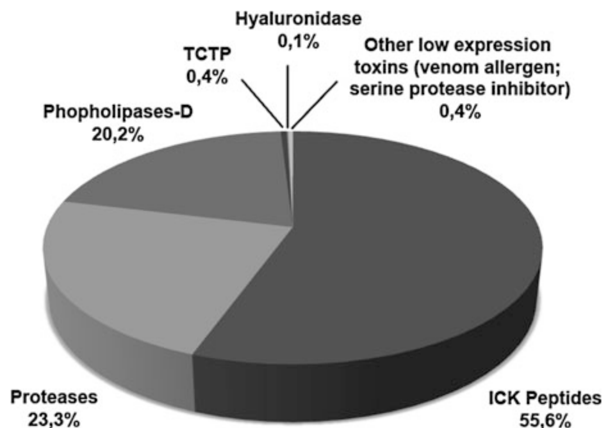

Fig. 2 Expression profile of *L. intermedia* venom toxin families over the total toxin-encoding sequences

metalloprotease LALP1 proteins, these recombinant toxins have been used as tools to provide further knowledge concerning venom activity and loxoscelism.

In this chapter, the relevance of these toxins to loxoscelism and the potential biotechnological application of these molecules are discussed.

Phospholipases-D

The phospholipase-D (PLD) family of toxins is the most well-studied and well-characterized component in the *Loxosceles* species venoms. These molecules have been reported to play an important role in the development of clinical sign and symptoms in loxoscelism. As these toxins induce dermonecrosis in vivo, the Brown spider PLDs are also known as dermonecrotic toxins (Chaim et al. 2011).

Gremski and coworkers (2010) revealed that 20.2 % of all the toxin-encoding ESTs from the *L. intermedia* venom gland correspond to PLDs, representing a significant proportion of the toxins present in the Brown spider venom (Fig. 2). Consistent with these data, Wille and coworkers (2013) conducted 2D electrophoresis analyses, showing at least 25 spots immunologically related to PLD toxins in the *L. intermedia* crude venom. Indeed, Machado and coworkers (2005) also identified at least 11 PLD isoforms, termed Loxonecrogin, in the venom of *L. gaucho*, suggesting the presence of several different PLDs in the Brown spider venom. Moreover, seven isoforms of PLD isoforms were generated as recombinant proteins, named LiRecDT (***L****oxosceles* ***i****ntermedia* ***rec****ombinant* ***d****ermonecrotic* ***t****oxin 1–7*), based on the *L. intermedia* venom gland transcriptome (Gremski et al. 2014), and these proteins have been classified as members of the Loxtox family (Kalapothakis et al. 2007). These recombinant PLDs were further examined to determine their roles in the inflammatory reaction, dermonecrosis, nephrotoxicity, and hemolysis observed in loxoscelism (Chaim et al. 2006; Chaves-Moreira et al. 2011; Luciano et al. 2004; Paludo et al. 2009).

Several PLD isoforms have also been identified in the venom of other *Loxosceles* species. In *L. reclusa* venom, the native PLDs were present at a molecular mass of approximately 32 kDa, and these four active isoforms induced dermonecrotic lesions, hemolysis, and platelet aggregation. Two PLD isoforms, at 32 and 35 kDa, were also described in *L. laeta* venom, and these proteins exhibited complement-dependent hemolysis, dermonecrosis, and sphingomyelin hydrolysis. In *L. laeta* venom two new PLD isoforms were reported: rLlPLD1, a dermonecrotic toxin with sphingomyelin hydrolysis activity, and rLlPLD2, a seemingly inactive protein, lacking the PLD region (i.e., rLlPLD2 lacked the initial amino acids of the catalytic site, such as His12). Several other PLD isoforms from the venom of other *Loxosceles* species, such as *L. gaucho*, have also been well characterized. It has been shown that recombinant isoforms of PLD from different species are able to reproduce most of the toxic effects observed after Brown spider bite and antigenic properties of the venom and can reverse venom effects with serum against them (Chaim et al. 2011; Gremski et al. 2014).

The PLDs are responsible for a large variety of disturbances in loxoscelism. Both native and recombinant PLDs trigger dermonecrotic lesions, increase vascular permeability, induce an intense inflammatory response at the inoculation site and at a systemic level, and promote platelet aggregation, hemolysis, nephrotoxicity, and even lethality in controlled experiments (Chaim et al. 2011; Gremski et al. 2014). In addition, recombinant *L. intermedia* phospholipase-D stimulates channel-mediated calcium influx into the cells via L-type calcium channels (Chaves-Moreira et al. 2011; Wille et al. 2013).

Initially, it was suggested that Loxtox protein family toxins played a role in the exclusive cleavage of the head-groups of sphingomyelin, the so-called sphingomyelinases. Further studies have shown that other substrates, such as glycerophospholipids and lysophospholipids, are also susceptible to PLD catalysis. Thus, the term phospholipase-D is more suitable for Brown spider dermonecrotic toxins (Chaim et al. 2010; Chaves-Moreira et al. 2011; Wille et al. 2013).

Studies comparing recombinant isoforms with distinct capacities for degrading substrates have shown differences in the intensity of the effects of these proteins (Chaim et al. 2010). Several recombinants of PLD isoforms from the *Loxosceles* genus have been heterologously produced in *E. coli*, and large amounts of the soluble and enzymatically active forms of these proteins are easily obtained. In addition, structural analysis of the catalytic site provided important insights into the enzymatic activities of each isoform (de Giuseppe et al. 2011; Murakami et al. 2005). Recently, de Giuseppe and coworkers (2011) published the crystal structure of LiRecDT1 from *L. intermedia*, indicating that this toxin contained an additional disulfide bond in the PLD structure catalytic loop compared with the previously described PLD from *L. laeta*. The structural details of PLD molecules reflect the distinct enzymatic behaviors of the venom from different species. PLDs with different structures could have different substrate affinities or enzymatic activities; therefore, these differences could explain the clinical symptoms or severity observed at the local bite site or the systemic effects during envenomation by different species of the *Loxosceles* genus.

Comparisons of the amino acid sequences of spider venom PLDs indicate that these proteins contain either 284 or 285 amino acids and display a significant degree of homology (de Santi Ferrara et al. 2009). The single polypeptide chain folds to form a distorted barrel, and the inner barrel surface is lined with eight parallel **β**-strands (termed A–H) linked by short flexible loops to eight **α**-helices (termed helices 1–8) that form the outer surface of the barrel (Murakami et al. 2005). The interconnecting loops are primarily hydrophilic and hydrophobic in the upper and lower sections, respectively. A short **β**-strand is inserted between strand B and helix 2, and two short helices are inserted between helix 3 and strand D and helix 4 and strand E. The catalytic loop is stabilized by a disulfide bridge (Cys51 and Cys57), and a second disulfide bridge (Cys53 and Cys201), which links the catalytic loop to the flexible loop to significantly reduce the flexibility of the latter loop, is present only in the class II enzymes (de Giuseppe et al. 2011). Because the **α**-helices, **β**-strands, and loops vary in length and character, the barrel is significantly distorted. The interior of the barrel is densely packed with hydrophobic amino acids, and in the short N-terminal section and the C-terminal extension, which contains a short helix, a **β**-strand and a random-coiled region cap the torus of the far side of the barrel. The surface loops forming the near side of the barrel are primarily hydrophobic, and a narrow cavity provides access to the catalytic site, characterized by a ring of negatively charged amino acids (de Giuseppe et al. 2011; Murakami et al. 2005). The catalytic and Mg^{2+} binding sites are located in a shallow depression and contain His12, Glu32, Asp34, Asp91, His47, Asp52, Trp230, Asp233, and Asn252, which are fully conserved in *Loxosceles* PLD isoforms (de Giuseppe et al. 2011; Murakami et al. 2005). Mutagenesis studies (de Giuseppe et al. 2011) and the crystal structure of PLDs (Murakami et al. 2005) indicate the involvement of two histidines in close proximity to the metal ion-binding site in the acid–base catalytic mechanism. Based on the structural results, His12 and His47 of PLD are key residues for catalysis and are assisted by a hydrogen bond network that involves Asp52, Asn252, and Asp233. Glu32, Asp34, Asp91, and solvent molecules coordinate the metal ion, which is essential for the catalytic activity of PLDs.

Furthermore, there are clear differences in the hydrolytic ability of PLD isoforms within the *Loxosceles* genus (Chaim et al. 2010; Chaim et al. 2011). Studies of LiRecDTs (isoforms 1–7) showed dermonecrosis at different levels in rabbit skin, consistent with the results of the spectrofluorimetric analysis of sphingomyelin hydrolysis (Chaim et al. 2011; Gremski et al. 2014). Ribeiro and coworkers (2007) reported that LiRecDT1 and LiRecDT2 were similar in all functional tests, including in vivo edema or cytotoxicity, while the effects of LiRecDT3 were significantly less intense. The observed amino acid alignment paralleled these results: LiRecDT1 and LiRecDT2 were markedly similar, but LiRecDT3 was slightly different. LiRecDT3 showed some important differences in hydrophobicity at the boundaries of the catalytic site, reflecting the differential performance of this enzyme (Ribeiro et al. 2007). In addition, site-directed mutagenesis of His12 in LiRecDT1, predicted to play a central role in catalysis, was not sufficient to completely abolish the catalytic activity of this enzyme. Moreover, the LiRecDT1H12A mutant isoform shows a dramatic reduction in enzymatic activity, but with no change in the secondary

structure, compared with LiRecDT1. Interestingly, although the mutant isoform did not induce the same level of any activity examined, attachment to the cell surface or to mobilized lipids remained unaltered (Chaim et al. 2010; Paludo et al. 2009; Wille et al. 2013). It is likely that other protein domains besides the catalytic cleft were preserved, and these domains might be relevant for interactions with the cell membrane or lipid substrates. PLD catalysis has primarily been associated with the release of lipid metabolites, which could modulate a wide range of biological events, such as the cell cycle, cell proliferation, cell differentiation processes, and cell death (Chaim et al. 2011; Gremski et al. 2014).

Dermonecrotic toxins are enzymes produced and secreted from Brown spider venom glands. These molecules catalyze the hydrolysis of phospholipids, such as sphingomyelin (SM), at a terminal phosphodiester bond to release choline and produce ceramide 1-phosphate (C1P) (Chaim et al. 2010; Chaves-Moreira et al. 2011; Wille et al. 2013). PLDs also mediate the Mg^{+2}-dependent hydrolysis of lysophosphatidylcholine (LPC) and release lysophosphatidic acid (LPA) (Chaves-Moreira et al. 2011; Horta et al. 2013; van Meeteren et al. 2004). These toxins are proteins that vary in molecular mass from 30 to 35 kDa and include a signal peptide followed by a propeptide. The amino acid sequences of PLDs are highly conserved (55–99 %), particularly in the residues around the catalytic cleft.

Studies have shown the upregulated expression of proinflammatory cytokines/chemokines after human fibroblasts are exposed to *L. reclusa* PLD (Dragulev et al. 2007), which hydrolyzes cell membrane SM into C1P, generating a receptor-dependent inflammatory response. This idea challenged the hypothesis that instead of C1P, LPA is a preferential product and bioactive metabolite, reflecting the relative abundance of LPC in the plasma as a substrate (van Meeteren et al. 2004). Recently, Horta and coworkers (2013) showed that cell death was induced through *L. similis* whole venom (LsV), and this effect was particularly observed with the recombinant isoform of *L. intermedia* PLD, recLiD1 (Kalapothakis et al. 2002). LPA released through the PLD activity of LsV and recLiD1 did not activate LPA receptors in the presence of an LPA1/LPA3 antagonist. This effect was indirectly observed through ELISA assays for IL-6, IL-8, CXCL1, and CXCL2. Moreover, these authors did not detect a role for LPA in the apoptosis induced by LsV or recLiD1 in fibroblasts and endothelial cells in vitro, which might be associated with other LPA-independent stimuli or the effect of C1P on the cell membrane receptors, as previously described. The variety of molecular mechanisms triggered through *Loxosceles* PLDs and their lipid metabolites should be further investigated as a complex event dependent on the types of cells involved, the abundance and availability of the lipid substrate, and intracellular signaling cascades. PLDs can serve as biotools to examine cell–cell communication via cell membranes in the context of inflammation. PLD isoforms have been proposed as potential models for designer drugs or other biotechnological applications (Chaim et al. 2011; Gremski et al. 2014). The production of more stable PLD isoforms with enhanced enzymatic activity would greatly contribute to many areas of toxinology and provide information to further the current understanding of the biochemical features, biological implications, and their related molecular mechanisms of PLDs.

Moreover, recombinant PLDs from *Loxosceles arizonica* also catalyze the transphosphatidylation of SM to generate cyclic ceramide phosphate (CC(1,3)P) and LPC to generate palmitoyl cyclic phosphatidic acid (CPA). The biological properties of cyclic phosphates are vastly different from their monoester counterparts, and these molecules might be relevant to the pathology of Brown spider envenomation (Lajoie et al. 2013).

Proteases

Proteases were first identified as components of *Loxosceles* venom in 1976 (Eskafi and Norment 1976). This study described the protease activity of *L. reclusa* crude venom against insect larvae using histochemical techniques. In addition, *L. reclusa* venom showed protease activity on distinct L-aminoacyl-β-naphthylamide derivatives, and L-leucyl-β-naphthylamide was more susceptible to the action of *L. reclusa* proteases. Subsequently, caseinolytic activity was reported in the venom of three *Loxosceles* species: *L. gaucho*, *L. laeta*, and *L. intermedia* (Chaim et al. 2011; Gremski et al. 2014). Two classes of proteases, metalloproteases and serine proteases, have been identified in *L. intermedia* venom (Feitosa et al. 1998; Veiga et al. 2000a).

Loxosceles metalloproteases were first described as two molecules of distinct molecular masses that degrade gelatin and exhibit clear substrate preferences (Feitosa et al. 1998). These metalloproteases were named Loxolysin A, a 20–28-kDa fibronectinolytic protease that is also responsible for the partial hydrolysis of fibrinogen (Aα and Bβ chains), and Loxolysin B, a 30–32-kDa gelatinolytic protease (Feitosa et al. 1998). Serine proteases have been detected in *L. intermedia* venom using zymography assays showing two gelatinolytic signals after trypsin activation (Veiga et al. 2000a). The biochemical nature of these proteases was characterized based on the total inhibition of gelatin hydrolysis using distinct serine-protease inhibitors, such as aprotinin, benzamidine, leupeptin, PMSF, and soybean-trypsin inhibitor (Veiga et al. 2000a). The biological functions of the proteases in *Loxosceles* venom remain unknown, but it has been suggested that these molecules might play a role in prey digestion and the deleterious effects observed after Brown spider envenomation. *Loxosceles* proteases might promote the diffusion of other venom toxins through the permeabilization of tissues and propagation of the toxic effects of other biologically active venom toxins. Moreover, these proteases could be important for processing other toxic proteins that become active after proteolysis (Barbaro et al. 2005; Chaim et al. 2011; da Silveira et al. 2002; Feitosa et al. 1998; Gremski et al. 2014). Notably, studies on the proteases in *Loxosceles* venom are scarce, and there have been more reports on the identification of metalloproteases than serine proteases.

Complementary data from *Loxosceles* serine proteases was obtained from transcriptome and proteome analyses (dos Santos et al. 2009; Fernandes-Pedrosa et al. 2008; Gremski et al. 2010). The transcriptome analysis of the *L. laeta* venom gland revealed fourteen ESTs that displayed similarity to serine-protease sequences

from the database (Fernandes-Pedrosa et al. 2008). In addition, the transcriptome analysis of the *L. intermedia* venom gland uncovered five transcripts with similarity to distinct members of serine-protease families (Gremski et al. 2010). These toxins are poorly expressed in *L. intermedia* venom, representing 0.3 % of the toxin transcripts (Gremski et al. 2010). These findings are consistent with the results of a proteome study of *L. intermedia* venom, in which five peptide sequences with similarities to snake venom proteases were identified (dos Santos et al. 2009). A previous analysis of the bidimensional protein profile of *L. intermedia*, *L. laeta*, and *L. gaucho* venoms showed spots with molecular masses corresponding to the identified serine-protease activities (Machado et al. 2005). However, the biological functions of *Loxosceles* serine proteases remain unknown. It has been suggested that this class of toxins are important not only for prey digestion but also for local tissue toxicity and hematological disturbances, such as blood coagulation and fibrinolysis, during Brown spider envenomation (Gremski et al. 2014; Veiga et al. 2000a). Indeed, the serine proteases in snake venom possess thrombin-like fibrinogenase and plasminogen-activating activities (Gremski et al. 2014). Because serine proteases present diverse structures, functions, and modes of action, further studies on *Loxosceles* serine proteases are imperative to obtain a better understanding of loxoscelism pathology and to develop potential novel therapeutic agents. Indeed, the potential therapeutic use of snake venom serine proteases in vascular diseases, such as in myocardial infarction, ischemic stroke, and thrombotic diseases, has been previously studied. Reptilase®, used to determine fibrinogen levels in the presence of heparin, is a thrombin-like serine protease purified from *Bothrops atrox* snake venom. Additionally, recombinant serine proteases have been studied in both basic and clinical research. A thrombin-like enzyme from the venom of an *Agkistrodon rhodostoma* viper improves the outcomes after cerebral stroke in humans, and Defibrase, an enzyme from the venom of *Bothrops*, is clinically beneficial in ischemic stroke (Chaim et al. 2011; Gremski et al. 2014).

Two metalloproteases have been identified in *L. rufescens* venom, a 23-kDa fibrinogenolytic protease and a 27.5-kDa gelatinolytic protease, and enzymatic activity was inhibited using 1,10-phenanthroline, thereby confirming the metalloprotease activity of these proteins (Young and Pincus 2001). The degradation of Aα and Bβ fibrinogen chains has been reported in the venom of both *L. reclusa* and *L. laeta*, and this activity was also inhibited with 1,10-phenanthroline. Other extracellular matrix molecules have been demonstrated as substrates of *Loxosceles* metalloproteases, such as entactin and heparan sulfate proteoglycans (Veiga et al. 2000b). It has been recently suggested that the *Loxosceles* metalloproteases identified in these studies were derived from gastric contents during venom extraction and not components of the venom. Nevertheless, the venom collected through electrostimulation and macerated venom glands were compared, and the protein profile and the protease activity of both samples were similar (da Silveira et al. 2002). Moreover, the distinct proteolytic profiles of the gastric content of *L. intermedia* and the venom obtained through both electrostimulation and macerated glands have been examined, and the results confirmed that metalloproteases are venom components (da Silveira et al. 2002). Consistently, the

proteolytic effect of *L. rufescens* venom was observed in venom obtained from the microdissection of the venom glands, a procedure that ensures the absence of gastric contaminants (Young and Pincus 2001). Evidence of *Loxosceles* metalloproteases has continued to increase, and *L. deserta*, *L. gaucho*, *L. intermedia*, *L. laeta*, and *L. reclusa* venoms showed in vitro protease activities primarily between 18.1 and 31.8 kDa (Barbaro et al. 2005). Thus far, all *Loxosceles* venoms have exhibited gelatinolytic, caseinolytic, and fibrinogenolytic activities, and most of these activities were abolished with 1,10-phenanthroline (Barbaro et al. 2005).

The analysis of a cDNA library of *L. intermedia* venom glands revealed a sequence encoding a metalloprotease presenting the hallmarks of astacin family enzymes: a catalytic domain of 18 amino acids (HEXXHXXGXXHEXXRXDR) and a conserved methionine involved in a sequence turn, met-turn (MXY) (Bond and Beynon 1995; da Silveira et al. 2007a). The zinc-dependent astacin (Merops M12A family) proteases have been described in most organisms from yeasts to humans. Although astacin family members are structurally related to metalloproteases, these enzymes present extremely diverse biological functions, including peptide hydrolysis, growth factor activation, and extracellular matrix component processing (Bond and Beynon 1995). In addition, these enzymes carry the name astacin based on the prototype enzyme isolated from the gastric fluid of the crayfish *Astacus astacus*, which played a role in digestion (Bond and Beynon 1995). The *L. intermedia* astacin LALP (*Loxosceles* astacin-like protease) was the first astacin family member identified as a constituent of animal venom. Two new astacin protease isoforms have recently been identified as toxins in *L. intermedia* venom, namely, LALP2 and LALP3. In addition, astacins have also been identified in *L. laeta* (LALP4) and *L. gaucho* (LALP5) venoms, suggesting the existence of an interspecies toxin family and revealing the importance of these metalloproteases as components of *Loxosceles* venom (Chaim et al. 2011; Gremski et al. 2014). Interestingly, the transcriptome analysis of *L. intermedia* and *L. laeta* venom glands revealed that astacin metalloproteases are highly expressed toxins (Fernandes-Pedrosa et al. 2008; Gremski et al. 2010). In the *L. intermedia* venom gland, astacin transcripts comprise 22.6 % of the toxin-encoding transcripts and 8 % of the total transcripts in the *L. laeta* venom gland (Chaim et al. 2010; Fernandes-Pedrosa et al. 2008). Thus, *Loxosceles* proteases (metalloproteases and serine proteases) account for 23.1 % of the total toxin-encoding transcripts in the *L. intermedia* venom gland, second only to insecticidal peptide sequences, which comprise the majority of expressed toxins (Fig. 2). In addition, the analysis of astacin content in *L. intermedia*, *L. laeta*, and *L. gaucho* venoms, using two-dimensional Western blotting and zymography, demonstrated a high content of active astacins among the three analyzed venoms, consistent with the high mRNA expression of astacins reported in the transcriptome analysis (Trevisan-Silva et al. 2013). Moreover, this study showed a distinct and complex proteolytic profile of *Loxosceles* astacin-like proteases, highlighting the importance of this toxin family to Brown spider biology and loxoscelism toxicity (Trevisan-Silva et al. 2013).

The functional characterization of isolated *Loxosceles* astacins has only been described for the first recombinant astacin isoform from *L. intermedia* venom

(da Silveira et al. 2007a). LALP hydrolyzes fibrinogen, fibronectin, and gelatin, consistent with previous data from distinct *Loxosceles* crude venoms (da Silveira et al. 2007a). Moreover, rabbit subendothelial cells exposed to LALP showed morphological alterations, such as the loss of substrate adhesion and cells that were fully rounded up and lysed, demonstrating the cytotoxic effect induced by LALP (da Silveira et al. 2007a). These results further suggest that metalloproteases could play a role in the toxic effects of Brown spider bites and also indicate the potential of these toxins for biotechnological applications (Chaim et al. 2011; da Silveira et al. 2007a; Gremski et al. 2014). Therefore, additional studies are required to explore the use of *Loxosceles* astacins as tools for the diagnosis of loxoscelism, basic research protocols, and the design of new drugs as agonist and/or inhibitor molecules. Additionally, astacins could be used as thrombolytic agents, and astacin inhibitors could be developed for use in the prevention of atherosclerosis.

Hydrolases (Hyaluronidases)

Hyaluronidases are a class of enzymes widely distributed throughout the animal kingdom. Hyaluronidases primarily degrade hyaluronic acid (HA), a ubiquitous component of the vertebrate extracellular matrix, and might also present hydrolytic activity upon chondroitin, chondroitin sulfate (CS), and dermatan sulfate (DS). Studies on several animal venoms, such as snakes, scorpions, spiders, bees, caterpillars, wasps, cone snails, fish, lizards, and stingrays, have described hyaluronidase activity. These matrix-degrading enzymes have been proposed to act as "spreading factors" in venom, as these enzymes promote tissue disorganization, facilitating the increased diffusion of other toxins throughout the tissues of the bite victim (Chaim et al. 2011; Gremski et al. 2014).

Cutaneous loxoscelism is characterized by edema, erythema, and necrosis, indicating extracellular matrix disturbances potentially associated with the actions of venom proteases and hydrolases. Indeed, a previous study of *L. reclusa* venom demonstrated hyaluronidase activity upon HA and CS types A, B, and C (Wright et al. 1973). The estimated molecular masses of the purified hyaluronidases were 33 and 63 kDa. Additionally, it has been demonstrated that rabbit antivenom serum inhibited both hyaluronidase activity in vitro and the spreading effect induced by the whole venom in vivo (Wright et al. 1973). HA-substrate SDS-PAGE was used to describe the hyaluronidase activity of a 32.5-kDa protein identified in *L. rufescens* venom (Young and Pincus 2001). Moreover, zymography assays revealed a 44-kDa hyaluronidase responsible for HA degradation in the venom obtained from five *Loxosceles* species of medical importance in the Americas (*L. deserta*, *L. gaucho*, *L. intermedia*, *L. laeta*, and *L. reclusa*) (Barbaro et al. 2005). These data suggest the biological conservation and significance of *Loxosceles* hyaluronidases (Barbaro et al. 2005). Two hyaluronidase molecules of 41 and 43 kDa were characterized as pH-dependent endo-β-*N*-acetyl-d-hexosaminidases in *L. intermedia* venom (da Silveira et al. 2007a). These enzymes degraded

HA and CS in vitro and HA in rabbit skin (da Silveira et al. 2007a). Consistently, a proteomics study also described the presence of hyaluronidases in *Loxosceles* venoms (dos Santos et al. 2009). *Loxosceles* hyaluronidases exhibit high activity, requiring few micrograms of the total venom to identify the enzymatic activity (Barbaro et al. 2005; da Silveira et al. 2007a). The transcriptome analysis of *L. laeta* and *L. intermedia* venom glands showed that this class of toxins is expressed at low levels (Fernandes-Pedrosa et al. 2008; Gremski et al. 2010). In the *L. laeta* venom gland, hyaluronidases represent only 0.13 % of the total expressed sequences (Fernandes-Pedrosa et al. 2008). The transcriptome of *L. intermedia* identified a transcript with similarity to hyaluronoglucosaminidase 1 from *Rattus norvegicus* (gbjEDL77243.1), which represents 0.1 % of the total toxin-encoding sequences (Gremski et al. 2010). The low expression of hyaluronidase transcripts might reflect the difficulties associated with the purification of this native enzyme from *Loxosceles* venom, leading to the lack of the biological and biochemical characterization of this protein. Using molecular biology techniques, Ferrer and coworkers (2013) generated a recombinant *L. intermedia* venom hyaluronidase, presenting a calculated molecular mass of 46.1 kDa. The active enzyme, named Dietrich's hyaluronidase, was obtained after refolding in vitro and was able to degrade HA and CS. These results are consistent with previous reports of native hyaluronidases that degrade glycosaminoglycans, demonstrating that the recombinant hyaluronidase can also be considered a chondroitinase (Ferrer et al. 2013). The biological characterization of Dietrich's hyaluronidase showed an increase in the erythema, ecchymosis, and dermonecrotic effect induced with the recombinant dermonecrotic toxin (LiRecDT1) in rabbit skin (Ferrer et al. 2013). This study greatly contributed to the understanding of loxoscelism pathology, showing that hyaluronidase is a spreading factor in *Loxosceles* venom (Ferrer et al. 2013). Additionally, active Dietrich's hyaluronidase can be used in further studies to examine the physiological and pathological role of hyaluronidases in HA degradation. The degradation of HA promotes tissue permeabilization and is involved in a great number of events, such as bacterial pathogenesis, fertilization, and cancer progression (Girish and Kemparaju 2007). Consequently, there are potential applications of hyaluronidases, including distinct surgical procedures, drug delivery, research oncology, and in vitro fertilization protocols (Chaim et al. 2011; Girish and Kemparaju 2007).

Inhibitor Cystine Knot (ICK) Peptides

Spiders are one of the most successful terrestrial predators, and the production of highly toxic venom to subdue prey and detain predators is one of the main features that contribute to the overall success of spiders (Windley et al. 2012). Most spider venoms contain small disulfide-rich peptide neurotoxins as major components, and these proteins are the largest and most extensively studied group of spider toxins (Windley et al. 2012).

Nearly 60 % of all spider venom toxins have three disulfide bridges, and these peptides primarily target voltage-activated ion channels. These peptides typically

contain a "disulfide pseudo-knot" and can be classified among toxins and inhibitory polypeptides with an "inhibitor cystine knot" (ICK) motif (Windley et al. 2012). The cystine knot is a structural motif with an embedded ring formed by two disulfide bonds that, together with their connecting backbone, are threaded by a third disulfide bond. This motif forms a structure comprising three antiparallel β-sheets (Daly and Craik 2011). In addition to ICK peptides, this same structural motif is present in two other families: the cyclic cystine knot (CCK) or cyclotide family and growth factor cystine knot (GFCK) family (Iyer and Acharya 2011). The ICK scaffold renders these peptides highly resistant to extremes of pH, organic solvents, high temperatures, and protease activities, which makes these molecules adequate targets for the design of drugs, molecular tools, and bioinsecticides (Daly and Craik 2011). The homology between the amino acid sequences of ICK peptides is typically low, but the distribution of cysteine residues is generally conserved. The consensus sequence of peptides exhibiting the ICK motif is $CX_{3–7}CX_{3–8}CX_{0–7}CX_{1–4}CX_{4–13}C$ – where X is any amino acid. The biological activities of spider ICK peptides are quite diverse, showing activity at voltage-activated sodium, calcium, and potassium channels, acid-sensing ion channels, transient receptor potential channels, and mechanosensitive channels (Windley et al. 2012).

Castro and coworkers (2004) were the first to describe ICK peptides in *Loxosceles* venom, and these authors isolated three peptides, LiTx1–3, with insecticidal activity from the venom of *L. intermedia*. The amino acid sequences of these toxins were demonstrated to contain an ICK motif, structurally defining these proteins as ICK peptides. These components are polypeptides with molecular masses ranging from 5.6 to 7.9 kDa, presenting insecticidal activity against highly destructive pests, such as *Spodoptera frugiperda* and *Spodoptera cosmioides*. Further analysis of the sequences revealed potential posttranslational modification regions, such as *N*-myristoylation, amidation, and casein kinase II phosphorylation sites, in the sequences of LiTx1–3. Based on the sequences of these toxins, the authors proposed that LiTx3 might act on NaV channels and both LiTx2 and LiTx3 might act on NaV or CaV channels (de Castro et al. 2004). Subsequently, these authors also described the sequence of another ICK peptide from *L. intermedia*, LiTx4. Analysis of the *L. intermedia* venom gland transcriptome showed that ICK peptides comprise 55,6 % of toxin-encoding mRNAs (Fig. 2). The most abundant toxin transcripts were similar to LiTx3 (32 %), followed by LiTx2 (11.4 %), LiTx1 (6.2 %), and LiTx4 (3.7 %) (Gremski et al. 2010). Additionally, 2.3 % of the toxin-encoding mRNAs represent transcripts similar to the ICK neurotoxin Magi3 from *Macrothele gigas*, which binds to site 3 of NaV channels. The transcriptome analysis of the *L. laeta* venom gland showed that 0,2 % of all toxin transcripts were similar to Magi3 (Fernandes-Pedrosa et al. 2008). Further analysis of the ICK peptide mRNAs from the *L. intermedia* venom gland revealed several sequences similar to LiTx3 that have not yet been described, potentially representing novel ICK neurotoxins (Gremski et al. 2010). Indeed, the cloning and production of a recombinant peptide from *L. intermedia* venom with high similarity to the ICK family of peptides, particularly LiTx3, has been described (Matsubara et al. 2013).

The recombinant peptide, named U2-sicaritoxin-Li1b (U2-SCRTX-Li1b), was used to demonstrate the antigenic cross-reactivity of antisera raised against the crude venom of *L. intermedia*, *L. gaucho*, and *L. laeta* with U2-SCRTX-Li1b. This cross-reactivity confirms the presence of ICK-like toxin members in these *Loxosceles* venoms, consistent with the idea that this toxin family is widespread throughout the genus (Matsubara et al. 2013).

Currently, there is no evidence of the involvement of Brown spider ICKs in the pathogenesis of spider bites (Gremski et al. 2014). There is increasing interest in toxins presenting the ICK motif, as the molecules exhibit marked stability and target ion channels. These features suggest that these molecules might play an important role in drug design and therapeutic applications and the development of bioinsecticides and pharmacological tools to study the pharmacology and biophysics of ion channels (Windley et al. 2012).

Translationally Controlled Tumor Protein (TCTP)

The *L. intermedia* venom gland transcriptome analysis described the sequence of a protein identified as a member of the TCTP family and revealed that this TCTP is present at relatively low levels in the venom, i.e., only 0,4 % of the toxin-encoding transcripts (Gremski et al. 2010). *Translationally controlled tumor proteins (TCTPs)* were first described as proteins that were regulated at the translational level. The *tumor* is derived from the first TCTP cDNA sequence described, which was obtained from a human mammary tumor (Bommer 2012). This protein has also been demonstrated as a histamine-releasing factor (HRF) and a fortilin (Bommer 2012). The *Loxosceles intermedia* TCTP was cloned and expressed as a heterologous protein in an *E. coli* expression system. The functional characterization of the recombinant protein, LiTCTP, showed that this toxin caused edema and enhanced vascular permeability (Sade et al. 2011). The cutaneous symptoms of envenomation with *Loxosceles* venom include erythema, itching, and pain. In some cases, *Loxosceles* spider bites can cause hypersensitivity or even allergic reactions. These responses could be associated with histaminergic events, such as an increase in vascular permeability and vasodilatation. LiTCTP could be associated with these deleterious venom activities, as this protein was identified in *L. intermedia* venom (Sade et al. 2011). Another *Loxosceles* TCTP has been described in the venom gland of *L. laeta* using transcriptome analysis (Fernandes-Pedrosa et al. 2008). A recent transcriptome analysis revealed a TCTP protein (named GTx-TCTP) in the venom gland and pereopodal muscle of the tarantula *Grammostola rosea* (Kimura et al. 2012).

Proteins of the TCTP superfamily have previously been described in the gland secretions of ixodid ticks and distinct spider species. In spiders, TCTP was first identified as the main pharmacological toxin in the venom gland of the wolf spider (*Lycosa godeffroyi*). The tarantula *Grammostola rosea* transcriptome analysis also identified the GTx-TCTP. Recently, TCTP proteins were identified in the venom gland of the spit spider (*Scytodes thoracica*). The three TCTP cDNA sequences identified in *S. thoracica* were 86 % identical to LiTCTP (Zobel-Thropp et al. 2014).

TCTP family members have been described as extracellular HRFs and are associated with the allergic reactions of parasites. The TCTPs are completely conserved among species from the same genus. The LiTCTP phylogeny tree demonstrates the similarities with the TCTPs from ixodid ticks, which were also characterized as HRFs (Sade et al. 2011). Ultrastructural studies of the *L. intermedia* venom gland revealed that TCTPs and other constituents of the whole venom are secreted through holocrine secretion (Gremski et al. 2010). TCTP secretion proceeds via an ER-/Golgi-independent or nonclassical pathway, most likely mediated through secreted vesicles, known as exosomes (Hinojosa-Moya et al. 2008). TCTP mRNAs do not encode a signal sequence, and no precursor protein has been described; however, a TCTP protein has been identified in the biological fluid of asthmatic or parasitized patients, the saliva of ticks (Hinojosa-Moya et al. 2008), and the crude venom of spiders. TCTPs represent a large, highly conserved, and ubiquitous protein family in eukaryotes, and these proteins are widely expressed in various tissues and cell types. TCTP protein levels are highly regulated in response to a wide range of extracellular signals and cellular conditions, suggesting that these proteins participate in various biological functions at diverse biochemical and signaling pathways. Indeed, multiple functions and biochemical roles for TCTPs have previously been examined (Chaim et al. 2011; Gremski et al. 2014).

TCTP proteins have already been described as calcium-binding proteins and as proteins that interact with the cytoskeleton through the binding and stabilization of microtubules. The involvement of TCTPs in the mitotic spindle has also been shown, and TCTPs are now considered as regulators of mitosis. A crucial role for TCTPs in early development has also been described. The loss of TCTP expression in mice leads to increased spontaneous apoptosis during embryogenesis and causes lethality. TCTP can be described as a multifunctional protein, reflecting the high number of protein partners and several areas/pathways of cell metabolism involving this protein (Amson et al. 2012). TCTPs are also a therapeutic target in cancers. The downregulation of TCTP has been implicated in biological models of tumor reversion, and these proteins are the targets of various anticancer drugs (Amson et al. 2012).

Studying LiTCTPs can elucidate the biological aspects of loxoscelism, particularly those aspects associated with histaminergic symptoms. Moreover, the analysis of LiTCTP can provide new insights into the functional characterization of the TCTP family. LiTCTP represents a promising tool for studies in toxinology and immunological and allergenic and experimental oncology.

Putative Toxins

The overall composition of *Loxosceles* venoms has been recently studied using proteome and transcriptome analyses (dos Santos et al. 2009; Fernandes-Pedrosa et al. 2008; Gremski et al. 2010). The results of these studies have not only uncovered the sequences of several toxins previously implicated in the pathophysiology of loxoscelism, such as phospholipases-D and metalloproteases, but sequences that

might represent proteins with putative roles in envenomation, i.e., putative toxins, have also been revealed.

Sequences with significant similarities to allergen-like toxins from other venoms were detected in the transcriptome studies of *L. laeta* and *L. intermedia* venom glands (Fernandes-Pedrosa et al. 2008; Gremski et al. 2010). In both cases, these transcripts are poorly expressed. The sequences identified in the *L. intermedia* transcriptome encode cysteine-rich venom allergens that show significant similarities to allergens from another spider genus (*Lycosa singoriensis*), as well as scorpions and mite allergens (Gremski et al. 2010). The amino acid sequence of a putative allergen from *L. laeta* venom is similar to venom allergen III (sp|P35779| VA3_SOLRI) and includes the presence of conserved cysteine residues (Fernandes-Pedrosa et al. 2008). In addition, the *L. intermedia* proteome analysis revealed a putative allergenic protein similar to a mite allergen (dos Santos et al. 2009). Indeed, allergic reactions following *Loxosceles* bites have been described in a few cases, as reviewed by Gremski and coworkers (2014). A fine macular or papular eruption appeared over the entire body in approximately 25 % of the published loxoscelism cases (Pippirs et al. 2009). Additionally, cases of AGEP (acute generalized exanthematous pustulosis) after accidents with *L. reclusa* and *L. rufescens* have been reported (Lane et al. 2011; Makris et al. 2009). A recombinant allergen from *L. intermedia* venom has previously been cloned, with a calculated molecular mass of 46.2 kDa and a predicted hydrophobic import signal to the endoplasmic reticulum (Ferrer VP and de Mari TL, 2014, personal communication). This recombinant protein might facilitate the investigation of the mechanisms underlying the allergic responses observed in loxoscelism cases and might be used for biomedical purposes in this field (Gremski et al. 2014).

Putative enzymatic inhibitors represented by transcripts with significant similarity to sequences encoding this group of proteins have been described in *Loxosceles* venom gland transcriptome analyses (Fernandes-Pedrosa et al. 2008; Gremski et al. 2010). The sequences identified in the *L. laeta* transcriptome showed significant similarities with serine (or cysteine)-protease inhibitors of diverse species with different functions and activities associated with the inhibition of factor Xa (Fernandes-Pedrosa et al. 2008). These studies also revealed transcripts with sequences similar to the serpin Bl-Spn-1, which inhibits the proprotein-processing proteases PC1/3 and furin. The *L. intermedia* transcriptome analysis revealed an EST similar to protease inhibitors from the serpin family, as described in the *L. intermedia* proteome, which also showed sequences similar to inhibitors from the cystatin family and Kunitz-type inhibitors (dos Santos et al. 2009; Gremski et al. 2010). Thus, *Loxosceles* venom might contain protease inhibitors belonging to different groups. It has been suggested that venom protease inhibitors might protect the integrity of the toxins through resistance to prey proteases (dos Santos et al. 2009; Zhao et al. 2011). Venom protease inhibitors are potential candidates to mediate certain biological processes, because proteases are involved in several physiological processes and are excellent therapeutic targets.

The transcriptome analysis of the *L. laeta* venom gland revealed 15 clones (0,5 % of the total) with sequence similarities to C-type lectins from different

species, i.e., *Bos taurus* (mammal) and *Tachypleus tridentatus* (arthropod), among others (Fernandes-Pedrosa et al. 2008). These clones were classified as "possible toxins." Indeed, a recent study showed that a C-type lectin isolated from *Bothrops atrox* snake venom exhibited glycan-binding characteristics similar to endogenous mammalian glycan-binding proteins and induced significant changes in leukocyte migration and activation that might contribute to host inflammation following *B. atrox* envenomation (Sartim et al. 2014). The authors point that previous studies have also implicated venom C-type lectins in the inflammatory response. In addition, venom C-type lectins have been suggested as prospects for potential use in cancer therapy because these factors promote antiangiogenesis and interfere with tumor cell proliferation (Calderon et al. 2014).

Transcripts with similarity to a salivary protein from *Ixodes scapularis* ticks have also been identified in the *L. laeta* transcriptome and included in the group of "possible toxins" (Fernandes-Pedrosa et al. 2008). The authors suggested that these sequences might be associated with substances that prevent blood clotting in the saliva of this tick for feeding functions. In addition, sequences similar to chitinolytic enzymes, which catalyze the hydrolysis of chitin, a polysaccharide in the exoskeleton and gut lining of arthropods and fungi, have also been identified in the *L. laeta* transcriptome and classified as "possible toxins" (Fernandes-Pedrosa et al. 2008).

A group of transcripts similar to "5′-nucleotidase" was also detected in the *L. laeta* venom glands (Fernandes-Pedrosa et al. 2008). The authors pointed that this group of enzymes might affect hemostasis through the inhibition of platelet aggregation, as these enzymes deplete the ADP present in the plasma. Although these transcripts were classified as encoding "possible toxins," Sales and Santoro (2008) showed that the venom of *Loxosceles gaucho* lacks most nucleotidase and DNase activities.

The presence of phosphate-releasing enzymes has been reported in *L. gaucho* venom (Sales and Santoro 2008). This study showed that alkaline phosphatases are primarily involved in the degradation of nucleotides in *L. gaucho* venom. Indeed, the authors point that previous studies demonstrated that the venom of *L. reclusa* shows ATPase and alkaline phosphatase activities. However, the role of alkaline phosphatase in *Loxosceles* venoms remains unknown.

Conclusion and Future Directions

Studies on *Loxosceles* toxins are continuously increasing. In recent years, several new toxins have been identified in *Loxosceles* venom through data from molecular biology techniques, proteomic studies, and the characterization of recombinant toxins. Indeed, the identification, biological characterization, and structural analysis of *Loxosceles* toxins improved the current knowledge of the venom composition and participation of these toxins in loxoscelism. However, several molecules remain unidentified, without biological characterization and/or unknown mechanisms of action, particularly the toxins with low expression levels.

Therefore, studies focusing on the recombinant production of novel toxins or the production of increased amounts of some known toxins are imperative to characterize the influence of these toxins on the toxic effects of Brown spider venoms and to explore the putative biotechnological applications of these molecules. Additionally, the design of inhibitor molecules for different toxins could be used as a tool to elucidate the underlying mechanisms and elaborate basic and clinical research protocols.

Cross-References

► Shotgun Approaches for Venom Analysis

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Venom Toxins of Fire Ants

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Eduardo Gonçalves Paterson Fox

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Abstract

Fire ants are aggressive invasive insects spread around the world via ship cargo, mainly originating from the United States. They can cause severe impacts on human activities and the environment. This chapter presents an overview of what is known about fire ant venom, ending with different open possibilities for investigation in this topic. For decades, studies on fire ant venoms have been limited in scope because of the technical difficulties in obtaining enough samples for analysis and bioassays; yet now there is one simple, effective, published method for extracting venom from whole colonies. Fire ant venom is mainly composed of a mixture of >95 % bioactive piperidine alkaloids and 0.01 % of proteins, which comprise mainly allergens, phospholipases, and neurotoxins. The alkaloids of fire ants, generally named solenopsins, are well known for their antifungal and insecticidal properties; however, many have also been suggested as promising alternatives for other biomedical applications, such as the treatment

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of parasitemia and various neurological diseases. The venom proteins of fire ants remain only superficially studied, as most published literature focuses on just four allergens. Crucially, others may contain compounds of interest to immunotherapy or even play a central role in aspects of social organization.

Introduction

Fire ants are a serious worldwide invasive pest originating from South America. Some of the first observations on the aggressiveness of fire ants were recorded by Henry Bates in his famous book *The Naturalist on the River Amazon*. As the author states,

> . . .Tapajos is nearly free from the insect pests [. . .] but the formiga de fogo is perhaps [the] greater. [. . .] They seem to attack persons out of sheer malice [. . .] whose sting is likened by the Brazilians to the puncture of a red-hot needle. (Bates 1856)

Fire ants will typically attack in great numbers, often while foraging, but most markedly when their fragile nests are disturbed. Yet, fire ants will not only attack man and also not only when defending their nests. Indeed, fire ants have been observed sending out foragers to locate nests and burrows of other animals, including vertebrates, and then recruit worker raids to attack, kill, and consume the nestlings and their food (Chalcraft and Andrews 1999). This results in considerable ecological impact from a fire ant infestation on local avian (Drees 1994) and reptile fauna (Allen et al. 2001) and can likewise affect human activities, livestock, and domestic animals. This behavior can affect humans, where fire ants have been reported to recurrently enter homes and attack sleeping people of various ages in their beds both in residences and hospitals in infested areas (author's observations in Brazil). Fire ant infestations have been a recurrent issue in small towns in the Amazon (reportedly from Novo Aripuanã, Envira, and Novo Airão), where people can be forced to abandon their homes until the infestation is controlled (Mendes 2008). Concerning other recurrent problems caused by fire ants in agriculture, they are known to attack several culture crops and some stored grains and can associate with sap-sucking pest insects, elevating their numbers, apart from being a constant nuisance to the exposed field worker. Moreover, they can frequently attack farm animals, especially young cubs.

Some remarks on the stinging behavior of fire ants while attacking are as follows: they often take some time climbing on the victim prior to stinging; thus, it is common for prey and victims to realize the attack only when many ants are already stinging at roughly the same time. Given their small size, fire ants must hold with their mandibles in order to insert their sting (Fig. 1), and the injection is rather slow as the venom is poorly soluble in water and their venom apparatus lacks muscles around the venom reservoir (Fox et al. 2010). There is one erroneous description of their stinging behavior that has been repeated many times over in the literature: that "fire ants sting repeatedly, many times rotating in a circular pattern, while maintaining a grip with their mandibles" (e.g., Hoffman 1995) – this notion

Fig. 1 A fire ant minor worker stinging the author (Picture courtesy of Roberto Eizemberg)

must be revisited, as logically an insect cannot rotate much while biting. Instead, a fire ant will quite often let go of the bite shortly after the sting was introduced into human skin and will remain waiting in place while venom is being injected; it generally will bite and sting again in the case of attempted removal or when eventually the grip of the inserted stinger comes lose. This pattern of behavior generally results in three or more different stings close together (usually not in circular pattern) delivered by each attacking fire ant while wandering, for instance, under the clothes of a human victim. On the other hand, during fights (usually against another insect), it will hold the bite hard and continuously with its mandibles (even postmortem) and will deliver venom whether or not the stinger is inserted, but this is generally like most other ants behave.

Fire ants are among the most common ants to be found in South America, where they typically occur in the lawns around houses and public areas (only one species being recurrently found in woods). Given their prevalence, quite often local ant queens and small colonies can be accidentally transported elsewhere by sea among commercial goods and ship ballast, and this has been a major determinant for the spread of invasive fire ants around the world (Tschinkel 2006). In some invaded areas, fire ant colonies have become even more abundant than in their original habitats in South America, and local fauna and human residents must physiologically and behaviorally adapt to deal with the new aggressive ants. Thus, quite often the arrival of fire ants can prove disastrous to local populations. At some point in the middle of the twentieth century, two species of fire ants were introduced to the Southern United States, probably among shipped goods originating from somewhere between Argentina and the south of Brazil (Tschinkel 2006). At least one other fire ant species may have arrived in the United States much earlier, perhaps coming from Central America (Wetterer 2011). Nowadays approximately 40 % of the territory in the United States is reported to contain fire ant colonies, and the estimated national spending surmounts over five billion dollars a year in losses and combative efforts, which are proving not quite effective (McDonald 2006). These figures are but a numeric indication of how fire ants can be dangerous to agriculture

and human welfare when their numbers get out of hand. The world spread of fire ants among shipped commerce goods, mainly originating from the United States, has become a serious concern to specialists around the globe, and the potential damage to regions suitable for invasion (for an interesting predictive map, refer to Morrison et al. 2004) is impossible to precise. As a piece of irony, referring again to Bates (1856), who wrote about the problem with fire ants in the Brazilian Amazon,

> This species is exclusively found in sandy soils, in open semi-cultivated or neglected places [where] they increase only in the neighbourhood of deserted houses or unweeded plantations; consequently they are a scourge only to the lazy and worthless people that inhabit the shores of this magnificent river.

As a result of the growing concern, fire ants are currently one of the most intensively studied insects in the world and as such have become a model species for studies of social insects. One of the most strongly emphasized aspects concerning fire ant biology is the effect of their stings and venom. This chapter aims to present a summarized overview of the state of knowledge on fire ant venom with special emphasis on some potential trends of future studies.

Fire Ant Venom: A Dangerous Mystery

As to illustrate how much of a threat fire ants can be to human populations, in some regions in the United States, over one fourth of the local residents may prove highly sensitive to fire ant stings (Ownby 2008). Quite often highly sensitive subjects live in heavily infested areas, where abundant fire ant nests can be found at house gardens and sidewalks where children and pets play. In such areas fire ants constitute a constant danger, especially to young toddlers and the elderly, who cannot react quickly and efficiently against aggressive insect attacks. The problem is fire ant stings can be often quite harmful, as will be explained in this section.

Among the stinging ants that recurrently induce anaphylaxis – *Hypoponera*, *Myrmecia*, *Odontomachus*, *Pogonomyrmex*, *Pachycondyla*, *Pseudomyrmex*, *Rhytidoponera*, *Solenopsis*, and *Tetramorium* – registered allergic reactions following accidents with fire ant stings are by far the most frequent prevalent in a world scale, with an estimated 50 % of residents being stung in endemic regions (Kemp et al. 2000). The onset of a serious anaphylactic reaction to fire ant stings in sensitized victims can occur within minutes after few stings, often unnoticed stings, and this can rapidly escalate from systemic itching and swelling to tachycardia and difficulty breathing. In rare cases, such reactions may culminate in death (Stablein et al. 1985; Prahlow and Barnard 1989; More et al. 2008). As such, sensitive patients who reside in infested areas are advised to undergo immunotherapy as a security measure to increase their resistance to likely future encounters with fire ants (Stafford 1996). However, fire ant immunotherapy is even today done using whole-body extracts of workers, given the traditional impracticability in obtaining pure venom protein as delineated below.

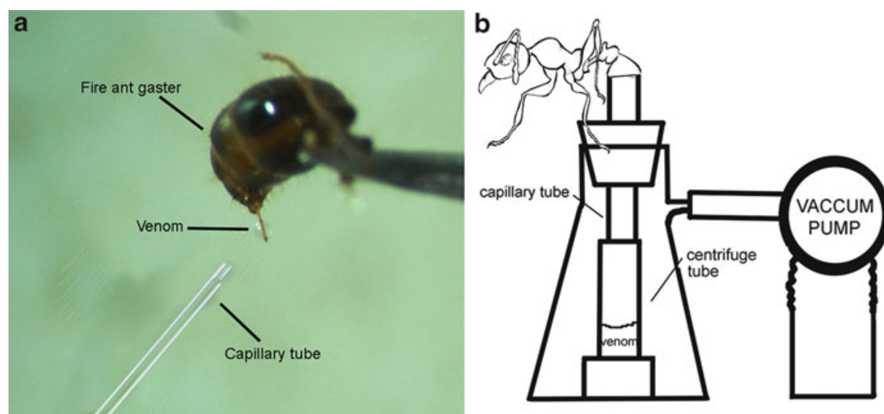

Fig. 2 Traditional labor-intensive methods for collection of fire ant venom. (**a**) Many studies have reported milking individual ants using a capillary tube, inducing venom extrusion by different means (e.g., excision of gaster, contact with another ant, electric shock). (**b**) A depiction of an intricate method described in Hoffman (1995), where pure venom is amassed into a tube by sucking the gaster of individual ants with a capillary tube, followed by centrifugation for separation of alkaloidal phase and aqueous phase (Adapted from Hoffman (1995), not drawn to scale)

Most studies about fire ant venoms are actually based on a small amount of general published information about the venom composition. For over 30 years, the limited sensitivity of methods employed and difficulties in extracting venom in quantities sufficient for biochemical characterization have hampered any ampler overview regarding the composition of fire ant venoms. Also, the unique composition of fire ant venom makes it hard to analyze: it is primarily composed (over 95 %) of a mixture of hydrophobic alkaloids somehow solubilized in conjunction with a comparatively minute amount of toxic proteins and peptides.

Given the small mass of each individual ant (ca. 1 mg), venom extracted from just a few thousand fire ants will deliver quantities sufficient for only superficial analysis of the most abundant venom alkaloids. Traditional methods for extracting fire ant venom are remarkably laborious, based on dissecting or milking each individual ant for venom over several weeks and saving the samples in a freezer until use. A few examples of such methods are depicted in Fig. 2, using fine capillary tubes or tissue paper, or even some suction apparatus adapted to collect venom from individual workers. Haight and Tschinkel 2003 reported employing direct repetitive contact of fire ant workers with a larger carpenter ant so as to stimulate their releasing venom droplets to keep them alive after milking.

However, one remarkable biochemical investigation from the late 1970s (Baer et al. 1979) reported obtaining as much as ~120 mg of pure venom, through milking individual amputated gasters (=posterior pedunculate body part of aculeate hymenopterans) of an estimated million ants – this feat having required three fully dedicated technicians over almost 3 years (personal communication by Murray S. Blum). This study by Baer et al. (1979) was the first to detect the existence and analyze proteins among the mixture of venom alkaloids, which were then partially

separated by size-exclusion liquid chromatography and superficially described in terms of their amino acid composition.

Unfortunately for scientific studies and immunotherapists, there is only one company from the United States selling pure venom extracts of fire ants for research, and they have not patented nor formally described their method of extraction. In some studies it was briefly stated that the ants can be milked by electrical stimulation (for a comprehensive overview on traditional methods for venom extraction and purification with fire ants, refer to Hoffman 1995), thus possibly done by placing a certain quantity of workers on tissue paper on an electric-pulse grid moistened with buffer solution and then recovering the extruded venom compounds from the tissue paper. Given such technical and practical impediments for collecting fire ant venom, research in this area has been delayed for decades, and as mentioned, even today immunotherapy with sensitive subjects is limited to the use of fire ant whole-body extracts.

Only recently a more practical method for extracting venom from large quantities of fire ants has been devised, enabling extraction of whole venom from fire ants in gram amounts within a few hours (Fox et al. 2013), based on a simple solvent extraction of live ants. This procedure has been shown to yield the very same results as obtained by the much more laborious methods employed by previous authors, as can be seen both by liquid chromatography and proteomic analysis. See Fig. 3 for a comparison of obtained results with previous studies employing extraction methods as shown in Fig. 2: compare 3A with similar profiles presented in Baer et al. (1979) and Hoffman et al. (1990), compare 3B with similar profiles presented in Pinto et al. (2012) and Sukprasert et al. (2012), and compare 3C with similar results shown in Hoffman et al. 1988, Hoffman et al. (1990), and Sukprasert et al. (2012).

Basically, the fire ants are obtained from field nests (or cultured in the laboratory), separated from the soil by flotation, and then they can be allowed to clean/groom themselves for some time or can be directly immersed into a biphasic mixture of water and some strongly apolar solvent, such as chloroform or pentane. The venom alkaloids and cuticular hydrocarbons will be extracted into the upper (organic) phase, while the lower aqueous phase will prove rich in active venom proteins and peptides (Fig. 4). Clean extracts of both fractions are obtained after centrifugation and evaporation of the solvents for further procedures or freeze storage. This new method has opened the possibility of obtaining fire ant venom in greater amounts, using minimum structure and enabling subsequent bioassays or purification steps. It has been hinted that the extraction method could be adapted to obtain venom from other aggressive animals, such as *Pseudomyrmex* ants or wasps and bees, but this still awaits direct experimentation (Fox et al. 2013).

Some of the Most Toxic Known Proteins: A Brief Molecular Overview

For decades, there were only a handful of proteins known from the venom of fire ants, given the general difficulty in obtaining enough samples for proper analysis. These few venom proteins were described for the first time in a comparative chromatographic

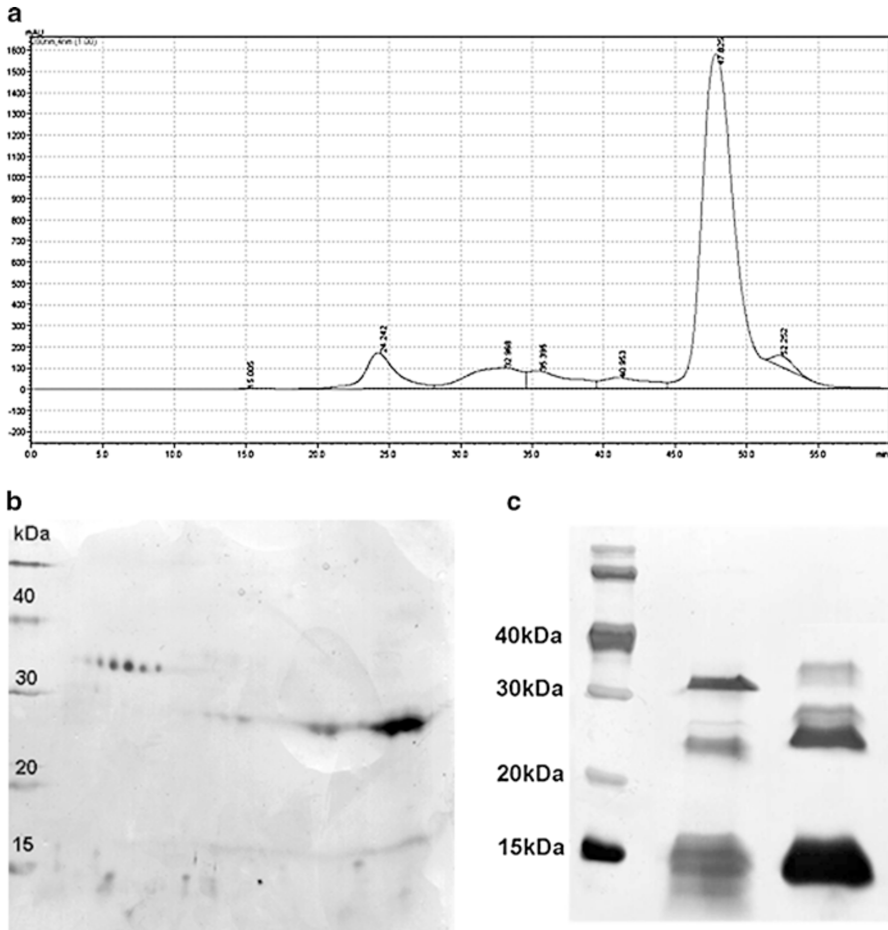

Fig. 3 Chromatographic laboratory analyses of fire ant venom proteins extracted by the fast method described by Fox et al. (2013). (**a**) Liquid chromatography of aqueous phase of *Solenopsis invicta* venom through a Sephadex G-75 gel exclusion column, as tracked by absorption at 256 nm. (**b**) Bidimensional SDS-PAGE scanned result gel of lyophilized venom proteins of S. invicta, revealed by silver staining reaction. (**c**) Unidimensional SDS-PAGE scanned result gel of venom proteins of *S. saevissima* (*middle lane*) and *S. invicta* (*right lane*); on the *left* lane the molecular weight marker is presented with respective protein band masses

study using purchased fire ant venom extract, and milked venom, shown in Hoffman et al. (1988). This study reported on four most abundant allergens, baptized Sol i 1–4. This pioneering study helped elucidate why the ant stings could often cause serious allergic reactions. Later, a series of studies focusing on each of these four allergens presented them in deeper detail (mainly Hoffman et al. 1990, 2005; Hoffman 1993; Hoffman 1995; Schmidt et al. 1996; Padavattan et al. 2008; Borer et al. 2012), for which the sequence, enzymatic activities, gene structure, and molecular structures were determined. This information will be briefly summarized below.

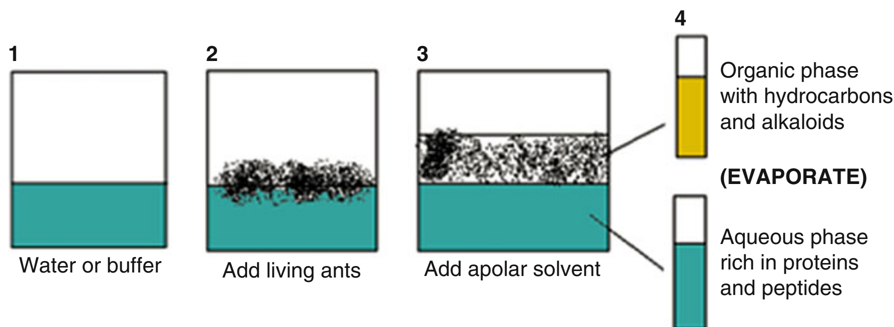

Fig. 4 A depiction of the fast method for massive venom extraction described by Fox et al. (2013)

Some insect allergens may also have enzymatic activity, as demonstrated here by Sol i 1, which is a phospholipase A_1B – a member of the lipoprotein lipase family that has homology with other hymenopterans. The other three described allergens in fire ant venom have no apparent enzymatic activity (although Hoffman et al. (1988) stated on page 826 that "Sol i II is a phospholipase with A and some B activity," as this activity was not mentioned in any subsequent papers except for Sol i 1 that was most probably a typographical mistake). Actually, venom allergen Sol i 2 was predicted as an odorant-binding protein (OBP) based on its sequence and modeled 3D structure, also suggesting that the similar protein Sol i 4 must be an OBP – OBPs are intermediary soluble molecules mediating the passage of pheromones or signal compounds between systems where they would be otherwise insoluble. The last described protein, venom allergen Sol i 3, has not yet demonstrated any alternate role, yet it belongs to one conserved class of known allergens known as cysteine-rich secretory proteins (CRISP), which is present in the venom of several aculeate hymenopterans, including the wasp antigen-5 and the ponerine ant allergen Pac c 3. The CRISP protein family also includes a series of cancer-related antigens and some inflammatory proteins known from sandfly saliva (Anderson et al. 2006).

For decades, these four allergens were believed to be the only proteins present in fire ant venom; however, recently the first proteomics study focusing on the same commercial venom sample of previous studies revealed an unfathomed diversity of other venom proteins (Pinto et al. 2012). Fire ant venom proteins were shown to be mainly a mixture of toxins and allergens and also self-protective components as to preserve the animal from its own venom, this way matching the typical pattern for other studied predator arthropods, such as scorpions and spiders, and in agreement with the habits of fire ants as active hunters. On this aspect it is worth stressing that some of the toxins reported by (Pinto et al. 2012) are unique among ants for specifically targeting vertebrates (e.g., U5-ctenotoxin Pk 1a and atrial neural peptide), illustrating why fire ants are capable of preying upon nests of alligators, lizards, birds, and even threaten humans and their livestock and brood. The most abundant protein in the fire ant venom – as based on the bidimensional gel

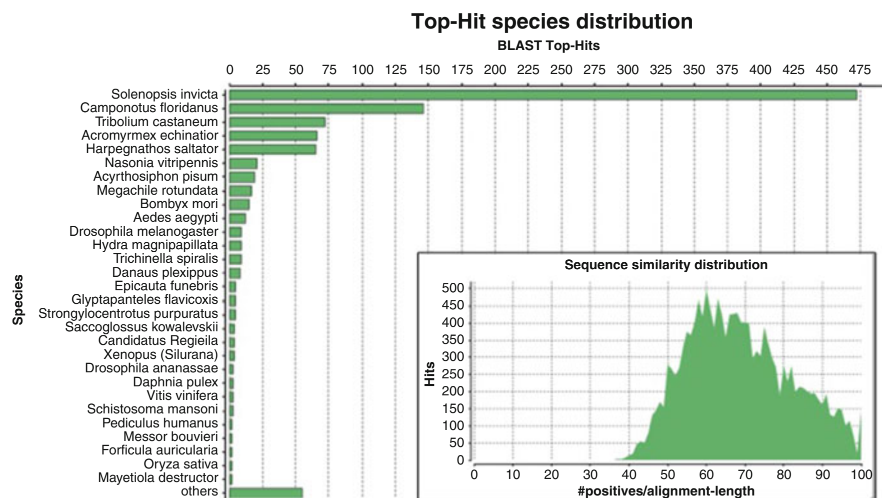

Fig. 5 Preliminary BLAST analysis of the transcriptome of the venom gland of the Brazilian fire ant *Solenopsis saevissima*. Random PCR-amplified transcripts from a cDNA library were sequenced by 454 GS FLX Titanium chemistry, and the resulting trimmed reads mapped to the available genome version of the fire ant *Solenopsis invicta*. Resulting contigs were automatically compared using blastx algorithm to online NCBI nr database

chromatography of *S. invicta* of (Pinto et al. 2012) – was Sol i 3, followed by Sol i 2. A remarkable set of new identifications by (Pinto et al. 2012) mentions toxins involved in causing local necrosis and increased microvascular permeability and cytolysis: myotoxin 2-like toxin (similar to phospholipases from Crotalidae snake venoms) and disintegrins and metalloproteinases, as well as a cytolytic protein similar to PSTx-60 from the sea anemone. Among neurotoxins, it is worth mentioning a protein similar to U5-ctenotoxin Pk 1a, one similar to millipede *Scolopendra* toxin, and the paralytic alpha-toxin Tc48a-like, which is possibly a blocker of Na^+ channels. Regarding self-protective factors, an identified vascular endothelial growth factor (VEGF) was also reported in honeybee and wasp venoms, as well as an inhibitor of phospholipases – it is believed that such factors are essential in keeping the venom apparatus integrity from the close contact with toxins.

Some preliminary results of an ongoing transcriptomic investigation of the venom gland of the Brazilian fire ant *Solenopsis saevissima* (Smith) further reveal interesting information about the metabolic particularities of this organ (Fig. 5). There seem to be further undetected protein toxins that can be present in the venom in such minute amounts that they preclude direct analysis, or perhaps are produced only under particular conditions. Venom peptides appear to be poorly diverse, if even present at all. Some remarkable toxin transcripts identified were a series of peptidases (markedly dipeptidases) and proteases, including prophenoloxidase, which likely have both a digestive function and a regulatory function in activating other venom secretions. Also identified were the paralyzing actin-depolymerizing factor cofilin, several mucin-like lubricant glycosylated proteins, the insecticidal

protein mulatexin, several chitinases, and a number of potential new allergens, including one new Sol i 3-like allergen, and finally odorant-binding proteins such as the social regulator protein gp-9. While several of the results confirm the finds by (Pinto et al. 2012), the existence of newly identified compounds could be indicative of species-specific venom variation. Such diversity would indicate that studying the venom proteins of other fire ant species might reveal a yet unimagined source of new potent bioactive compounds. Apart from revealing new venom compounds, a transcriptome can provide insights into the biology and physiology of the targeted organ. In summary, preliminary data suggests that the venom gland of fire ants is a highly dynamic organ under strict physiological regulation that will immediately respond to metabolic and environmental cues. This is clearly illustrated by the fact that the most diverse of the expressed transcripts are related to transcription factors (mainly transmembrane receptors), followed by signaling-pathway proteins. The observed proportions of classes of identified transcripts contrast with those described for venom glands of other organisms; thus, this is an interesting aspect of this organ to be further investigated. Indeed there are very few published studies assessing variation in venom composition resulting of different situations, yet one study has already demonstrated that fire ants will produce more venom during the flooding of their colonies (Haight 2006), reflecting a metabolic response of the venom gland to external stimuli. It would be interesting to check for the plasticity of venom profile variation within the same species and among individuals of different ages, as further insights into the venom nature and their pattern of synthesis could be drawn.

Finally, while opening the topic of the next section, the preliminary results from the venom gland transcriptome have revealed potential candidates for the synthesis of venom toxins, including the synthesis of venom alkaloids. Among the transcripts of the fire ant venom gland, one can identify the presence of some enzymes related to the mevalonate pathway of the synthesis of polyketides, which are known only from some microorganisms and plants – such enzymes could be involved in the production of alkaloids, which have not been shown to be produced by other animals. Further knowledge on how such compounds are produced can be useful for their study. Piperidine alkaloids are of great biotechnological interest, as described in the next section.

Worse than a Cigarette

As previously mentioned, the venom of fire ants is almost completely composed of a mixture of hydrophobic alkaloids. These are essentially compounds with a piperidinic ring attached to a side hydrocarbon chain of variable length and with varied degrees of unsaturation (Fig. 6). These alkaloids in fire ants are hydrophobic dialkylpiperidines, generally called solenopsins (under terminologies as isosolenopsins, dehydrosolenopsins, or dehydroisosolenopsins according with their molecular conformation), similar in structure to coniine and nicotine.

The solenopsins include ten different substances termed 2,6-methyl-6-alkylsolenopsins A-E and their unsaturated counterparts and isomers (for a general

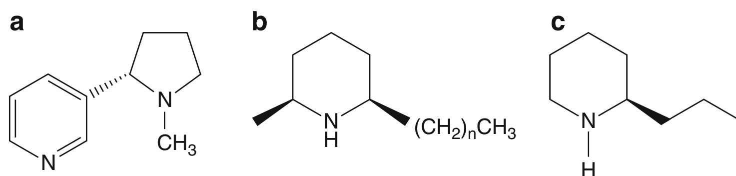

Fig. 6 Examples of chemical structure of some well-studied piperidine alkaloids. (**a**) Nicotine, extracted from tobacco plants. (**b**) Solenopsin, extracted from fire ants (n = even number, varying according with solenopsin species). (**c**) Coniine, extracted from hemlock plants

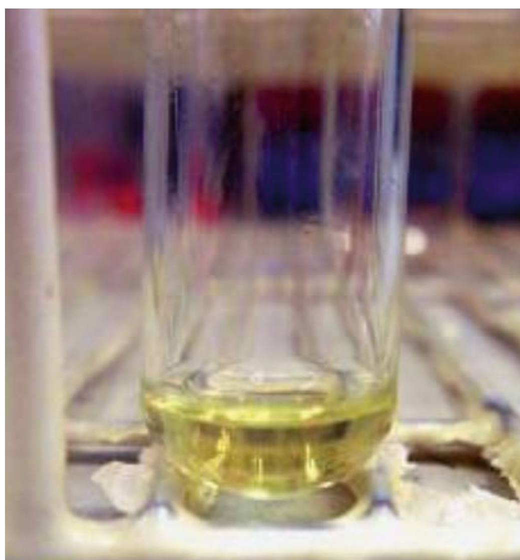

Fig. 7 Freshly extracted alkaloids from a whole colony of *Solenopsis invicta*, by the method described by Fox et al. (2013)

overview on their structures, refer to Chen and Fadamiro (2009)). Each of these compounds has slightly different chemical properties and biological activities. Given the fact that solenopsins are a class of alkaloids unique to fire ants and are thus compounds relatively easy to obtain and partially purify in endemic areas, there are numerous studies on their chemistry and physiological effects. Solenopsins are overall highly bioactive compounds, which have been demonstrated to be of interest to several biotechnological and biomedical applications. Working with solenopsins is much more straightforward than working with venom proteins: fire ant alkaloids can be easily obtained by immersing any amount of these ants in organic solvents, and from which they can be partially purified from contaminants by their relative affinity with silica powder, either by thin-layer chromatography or common resin columns. The mixture of purified venom alkaloids appears as a translucent yellowish oil (Fig. 7), and compounds are stable at room temperature. As much as 1.0 g can be obtained from one large nest within few hours of work.

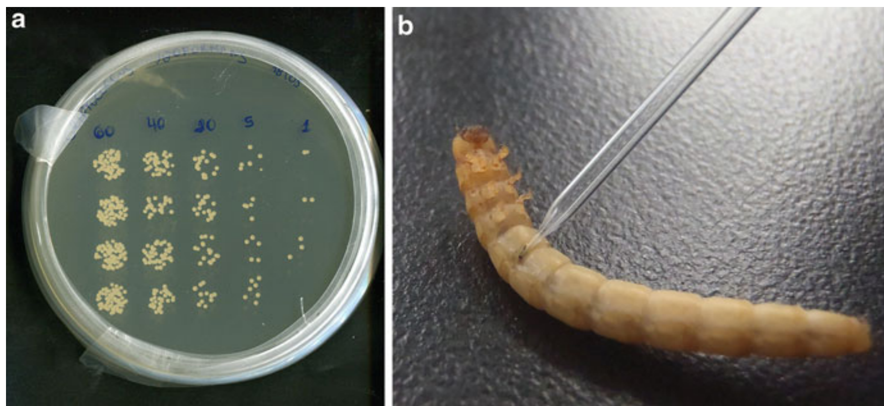

Fig. 8 Bioassays with ethanolic suspensions of solenopsins at varied concentrations against different organisms. (**a**) Antimicrobial test against *Micrococcus luteus* by counting number of colony-forming units in a Petri dish. (**b**) Topical application/injection into hemolymph of *Tenebrio molitor* larvae

How can ants produce the venom alkaloids is presently a mystery. Several laboratory synthesis methods have been designed, and at least one biochemical pathway has been proposed (Leclercq et al. 1996a). When first discovered, it was supposed that the ants were acquiring these compounds from feeding on vegetable sources; however, it was soon made clear that they somehow synthesize alkaloids – given that venom alkaloids are species specific, general proportions and composition are maintained over the ant's life and finally that ants can produce the compounds when cultured in a laboratory without any access to vegetable matter. Among solenopsins, there are also minor amounts of similar alkaloids named piperidines, which are currently regarded as unstable intermediates in the synthesis of alkaloids, but these are essentially unstudied compounds (Chen et al. 2009), except for two recent studies (Li et al. 2012; Rashid et al. 2013).

Regarding their activities as antimicrobials in general, solenopsins have proved to be potent antifungals and insecticides after being tested against a range of microorganisms (Jouvenaz et al. (1972), e.g., *Candida albicans*, *Escherichia coli*, and *Pseudomonas aeruginosa*) and several insects (mainly lepidopterans, see Blum et al. (1958) and Lai et al. (2010)). Some ongoing tests are presented in Fig. 8.

As mentioned, some isomers prove more effective than others depending on the bioassay conditions, and this must correlate with the biological adaptations of the different fire ant species, given that they possess unique proportions of solenopsins (Fox et al. 2012). One aspect that should be emphasized is that by far the most intensively tested compound was solenopsin A, and much information is lacking about the other compounds and isomers, including one existing solenopsin still currently lacking a name with a side hydrocarbon chain shorter than solenopsin A, reported from poorly studied species of fire ants (e.g., *Solenopsis virulens*).

As for reported physiological activities in mammals, solenopsins have been reported to trigger histamine production in mastocytes (Read et al. 1978), block neuromuscular junctions (Yeh et al. 1975), and inhibit ATP-dependent sodium-potassium pumps and respiratory chains (Koch et al. 1977; Lind 1982). Javors et al. (1993) also observed that solenopsins are capable of activating platelets and neutrophils, and Yi et al. (2001) and Yi et al. (2003) reported that they can inhibit three isoforms of neuronal nitric oxide synthases. One particular aspect that has made a greater impact in the mainstream media is that solenopsin A was demonstrated to be a potent inhibitor of class-1 phosphatidylinositol-3-kinase signaling and angiogenesis in embryonic fibroblasts of mice, and also angiogenesis in zebra fish (e.g., Arbiser et al. 2007), making it a promising anticancer candidate. Still on the physiological effects, yet concerning the toxicity of venom alkaloids towards mammals (for instance, if to be administrated as a drug), it was reported that intravenous application of solenopsins is capable of affecting the central nervous and cardiovascular system (Howell et al. 2005) of mice, as it is capable of crossing the hematoencephalic barrier. However, it becomes significantly toxic 3–30 mg/kg in a dose-dependent manner, causing dizziness, then cardiorespiratory complications, and death (Howell et al. 2005). The least toxic means of administration seems to be oral intake. In fact, some controversial applications for fire ant venoms have been proposed by recent patents deposited in the United States, for instance, one patent suggesting the oral administration of milligram amounts of venom to domestic cats and dogs and humans, for parasite control (Patent US 5098914 A); another patent recommends oral intake for humans (elderly people) as to revert aging-associated decline of motor skills, particularly in patients with Alzheimer's disease (Patent EP 2043642 A2).

The wide diversity of existent alkaloids in fire ant venoms, and the present paucity of published investigations into most of them, is but emblematic of the poorly explored biotechnological potential of such compounds. However, as all existent compounds are chemically similar substances, greatly due to isomers, obtaining pure natural fractions of individual solenopsins is technically challenging. Obtaining pure compounds heavily relies upon finding the ant species in which they are prevalent and also perfecting the current purification methods, or else relying on laboratory synthesis of solenopsins. Unfortunately, currently available methods for synthesizing solenopsins are expensive and time-consuming and yield small amounts of product, even with racemic mixtures of product compounds (e.g., Leclercq et al. 1996b; Pianaro et al. 2012). Adjusting the synthesis to yield enantiomerically pure end products greatly increases the methodological challenge and associated costs (e.g., Pelletier et al. 2014). These difficulties have limited tests with these compounds to small-scale essays with mixtures of compounds and microscale in vitro tests with synthetic compounds. Given the ease of obtaining venom alkaloids from ants and the difficulties in synthesizing them, finding effective methods for their purification or practical biological synthesis will greatly speed up breakthroughs in this field. There is a complete lack of papers published towards this direction.

Conclusion and Future Directions

Taken overall, this chapter aimed at presenting an updated overview about the state of knowledge about fire ant venoms and indicated possibilities of research and developments on this topic. Recent finds and test results emphasize that ant venom secretions of ants can be rich in bioactive factors useful to many different fields of application and provide a yet essentially untapped rich source of knowledge about new biochemical pathways and potential new pharmacological compounds. Given the broad nature of possible applications and the necessary background knowledge to deal with the different chemical biochemical and physical aspects of the venom alkaloids and the immunological and metabolic questions about fire ant proteins and peptides, interdisciplinary research teams are necessary to advance in these topics. Many young scientists may find in this a hot topic for relevant discoveries. Some possible future directions are suggested below as a mind-provoking invitation to young researchers and investors to this field of research.

Future Directions: Possible Uses for Fire Ant Venom

A Social Role of Ant Venoms?

There is some timid evidence suggesting that the venom may play an important central role in fire ant social organization. Not only are all ants in the nest covered with venom, which is recurrently sprayed by workers inside the colonies through a behavior known as gaster flagging, but the venoms of female ants of different castes are markedly different. Moreover, the venoms of fire ants are species specific, and workers have been seen to immediately present their gasters when introduced to other workers of a different colony (author's personal observations). Given the abundance and relative volatility of fire ant alkaloids, it would be reasonable to assume that they may work as pheromones among the ants and towards other species. Additionally, the presence of abundant odorant-binding proteins in the venom as exemplified by some of the described allergens and also other molecules detected by proteomics (like the social pheromone protein gp-9) suggests a social role for venom secretions. Such odorant-binding proteins could be carrying a message among the ants covered in secreted venom. Currently, studies with manipulation of fire ant venom in association with behavioral observations are still lacking, and this is still a field to be evaluated.

Derivatives of Solenopsins to Prevent Biofilm Formation?

Great industrial expenses are directed towards fighting off microbial biofilm formation, which cause continuous duct obstruction and corrosion and inutilization of surfaces in factories and refineries. In their attempt to prevent biofilm formation inside ducts, industries release highly toxic pollutants into the environment,

resulting in the appearance of resistant microbial strains and causing massive environmental impact. There is thus considerable interest in the discovery of new (cheaper and less toxic) chemicals or surface materials that will delay or eliminate biofilm formation. This can be attained by preventing fixation of early-succession microbes or by disrupting biosignaling among the biofilm-secreting microbes. Treating exposed surfaces with several chemicals and paints – termed surface conditioning – has been proposed as an alternate solution. Park et al. (2008) have demonstrated that fire ant venom alkaloids can inhibit biofilm formation by gram-negative *Pseudomonas aeruginosa*, possibly from its structural resemblance of a quorum-sensing molecule termed 3-oxo-C12-HSL. As mentioned, solenopsins strongly adsorbs to silica, and there are several reports of alkaloids adsorbing to surfaces (e.g., chinchonines adhering to metal layers) and plastics. Thus, maybe artificial solenopsin derivatives could be added to paints for surface conditioning as an alternative environmentally friendly measure against biofilm formation. This is an interesting aspect open for investigation.

Venom Alkaloids as Sustainable Pesticides?

As mentioned earlier in this chapter, solenopsins can be partially purified from contaminants based on their relative affinity to silica. During the process of solenopsin purification, some inevitably remain adsorbed to the silica phase employed (termed phase saturation of the system), which must be then replaced for continued use. However, as silica is an inert cheap material, one could picture practical utilities for alkaloid-saturated silica, given that it is impregnated with bioactive compounds. One idea, for instance, could be using solenopsin-saturated silica for delaying the growth of filamentous soil fungi on collected fruit. One of the greatest losses of commercial fruit is due to mold formation during its transport to points of sales or during its shelf life, and the usual method for preventing this is to apply toxic pesticides after harvesting fruits. Perhaps an inert thin layer of alkaloid-bound silica would provide good protection, while being less toxic and easier to wash away. How about adding alkaloid-bound minerals to the soil of crops in an attempt to make them obtain the insecticidal compounds? Such agricultural possibilities are worthy of experimentation.

Fire Ant Venom for Immunotherapy

As mentioned previously, immunotherapy of patients sensitive to fire ant stings is currently done with whole-body ant extracts. Logic entails that the new extraction method for pure venom protein could be used for immunotherapy, with the potential of more efficient and specific results. However, farming fire ants for venom extraction could prove impracticable and environmentally risky. Thus, further knowledge about the characteristics and diversity of the main allergens in fire ant venom could enable the recombinant production of antigen sites as to make

immunotherapy less risky and also independent of biological samples. Combining recombinant antigens similar to wasp/bee/ant venoms could perhaps provide a broad-range immunizing therapy. Moreover, it has been demonstrated in some vertebrates (as illustrated by the use of bee or ant stings against arthritis) that adjuvant-mediated stimulation with hymenopteran venom phospholipases can induce interesting immunological reactions, such as anti-inflammatory effects, or acquired immunological resistance to certain parasites. It seems reasonable that fire ant venom could prove to have the same properties given that it is rich in phospholipases.

Alkaloids as Future Antibiotics?

Previously in this chapter, it was mentioned that venom alkaloids were administered orally to mammals without any apparent significant side effects. Also, their potent activity as antifungals is widely reported in the scientific literature. Fungal infections are among the most difficult to treat clinically, as common fungi are naturally resistant to most known antibiotics, and most can easily spread to different tissues in the body. There is already some research on the use of microencapsulated preparations of solenopsins (as creams) for topical application as an alternative treatment against skin fungal infections. The reported possibility of oral administration is also suggestive that internal application in cases of respiratory or oral diseases should be feasible. Such alkaloids or artificial derivatives could be employed in association with other drugs as to maximize their effects and prevent the onset of resistant strains. As the antimicrobial effects of piperidinic alkaloids are yet unknown, it is hard to precise how enhanced drugs could be generated from simple modifications in the original molecules, and this is a hot topic in lack of further research.

Cross-References

► Automated Mass Fingerprinting of Venoms in Nanogram Range: Review of Technology
► Shotgun Approaches for Venom Analysis

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The Platypus: A Venomous Mammal

8

Camilla M. Whittington and Katherine Belov

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Abstract

The platypus is a monotreme, an egg-laying mammal, found only in Australia. Males are venomous. During the breeding season they are able to deliver venom through spurs located on their hind legs. Venom delivery is believed to provide individuals with an advantage over conspecifics throughout the breeding season. This paper reviews the current literature on platypus venom, focusing primarily on recent advances which have been made since the sequencing of the platypus genome and venom gland transcriptome. It first provides an overview of the genes and molecules involved in venom production and focuses on how these molecules explain the symptoms of envenomation: allodynia, hyperalgesia, swelling and changes to blood pressure. The paper concludes by providing insights into how these venom peptides could be developed into novel therapeutics for human use.

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Introduction

The Platypus

History

Since its discovery by Europeans in 1797, the venomous platypus (*Ornithorhynchus anatinus*) has remained an enigma. At first, when a dried skin was sent back to England, it was thought to be the trick of a taxidermist (Grant 2007), and even once it was proven to be real, controversy raged over its mode of reproduction and its classification. It was not until 1884 that questions about the reproductive mode of the platypus were definitively answered. Biologist William Caldwell, after the wholesale slaughter of hundreds of platypuses, declared in his famous telegram to the Montreal Meeting of the British Association for the Advancement of Science "Monotremes oviparous, ovum meroblastic" (Burrell 1927; Grant 2007). Platypuses, along with the echidnas (*Tachyglossus aculeatus* and *Zaglossus* sp.), became the monotremes (Grant 2007), the egg-laying mammals, true evolutionary oddities.

The platypus and echidnas are the only remaining members of mammalian subclass Prototheria, a group that once contained a diverse range of animals that are now extinct (Musser 2003). The living monotremes are sometimes referred to as "primitive" mammals. This is because subclass Prototheria was the first divergence from the mammalian lineage; it is estimated that this split occurred around 166 Mya (Warren et al. 2008). A schematic phylogenetic tree showing the evolutionary position of the monotremes is displayed in Fig. 1. The echidna family probably evolved from the platypus family, with the two groups diverging ~32 Mya (Phillips et al. 2009).

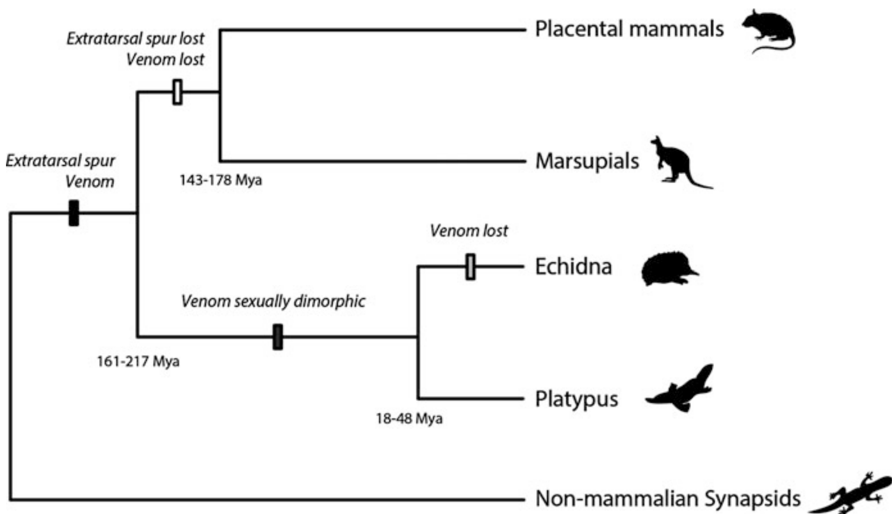

Fig. 1 Phylogenetic tree indicating the evolution of venom and extratarsal spur in monotremes (Divergence dates taken from Phillips et al. 2009)

General Features

The platypus is semiaquatic and semi-fossorial, living and nesting in burrows in riverbanks and emerging into the river or stream to feed on benthic invertebrates. As a result of its membership of the earliest mammal group, it has a number of unusual features that are plesiomorphic (representative of the ancestral state) and more often associated with reptiles than with mammals. For example, platypuses lay eggs; however, they also possess mammary glands and suckle young (from milk patches rather than nipples) (Grant 2007). Like other mammals, the platypus is furred, but its skeleton has several reptilian features, including reptilian-like ribs and a pectoral (shoulder) girdle more similar to that of fossil reptiles than other modern mammals (Grant 2007). The platypus has a lower body temperature (32 °C) than other mammals, but it does regulate this and is able to maintain a constant body temperature even while feeding in water close to freezing point (Grant 2007). The karyotype of the platypus (2n = 52) is also unusual, as it contains macro-chromosomes as well as chromosomes resembling the micro-chromosomes seen in karyotypes of reptiles and birds (reviewed in Grützner et al. 2003). However, the most unusual feature of the platypus may be the fact that it is venomous. Venom in a mammal is extremely unusual, and the platypus possesses a complex venom system that is only just beginning to be understood.

Platypus Genomic Research

The unique characteristics of the platypus, along with the fact that many of these are poorly understood, make the animal an intriguing resource for genomic studies. The recent publication of the platypus genome sequence affords an unprecedented opportunity to do this (Warren et al. 2008). The platypus is shy and difficult to study, and genomic techniques can allow researchers to understand the basic mechanisms underpinning the unique biology of the platypus. Examinations of monotreme genomes also have the potential to increase current understanding of the evolution of mammals, providing insights into the genomes of extant mammals as well as ancestral mammals (e.g., Margulies et al. 2005). This is mediated by comparisons with genomes of other species, in a field known as comparative genomics. These studies of the similarities and differences between genomes can reveal genomic regions that are conserved between divergent organisms to provide clues as to their functions and evolutionary histories (e.g., Margulies et al. 2005; O'Brien 2009). Importantly, these conserved regions can reveal functionally important genes, gene products, and regions within noncoding areas; show changes in genome organization and the dynamics of gene families during evolution; reconstruct ancient genomes; and shed light on genomic regions important in inherited genetic disorders and even human disease (O'Brien 2009). The monotremes, as members of the earliest extant offshoot from the mammalian lineage, effectively straddle the phylogenetic gap between reptiles and therians (placental mammals) (O'Brien 2009). The platypus genome thus has the potential to allow valuable comparisons between lineages and represents a powerful tool for reconstructing the evolutionary history of mammals.

The draft platypus genome sequence was released in 2008 and represents the first monotreme genome sequence (Warren et al. 2008). It was constructed from

whole-genome shotgun sequence of the DNA of a single female platypus, collected at Glenrock Station, NSW. DNA segments were localized to chromosomes using fluorescence in situ hybridization (FISH). Around 18,000 protein-coding genes were predicted in the original assembly, similar to the number in the marsupial opossum (*Monodelphis domestica*) and human.

The platypus genome can be used to provide insight into the genetic development of mammalian characteristics such as lactation, along with the disappearance of traits such as egg laying, relevant to the basic biology of this animal. The genome paper examined, via homology, genes encoding some important biological traits. For example, it was found that the platypus genome includes two *ZPAX* genes, which encode egg envelope proteins in birds, amphibians, and fish, and one vitellogenin gene plus a pseudogene, present as three genes in chicken and none in therian mammals. However, it also includes four genes encoding mammalian zona pellucida proteins, indicating that the platypus has an amalgamation of reptilian and mammalian genes (Warren et al. 2008). The genome also encodes a cluster of casein genes involved in lactation, and tooth enamel matrix protein genes presumably involved in the presence of teeth in juvenile platypuses (Warren et al. 2008). In particular, as will be discussed here, genomics has allowed a much better understanding of platypus venom.

Platypus Crural System

Platypus Venom Gland and Spurs

Platypus venom is sexually dimorphic, as only males are venomous. Their venom (crural) system consists of paired venom glands on the dorsocaudal sides of the abdomen, each of which is connected via a venom duct to an extratarsal spur on each hind leg (Grant 2007). The spur is a hollow sheath made of keratin and is associated with a small articulating bone (*os calcaris*) that is attached to tendon and muscle to allow erection of the spurs prior to envenomation (Temple-Smith 1973). Juvenile females have a remnant spur sheath that is lost within a year of hatching, and spurs develop only in males (Grant 2007). The venom glands are thought to be derived from sweat glands and migrate during male development from the inner surface of the thigh to the dorsocaudal surface over the pelvis (Temple-Smith 1973). At maturity, during the spring breeding season, the venom gland increases to peak size and venom production increases (Temple-Smith 1973). Outside of the breeding season, the venom gland secretory epithelium becomes inactive and the gland regresses, and the behavior of the males changes to displaying little spurring activity (Temple-Smith 1973). This is the only known example of temporally variable (seasonal) venom production.

Function of Platypus Venom

Europeans first discovered the spur of the platypus in 1799; spurs were originally thought to be used by the male to hold the female during mating, but it was also

noted early on by some naturalists that the use of these spurs could cause serious wounding (Burrell 1927). The function of the platypus crural system is unclear. Although foxes and dogs can kill platypuses, the platypus has very few native predators (crocodiles, Tasmanian devils, and raptors being the only, occasional, exceptions) (reviewed in Whittington et al. 2009). This and the seasonal nature of venom production suggest that the crural system may have evolved to have a reproductive role such as territory defense [assertion of dominance over other male platypuses during the breeding season (Grant 2007)] rather than a defensive function. The trapping of male platypuses with healed spur marks suggests that intraspecific envenomation is not usually fatal (Grant 2007); envenomated male platypuses display edema and temporary limb paralysis (Temple-Smith 1973).

Evolution of the Crural System

Like the platypus, modern echidnas also have a crural system; male echidnas have spurs (0.5–1.0 cm) smaller than those of the male platypus, and females have vestigial spurs that are usually lost in later life (Griffiths 1978). The spurs are connected via a duct to a gland below the knee that is active during the breeding season (reviewed in Griffiths 1978; Krause 2010). The echidna spur cannot be erected or everted from beneath the covering protective skin flap. Echidna spurs are also in a different arrangement to platypus spurs with respect to the attachment of the *os calcaris* to the tarsal bones, meaning that the spurs cannot be rigidly locked into place for spurring (Hurum et al. 2006; Krause 2010). This suggests that the echidna possesses a nonfunctional, regressed venom system.

Unfortunately, as much of the monotreme fossil record is in the form of tooth and jaw fragments (e.g., reviewed in Musser 2003), it is not known whether the ancestral monotreme had a crural system and venomous spurs on the hind limbs. However, given the presence of either functional or regressed crural systems in modern monotremes, and recent molecular data indicating that at least one platypus venom component arose before to the divergence of platypuses and echidnas (Whittington et al. 2008a), the basal monotreme was probably also venomous (Fig. 1).

It is unclear why venom would have evolved in the monotremes, as unlike many other venomous species, modern monotremes do not use their venom systems to capture or digest prey (reviewed in Whittington and Belov 2007). The fact that spurs (functional or vestigial) are present in both sexes of platypuses and echidnas indicates a probable defensive function for the ancestral venom system. The divergence of monotremes occurred in the Jurassic ~166 Mya, and there are fossil monotremes from the Early Cretaceous period (~110–115 Mya, reviewed in Musser 2003). The first fossils of modern monotremes appear in the Pleistocene (1.78 Mya onwards, reviewed in Musser (2003)). The earth from the Jurassic to the Pleistocene was inhabited by dinosaurs and then a diversity of mammals, some of them large carnivores. These potential predators might have meant that defensive venom provided the ancient monotremes with a survival advantage. Perhaps once the

selective pressure of predation was lifted, the crural system was instead used in a reproductive context, and the energetically expensive venom then became a sexually dimorphic trait.

Platypus Venom

Symptoms of Envenomation

To envenomate, the male platypus wraps the hind legs around the victim, drives the spurs into the flesh, and injects the venom. The spurs and attachments are very strong, and males are able to support their full weight by the spurs (Fenner et al. 1992). Up to 4 mL of venom is available to be injected (Temple-Smith 1973), although the small diameter of the duct and the high pressure required for injection means that much smaller amounts would be injected each time (Whittington et al. 2009).

Platypus envenomation has been known to kill dogs (e.g., Burrell 1927), and although no human fatalities have been reported, the venom produces swelling and immediate and excruciating pain that cannot be relieved through normal first-aid practices including morphine administration (Fenner et al. 1992). It also produces nausea, gastric pain, cold sweats, and lymph node swelling (Temple-Smith 1973). One case of platypus envenomation has been clinically reported, recording a high erythrocyte sedimentation rate and low total serum protein and albumin levels in an envenomated human patient, as well as generalized and persistent localized pain and muscle wasting of the spurred limb (Fenner et al. 1992).

As humans rarely fall victim to platypus envenomation and fatalities have never been recorded, no antivenom has been developed.

The Contents of Platypus Venom

The unusual nature of the symptoms of platypus envenomation may be due to the complex mixture of many different peptides and proteins in the venom, some of which have unknown function (de Plater et al. 1995) and some of which may be potentially novel and clinically useful. However, platypus venom research has been hindered because of the limited quantities of venom available for study (platypuses cannot be easily bred in captivity and produce little venom for collection) (Whittington et al. 2009). The research that has been done on platypus venom can be broadly separated into two categories: pharmacological studies of the crude venom and proteomic studies. More recently, genomic techniques have also been used to elucidate the venom components.

Pharmacological Studies of Crude Platypus Venom

The venoms of other species such as snakes are often tested in rodent models, but this type of platypus venom research has been very limited.

Venom injection into rabbits produces coagulation, lowered blood pressure (probably due to vasodilation), hemorrhagic edema, and death (Martin and Tidswell 1895; Kellaway and Le Messurier 1935).

These effects may be a result of disruption of cell membrane ion transport pathways or the creation of abnormal ion channels, which occurs when platypus venom is applied onto cell membrane-mimicking artificial lipid bilayers (Kourie 1999b; Torres et al. 2002a) and onto putative nociceptors (sensory neurons able to send pain signals to the brain) (de Plater et al. 2001). This would disrupt ion concentrations and cause symptoms such as edema (Kourie 1999b); disrupted ion concentrations could cause nerve firing in vivo and thus the pain that is characteristic of platypus envenomation (Kourie 1999b; de Plater et al. 2001).

The venom has mild proteolytic activity. In laboratory animals, injections of venom cause histamine release (rabbits) and cutaneous anaphylaxis (localized skin allergic reaction) and rapid death following intravenous doses of 75–90 mg kg^{-1} (mice) (Temple-Smith 1973). In vitro, the venom causes smooth muscle relaxation (Kellaway and Le Messurier 1935; de Plater et al. 1995), feeble hemolysis (red blood cell breakdown) (Kellaway and Le Messurier 1935), and calcium-dependent nonspecific cation current into neuronal cells, which in vivo may produce edema, nerve firing, and pain (de Plater et al. 2001).

Proteomic Studies

The venom includes nineteen different peptide fractions along plus non-peptide components (de Plater et al. 1995; Kourie 1999b). The identification of these components began with protein characterization techniques. The reverse-phase high-performance liquid chromatography (HPLC) chromatogram of platypus venom reveals many novel polypeptides ranging in size from 4 to 6 kDa (Torres et al. 1999, 2000) and proteins with sizes above 12 kDa. Until recently, three types of peptides had been identified and fully sequenced. These were named after peptides with which they share homology: C-type natriuretic peptides (OvCNPs, de Plater et al. 1998a), defensin-like peptides (OvDLPs, Torres et al. 1999), and nerve growth factor (OvNGF; A. Torres and P.W. Kuchel, unpublished data; described in de Plater 1998). Their exact functions are not known. A venom peptide isomerase and hyaluronidase have also been discovered, but their full sequences have not been determined, and the venom has protease activity (de Plater et al. 1995). The isomerase catalyzes the conversion of the second L-amino acid from the *N*-terminus to a D-form in at least two of the platypus venom components (reviewed in Whittington et al. 2009).

OvDLPs

OvDLPs (*Ornithorhynchus* venom defensin-like peptides) are the most abundant peptides in platypus venom (Torres et al. 2000). They share structural and sequence similarities with antimicrobial beta-defensins. They are characterized by having six paired cysteine residues (Torres and Kuchel 2004) in a similar arrangement to those of the beta-defensins. For example, five cysteine residues match those found in bovine beta-defensins (Torres et al. 2000). This suggests that their tertiary structures are similar. The OvDLPs also have some similarity in folding structure to ShI, a sea anemone (*Stichodactyla helianthus*) sodium channel neurotoxin, but OvDLP-C, unlike ShI, does not affect rat vas deferens or dorsal root ganglia sodium channels

(Torres et al. 1999); the OvDLPs show similarity to two snake venom peptides [rattlesnake myotoxin a and crotamine (Torres and Kuchel 2004)]. In addition, defensin peptides have been isolated from the other venoms, e.g., from the ectoparasitic wasp *Nasonia vitripennis* (Ye et al. 2010).

The side chains of OvDLPs and the homologous venom peptides in other species are different, suggesting that they may have different modes of activity (Torres et al. 1999; Torres and Kuchel 2004). The similarity of OvDLPs to the beta-defensins suggests that they may have similar functions, but there is no evidence that OvDLP-C acts antimicrobially, nor has any myotoxic (muscle toxicity) activity been found (Torres et al. 1999).

OvCNPs

OvCNP (*Ornithorhynchus* venom C-type natriuretic peptide) is present in two isomeric forms in platypus venom, OvCNPa and OvCNPb, which have identical amino acid sequences (Torres et al. 2002b), the significance of which is discussed below. They are the most biologically active peptides identified in the venom so far (Torres et al. 2002a). Natriuretic peptides [atrial (ANP), brain (BNP), and C-type (CNP)] are normally produced in mammalian non-venom tissues, where they are believed to play a role in blood pressure regulation (de Plater et al. 1998b). OvCNP in platypus venom shares homology with C-type natriuretic peptides (in particular, CNP-53 and CNP-22), which are produced in the brain and endothelium but lack natriuretic activity (de Plater et al. 1998a). CNPs have also been found in the venom of some snakes, e.g., the rattlesnake *Crotalus durissus collilineatus* (Boldrini-França et al. 2009).

OvCNP has been shown to form fast cation-selective channels in lipid bilayer membranes (Kourie 1999a) and causes relaxation of smooth muscle (de Plater et al. 1995), uterus, and vas deferens (de Plater et al. 1998b). It produces mast cell degranulation (histamine release) (de Plater et al. 1998b), which may cause swelling. OvCNP also activates calcium-dependent cationic currents which may lead to nerve depolarization (firing) (de Plater et al. 2001) and thus the intense pain that is also characteristic of platypus envenomation (Fenner et al. 1992); 11 analogues of fragments of OvCNP have recently been found in platypus venom, and these also cause calcium influx into neuroblastoma (neuroendocrine tumor) cells (Kita et al. 2009). It has been suggested that OvNGF may cause allodynia (pain due to a stimulus that is not normally painful) and hyperalgesia (increased sensitivity to pain) (de Plater et al. 2001), but the role of the OvDLPs is unclear.

OvNGF

The role of OvNGF (*Ornithorhynchus* venom nerve growth factor), which is homologous to endogenous nerve growth factors (NGFs), is also unknown, and little research has been carried out. NGFs have also been found in snake venoms, and when recombinant human NGF has been injected into humans, some of the symptoms produced (such as allodynia and hyperalgesia) (Dyck et al. 1997) are similar to those produced by platypus envenomation. Other researchers have suggested that OvNGF and OvDLPs may act synergistically to produce pain.

For example, it is possible that NGF renderers cells more susceptible to other venom components, in part because during RP-HPLC, DLP-3 co-elutes with NGF (Torres et al. 2000), although this has not been investigated further.

L-to-D-Peptide Isomerase

As previously mentioned, OvCNP is found in two isomeric forms in platypus venom. These have identical amino acid sequences and differ only by a D-amino acid residue at position two (OvCNPb) that is in L-form in OvCNPa (Torres et al. 2002b). DLP-2 and DLP-4 also have identical amino acid sequences, with a D-form amino acid residue at position two of DLP-2 (Torres et al. 2005). Torres et al. began searching for an isomerase in platypus venom that was responsible for posttranslational conversion of the second amino acid from L- to D-form (Torres et al. 2002b). The isomerase (referred to as L-to-D-peptide isomerase) was partially purified and found to be ~55–65 kDa in size (Koh et al. 2009). Subsequently, a two-base mechanism of isomerization has been proposed, in which two histidine residues of the active site of the isomerase are positioned on either side of the second amino acid of the venom peptide substrate and act as proton donors and acceptors to cause isomerization (Koh et al. 2009).

D-Amino acid residues do not occur frequently in nature and have previously been found only in prokaryotes, yeasts, and some invertebrates (crustaceans, mollusks, spiders) (Torres et al. 2002b, 2006). In some cases, these residues are also present at the second amino acid position (Torres et al. 2002b), raising the possibility that there are similar isomerases between these species. Work is currently underway to search for the presence of similar isomerases in higher mammals (P.W. Kuchel, pers. comm.), and isomerase activity has been found very recently in mouse heart (Koh et al. 2010). The effect of the D-form amino acids in OvCNPb and DLP-2 is unknown, but in other species, the D-form is more biologically active (reviewed in Torres et al. 2006). It has been suggested that the D-form peptides may be more resistant to protease degradation during storage in the venom gland, and perhaps also in the tissues of the victim, thus prolonging envenomation symptoms (Torres et al. 2002b, 2006, reviewed in Koh et al. 2009); this could account for the persistent pain that is characteristic of platypus envenomation (Fenner et al. 1992). Recently, similar isomerase activity has been discovered in mouse heart (Koh et al. 2010), raising the possibility that this isomerase is far more widespread and biologically significant than previously thought.

Genomic Studies

The platypus genome has provided a new avenue for platypus venom research, resulting in a leap forward in the current understanding of platypus toxins. From the genome, researchers decoded the gene sequences for the toxins identified by previous proteomics research, allowing them to investigate the tissue expression patterns of these genes (Whittington et al. 2008b; Whittington and Belov 2009). From here, it was discovered that a number of platypus toxin genes are expressed in non-venom tissues in both males and females, raising the possibility that these genes function in broader, non-venom roles including OvDLP-mediated immunological protection of

the venom gland, which is open to the external environment via the venom duct and spur (Whittington et al. 2008b), and suggesting that these genes may be processed posttranslationally in the venom gland (possibly by the venom isomerase) to confer toxin function (Whittington and Belov 2009).

In 2010, knowledge of platypus venom was vastly increased with the publication of platypus venom gland transcriptome sequence (Whittington et al. 2010). Using technologies similar to those employed to sequence the platypus genome, researchers were able to sequence all of the genes that are expressed in a breeding season platypus venom gland, gaining a snapshot of the sequences of toxins produced in the gland during this time. This new technique identified 83 new putative toxins classified into 13 different toxin families: serine protease, stonustoxin-like, Kunitz-type protease inhibitor, zinc metalloproteinase, latrotoxin-like, CRiSP (cysteine-rich secretory protein), sea anemone cytolytic toxin-like, unknown (IG domains), mamba intestinal toxin-like, C-type lectin domain containing, sarafotoxin-like, VEGF, and DNAse II (Whittington et al. 2010). The putative toxins, which require further functional testing to confirm toxin activity, were identified on the basis of DNA sequence similarity to toxins in other venomous species (including arachnids, marine invertebrates, fish, snakes and lizards, and insectivores). Based on this similarity, the researchers were also able to speculate about the functions of these putative toxins in the venom, which included inflammation, pain, edema, muscle wasting, and coagulation (Whittington et al. 2010).

A limitation to the methodology of the 2010 transcriptomic study is that identifying platypus toxins on the basis of similarity to venom toxins in other species would miss completely novel platypus toxins. In order to discover completely novel toxins, researchers sequenced the genes expressed in both a breeding season and an out-of-breeding season venom gland and compared the levels of each expressed gene. In tandem with this, they also utilized protein sequencing techniques to directly sequence the toxins in platypus venom samples. In this way, they were able to identify novel toxin genes that were strongly upregulated during the breeding season as well as toxin proteins present in the venom (Wong et al. 2012). This uncovered five new platypus toxins: growth differentiation factor 15, nucleobindin-2, CD55, a CXC-chemokine, and corticotropin-releasing factor-binding protein (Wong et al. 2012).

Venom Gene Evolution

The genomic studies of platypus venom have not only revealed a large number of new toxins, as they have also allowed a glimpse into the evolutionary history of platypus toxins. One important factor in platypus venom gene evolution is gene duplication. Gene duplication occurs when a gene is replicated, for example, by unequal crossing over during meiosis, to form two copies. One copy is able to fulfill the original function of the gene, effectively removing the functional constraints from the other copy and leaving it free to vary and take on new functions (e.g., Nei and Rooney 2005). Gene duplication is an important source of possibilities for adaptive evolution, as is evident in the evolution of platypus venom genes, where, as in other animals, venom peptides have been derived via duplication from genes

for nontoxic peptides. For example, the OvDLPs were found to have evolved via gene duplication from antimicrobial beta-defensins ~192 Mya, after which they underwent neofunctionalization to become toxins (Whittington et al. 2008a). Gene duplication has also been responsible for venom gene diversification, generating large multigene families of platypus toxins that may serve to increase expression levels of a particular toxin type (Whittington et al. 2010).

Another important feature of platypus toxin development is convergent evolution. The similarity of platypus venom toxins to the venom toxins of other species (Whittington et al. 2008a, 2010) adds to previous studies finding independent recruitment of venom components in such widely divergent species as cephalopods, cnidarians, cone snails, fish, insects, scorpions, shrews, spiders, reptiles, Hymenoptera, and ticks, in fact across all of the major phyla of venomous animals (e.g., reviewed in Fry et al. 2009). The concept of convergent toxin evolution is important, as it raises the idea that there are certain peptide motifs that appear to be preferentially selected for evolution to venom peptides, possibly due to structural or functional constraints (e.g., reviewed in Fry et al. 2009). This allows speculation into the features that predispose a protein to evolve into a venom toxin. These features include proteins from families with extensive cysteine cross-linkages, secretory proteins, protein scaffolds that allow diversification into multimember toxin families with slightly differing activities, and protein scaffolds that have similarities to endogenous proteins, allowing a toxin to take advantage of one of the three different mechanisms for immediate disruption of the victim's homeostasis (physical damage, agonistic targeting, or antagonistic targeting) (e.g., Fry et al. 2009). Many of the platypus venom peptides satisfy these conditions: cysteine cross-linkages (e.g., OvDLPs), multigene toxin families (e.g., Kunitz-type protease inhibitors), and similarities to existing nontoxin proteins (e.g., OvDLPs and beta-defensins) (Whittington et al. 2008a, 2010).

The independent origin of venom molecules in platypuses and other species also appears to coincide with the independent origin of venom glands, for example, in snakes and platypuses. Snake venom glands are specialized salivary glands, which may have been derived from the pancreas (Kochva 1987), whereas platypus venom glands evolved from modified sweat glands (Temple-Smith 1973). These origins appear to be reflected in tissue expression patterns of venom genes; many of the platypus venom genes identified are derived from endogenous genes that are expressed in the skin, such as kallikreins and defensins (Whittington et al. 2008a, b, 2010). In contrast, there are several examples of snake venom molecules that have evolved from peptides expressed in the salivary gland and pancreas (Kochva 1987).

The Importance of Platypus Venom Research

The platypus is one of Australia's unique native species. Identifying the constituents of platypus venom has constituted an improvement in knowledge of the basic biology of this animal. For effective conservation measures, it is essential to understand all aspects of the biology of the species in question, and so any further

progress in this area would be beneficial. One example is that the effect of intraspecific platypus envenomation is unknown; knowledge of the platypus venom toxins and their functions could provide key improvements in this area.

The utility of platypus venom research should also extend to having human benefits. An understanding of mammalian venoms may improve knowledge of intra- and intercellular signaling in mammals. For example, further research into the platypus venom peptide isomerase may lead to its identification in higher animals including humans, where D-amino acids possibly participate in the etiology of altered-protein-turnover diseases like Alzheimer's and amyloidosis (Shanmugam et al. 2005).

Even more promising is the possibility of discovering novel therapeutic agents in platypus venom. Many venoms have already been used in pharmaceutical applications (reviewed in Escoubas and King 2009), and platypus venom is an untapped resource. The identification of new platypus venom toxins now allows their synthesis and subsequent functional characterization, and it is anticipated that this will provide another rich source of new molecules for pharmaceutical design. Of course, drug development from the platypus toxins is a long way off; not only do the putative toxins need to be confirmed by expression or synthesis of the toxin and then testing in functional assays, but the road to drug development, testing, and regulatory approval is a long one. However, this research is an important first step toward identifying sources of new molecular scaffolds in platypus venom for possible drug design.

The unusual symptoms of platypus envenomation suggest that platypus venom does contain many substances which may be clinically useful. For example, given that D-amino acid-containing peptides and proteins are more stable in vivo, medically useful therapeutic peptides with greater stability in vivo may eventuate from platypus venom peptide isomerase research. The platypus stonustoxin-like toxins, serine proteases, and protease inhibitors, like their functional analogues in snake venom, have potential applications for treating vascular disease in humans (Felicori et al. 2003). The platypus protease inhibitors with similarities to bikunin could be used to gain further insight into the inflammatory process and develop anti-inflammatories to treat organ injuries, cancers, and inflammatory disorders (Kobayashi et al. 2003). The platypus VEGF-like toxin could be used to increase permeability of tumor cells to anticancer drugs without increasing angiogenesis in the proliferating cells (Takahashi et al. 2004). The platypus toxins that cause the extreme pain could be used to probe new pain pathways and thus identify targets for novel painkillers.

Conclusion and Future Directions

The platypus holds an important position at the base of the mammalian phylogenetic tree. Its genome provides a treasure trove of novel evolutionary innovations that may yield unique human therapeutics. The first steps have been taken – the genome and transcriptome are now available. The door is now open for understanding the functional role of these genes and ultimately for the development of novel therapeutics.

Cross-References

▶ Snake Venom Phospholipase A_2: Evolution and Diversity

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Structure-Function Relationship of Modular Domains of P-III Class Snake Venom Metalloproteinases

9

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Abstract

Snake venom metalloproteinases (SVMPs) are among the most abundant toxins in many venoms of the Viperidae. SVMPs are primarily responsible for the hemorrhagic activity of venoms and may also interfere with the hemostatic system. Phylogenetically, SVMPs are most closely related to the mammalian ADAM (a disintegrin and metalloproteinase) protein family, a major class of membrane-bound sheddases. Together with ADAMs and the related ADAMTS (ADAM with thrombospondin type-1 motif) family of proteins, SVMPs constitute the M12B clan of zinc metalloproteinases. SVMPs are zinc-dependent proteinase ranging in size from 20 to 110 kDa, and they are categorized into classes P-I, P-II, and P-III according to their domain organization. Although all SVMPs share a catalytic metalloproteinase (M)-domain structure that is topologically similar to those of matrix metalloproteinases (MMPs), large P-III SVMPs have a modular structure with multiple non-catalytic ancillary domains that are not found in MMPs.

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P-III SVMPs generally have higher hemorrhagic activity and more diverse functions than P-I SVMPs, which only contain the metalloproteinase domain, suggesting that the non-catalytic domains are functionally important. Recent crystallographic studies of various subclasses of P-III SVMPs and mammalian ADAM/ADAMTS proteins have shed new light on the structure-function relationships of these modular proteinases. This chapter will focus on the three-dimensional structures of P-III SVMPs, particularly their non-catalytic domains, which may participate in directing these proteinases to specific substrates.

Introduction

Snake venom is a complex mixture of bioactive enzymes and nonenzymatic proteins used for the immobilization, killing, and digestion of prey (Calvete 2010b; Kang et al. 2011). Proteomic analyses of snake venoms have shown that snake venom metalloproteinases (SVMPs) constitute more than 30 % of the total proteins in many viper venoms; SVMPs are also present in the venoms of some species of Colubridae and Elapidae (Calvete et al. 2007; Fox and Serrano 2008a; Junqueira-de-Azevedo Ide and Ho 2002). These observations suggest that SVMPs play potentially significant roles in envenomation-related pathologies, such as local and systemic hemorrhage, intravascular clotting, hypovolemia, hypotension, edema, inflammation, and necrosis (Gutierrez and Rucavado 2000; Moura-da-Silva et al. 2007).

SVMPs are the primary factors responsible for the hemorrhagic effects of venoms, and they may also interfere with the hemostatic system through fibrinogenolytic or fibrinolytic activities, activation of prothrombin or factor X, and inhibition of platelet aggregation (Bjarnason and Fox 1994; Fox and Serrano 2005, 2009; Markland and Swenson 2013). The hemorrhagic activities of SVMPs contribute prominently to the lethality of viper snakebites. SVMPs degrade the components of basement membranes underlying capillary endothelial cells, thereby disrupting the vessel wall and allowing the escape of blood components into the stroma (Gutierrez et al. 2005). Therefore, before the advent of the first complete sequence determination of SVMPs, these proteins were believed to belong to the matrix metalloproteinase (MMP) family of proteinases.

It soon became apparent, however, that SVMPs were sufficiently different to warrant consideration as a separate structural class, even though they share some topological similarity with MMPs in the structure of the catalytic domain (Gomis-Ruth 2003). SVMPs of the high molecular weight P-III class have modular structures clearly distinct from those of MMPs and other metalloproteinases. SVMPs comprise the M12B clan of zinc metalloproteinases (MEROPS classification, http://merops.sanger.ac.uk/) together with proteins of the ADAM (a disintegrin and metalloproteinase) and ADAMTS (ADAM with thrombospondin type-1 motif) families. Metalloproteinases in this clan are also referred to as "reprolysins," a nomenclature chosen to reflect the two distinct origins of proteins in this class: some members are from reptiles (*rep*), whereas others belong to a group of proteinases initially described in mammalian reproductive tissues (*repro*) (Bjarnason and Fox 1995).

Recent crystallographic studies of P-III SVMPs and ADAM/ADAMTS proteins have shed new light on the structure-function relationships of these modular proteinases. This chapter will provide an overview of the current structural knowledge regarding SVMPs.

Classification and Modular Organization of SVMPs

SVMPs are classified into three major classes, P-I, P-II, and P-III, according to their domain organization (Fig. 1; Fox and Serrano 2008b, 2009). P-I SVMPs are composed of a single catalytic metalloproteinase (M) domain. P-II SVMPs are synthesized as an M domain and a disintegrin (D) domain. P-III SVMPs are synthesized as the M and D domains in addition to a cysteine-rich (C) domain (together, the MDC domains). In venoms, P-I and P-III SVMPs are abundant, but P-II SVMPs are frequently found in processed forms containing only their disintegrin domains, i.e., the classical disintegrins. P-II and P-III SVMPs can be divided further into several subclasses depending on the posttranslational modifications they undergo, such as proteolytic processing between the M and D domains (P-IIa and P-IIIb) or dimerization (P-IIc and P-IIIc) (Fox and Serrano 2008b). Previously called P-IV SVMPs, the heterotrimeric SVMPs that contain two additional snake C-type lectin-like (snaclec) domains (Clemetson 2010) are now categorized as a subclass of P-III SVMPs (P-IIId) because no P-IV mRNA transcript has been identified to date. The P-IIId SVMPs are therefore considered to be another type of posttranslationally modified derivatives of the canonical P-IIIa SVMPs (Fox and Serrano 2008b).

The genes for all SVMPs encode signal peptides, which mark the protein for the secretory pathway and a pro-domain sequence that appears before the M domain; however, no SVMP containing either of these sequences has been isolated from the venom. The pro-domain has been suggested to assist with correct folding of the protein and to maintain the proteinase in a latent state by a cysteine-switch mechanism (Grams et al. 1993) until cleavage, in the Golgi apparatus either by a pro-protein convertase or by autocatalysis. SVMPs of different classes are often present in the same venom. The P-III SVMPs are characterized by higher hemorrhagic activities and more diverse biological effects than the P-I SVMPs, implying that the non-catalytic ancillary domains are functionally important, e.g., by targeting the enzymes to specific substrates.

ADAM/ADAMTS Family Proteins

ADAMs, also called metalloproteinase-disintegrins, adamalysins, or MDC proteins, are mammalian glycoproteins implicated in cell-cell and cell-matrix adhesion and signaling (Edwards et al. 2009). ADAMs are phylogenetically most closely related to the P-III SVMPs, but are mostly expressed as type-1 integral membrane proteins. In addition to the MDC domains that they share with P-III SVMPs, most

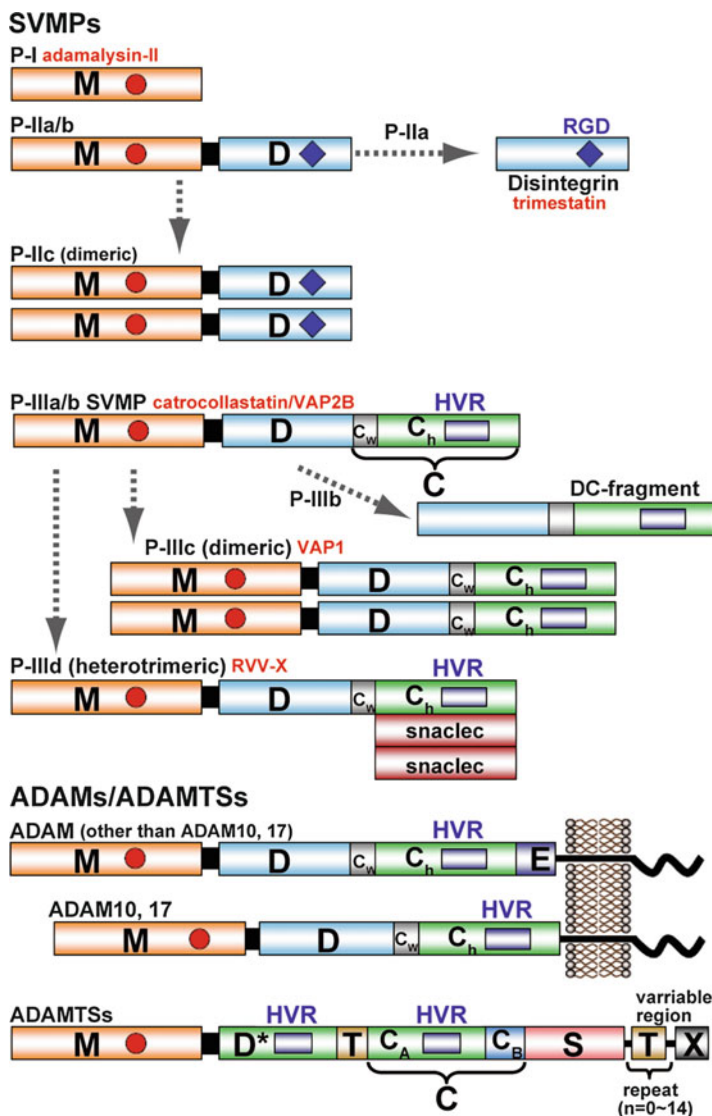

Fig. 1 Schematic diagram of the domain structure of SVMPs, ADAMs, and ADAMTSs. Each domain or subdomain is represented by a different color. *M* metalloproteinase domain, *D* disintegrin (or disintegrin-like) domain, *C* cysteine-rich domain, C_w cysteine-rich "wrist" subdomain, C_h cysteine-rich "hand" subdomain, snaclec, snake venom C-type lectin-like domain, *E* epidermal growth factor (*EGF*) domain, *T* thrombospondin type-1 repeat motif domain, *S* spacer domain, *X* a domain that is variable among ADAMTSs. Resent crystallographic studies have revealed that the D domain of ADAMTSs does not have disintegrin-like structure, but instead adopts the C_h-subdomain fold. In addition, the N-terminal region of the C domain of ADAMTSs (the C_A subdomain) also adopts the C_h-subdomain fold; therefore, these two regions in ADAMTSs are represented in the same color as the C_h subdomain of P-III SVMPs. The definitions of the subclasses of P-II and P-III SVMPs are as described in previous publications (Fox and Serrano 2008b, 2009)

ADAMs also contain an epidermal growth factor-like (EGF) domain, a transmembrane segment, and a cytoplasmic domain (Fig. 1). With regard to their cellular interactions, fertilins (ADAMs1 and 2) (Blobel et al. 1992) have been identified as sperm-surface molecules essential for fertilization, and meltrins (ADAMs9, 12, and 19) were initially identified as molecules that function in myoblast fusion (Yagami-Hiromasa et al. 1995). The best-characterized in vivo activity of ADAMs is their ectodomain shedding activity. ADAM17, which is also known as tumor necrosis factor-α (TNF-α)-converting enzyme (TACE), was initially identified as the physiological convertase for TNF-α (Black et al. 1997; Moss et al. 1997). ADAMs play key roles in normal development and morphogenesis through their function in shedding ectodomains of cell-surface proteins, including the latent forms of growth factors, cytokines, their receptors, and adhesion molecules; consistent with these diverse roles, ADAMs are associated with numerous diseases, including rheumatoid arthritis, Alzheimer's disease, heart disease, and cancer (Edwards et al. 2009; Mochizuki and Okada 2007; Murphy 2008). The human genome expresses 20 ADAM genes (excluding pseudogenes). However, unlike SVMPs and ADAMTSs, which are all active enzymes, only ~60 % of membrane-bound ADAMs contain a functional catalytic sequence (see below). The physiological functions of the proteinase-inactive ADAMs remain largely unknown, although several members of this group play important roles in development (Blobel et al. 1992).

The ADAMTS family is a branch of the ADAM family whose members contain various numbers of C-terminal thrombospondin type-1 repeats instead of a transmembrane/cytoplasmic segment (Fig. 1); consequently, these proteins function as secreted proteinases. There are 19 ADAMTS proteins in humans, and they function variously as aggrecanases, procollagen *N*-proteinases, and von Willebrand factor (VWF)-cleaving proteinases (Apte 2009). Recent crystallographic studies have revealed that the D domains of ADAMTS proteins exhibit no structural homology to classical disintegrins, but are very similar in structure to a part of the C domains of P-III SVMPs (the C_h subdomain; see below) (Gerhardt et al. 2007; Takeda 2009). In addition, the *N*-terminal region of the C domain of ADAMTSs (the C_A subdomain) possesses essentially the same fold as the C_h subdomains of P-III SVMPs and ADAMs, even though it shares no apparent amino-acid sequence similarity with them (Akiyama et al. 2009; Takeda et al. 2012). Thus, while the "disintegrin" nomenclature has been used, ADAMTSs actually contain no disintegrin-like structures, but instead have two C_h-subdomain-fold domains (Fig. 1).

Crystal Structures of SVMPs

Adamalysin II is a P-I SVMP isolated from *Crotalus adamanteus* and is the first SVMP for which a crystal structure was solved (Gomis-Ruth et al. 1993, 1994). To date, crystal structures of 10 P-I SVMPs have been deposited in the PDB (Table 1). In addition, the structures of the M-domain-containing fragments of seven ADAM/ADAMTS proteins are currently available in the PDB. Vascular apoptosis-inducing protein-1 (VAP1) from *Crotalus atrox* is the first P-III SVMP for which a

Table 1 Selection of the X-ray structures of SVMPs and related mammalian proteins deposited in the PDB

Protein	Source	Domains	PDB code	Publication year
SVMPs				
P-I				
Adamalysin II	*Crotalus adamanteus*	M	1IAG, 2AIG, 3AIG	1993
Atrolysin C	*Crotalus atrox*	M	1ATL, 1HTD	1994
Acutolysin A	*Agkistrodon acutus*	M	1BSW, 1BUD	1998
BaP1	*Bothrops asper*	M	1ND1	2003
H2-proteinase	*Trimeresurus flavoviridis*	M	1WNI	1996
TM-1	*Trimeresurus mucrosquamatus*	M	4J4M	2013
TM-3	*Trimeresurus mucrosquamatus*	M	1KUF, 1KUG, 1KUI, 1KUK	2002
Acutolysin C	*Agkistrodon acutus*	M	1QUA	1999
FII	*Agkistrodon acutus*	M	1YP1	2005
BmooMPα-I	*Bothrops moojeni*	M	3GBO	2010
P-IIIa/b				
Catrocollastatin/ VAP2B	*Crotalus atrox*	MDC	2DW0, 2DW1, 2DW2	2007
Bothropasin	*Bothrops jararaca*	MDC	3DSL	2008
AaHIV	*Agkistrodon acutus*	MDC	3HDB	2009
Atragin	*Naja atra*	MDC	3K7L	2010
K-like	*Naja atra*	MDC	3K7N	2010
P-IIIc				
V AP-1	*Crotalus atrox*	2 × (MDC)	2ERO, 2ERP, 2ERQ	2006
P-IIId				
RVV-X	*Daboia russelli*	MDC + snaclec	2E3X	2007
Mammalian proteins				
ADAMs				
ADAM8	Human	M	4DD8	2012
ADAM10	Bovine	DC	2AO7	2005
ADAM17	Human	M	1BKC	1998
ADAM17	Human	C	2M2F (determined by NMR)	2013
ADAM22	Human	MDCE	3G5C	2009
ADAM33	Human	M	1R54, 1R55	2004

(*continued*)

Table 1 (continued)

Protein	Source	Domains	PDB code	Publication year
SVMPs				
ADAMTSs				
ADAMTS1	Human	MD*	2JIH, 2V4B	2007
ADAMTS4	Human	MD*	2RJP, 3B2Z	2008
ADAMTS5	Human	M	3B8Z	2008
ADAMTS5	Human	MD*	2RJQ	2008
ADAMTS13	Human	D*TCS	3GHM, 3GHN	2009

three-dimensional structure was determined (Takeda et al. 2006). To date, structures of seven P-III SVMPs have been deposited in the PDB, including structures from almost all P-III subclasses. Although no structures of full-length molecules are available for ADAMs and ADAMTSs, structures of several non-catalytic exosite-containing fragments of ADAM/ADAMTS proteins have also been deposited in the PDB. No complete P-II structures are currently available, although an increasing number of crystal and solution structures of disintegrins are being added to the PDB.

C-Shaped MDC Domains of P-III SVMPs

Figure 2a depicts the crystal structure of catrocollastatin/VAP2B from *Crotalus atrox* venom, the first monomeric P-III structure to be solved, representing a structural prototype of the P-III class of SVMPs and ADAMs (Igarashi et al. 2007). The crystal structures of P-III SVMPs reveal that the MDC domain folds into a C-shaped configuration in which the distal portion of the C domain comes close to, and faces toward, the catalytic site in the M domain. The M domain follows and is adjacent to the D domain, which protrudes from the M domain close to the Ca^{2+}-binding site I (see below), opposite the catalytic zinc ions. The D domain is linked to the M domain by a short linker that allows variable orientation between the M and D domains. Consistent with this, comparison of the available P-III SVMP structures reveals substantial diversity in the relative orientation of the M and D domains. For example, the MDC domains of RVV-X form a rather closed C-shaped structure, unlike the open C-shaped structures of VAP1 and catrocollastatin/VAP2B (Fig. 2b). Such variability is also observed even among different crystal structures of the same SVMPs (Igarashi et al. 2007). The side chain of Leu408 in the D domain of catrocollastatin/VAP2B (Phe410 and Ile220 in VAP1 and RVV-X, respectively) is located at the pivot point of the structural variations. The main-chain carbonyl oxygen atom of Leu408 coordinates the Ca^{2+} at site II (see below), whereas its side chain protrudes from the D domain

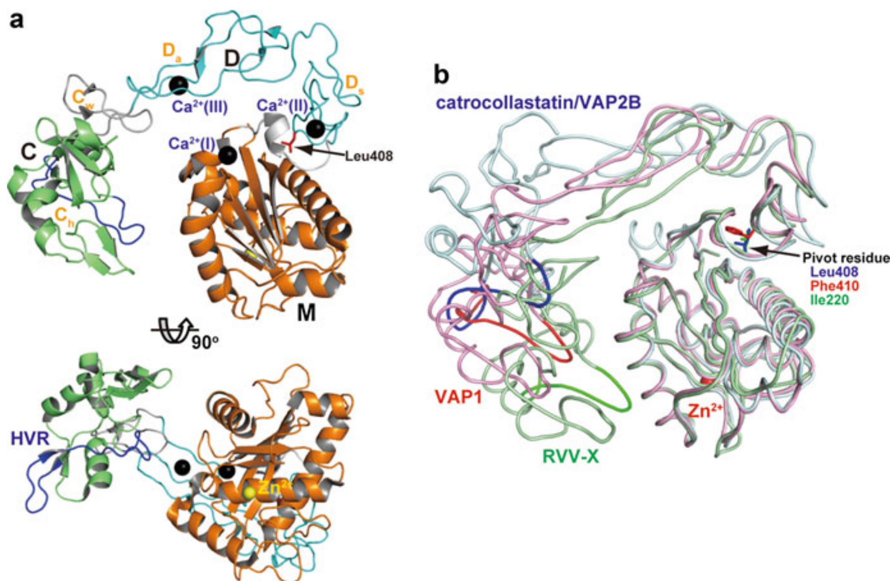

Fig. 2 C-shaped MDC-domain structure of typical P-III SVMPs. (**a**) Two orthogonal views of the structure of catrocollastatin/VAP2B (PDB ID: 2DW0), a structural prototype of P-III SVMPs. Each domain or subdomain is colored as in Fig. 1. Bound Zn^{2+} and Ca^{2+} ions are represented as *yellow* and *black* spheres, respectively. (**b**) Superimposition of the M domain of catrocollastatin/VAP2B (in *cyan* and *blue*), VAP1 (in *salmon* and *red*), and RVV-X (in *light green* and *green*). The side chains of the residues located at the pivot points are shown

and interacts with the small hydrophobic cavity on the surface of the M domain. A bulky hydrophobic residue at this position is highly conserved among P-III SVMPs and ADAMs, and its side chain probably functions as a universal joint (shoulder joint) that allows the “shoulder” domain (D_s subdomain, see below) to adopt various orientations with respect to the M domain (Igarashi et al. 2007; Takeda et al. 2012). The complete ectodomain (M/D/C/EGF domains) structure of ADAM22 reveals that both the C-shaped configuration of the MDC domains and the shoulder-joint mechanism are also conserved in mammalian ADAMs (Liu et al. 2009). The additional EGF domain in ADAM22 is tightly associated with both the D and C domains and may form a rigid spacer that properly positions the MDC domains against the membrane for the purpose of shedding membrane-anchored molecules.

Catalytic Metalloproteinase Domain

The M domains of SVMPs and ADAMs/ADAMTSs range from 180 to 260 (typically 200–210) residues in length (Fox and Serrano 2005; Igarashi et al. 2007). Figure 3a depicts the M-domain structure of catrocollastatin/VAP2B in complex

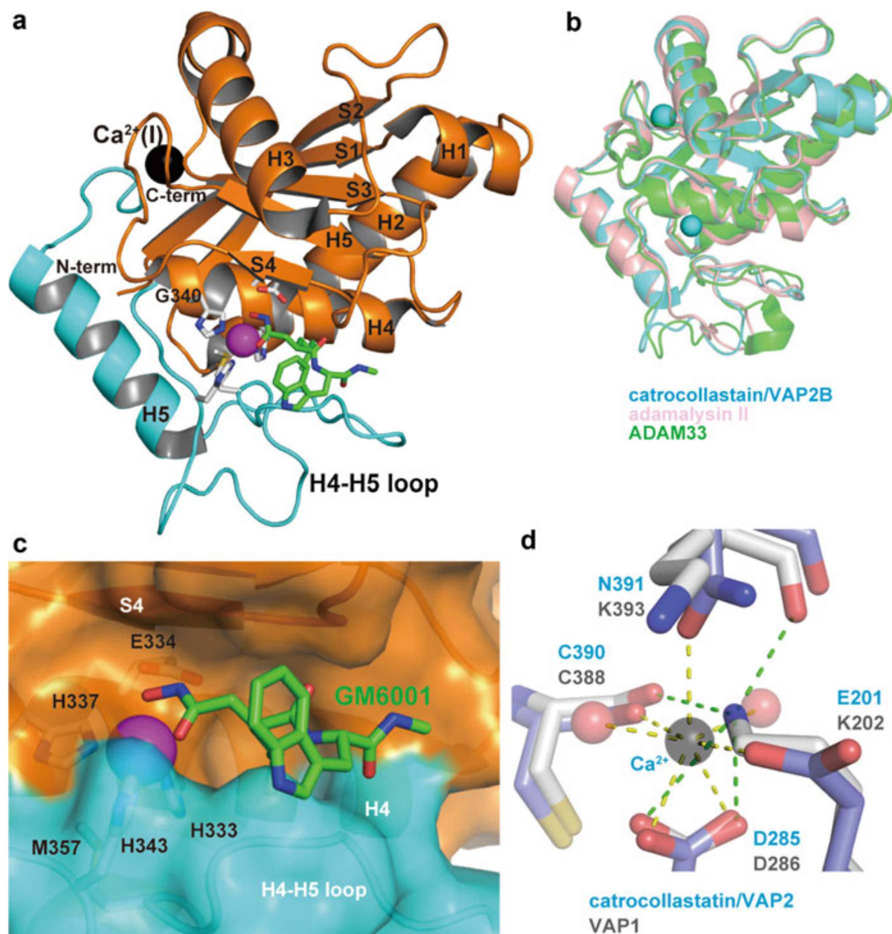

Fig. 3 M-domain structure. (**a**) Structure of the M domain of catrocollastatin/VAP2B (2DW0). The *upper* and *lower* domains are colored in *orange* and *cyan*, respectively. Bound Zn^{2+} and Ca^{2+} ions are shown in *magenta* and *black*, respectively, and bound inhibitor GM6001 is shown in *green*. (**b**) Superimposition of the M domains of catrocollastatin/VAP2B (in *cyan*), adamalysin II (1IAG, in *salmon*), and human ADAM33 (1R54, in *green*). (**c**) Closeup view of the catalytic site of catrocollastatin/VAP2B. (**d**) Comparison of the Ca^{2+}-binding site I structures of catrocollastatin/VAP2B (*blue*) and VAP1 (2ERO, *gray*). The residues in catrocollastatin/VAP2B and VAP1 are labeled in *cyan* and *gray*, respectively. A Ca^{2+} ion and a water molecule bound to catrocollastatin/VAP2B are represented as *black* and *red* spheres, respectively. The Nε atom of Lys202 in VAP1 occupies the position of the Ca^{2+} ion in catrocollastatin/VAP2B

with GM6001, a member of the hydroxamic acid class of inhibitors, viewed from the so-called "standard" orientation (Gomis-Ruth et al. 2012). The M domain has an oblate ellipsoidal shape with a notch in its flat side that separates the upper main molecular body (about 150N-terminal residues, shown in orange) from an irregularly folded lower subdomain (about 50 C-terminal residues, shown in cyan).

The active site cleft extends horizontally across the flat surface of the M domain, and the peptidic inhibitor binds to the cleft so as to closely mimic a peptide substrate lying from left to right. Currently available M-domain structures of all classes of SVMPs and ADAMs/ADAMTSs are very similar to each other, with a conserved molecular topology consisting of a five-stranded β-sheet, four long α-helices, and one short N-terminal α-helix (Fig. 3b). This topology was originally identified in the adamalysin II structure, although different disulfide-bond patterns have been observed in different proteins. In addition, the structures vary from one another in the loop regions that connect conserved helices and strands, most prominently in the loop connecting the H4 and H5 helices in the lower subdomain. The amino-acid sequence of this irregular region is also highly divergent among SVMPs and is therefore important for substrate recognition because it forms part of the wall of the substrate-binding pockets (Fig. 3c). The catalytic zinc ion is situated at the bottom of the active site cleft, where it is coordinated by the Nε2 atoms of the three conserved histidine residues (His333, His337, and His343). In the absence of the inhibitor, a water molecule also coordinates the zinc ion in a tetrahedral manner and is anchored to Glu334 (Gomis-Ruth et al. 1994). This bound water molecule is polarized by Glu334 and is involved in the nucleophilic attack at the scissile peptide bond. The long H4 helix, which contains the active site, extends to Gly340, where it turns sharply toward His343 (Fig. 3a). The active site is characterized by a catalytic consensus sequence, HEXXHXXGXXH, that is conserved not only in SVMPs and in ADAMs/ADAMTSs but also across the metzincin superfamily of metalloproteinases, which also contains MMPs, astacins, and serralysins (Gomis-Ruth 2003, 2009). The conserved Met357 downstream of the catalytic consensus sequence folds into a so-called Met-turn and forms a hydrophobic basement for the three zinc-binding imidazoles, the hallmark of the metzincin proteinases.

Close to the crossover point of the N- and C-termini of the M domain is a calcium ion-binding site, located in opposition to the active site cleft (Fig. 2a). In catrocollastatin/VAP2B, the bound Ca^{2+} ion is coordinated by the side chains of Asp285, Asn391, and Glu201; the backbone carbonyl oxygen atom of Cy388; and two water molecules in a pentagonal bipyramidal arrangement (shown with yellow dotted lines in Fig. 3d). The three side chains that coordinate the Ca^{2+} ion are largely conserved among SVMPs and ADAMs (Fox and Serrano 2005; Takeda et al. 2006). However, some SVMPs and ADAMs have substitutions in these three residues. In VAP1, Glu201 and Asn391 are replaced by Lys202 and Lys392, respectively, and the distal Nε atom of Lys202 substitutes for the Ca^{2+} ion (Fig. 3d) (Takeda et al. 2006). Replacement of the calcium-coordinating Glu residue with Lys is also observed in other SVMPs and ADAMs (Fox and Serrano 2005; Igarashi et al. 2007; Takeda et al. 2006). The high degree of conservation of residues involved in Ca^{2+} binding (or in mimicking Ca^{2+} binding) might reflect the importance of this region for the structural link between the M and D domains. In addition, Ca^{2+} protects against autoproteolysis at this M-/D-domain junction (Gomis-Ruth et al. 1994; Takeya et al. 1993).

As mentioned above, the P-I SVMPs generally exert lower hemorrhagic activity than the P-III SVMPs; indeed, some P-I SVMPs are essentially devoid of this

activity. Structural comparisons among SVMPs have revealed differences in the features of the substrate-binding cleft in the M domain. However, despite intensive structural studies of both hemorrhagic and nonhemorrhagic P-I SVMPs, the detailed structural determinants of hemorrhagic activity remain unknown.

Disintegrin-Like Domain

The D domain of P-III SVMPs can be structurally further divided into two subdomains, the "shoulder" (D_s) and the "arm" (D_a) subdomains. Both the D_s and D_a subdomains consist largely of a series of turns and short regions of antiparallel β-sheet and constitute an elongated C-shaped arm structure together with the immediately subsequent region of the primary sequence, the N-terminal region of the C domain or "wrist" (C_w) subdomain (Fig. 4a). There are three disulfide bonds in D_s, three in D_a, and one in the C_w subdomain, and the segments are connected by single disulfide bonds. The numbers and spacing of the cysteine residues involved in these disulfide bonds are strictly conserved among P-III SVMPs and ADAMs (Igarashi et al. 2007; Takeda et al. 2006; Fig. 4f), with a few exceptions, one of which is the kaouthiagin-like (K-like) proteinase from *Naja atra*. The K-like proteinase lacks the 17-amino-acid segment at the junction of the D_s and D_a subdomains, resulting in a different disulfide-bond pattern in the D_s/D_a subdomain (Fig. 4f) and a different orientation between the D_s and D_a/C_w domains compared to other P-III SVMP structures (Fig. 4b). These changes force the MDC domains of K-like proteinase to adopt a more elongated, I-shaped configuration (Guan et al. 2009). However, how this I-shaped structure correlates with the protein's function remains to be elucidated.

The structures of both the D_s and D_a subdomains of P-III SVMPs contain Ca^{2+}-binding sites that were not predicted from the primary structures. In the D_s subdomain, side-chain oxygen atoms of residues Asn408, Glu412, Glu415, and Asp418 and the main-chain carbonyl oxygen atoms of Val405 and Phe410 (catrocollastatin/VAP2B sequence) are involved in pentagonal bipyramidal coordination of Ca^{2+}-binding site II (Fig. 4c). Those residues are strictly conserved among all known P-III SVMPs and ADAMs (Igarashi et al. 2007; Takeda 2011; Takeda et al. 2006) and are represented by the consensus sequence XCGNXXXEXGEECD (residues 405–418; underlined residues are involved in the Ca^{2+} coordination). On the other hand, side-chain oxygen atoms Asp469, Asp472, and Asp483 and the main-chain carbonyl oxygen atoms of Met470 and Arg484, as well as a water molecule, coordinate the binding of a Ca^{2+} ion at the corner of a pentagonal bipyramid and constitute Ca^{2+}-binding site III in the D_a subdomain (Fig. 4d). These residues are also strictly conserved among all known P-III SVMP and ADAMs, with the exception of ADAM10 and ADAM17, and are represented by the consensus sequence CDXX(E/D)XCXG$(X)_4$C$(X)_2$(D/N)X (residues 466–483) (Igarashi et al. 2007; Takeda 2011; Takeda et al. 2006). In sharp contrast to Ca^{2+}-binding site I, due to the deeply buried positions and tight coordination of sites II and III, the Ca^{2+} ions bound at these sites cannot be stripped from

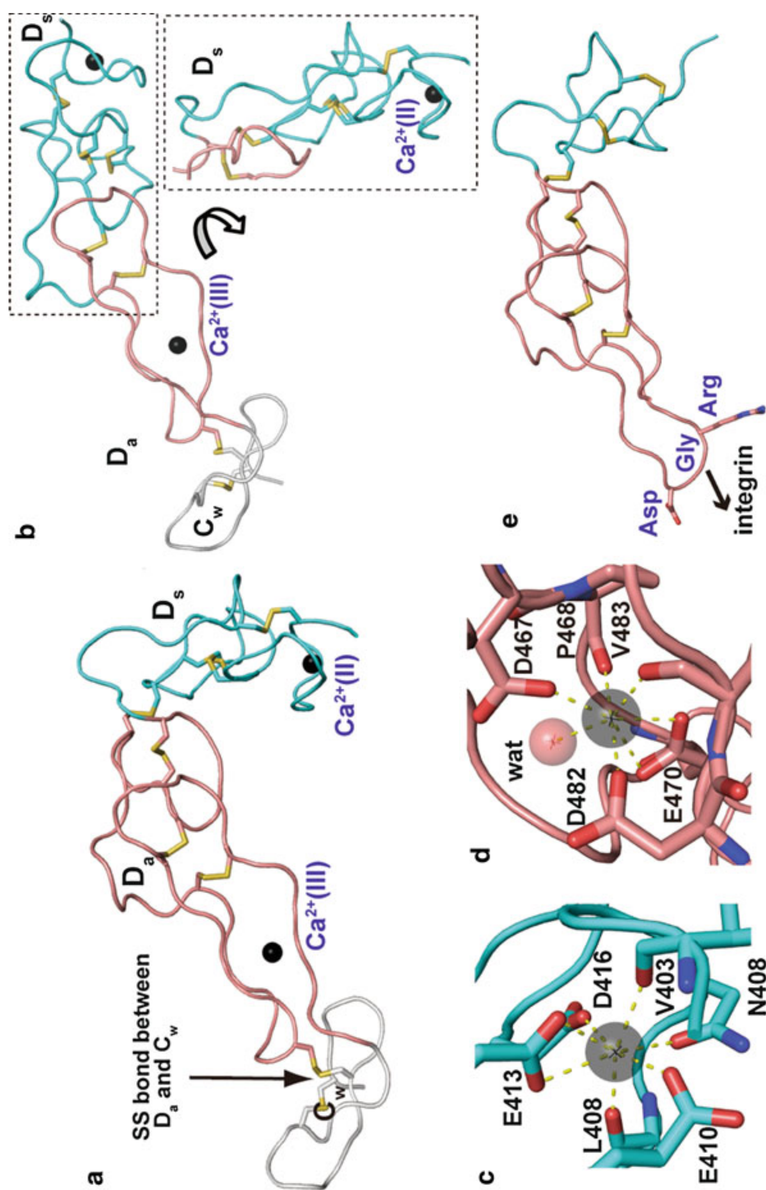

Fig. 4 (continued)

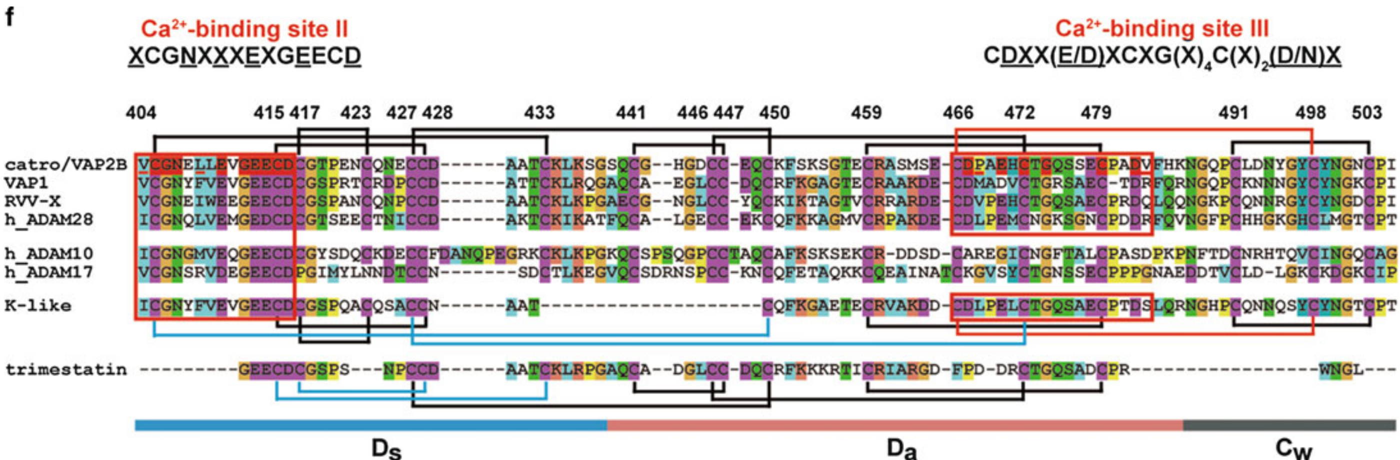

Fig. 4 D-domain structure. (**a**) The D_s, D_a, and C_w subdomains of catrocollastatin/VAP2B (2DW0) are shown in *cyan*, *salmon*, and *gray*, respectively. Disulfide bonds and bound Ca^{2+} ions are shown in *orange* and *black*, respectively. (**b**) The D domain of K-like protein (3K7N) with two different views of the D_s subdomain (in *dotted line boxes*). The D_s and D_a subdomains of P-III SVMPs contain Ca^{2+}-binding sites II (**c**) and III (**d**), respectively. The catrocollastatin/VAP2B residues and a water molecule involved in the Ca^{2+} coordination are indicated. (**e**) Structure of an RGD-containing disintegrin, trimestatin (1J2L). Suggested integrin-binding residues are indicated. (**f**) Amino-acid sequence alignment of catrocollastatin/VAP2B (PDB: 2DW0_A), VAP1 (PDB: 2ERO_A), RVV-X heavy chain (PDB: 2E3X_A), human ADAM28 (GI: 98985828), human ADAM10 (GI: 29337031), human ADAM17 (GI: 14423632), K-like (PDB: 3K7N_A), and trimestatin (1J2L_A) generated using ClustalX (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/). Disulfide bonds and the boundaries between subdomains are schematically indicated. Ca^{2+}-binding sites II and III are boxed in *red*

ADAM22, even using EDTA; consequently, these Ca^{2+} ions are likely to remain permanently in place once the D domain is folded (Liu et al. 2009). Because there are few secondary structural elements, the disulfide bonds and bound Ca^{2+} ions are essential for the structural rigidity of the C-shaped arm structure.

As predicted from its amino-acid sequence, the structure of the D domain of P-III SVMPs is quite similar to that of trimestatin, an RGD-containing classical disintegrin (Fujii et al. 2003; Fig. 4e). The integrin-binding ability of disintegrins has been attributed to a highly mobile loop (disintegrin loop) that contains a cell-adhesion sequence (e.g., RGD) at the tip of the hairpin. In the P-III SVMPs, the RGD sequence is usually replaced by an (D/S)XCD sequence (residues 466–469 in the catrocollastatin/VAP2B sequence, Fig. 4f), the disintegrin loop is packed against the subsequent C_w subdomain, and a disulfide bond (Cys468–Cy499) and bound Ca^{2+} at site III further stabilize the continuous and rather rigid arm-like structure. Thus, the disintegrin loop in P-III SVMPs is unavailable for protein-protein interactions due to steric hindrance. No structural Ca^{2+} binding has been identified in classical disintegrins, and the D_s region of disintegrins is generally shorter and less ordered than the corresponding regions of P-III SVMPs. Because of the lack of structural Ca^{2+} ions, disintegrins have a more flexible structure, throughout the entire molecule, than the D domain of P-III SVMPs. The flexibility of the disintegrin loop is probably important for the target-binding function of integrins. P-II SVMPs, the precursors of most of disintegrins, may have evolved from ancestral P-III SVMP genes after losing the genetic information encoding the protein regions downstream of the D domain (Calvete 2010a). Removal of structural constraints (disulfide bonds and structural Ca^{2+}-binding sites), imposed both on the disintegrin loop and the D_s subdomains in the ancestral P-II SVMPs, has been postulated as the key event that permitted the subsequent evolution of both integrin-binding activity and the proteolytic release mechanism.

Cysteine-Rich Domain and Hypervariable Region

The C-terminal region of the P-III SVMPs, typically about 120 amino-acid residues, contains 13–15 cysteine residues and has therefore been termed the "cysteine-rich" domain. Neither the D nor C domain of any P-III SVMP has been separately isolated from venom. In some cases of the P-IIIb subclass, however, substantial quantities of the processed DC domain fragments have been identified in venom along with the unprocessed molecules (Shimokawa et al. 1997; Usami et al. 1994). Although lacking proteolytic activity, such isolated DC fragments display diverse biological activities, such as inhibition of collagen-stimulated platelet aggregation and the modulation of integrin-mediated cell adhesion, migration, and proliferation, implying that the DC domains of P-III SVMPs are also important in the biological functions of the toxins. Most of these studies, however, have not yet identified specific regions of the DC domain involved in protein-protein interactions, and the molecular mechanisms underlying their function remain largely unknown. The role of the C domain has not received the same

level of attention as the D domain, mostly because it lacks known structural/functional motifs; however, crystallographic studies have revealed potentially important functions of the C domain.

The C domain of P-III SVMPs can be structurally subdivided into the C_w and "hand" (C_h) subdomains (Takeda et al. 2006; Fig. 2a). As mentioned before, the C_w subdomain tightly associates with the D domain, and the two are structurally integrated into a continuous structure. On the other hand, the C_h subdomain constitutes a separate unit and has a unique structure consisting of irregularly folded loops with a core of α-/β-fold and disulfide bonds. In the P-III SVMPs, the C_h subdomain has a novel fold with no structural homology to currently known proteins, with the exception of the corresponding segments of ADAM and ADAMTS proteins. The whole C domain of the P-III SVMPs and ADAMs has been deposited into the Conserved Domains Database (CDD, http://www.ncbi.nlm.nih.gov/cdd) as a member of the ADAM_CR superfamily (cl15456).

Figure 5 depicts the crystal structure and the topology diagram of the C_h subdomain of catrocollastatin/VAP2B (**A**) and a gallery of the currently available C_h-subdomain structures of SVMPs and some mammalian counterparts (**B**). In these figures, the conserved core α-helix is shown in red, and the two sets of β-sheets are shown in yellow. The overall structure of the C_h subdomain of catrocollastatin/VAP2B is very similar to those of other SVMPs and that of ADAM22, with the variability occurring mostly in loop regions. The loop encompasses residues 561–582 and extends across the central region of the C_h subdomain in catrocollastatin/VAP2B. This is the region in which the amino-acid sequences are most divergent and variable in length among SVMPs (16–22 amino acids), human ADAMs (27–55 amino acids), and ADAMTSs (13–17 amino acids). Therefore, this region (shown in blue in Fig. 5) has been designated as the hypervariable region (HVR) (Takeda et al. 2006). Furthermore, comparison between the structures resulted in identification of the variable (V) loop (gray region in Fig. 5) located beside the HVR. The V-loop also exhibits quite a high level of variability among ADAMTSs and the noncanonical ADAMs (ADAM10 and ADAM17), comparable to that of HVR, and their V-loops generally have quite mobile structures (Akiyama et al. 2009; Takeda et al. 2012). The V-loop in the P-III SVMPs and canonical ADAMs commonly consists of an α-helix (H8) and two solvent-exposed hairpin loops. Considering the V-loop structures, the C_h-subdomain structures can be categorized into two groups: catrocollastatin/VAP1B type (group A) and ADAM10 type (group B). Although there are common structural elements among those C_h-subdomain structures, the degrees of amino-acid identity between the C_h subdomains of group A molecules and those of group B molecules, and also among the group B molecules, are negligible (e.g., ~15 % between catrocollastatin/VAP2B and ADAM10, ~16 % between catrocollastatin/VAP2B and ADAMTS13 [D domain], and ~17 % between the D and C_A domains of ADAMTS13).

In the P-III SVMP structures, the HVR is present at the distal end of the C-shaped MDC domains, and it points toward and comes close to the catalytic site of the M domain (Fig. 2). This raises the intriguing possibility that the HVR

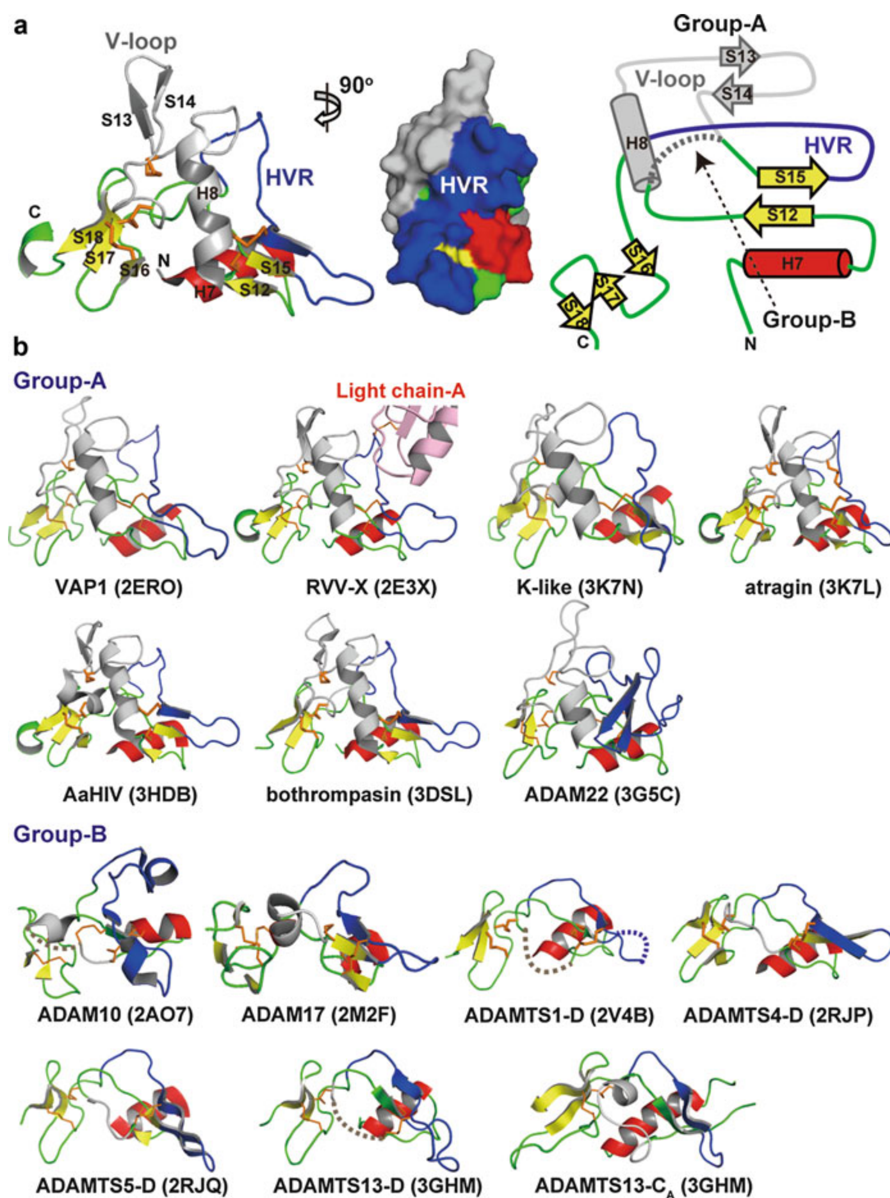

Fig. 5 C_h-subdomain structures. (**a**) Ribbon (*left*), molecular surface (*middle*) representation, and topology diagram (*right*) of the C_h-subdomain structure of catrocollastatin/VAP2B (2DW0). Conserved β-strands and an α-helix are shown in *yellow* and *red*, respectively. Disulfide bonds, residues in the HVR, and residues in the V-loop are shown in *orange*, *blue*, and *gray*, respectively. (**b**) Ribbon representation of the C_h subdomains of SVMPs and ADAMs, and the D and C_A domains of ADAMTSs. PDB ID for each protein is indicated in parentheses

creates an exosite for binding of substrate molecules (Takeda 2009; Takeda et al. 2006). Different SVMPs have distinct HVR sequences, resulting in distinct molecular surface features. They might, therefore, function in specific protein-protein interactions, which could explain the diversity of biological activities characteristic of the P-III SVMPs and ADAMs/ADAMTSs (Takeda 2009; Takeda et al. 2006). The D-domain portion is located opposite to and apart from the catalytic site and thus might play a primary role as a scaffold that spatially positions two functional units, the catalytic site and exosite. The V-loop may also function as an exosite. The intrinsic flexibility between the domains may be important for the fine-tuning of substrate recognition, probably by adjusting the spatial alignment of the catalytic site and exosite during catalysis (Igarashi et al. 2007; Takeda et al. 2012).

Several reports suggest that the HVR region directly contributes to the function of P-III SVMPs. Most of these studies, however, used synthetic peptides derived from the HVR region or *E. coli*-expressed protein domains for functional assays. Therefore, although these studies shed some light on the functions of HVR, the significance of these findings is still uncertain. It should be noted that short peptides or *E. coli*-expressed cysteine-rich proteins do not always mimic their counterparts in the intact proteins. Although there is as yet no evidence that HVR in P-III SVMPs actually functions as an exosite, systematic structure-based site-directed mutagenesis studies on ADAMTS13 have revealed that both the HVR and the V-loop in the D and C_A domains constitute the VWF-binding exosites (Akiyama et al. 2009; de Groot et al. 2009). One study proposed that P-III SVMPs could be classified into two or more subgroups according to their HVR sequences (Muniz et al. 2008), but the relationships between different HVR sequence classes and their biological activities remain to be elucidated.

The mechanism by which DC fragments and unprocessed P-IIIb SVMPs coexist in venom still remains unclear. It is possible that multiple structural isoforms of the same P-III SVMPs exist in the venom, perhaps the result of alternative disulfide-bond pairing (Moura-da-Silva et al. 2003). Recently, protein-disulfide isomerase (PDI) has been implicated in the regulation of shedding activity of ADAM17, and a structural analysis of the C_h subdomain of ADAM17 revealed that PDI can indeed act on this subdomain and convert it from the inactive to the active conformation by disulfide-bond isomerization (Düsterhöft et al. 2013).

Dimeric P-IIIc Class

Some P-III SVMPs, such as VAP1, HV1 (GenBank ID (GI): 14325767), halysase (GI: 60729695), VLAIP (GI: 82228619, 82228618), and VaH3 (GI: 496537199), exist in their native states as homo- or heterodimers. Examination of their primary structures reveals that a cysteine residue at position 365 (in VAP sequence) is conserved, whereas none of the other SVMP classes have cysteine residues at this

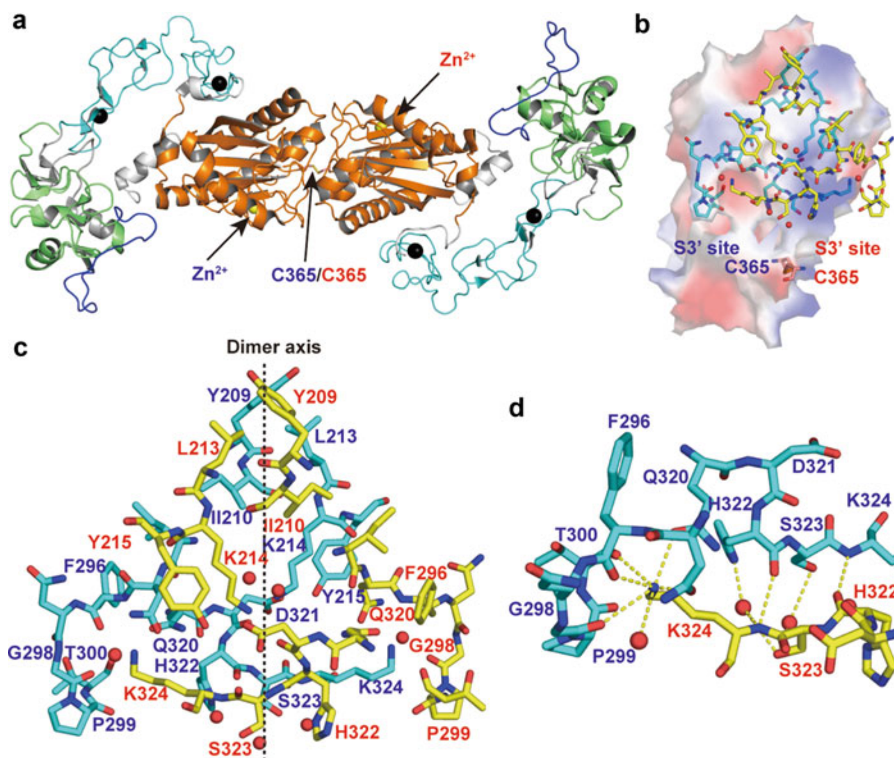

Fig. 6 Structure of the dimeric P-IIIc class of SVMPs. (**a**) Structure of VAP1 (2ERO) viewed from the dimer axis. (**b**) Dimer interface is viewed from a direction nearly perpendicular to the dimer axis. The molecular surface of the *cyan* molecule is shown with electrochemical potential (*red* to *blue*). (**c**, **d**) Closeup views of the dimer interface. The residues involved in the interchain interactions are indicated with *blue* and *red* letters for *cyan* and *yellow* molecules, respectively. The water molecules involved in the interaction are represented as *red* spheres

position (Fox and Serrano 2005, 2008b; Takeda et al. 2006). The crystal structure of VAP1 revealed an intermolecular disulfide bond that is formed between symmetry-related Cys365 residues and provides the basic architecture for a dimeric interface formed exclusively by the M domains (Takeda et al. 2006; Fig. 6a). The top of the dimer interface is capped by hydrophobic interactions involving Tyr209, Ile210, Leu213, and Tyr215 and the aliphatic portion of Lys214 (Fig. 6b, c). The dimer interface is best characterized by the recognition sequence QDHSK (residues 320–324 in VAP1) and the presence of Cys365. Residues 322–324 form an antiparallel β-sheet with their counterpart (Fig. 6d). Three water molecules are bound to the side chains of His322 and Ser323 and are involved in the hydrogen-bond network that is formed between the monomers. Lys324 plays a pivotal role in this dimer recognition: its Nε amino group is coordinated by six oxygen atoms, which belong to the symmetry-related monomer and are located at the corners of a pentagonal pyramid. These six atoms include the side-chain oxygen atoms of

Asn295 and Gln320; the carbonyl oxygen atoms of Phe296, Gly298, and Thr300; and a water molecule (Fig. 6d). Alignment of the amino-acid sequence of 40 P-III SVMPs revealed that three proteins in addition to the five SVMPs listed above have both this unique cysteine residue and the QDHSK sequence suggestive of the P-IIIc class (Igarashi et al. 2007).

The disulfide bond formed between symmetry-related Cys365 residues and the residues in the consensus sequence also constitute the side wall of the substitute recognition S3′ pocket, which merges with its counterpart inside the molecule (Fig. 6b). In the P-IIIc SVMP structure, the two catalytic sites in the dimer are thus located back-to-back but share their S3′ pockets, suggesting that the two catalytic sites may work in a cooperative manner. However, how this dimeric structure relates to the substrate preference and/or the activity of the P-IIIc SVMPs remains to be elucidated.

Bilitoxin I (GI: 172044534), which has been suggested to belong to homodimeric P-IIc class (Fox and Serrano 2008b), has neither a cysteine residue at position 365 nor the QDHSK sequence, suggesting that its dimer interface is different from that of VAP1.

Heterotrimeric P-IIId Class

RVV-X and VLFXA, coagulation factor X (FX) activators isolated from Russell's viper *Daboia russelii* and *Vipera lebetina*, respectively, are the representatives of the P-IIId class of SVMPs, which are composed of an MDC-containing heavy chain and two light chains with the snaclec fold (Morita 1998; Siigur and Siigur 2010; Takeda 2010; Takeya et al. 1992). Because of its extremely high specificity, RVV-X is widely used in coagulation research and in diagnostic applications. RVV-X activates FX by cleaving the Arg194–Ile195 bond in FX, which is also cleaved by factors IXa and VIIa during physiological coagulation (Morita 1998; Tans and Rosing 2001). Cleavage removes the heavily glycosylated amino-terminal 52 residues (activation peptide, AP) of the FX heavy chain, which results in maturation of the catalytic triad in the active site. Activated FX (FXa) in turn converts prothrombin to thrombin, which ultimately leads to formation of hemostatic plugs.

The crystal structure of RVV-X determined at 2.9 Å resolution revealed its unique hook-spanner-wrench configuration (Fig. 7a), in which the MD domains constitute the hook, and the remainder of the molecule forms the handle (Takeda 2010; Takeda et al. 2007). The backbone structure of the heavy chain is essentially the same as those of other P-III SVMP structures, although the subdomain orientations are different (Fig. 2b). RVV-X has a unique cysteine residue (Cys389), not found in other classes of SVMPs (Figs. 5c and 7b), in the middle of the HVR in the C_h subdomain (Fox and Serrano 2005; Takeda 2010). Cys389 forms a disulfide bond with the C-terminal cysteine residue (Cys133) of the light chain A (LA). In addition to this disulfide bond, the residues in the HVR and the surrounding regions in the heavy chain form multiple aromatic and hydrophobic interactions and hydrogen bonds with the N- and C-terminal residues in LA, further stabilizing the

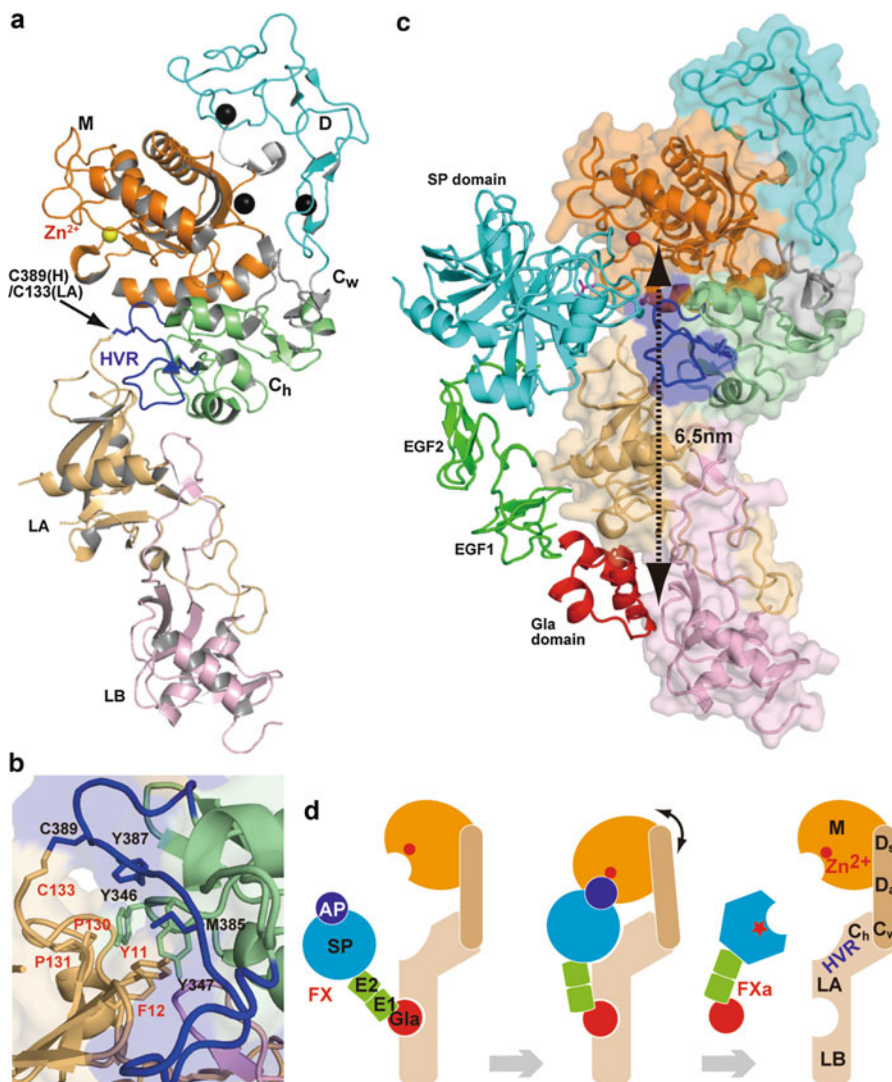

Fig. 7 RVV-X, a heterotrimeric P-IIId SVMP. (**a**) Ribbon representation of the structure of RVV-X (2E3X). Bound Zn^{2+} and Ca^{2+} ions are represented as *yellow* and *black* spheres, respectively. Each domain (or subdomain) is colored as in Fig. 1, and light chains A (LA) and B (LB) are colored in *brown* and *pink*, respectively. HVR is shown in *blue*. (**b**) Closeup view of the interactions between the heavy chain and LA. The residues involved in the interchain interactions are indicated with *black* and *red* letters for the heavy chain and LA, respectively. (**c**) FX docking model. The molecular surface of RVV-X is colored as in A. FXa is shown in ribbon representation. The side chain of Ile195 of FXa is shown in magenta. (**d**) Schematic model of FX activation by RVV-X

continuous C/LA structure (Fig. 7b). Thus, the RVV-X structure provides the first example of a protein-protein interaction mediated by the HVR. The structure of the pseudosymmetrical RVV-X light-chain dimer is quite similar to the structure of the FX-binding protein isolated from *Deinagkistrodon acutus* venom in complex with the γ-carboxyglutamic acid (Gla) domain of FX (PDB ID: 1IOD). This structural similarity, along with surface chemical properties and previous biochemical observations, suggests that the concave gorge formed by the light chains in RVV-X may function as an exosite for FX. The 6.5 nm separation between the catalytic site and the Gla-domain-binding exosite suggested a docking model for FX (Fig. 7c; Takeda 2010; Takeda et al. 2007).

Figure 7d depicts a model of FX activation by RVV-X, based on the crystal structure and the docking model. The light chains form a Gla-domain-binding exosite that may serve as the primary capture site for FX in the circulation. This interaction also plays a regulatory role in the Ca^{2+}-dependent activation of FX by RVV-X (left). The handle portion (C_h/LA/LB) of RVV-X may be rigid enough to act as a scaffold to accommodate the elongated FX molecule while separating the Gla domain and the scissile peptide bond in the serine-proteinase (SP) domain (middle). The mobile hook portion (M/D/C_w) of RVV-X not only catalyzes cleavage but may also help regulate the binding between molecules, thus driving the catalytic cycle; the M domain may associate with the AP region of zymogen FX and release it upon activation (right). Conformational changes on the surface of the SP domain, which is driven by the internalization of the newly appeared amino-terminal group of Ile195 following cleavage, may also promote FXa release from RVV-X. The relatively large separation (~65 Å) of the catalytic site and the Gla-domain-binding exosite effectively explains the high specificity of RVV-X for FX. The RVV-X structure provides a good example of the evolutionary acquisition of ligand-binding specificity by a snake venom protein complex (Doley and Kini 2009).

Carinactivase-1 and multactivase, potent prothrombin activators isolated from the venoms of *Echis carinatus* and *Echis multisquamatus*, respectively, also contain snaclec domains in addition to MDC domains, and they also use their snaclec domains for substrate recognition (Yamada and Morita 1997; Yamada et al. 1996). These two proteins are additional examples of P-IIId SVMPs, although they do not have a disulfide bond between the heavy and light chains. Their X-ray structures remain to be elucidated.

Conclusion and Future Direction

SVMPs are the key toxins involved in venom-induced pathogenesis. Due to their contribution to venom complexity and their versatile functions, the P-III SVMPs have been the most intriguing of the SVMP category. However, fundamental aspects of their functions, such as how non-catalytic domains of P-III SVMPs are involved in their toxicity and why P-III SVMPs exhibit higher hemorrhagic activity

than P-I SVMPs, remain to be elucidated. Recent crystallographic studies shed new light on the structure-function relationships of the modular domains of P-III SVMPs, revealing potentially novel protein-protein interaction sites. Furthermore, these crystal structures provide data pertinent to the working hypothesis that the HVR constitutes an exosite that captures targets or their associated proteins. The RVV-X structure is consistent with this working hypothesis and provides additional insights into the molecular basis of HVR-mediated protein-protein interactions and cleavage-target recognition by P-III SVMPs. Additional structural and biochemical studies, including functional expression and site-directed mutagenesis, will facilitate the identification of the key substrates of individual SVMPs and enable a better understanding of the molecular mechanisms of action of P-III SVMPs.

Cross-References

▶ Biological Activities and Assays of the Snake Venom Metalloproteinases (SVMPs)

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Biological Activities and Assays of the Snake Venom Metalloproteinases (SVMPs)

10

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Abstract

Snake venom metalloproteases (SVMP) are a key group of enzymes abundant in Viperidae venoms. Structurally, secreted SVMPs are typically organized into three main groups based on the presence or absence of domains: PI – which contains only a metalloproteinase domain; PII – includes also a disintegrin domain; and PIII – in addition to the first two domains, possesses a cysteine-rich domain. Diverse functions have been described to this group of proteases including their well-known hemorrhagic activity. Fibrin(ogen)olysis, prothrombin activation, interaction and lysis of von Willebrand factor, cytotoxicity, obstruction of angiogenesis, interference with platelet aggregation, myotoxicity, and proinflammatory

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are some of the other activities described by SVMPs. The SVMPs possess a broad range of biological activities, many with pathological consequences, that a brief description of the more common assays for biological activity associated with these proteins is of value to those entering the field. This chapter discusses the numerous activities attributed to the SVMPs and outlines the major assays utilized in systematic investigations of SVMPs.

Introduction

Snake venoms are complex mixtures of biologically active proteins and peptides that induce a wide variety of biological effects, many of which give rise to pathological outcomes (Tu 1996). In snake venoms, there are many different toxins organized into a relatively smaller number of toxin families associated with molecular targets that impact a variety of physiological systems. The key systems affected by Viperidae and Elapidae families of snake venoms include the hemostatic system, neurological system, renal system, as well as tissue integrity (Warrell 2010). It is the somewhat unique combination of the toxins in each venom and subsequently their molecular targets that give rise to the varied pathologies observed in patients envenomated by snakes from different genera and species.

Snake venom metalloproteinases (SVMPs) are an important group of enzymes with molecular masses ranging from approximately 20–100 kDa (Jia et al. 1996). They have been characterized according to both their function and structure. Structurally, the SVMPs have been organized into three main groups primarily based on the presence or absence of nonproteinase domains in context with the metalloproteinase domain. The three major classes are: PI – containing only a metalloproteinase domain; PII – including the metalloproteinase domain and a disintegrin domain; and PIII – which possesses metalloproteinase, disintegrin-like and cysteine-rich domains and in some cases additional lectin-like domains linked posttranslationally to the canonical PIII cysteine-rich domain structure. This classification can be further divided into 11 subclasses (PIa, PIIa, PIIb, PIIc, PIId, PIIe, D-I, PIIIa, PIIIb, PIIIc, and PIIId) according to the proteolytic processing the structures undergo and/or formation of dimeric structures (Fig. 1) (Fox and Serrano 2008).

Over the past 10 years, numerous new reports of SVMPs structures, primarily at the cDNA level, have been reported of which 35 have been isolated and assayed for biological activities (Table 1). While it is generally recognized that the *in vitro* activities associated with toxins in general and SVMPs in particular may only partially recapitulate the function of the toxin in the context of the whole venom in the course of envenomation, such assays are of critical value in terms of assisting with the isolation of the toxins as well as providing some level of understanding as to what role the toxin may be playing in actual envenomations (Gallagher et al. 2005). With this in mind, we have assembled some of the key assays associated with SVMPs' function (*in vitro* and *in vivo*) with the aim of providing the field with the technological landscape available when characterizing SVMPs.

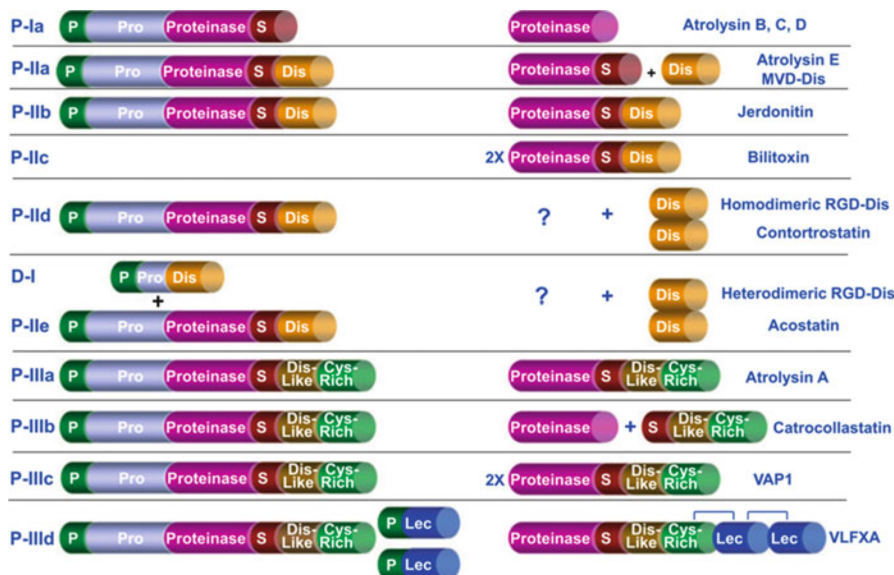

Fig. 1 Schematic of SVMP classes. Question marks (?) in the figure indicate that the processed product has not been identified in the venom (Fox and Serrano 2008)

SVMP: Proteolysis Assays *In Vitro*

Of the pathologies associated with envenomation by snake venoms, the SVMPs have been established as primarily contributing to the hemostatic disorders including hemorrhagic activity and the proteolytic and nonproteolytic disruption of key elements of the coagulation pathway. The hemorrhagic activity is strictly attributed to the proteolytic degradation of components of basement membrane by hemorrhagic SVMPs, since zinc chelation by EDTA, *o*-phenanthroline, or synthetic peptidomimetic hydroxamates completely abrogates this effect (Bjarnason and Fox 1994; Escalante et al. 2000). As mentioned, SVMPs may also disrupt hemostasis by perturbation of the coagulation pathways with a pro- or anticoagulation outcome which in general is accomplished by degrading plasma proteins such as fibrinogen and von Willebrand factor as well as other key proteins involved in these pathways (Baramova et al. 1989; Serrano et al. 2007).

Caseinolytic Activity – One of the first methods published for measuring nonspecific proteolytic activity was developed by Kunitz (1947) using the milk protein casein as the protein substrate. Using this assay, the SVMP is incubated with casein and after a set reaction time the mixture is subjected to acid precipitation with trichloroacetic acid and the resultant soluble digestion fragments are then spectrophotometrically quantitated at 280 nm, and the value used as a measure of the proteolytic action of the enzyme on the substrate.

Table 1 List of SVMPs fully sequenced in the last 10 years and their respective biological activities

Name	Accession #	*Species*	Activities	MHD	References
PI class					
Atroxlysin-I	P85420.2	*B. atrox*	Fibrinolytic and fibrinogenolytic (6.53 U/mg and 16.8 U/lg), digests fibronectin, binds to integrins α7β1 and α1β1 but not α2β1 and α3β1. Also inhibits platelet aggregation	19.9 μg	(Sanchez et al. 2010)
Batx-1	P0DJE1.1	*B. atrox*	Proteolytic activity (minimal and maximal doses: 4,800 U/mg and 1,670 U/mg), Fibrinogenolytic activity, induces a mild myotoxicity, lacks coagulant activity on human plasma or bovin fibrinogen and defibrinating activity	17 μg	(Patiño et al. 2010)
BjussuMP-II	Q7T1T4.1	*B. jararacussu*	Did not induce hemorrhage, myotoxicity or lethality, but dis- played proteolytic activity on fibrinogen, collagen, fibrin, casein and gelatin and does not show any clotting or anticoagulant activity, in contrast to its inhibitory effects on platelet aggregation	N.H.	(Marcussi et al. 2007)
BleucMP	P0DJJ6.1	*B. leucurus*	Acts on fibrinogen and fibrin, provoking blood incoagulability, however, devoided of hemorrhagic activity and did not induce relevant biochemical, hematological and histopathological alterations in mice	N.H.	(Gomes et al. 2011)
BmHF-1	P86802.1	*B. marajoensis*	Fibrinogenolytic, caseinolytic, induces dose-dependent edema and is weakly (or non)-hemorrhagic	41.14 μg	(Torres-Huaco et al. 2010)
BmooMPalpha-I	P85314.2	*B. moojeni*	Proteolytic activity towards azocasein, fibrinogen and fibrin, provoking blood incoagulability, but devoided of hemorrhagic and thrombin-like activities	N.H.	(Bernardes et al. 2008)
BnP1	P0C6S0.1	*B. pauloensis*	It is able to hydrolyze fibrinogen and fibrin, but devoided of significant myotoxic and hemorrhagic activities. Induces cell detachment, a decrease in the number of viable endothelial cells and apoptosis	>50 μg	(Baldo et al. 2008)

BnP2	P0C6S1.1	*B. pauloensis*	It is able to hydrolyze fibrinogen and fibrin, but devoid of significant myotoxic and hemorrhagic activities	N.H.	(Baldo et al. 2008)
Bothrojaractivase	P0C7A9.1	*B. jararaca*	Is a cofactors-independent prothrombin activator. Also has fibrinolytic and fibrinogenolytic activity. A dose-dependent procoagulant activity is shown in human plasma	N.D.	(Berger et al. 2008)
BpirMP	P0DL29.1	*B. pirajai*	Proteolytic activity on azocasein, and weak (or no) hemorrhagic activity. Degrade fibrin, fibrinogen and blood clots *in vitro*	50 μg	(Bernardes et al. 2013)
Leucurolysin-A	P84907.2	*B. leucurus*	Nonhemorrhagic, proteolytic activity on dimethylcasein, fibrin, fibrinogen, and plasma fibronectin, but not laminin. Inhibits ADP-induced platelet aggregation and is thrombolytic. Moreover, it induces changes in cell morphology and detachment followed by cell death, increases microvessel permeability and edema in mouse paw	N.H.	(Bello et al. 2006; Gremski et al. 2007)
[a]VMP1	B7U492.1	*A. piscivorus leucostoma*	*Recombinant protein* show proteolytic activity on fibrinogen but is devoid of hemorrhagic activity and had no capacity to inhibit platelet aggregation	N.H.	(Jia et al. 2009)
PII class					
BlatH1	U5PZ28.1	*Bothriechis lateralis*	Potent local and systemic hemorrhagic activity in mice, hydrolyzed azocasein, gelatin and fibrinogen, and inhibits human platelet aggregation	0.23 μg	(Camacho et al. 2014)
Stejnitin	P0DM87.1	*Trimeresurus stejnegeri*	It is fibrinogenolytic, inhibits platelet aggregation in human platelet-rich plasma (IC50 = 175 nM). It is also able to inhibit proliferatin of ECV304 cells by inducing apoptosis	N.D.	(Han et al. 2007)
[a]CamVMP-II	J9Z332.1	*C. adamanteus*	Expressed disintegrin domain (r-Cam-dis) and was able to inhibit all processes of platelet thrombus formation including platelet adhesion with an estimated IC50 of 1 nM, collagen- and ADP-induced platelet aggregation with the estimated IC50s of 18 and 6 nM, respectively, and platelet function on clot retraction	N.D.	(Suntravat et al. 2013)

(*continued*)

Table 1 (continued)

Name	Accession #	*Species*	Activities	MHD	References
PIII class					
AAV1	P0DJH3.1	*Deinagkistrodon acutus*	Induced platelet aggregation and the tyrosine phosphorylation of intracellular signaling proteins (LAT, SYK, p76SLP76, PIK3C, and PLCG2) that follows GP6 activation. Interestingly, blocks platelet aggregation but does not affect shape change	N.D.	(Wang 2007)
Ammodytagin	P0DJE2.3	*V. ammodytes ammodytes*	Fibrinogenolytic and hemorrhagic	10.86 μg (rat) 0.65 μg (mice)	(Kurtović et al. 2011)
Atragin	D3TTC2.1	*N. atra*	Exhibits an inhibitory activity toward cell migration of both NIH3T3 and CHOK1	N.D.	(Wei et al. 2006; Guan et al. 2010)
Atrahagin	P0DJJ1.1	*N. atra*	Failed to induce skin hemorrhage at doses of 5 or 50 μg per injection point. Causes mast cell degranulation and histamine release. Selectively degrades the alpha-chain of human fibrinogen (FGA)	N.H.	(Leonardi et al. 2008)
Atrase-A	D5LMJ3.1	*N. atra*	Fibrinogenolytic, edematogenic and bactericidal activity against *Staphylococcus aureus*. Did not show cytotoxicity on A549 and K562 cells, but detached adherent A549 cells. Also did not show significant inhibition of platelet aggregation, proteolytic activities towards fibrin, azocasein and *N*-alpha-benzoyl-L-arginine ethyl ester (BAEE), nor hemorrhagic activity. On the other hand, it significantly inhibited the growth and caused detachment of adherent HMEC, increased releasing of IL-8, ICAM-1 and MCP-1 and expression of caspase-3/7 and caspase-8	N.H.	(Qianyun et al.; Ye et al. 2009; Sun and Bao 2010)

Atrase-B	D6PXE8.1	*N. atra*	Non hemorrhagic	N.H.	(Sun and Bao 2010)
BjussuMP-1	Q1PHZ4.1	*B. jararacussu*	Hemorrhagic, inhibits platelet aggregation induced by collagen and ADP. Has moderate edema activity, but no myotoxic activity. It is unable to clot plasma. It also shows bactericidal activity against *E. coli* and *S. aureus*	4.0 μg	(Mazzi et al. 2004)
Daborhagin-K	B8K1W0.1	*Daboia russellii russellii*	High hemorrhagic activity when subcutaneously injected into mice. May also potently degrade alpha chain of fibrinogen (FGA)	0.82 μg	(Chen et al. 2008)
Daborhagin-M	P0DJH5.1	*D. siamensis*	High hemorrhagic activity when subcutaneously injected into mice. Has potent fibrinogenolytic activity on alpha-chain of fibrinogen (FGA). Hydrolyzes model substrate (beta-chain of insulin) at Ala(14)-Leu(15) and Tyr(16)-Leu(17) followed by His(10)-Leu(11) and Phe(24)-Phe (25)	0.86 μg	(Chen et al. 2008)
EoVMP2	Q2UXQ5.1	*Echis ocellatus*	Hemorrhagic, inhibits collagen-induced platelet aggregation and activates prothrombin (F2)	10 μg	(Howes et al. 2005)
Jerdohagin	P0DM88.1	*Protobothrops jerdonii*	High hemorrhagic activity. It may also inhibit platelet aggregation	0.04 μg	(Chen et al. 2004)
Kaouthiagin-like	D3TTC1.1	*N. atra*	Exhibits an enzymatic specificity toward pro-TNF-alpha with low inhibition of cell migration	N.D.	(Guan et al. 2010)
Leucurolysin-B	P86092.1	*B. leucurus*	Hemorrhagic	30 ng (rabbit)	(Sanchez et al. 2007)
Moojenin	P0DKR0.1	*B. moojeni*	Fibrinogenolytic and coagulant. Nonhemorrhagic	N.H.	(de Morais et al. 2012)
Ohanin	A3R0T9.1	*Ophiophagus hannah*	Has hemorrhagic activity. Inhibits ADP-, TMVA- and stejnulxin-induced platelet aggregation in a dose-dependent manner (on washed platelet, but not on platelet rich plasm)	N.D.	(Guo et al. 2007)

(continued)

Table 1 (continued)

Name	Accession #	*Species*	Activities	MHD	References
Oxiagin	P0DJJ4.1	*N. oxiana*	Inhibits the classical complement pathway dose-dependently. Also induces cation-independent hemagglutination that can be prevented by D-galactose pretreatment	N.D.	(Shoibonov et al. 2005)
TSV-DM	Q2LD49.1	*T. stejnegeri*	Non hemorrhagic, inhibits cell proliferation and induces cell morphologic changes transiently on human umbilical vein endothelial cells	N.H.	(Wan et al. 2006)
VAFXA-I	P0C8I7.1	*V. ammodytes ammodytes*	Activates coagulation factor X (F10) in a calcium-dependent manner	N.H.	(Wei et al. 2006)
VLAIP-A	Q4VM08.1	*M. lebetina*	Induces apoptosis in vascular endothelial cells and inhibits endothelial cell adhesion to extracellular matrix proteins such as fibrinogen, fibronectin, vitronectin, collagen I, and collagen IV. Also hydrolyzes azocasein, and oxidized insulin B-chain	N.D.	(Trummal et al. 2005)
VLAIP-B	Q4VM07.1	*M. lebetina*	Inhibits endothelial cell adhesion to extracellular matrix proteins such as fibrinogen, fibronectin, vitronectin, and collagen. Induces apoptosis in vascular endothelial cells	N.D.	(Trummal et al. 2005)

NH non hemorrhagic
ND non determined
[a]Sequence obtained from cDNA library

A colorimetric version of the caseinolytic activity utilizes azocasein which upon digestion releases the soluble azo dye which is measured colorimetrically. The reaction is stopped by the addition of perchloric acid and the solution is then filtered and the absorbance measured at 390 nm.

Fibrinogenolytic Assay – Fibrinogen or coagulation factor I is a 340 kDa glycoprotein synthesized in the liver and found with a plasma concentration of 1 to 5–4.0 g/L. Fibrinogen is a potent hemostatic agent playing an essential role in controlling bleeding by bridging activated platelets and being the key substrate for thrombin in establishing a fibrin network (Sorensen et al. 2012). The fibrinogenolytic activity of SVMPs can be classified according to their specificity of hydrolysis of fibrinogen chains. Proteases that are responsible for cleaving Aα chains are α-fibrinogenases and those responsible for cleaving Bβ chains are termed β-fibrinogenases (Markland 1998b). It should be noted that this activity is not directly related with hemorrhage since some SVMP may contain a high fibrinogenolytic activity yet are devoid of hemorrhagic activity (Girón et al. 2013).

Fibrinogenolytic activity is typically evaluated by SDS-PAGE on the basis of proteolytic activity on fibrinogen (Edgar and Prentice 1973) with slight modifications. Fibrinogen and SVMP are mixed and incubated in buffer with different pH values for different time intervals. Substrate to SVMP ratios should be adjusted for optimal results. The reaction is stopped by the addition of a denaturing buffer containing 2 % sodium dodecyl sulfate (SDS) and 10 % β-mercaptoethanol. Reaction products are then analyzed by SDS-PAGE. Heat stability can also be tested dissolving the SVMP in 50 mM Tris-HCl buffer, pH 8.0, then incubating for 15 min at 0–70 °C. The remaining fibrinogenolytic activity is followed as described above. Similarly, different inhibitors of fibrinogenolytic activity can be determined by incubating the enzyme in buffer containing inhibitors such as: EDTA, aprotinin, benzamidine, and β-mercaptoetanol for 15 min. In the Fig. 2a, the SVMP named BmooMPalpha-I, isolated from the venom of *Bothrops moojeni*, was evaluated by this method and showed the cleavage of the Aα-chain of fibrinogen first, followed by the Bβ-chain, and showed no effects on the γ-chain. Studies with inhibitors demonstrated that BmooMPa-I is a metalloproteinase (Fig. 2b) and revealed the importance of the disulfide bonds in the stabilization of the native structure and retention of activity. Like many venom SVMPs, BmooMPalpha-I was observed to be stable at pH values between 4 and 10 and exhibit its maximum fibrinogenolytic activity between 20 °C and 50 °C (Fig. 2c) (Bernardes et al. 2008).

Fibrinolyic Assay – The action of the protease thrombin on fibrinogen generates fibrin (Factor Ia), a fibrous protein that participates in blood coagulation. As it polymerizes, it traps platelets and forms a clot that functions as a hemostatic plug at the wound site (Mosesson 2005). Many of the venom fibrinolytic enzymes are metalloproteinases, and similarly most of the SVMP are fibrinolytic (Markland 1998a). The fibrinolytic activity of an SVMP may be evaluated by the same principle as the fibrinogenolytic assay described above by observing the fibrin degradation pattern in SDS-polyacrylamide electrophoresis (Fig. 3) (Bernardes et al. 2008). It can also be assayed based on the fibrin plate method described by Marsh and Arocha-Piñango (1972). The assay consists of measuring the diameter of

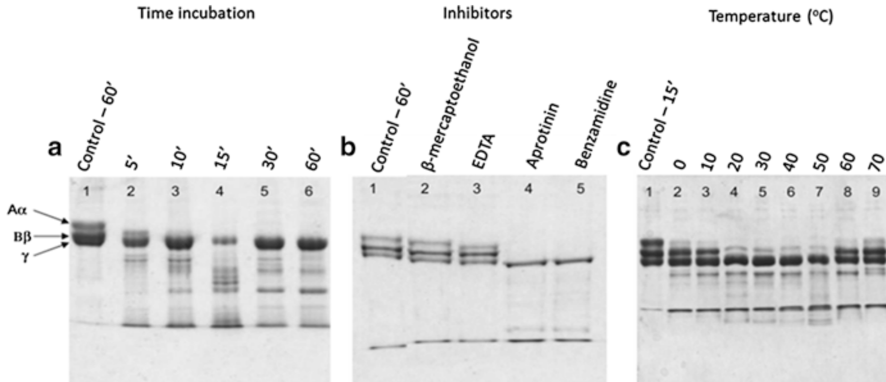

Fig. 2 Fibrinogenolytic activity evaluated by the fibrinogen degradation pattern on SDS-polyacrylamide electrophoresis. (**a**) Proteolysis of bovine fibrinogen by the BmooMPa-I in different times and (**b**) effect of inhibitors on BmooMPa-I. Lanes *1* – control fibrinogen incubated without enzyme for 60 min; (**c**) Effect of temperature on BmooMPa-I. Lanes *1* – control fibrinogen incubated without enzyme for 15 min; Lanes *2–9* – control fibrinogen incubated with enzyme for 15 min at 0, 10, 20, 30, 40, 50, 60 and 70 °C, respectively (Adapted from Bernardes et al. 2008)

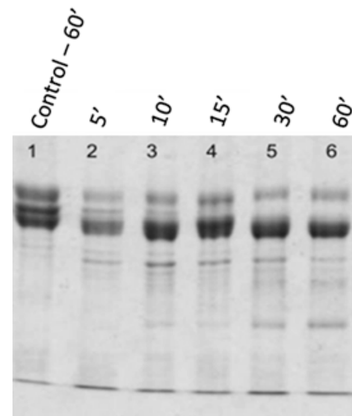

Fig. 3 Fibrinolytic action by BmooMPa-I SVMP. Lanes: *1* – Control incubated without enzyme for 60 min; *2–6* – control incubated with enzyme for 5, 10, 15, 30 and 60 min, respectively (Adapted from Bernardes et al. 2008)

the halos of lysis produced after 24 h of incubation with aliquots of the SVMP solution in different concentrations (Table 2). A solution of purified fibrinogen (containing 10 % added plasminogen) is coagulated by adding bovine thrombin at room temperature for 30 min. Human plasmin, single-chain t-PA (sct-PA), and two-chain u-PA (tcu-PA) may be used as controls. Fibrinolytic activity may also be evaluated in the presence of inhibitors such as, serine protease (SBTI, PMSF, benzamidine/HCl, and aprotinin), metalloprotease (EDTA, EGTA, and 1,10-phenanthroline), DTT, and cysteine protease (iodoacetic acid) (Marsh and Arocha-Piñango 1972; Da Silva et al. 2009; Girón et al. 2013).

Table 2 Purified fibrinolytic metalloproteinases activity yields from *Bothrops colombiensis* purification steps Purification step (Adapted from Girón et al. 2013)

	Protein (mg)	Active fractions	Fibrinolytic activity ($mm^2/\mu g$)	Hemorrhagic activity
Crude venom	127.00		30.6	+
Mono S 10/10	12.11	F1		
		F2	96.1	+
		F3		
Phenyl Sepharose	3.05	F2-A	40.8	–
	0.39	F2-B	162.2	–
Superose F 2-A	2.44	Colombienase-1	94.6	–
Bio-Select SEC F 2-B	0.33	Colombienase-2	211.57	–

Extracellular Matrix Protein Degradation – Many biologically relevant substrates for proteolytic assays with SVMPs have been described. Some of these substrates include type IV collagen, fibronectin, laminin, plasminogen, and protein mixtures such as matrigel. Detection of proteolysis is generally accomplished by SDS-polyacrylamide electrophoresis as described above (Cintra et al. 2012). The obvious caveat with these assays is that they are *in vitro* and not in the context of other proteins which may be interacting with them and altering their proteolysis susceptibility. An excellent example would be the extracellular matrix proteins which are know to be involved in numerous protein–protein interactions which contribute to their structure and function (Hynes and Naba 2012). One approach to attenuate those concerns is the use of matrigel, which represents an extract of proteins found in the specialized extracellular matrix and basement membranes (Kleinman and Martin 2005). While this mixture may better mimic *in vivo* substrate organization, it lacks authentic basement structure composition in that most of the collagen IV is lacking. Nevertheless, matrigel as a complex substrate is better than individual components. Matrigel proteolysis by SVMPs has demonstrated that different P classes of SVMPs generate different patterns of limited proteolysis and that the component basement membrane proteins show a somewhat different digestion pattern when in complex compared with their digestion as a lone substrate.

Thrombolytic Activity – The ability of some SVMPs to target different factors (fibrin or fibrinogen) gives them the ability to degrade fibrin-rich clots and prevent progression of clot formation (Markland 1998a). The thrombolytic activity of SVMPs assayed *in vitro* by clot formation can be evaluated by incubation in 24-well plates of SVMPs with whole rabbit blood (Gremski et al. 2007) or human blood (Cintra et al. 2012). After incubation, samples can be the quantitatively evaluated based on the clot density. Figure 4 illustrates the thrombolytic activity of Batroxase in a 24-well plate assay showing thrombus clot reduction.

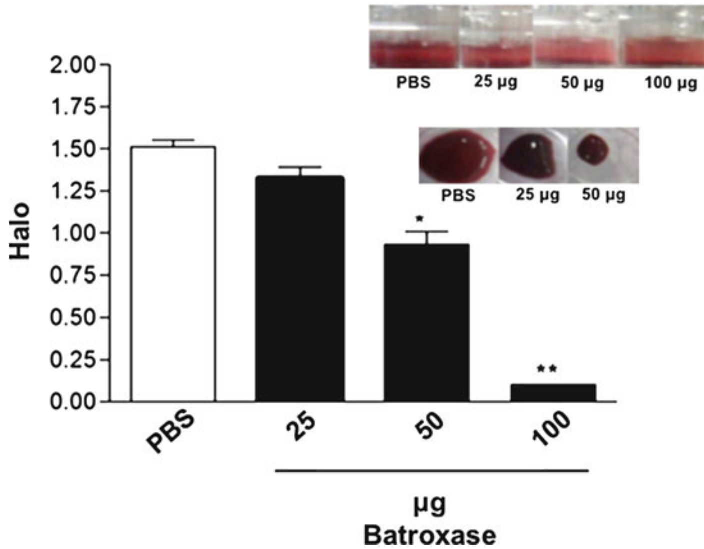

Fig. 4 Thrombolytic activity of Batroxase (25, 50 and 100 µg). The results are expressed as the mean thrombus diameter (cm) ± SD ($n = 3$). The Tukey test was used for statistical analysis. *p <0.05 and **p <0.001 compared with the control (PBS). (*B insert*) Side and upper views of a 24-well plate showing thrombus clot reduction. *Upper panel*, side view; *Lower panel*, upper view after supernatant removal for thrombus measurement (Cintra et al. 2012)

Protein Interaction/Proteolysis Assays

von Willebrand factor (vWF) Cleavage – von Willebrand factor is a multimeric glycoprotein involved in endothelial cell–matrix interactions (Cheresh 1987; Whittaker and Hynes 2002) that plays an essential role in hemostasis and thrombosis (Wagner 1990; Zhou et al. 2012) and is also involved in the angiogenesis regulation (Starke et al. 2011). The binding of vWF to platelets occurs through binding with platelet glycoprotein Ib (gpIb), one of the major platelet receptors for ligands such as vWF and thrombin. This binding is known as the first event in the formation of hemostatic plugs (Kroll et al. 1991).

To examine whether SVMPs may affect vWF–glycoprotein Ib interaction and subsequent platelet aggregation, vWF is pretreated with the SVMP for different time intervals, and then ristocetin is added to trigger platelet aggregation of washed platelets from human blood. The platelet aggregation levels are continuously monitored by turbidimetry. Also, proteolytic degradation of vWF may be evaluated and analyzed utilizing SDS-PAGE. The reactions are carried out with different substrate:enzyme ratios and time intervals and then stopped by adding reducing or nonreducing buffer and boiling at 95 °C for 5 min. The products of the reactions are then subjected to 12 % SDS-PAGE (Wang et al. 2004).

Cell-Based Assays

In addition to the ability to degrade extracellular matrix proteins (Souza et al. 2000; Baldo et al. 2010), some of the PII and PIII class members can also interact with cell surface integrins (Isabelle Tanjoni et al. 2010). Thus, it is likely that some of the pathologies associated with SVMPs may involve interference or disruption of cellular activities (Moura-da-Silva et al. 2007). Accordingly, assays designed to assess the ability of SVMPs to interfere in diverse biological processes, such as platelet aggregation, cell viability, migration, adhesion, and angiogenesis have been developed.

Platelet aggregation – Platelet aggregation is a well-studied process by which platelets adhere to each other at sites of vascular injury and has been recognized as critical for plug formation and thrombosis (Osler 1886). As fibrinogen is required for platelet aggregation and metalloproteinases possess α-fibrinogenase activity, it was initially proposed that fibrinogen degradation is responsible for the abnormalities of platelet function (Teng and Huang 1991). However, a study using jararhagin, a hemorrhagic PIII SVMP, showed that the cleavage of fibrinogen Aα-chain by this enzyme does not interfere with the binding of fibrinogen to the platelet integrin and therefore, jararhagin-induced inhibition of platelet function is not caused by proteolysis of fibrinogen (Kamiguti et al. 1994).

Subsequently, it was proposed that the inhibition of platelet response by jararhagin is mediated by its disintegrin-like domain by binding to integrin α2-subunit of platelets followed by cleavage of the β1-subunit, causing loss of the integrin native conformation, necessary for binding of macromolecular ligands (Kamiguti et al. 1996). Since the disintegrin-like domains in SVMP are similar to disintegrins (Jia et al. 1997), it was suggested that this domain would be most likely responsible for the inhibition of platelet aggregation. However, the recombinant cysteine-rich domain of atrolysin A was demonstrated to inhibit collagen-stimulated platelet aggregation, showing that this domain by itself is able to bind to α2β1 receptors on platelets and inhibit platelet aggregation (Jia et al. 2000). Moreover, another study demonstrated two complete peptide sequences from the homologous cysteine-rich domains of atrolysin A and jararhagin interact with α2β1 integrin and interfere with platelets aggregation response as well as the interaction of other cells to collagen, thus clarifying the mechanism of action of SVMPs in relation to platelet aggregation (Kamiguti et al. 2003).

Inhibition of platelet aggregation by SVMPs may be tested using the protocol described by Howes and colleagues (Howes et al. 2005). Platelet-rich plasma (PRP) is initially separated by centrifugation of fresh human blood mixed with sodium citrate. The platelet pellet is resuspended in Tyrode's HEPES buffer and platelets are then isolated by gel filtration on a Sepharose column. To evaluate the action of the SVMP on platelets, the washed platelet suspension is stirred in a dual-channel aggregometer with increasing amounts of SVMP added and incubated for 5 min. Platelet aggregation is stimulated by introduction of an agonist (such as thrombin, epinephrine, platelet-activating factor, collagen, or ADP to specific platelet membrane receptors) and the aggregation monitored as a change in optical density. Figure 5 provides an

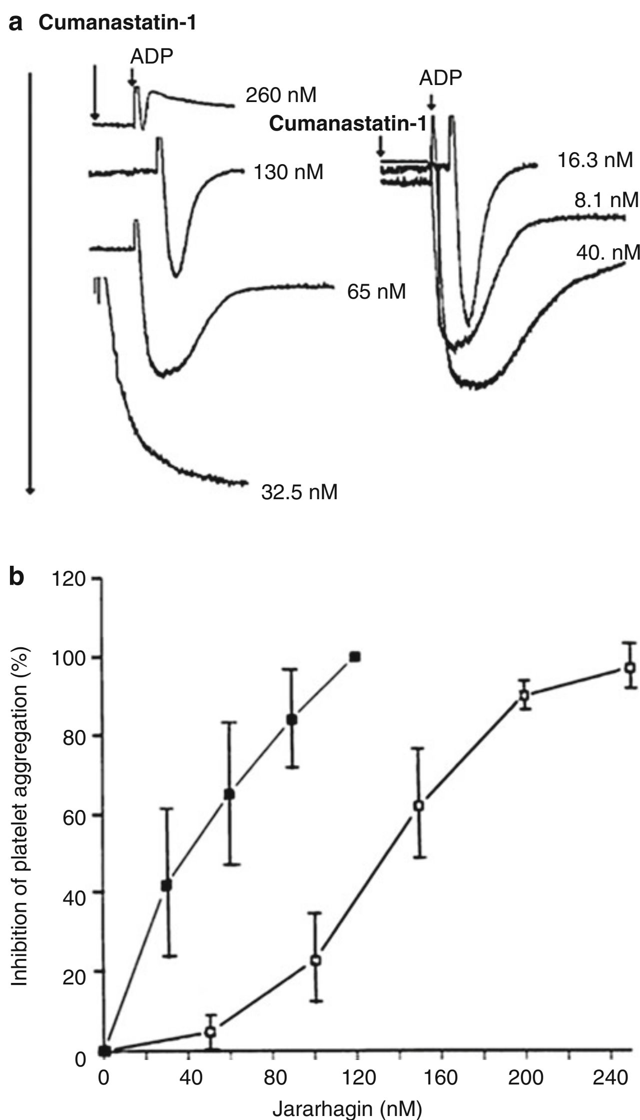

Fig. 5 Inhibition of platelet aggregation induced by two different mechanisms. (**a**) Inhibition of ADP-induced platelet aggregation by cumanastatin-1. The percentage of inhibition was calculated by comparing light transmittance obtained in presence of venom against the control sample. The IC50 value determined from the curve is 158 nM (Da Silva et al. 2009). (**b**) Inhibition of collagen-induced platelet aggregation by native and inactivated jararhagin. Percentage inhibition was calculated relative to control suspensions incubated with buffer only., ■ Native jararhagin; □ 1,10-phenanthroline-treated jararhagin (Kamiguti et al. 1996)

example of the inhibition of platelet aggregation induced by two different mechanisms, collagen-induced, which is inhibited by native and inactivated jararhagin (Kamiguti et al. 1996) and ADP-induced which is inhibited by cumanastatin-1 (Da Silva et al. 2009).

Cell viability – The viability of cultured cells (which implies in the ability of growing and dividing) is frequently evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method, a colorimetric technique based on the ability of mitochondrial reductases in viable cells to convert MTT into purple formazan crystals (Mosmann 1983). In this assay, the cells are seeded on a 96-well plate. Following adhesion, the medium is changed to media containing different concentrations of the SVMP. After 24 h, cells are incubated with MTT for 3 h and then formazan crystals are solubilized using DMSO and the intensity measured by optical density. Another method is an adaptation of the neutral red uptake method (Chaim et al. 2006), adapted by Gremski and collaborators (2007). In this protocol, the neutral red solution in PBS is added after the cell incubation with SVMP, and allowed to sit for 3 h. Then, the plate is washed with PBS followed by the addition of a fixing solution. The incorporated dye is finally released from cells with 1 % acetic acid in 50 % ethanol and the absorbance measured at 540 nm.

Cytotoxicity activity – Cytotoxicity of SVMPs *in vitro* with cultured cells may also be evaluated by using dyes and flow cytometry, in order to check cell membrane integrity and DNA fragmentation. Incubation of treated cells with the nucleic acid dye Sytox Green (Molecular Probes), with subsequent cell counting by flow cytometer, allows the measurement of cell death (Kim et al. 2006). Alternatively the use of annexin V-fluorescein isothiocyanate (annexin V-FITC) and propidium iodide (PI) allows the measurement of apoptotic and necrotic cells respectively (Teklemariam et al. 2011).

The disruption of cell adhesion and subsequently initiation of apoptosis seems to be dependent on the metalloproteinase domain of the SVMP. Treatment with jararhagin, a SVMP from *Bothrops jararaca*, induces changes in cell shape and causes anoiksis following loss of cell adhesion. However, when the catalytic activity of jarahagin was inhibited, these effects were abolished, suggesting the importance of the catalytic domain (Tanjoni et al. 2005).

Angiogenesis – The influence of SVMPs on endothelial cell tube formation may be evaluated *in vitro* by matrigel tube formation assays. In the protocol described by Yeh and collaborators (2001), matrigel is diluted with or without basic fibroblast growth factor (bFGF) as a chemoattractant and added to 24-well plates to form a gel layer. HUVECs are then incubated with or without the toxin and then stained with toluidine blue and analyzed microscopically. Fields are chosen randomly and the total tube length is quantified using an image analysis software (Fig. 6).

Cell migration – All nucleated cell types during at least one point in their development undergo cell migration. In most cases, this process occurs during morphogenesis, stops at the terminal differentiation, and may be restarted only during tissue regeneration or neoplastic processes (Friedl and Wolf 2010).

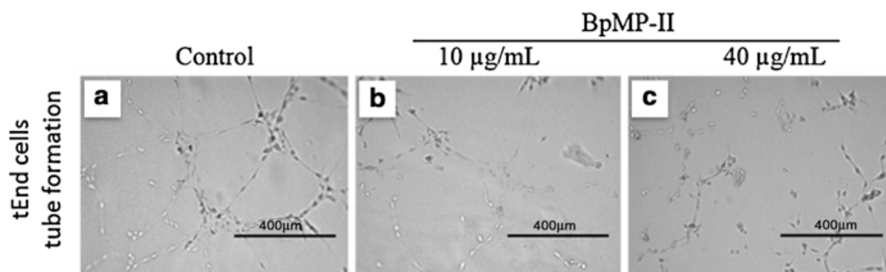

Fig. 6 Effect of BpMP-II on tube formation of endothelial cells inmatrigel. (**a**) tEnd cells preincubated with medium (Control) arranged capillary-like form. Samples containing 10 mg/mL (**b**) or 40 mg/mL (**c**) of BpMP-II were preincubated with tEnd cells for 30 min at room temperature (Achê et al. 2014)

The migration of tumor cells *in vitro* is evaluated by measuring the migration of cells on the bottom of the well after scratching a line through them. The experiment is performed in a 24-well plate. Cells are plated and after 24 h of incubation, the medium is discarded and the confluent monolayer is scratched at the center of the well. The detached cells are then washed with medium and 900 µL of new medium containing the SVMP is added. A disintegrin that blocks migration of the cells (such as the disintegrin echistatin) must be used as a positive control and PBS may be used as a negative control. After incubation, microscopic images at different times (0, 3, 6, 12, and 24 h) are taken and the percent of closure of the scratch is calculated.

In Vivo Assays

Considering the multitude of physiological mechanisms involved in the maintenance of hemostasis, it is clear that tissue, physical and biological factors play an important role in this process. Thus, performing *in vivo* assays is extremely important to better understand the action of these enzymes in envenomation. Despite the knowledge about the role of SVMPs on hemostasis *in vitro*, a few studies have evaluated its effects *in vivo*.

Defibrinating Activity – Many snake envenomations are characterized by a defibrination syndrome which is a systemic event (Fig. 7) and may be clinically utilized to monitor the time course of envenomation and success of treatment (Barrantes et al. 1985).

In vivo, this activity may be tested by the method based on Gené and collaborators (1989) with slight modifications. The minimum defibrinating dose (MDD) is defined as the amount of venom capable of making the plasma incoagulable. In this method, mice are injected i.p. with 200 µL of saline and increasing doses of the SVMP. After 1 h, animals are anesthetized and whole blood is kept at 25–30 °C until clotting occurs (Bernardes et al. 2008).

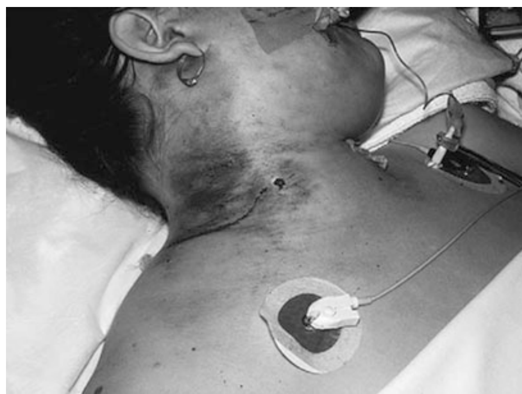

Fig. 7 A case of severe defibrination coagulopathy after a taipan bite causing extensive bleeding following failed IV insertion into the right jugular vein. The resultant hemorrhage resulted in a halving of hemoglobin levels in 3 h (Photo copyright q Dr. Julian White) (White 2005)

Fibrinogenolytic activity – The fibrinogenolysis provoked by proteinases in *B. jararaca* venom was also studied *in vivo* by Yamashita and colleagues (Yamashita et al. 2014). The venom was preincubated with Na_2-EDTA or 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), which are inhibitors of SVMPs and serine proteases, respectively, and injected subcutaneously or intravenously into rats. Samples of blood, lung, and skin were analyzed at 3 and 6 h. Degradation levels of fibrin(ogen) in plasma were estimated by ELISA assay using three different antibodies (rabbit antirat fibrinogen IgG for coating, rat fibrinogen as standard, and biotinylated rabbit antirat fibrinogen IgG). The preincubation with the chelating agent markedly reduced plasma fibrinogen consumption. SVSP, on the other hand, did not seem to be important *in vivo*. Another effect was the elimination of the marked elevation in tissue factor (TF – a cellular receptor essential for initiating blood coagulation *in vivo*) levels in plasma by both administration routes.

Myotoxicity – Studies have recognized that SVMPs are also able to induce acute muscle damage (Rucavado et al. 1999). Myotoxic activity can be assayed by intramuscular injection of the SVMPs in the gastrocnemius muscle of mice and after 3 h, the animals are anesthetized, bled by cardiac puncture, and their sera is assayed for creatine-kinase activity with a commercial kit (Baldo et al. 2008). The skeletal muscle alterations induced by SVMPs may also be assayed using transmission electron microscopy. In this case, mice are injected intramuscularly in the gastrocnemius and at different times postinjection, the animals were sacrificed and the gastrocnemius muscle dissected out and processed for microscopy, thus allowing a histological examination of the damage (Baldo et al. 2010). However, one must take care in the interpretation of these experiments as it is not clear if this effect is owing to direct cytotoxic action on muscle cells or weather other indirect mechanisms related to ischemia due to microvasculature damage and hence tissue death (Gutiérrez and Rucavado 2000).

Hemorrhagic activity – Binding to and subsequent hydrolysis of the ECM components by SVMPs are central to their hemorrhagic action, since these constituents ensure the mechanical stability of microvessels (Escalante et al. 2011). Experimental evidence indicates a two-step mechanism of SVMP-induced hemorrhage

(Gutiérrez et al. 2005). The first comprising of proteolysis of the major components of basal membrane (BM), laminin, type IV collagen, nidogen, and perlecan (Escalante et al. 2006) and of proteins that play a key role in the mechanical stability of BM, such as nonfibrillar collagens weakens the capillary wall (Escalante et al. 2009).

The second step involves the transmural pressure acting on the weakened capillary wall causing distention and eventually disruption and as consequence; extravasation of blood occurs (Gutiérrez et al. 2005). In addition, the hemorrhagic effect of SVMPs is further potentiated by their ability to inhibit platelet aggregation and to hydrolyze plasma proteins involved in the process of blood clotting, such as different coagulation factors and von Willebrand factor (vWF) (Sajevic et al. 2011), which have been described previously.

Determination of the hemorrhagic activity was initially described by Kondo and colleagues (1960) in which solutions of toxin or saline are subcutaneously injected into the dorsal skin of mice. Hemorrhagic spots are measured on the inside surface of the skin. The minimum hemorrhagic dose (MHD) is defined as the least amount of protein that causes a hemorrhagic spot 5 mm in diameter 6 h after injection (Bjarnason and Tu 1978). Modifications of this protocol were proposed by Gutiérrez and collaborators (1985) in which the animals are sacrificed after 2 h and the minimal hemorrhagic dose as the amount of toxin that causes a 10 mm hemorrhagic spot (Fig. 8).

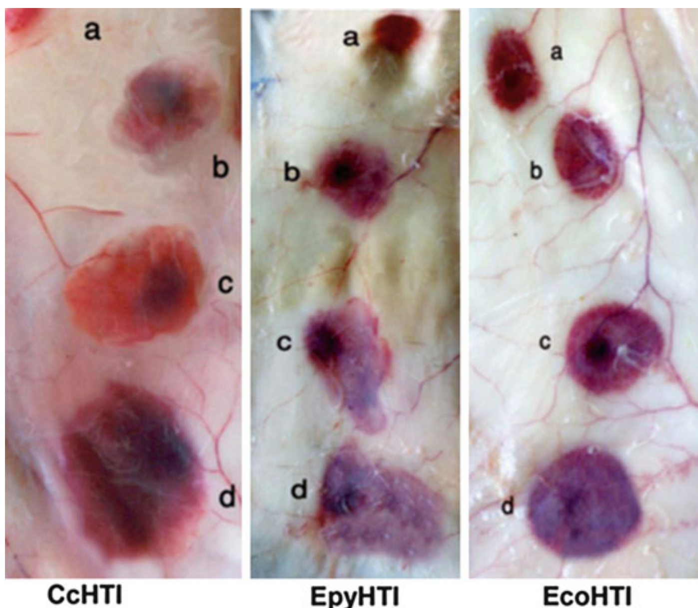

Fig. 8 Determination of the SVMPs hemorrhagic activity. Calculation of the hemorrhagic dose using 1 mg (*a*), 2 mg (*b*), 4 mg (*c*), and 8 mg (*d*) of three viper P-III hemorrhagic SVMPs: EpyHTI, EcoHTI, and CcHTI of the vipers *Echis pyramidum*, *Echis coloratus*, and *Cerastes cerastes*, respectively (Wahby et al. 2012)

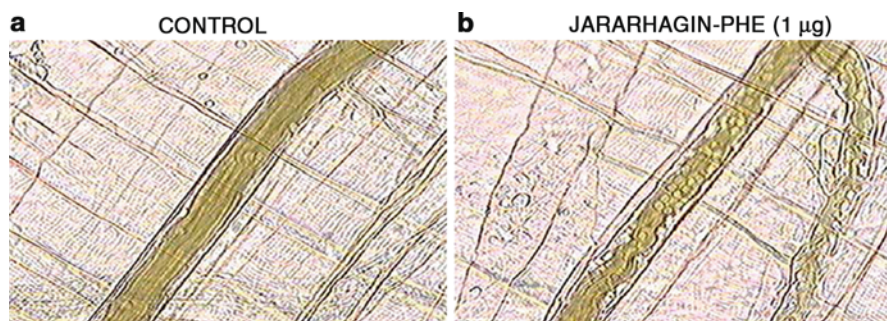

Fig. 9 Intravital micrograph of cremaster muscle after topical application of inactivated jararhagin: (**a**) control; (**b**) inactivated jararhagin. The presence of PMN cells rolling in the vascular well after topical application of inactivated jararhagin can be observed (Clissa et al. 2006)

The hemorrhagic activity may also be evaluated *in vivo* by intravital microscopy (Fig. 9). Mice are anesthetized and placed on a water-heated bed. The *cremaster* muscle is exposed and the SVMP placed onto the muscle and immediately covered with Mylar® to prevent dehydration. The alterations are then observed for a period of 30 min (Lomonte et al. 1994).

Miscellaneous Assays

Inflammation – Inflammation can be initially defined as a beneficial response to injury that gives rise to a combination of clinical signs and symptoms. The four well-known signs of inflammation are redness, swelling, heat, and pain, which may end up in loss of function, characterized by loss of mobility in a joint due to the edema and pain or to the replacement of functional cells with scar tissue (Punchard et al. 2004; Scott 2004).

However, this clinical view of inflammation includes many cellular and molecular implications, which makes possible the analysis of inflammation by different approaches, such as counting of inflammatory cells, measurement of proinflammatory transcripts such as interleukins, chemokines, and tumor necrosis factors, in addition to quantifying edema.

The formation of blisters and infiltration of leukocytes into dermis may be accessed through macroscopic and histological observations following intramuscular injection of the toxin in the gastrocnemius of mice (Rucavado et al. 1998). Also, the increase of blood neutrophil numbers and their accumulation in the peritoneal cavity in the early phase followed by an increase in mononuclear cells in the late period is another way to assess the inflammatory effect of SVMPs. For this approach, mice are injected with the SVMP or sterile saline (control) into the mouse peritoneal cavity. Total leukocyte, polymorphonuclear, and mononuclear cells are then determined in peritoneal washes harvested at different times after injections. For determination of the number of leukocytes in the blood different

times after SVMP injection, blood aliquots are collected from the tail vein and the leukocyte number is determined with a Neubauer chamber (Fernandes et al. 2006).

The induction of TNF-α, interferon-γ (IFN-γ), and interleukins may be quantitated by enzyme immunoassays. Basically, mice are injected in the gastrocnemius and at different time intervals; the injected muscle is then excised, weighed, and homogenized in saline solution. After centrifugation, cytokine levels are quantitated by enzyme-linked immunosorbent assay (Rucavado et al. 2002).

Inflammation caused by SVMPs was described for all of the different classes of SVMPs. The first studies showed that inflammation induced by BaP1, a P-I class SVMP from *Bothrops asper* venom (Gutiérrez et al. 1995) is able to induce formation of blisters and infiltration of leukocytes into the dermis and peritoneal cavity as well as increasing their number in blood circulation (Rucavado et al. 1998; Fernandes et al. 2006). Also, BaP1 induces a prominent increase of IL-1b and IL-6, but no elevation of TNF-α and IFN-γ were detected (Rucavado et al. 2002).

Edema induced by SVMPs treatment is assayed by injecting the toxin subcutaneously into the subplantar area of a mouse paw along with the same volume of vehicle in the contra paw as a control. The paw edema is then determined by measuring paw thickness with a digital caliper or a digital water plethysmometer at different times after injection, and the results calculated as the difference in thickness between the right and left paws (Sharma et al. 2004).

The PIII class SVMPs are also important proinflammatory molecules. Jararhagin, for example, in addition to directly stimulating TNF-α, IL-1β, and IL-6 expression (Clissa et al. 2001), has been shown to induce influx of leukocytes into mouse air pouch and its activity seems to be dependent upon proteolytic activity and the presence of macrophages (Costa et al. 2002). Gene expression profiles of human fibroblasts *in vitro* and mouse tissue *in vivo* also revealed significant upregulation of proinflammatory transcripts such as IL-1β, IL-6, CXCL1, CXCL2, IL-8, and apoptosis and inflammation-related transcripts such as TNF-α induced protein 6 (Gallagher et al. 2005).

SVMPs belonging to PI-class trigger inflammatory signals *in vivo* and *in vitro* models and therefore provides evidence that proteolytic domain is able to cause inflammation, while the disintegrin-like domain in turn, seems to stimulate leukocyte functions through integrin-mediated pathways (Teixeira et al. 2005). In order to evaluate the importance of disintegrin-like/cysteine-rich domains, a study using either inactivated jararhagin and jararhagin-C, which lacks the catalytic domain, showed that both are able to increase the number of rolling leukocytes without interfering with microvasculature (Fig. 9), thus suggesting that the disintegrin-like/cysteine-rich domains are sufficient to locally activate the early events of an acute inflammatory response (Clissa et al. 2006).

The cysteine-rich domain via its hypervariable region (HVR) were shown to play a role in inflammation by analyzing the effect of recombinant proteins and peptides of HF3, a hemorrhagic P-III SVMP, containing the cysteine-rich but not the disintegrin-like domain. These proteins were able to significantly increase the leukocyte rolling in the microcirculation. The proinflammatory effect of HF3 and its disintegrin-like/cysteine-rich domains expressed together or individually were

also evaluated by analyzing the alterations on the microcirculatory network through intravital microscopy and the results corroborate the role of the cysteine-rich domain in SVMPs targeting *in vivo* as well as confirming the role of integrin $\alpha_M\beta2$ in the proinflammatory effects of HF3. Taken together, these results show for the first time that the cysteine-rich domain and its HVR play a role in triggering inflammation which is mediated by integrins (Menezes et al. 2008).

Conclusion

Understanding the structure and the mode(s) of action of the SVMPs has long been the focus of venom studies due to the important role of these enzymes in the pathophysiology of envenomation. In spite of the decades of research conducted in the characterization of SVMP structure and function, there are still numerous unanswered questions, some of which we know and certainly some as yet to be asked. As new technologies become available in conjunction with the rich databases being generated on these toxins as well as others, we are optimistic that a more complete understanding of SVMP activities *in vitro* and more importantly *in vivo* will be gained. Furthermore, novel approaches for understanding SVMP activity in conjunction with other bioactive toxins in the venoms must be developed in order to have a complete system-based understanding of envenomation which will in turn hopefully lead to novel modes of therapeutic intervention.

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Three-Dimensional Structures and Mechanisms of Snake Venom Serine Proteinases, Metalloproteinases, and Phospholipase A_2s

11

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Abstract

High-resolution crystal structures provide accurate information of the positions of the atoms which can be used to understand substrate specificity, secondary binding sites, and catalytic mechanisms. Detailed structural information and mechanisms of serine proteinases, metalloproteinases, and phospholipases A2s are presented.

Introduction

Structural information gleaned at the molecular and atomic levels, when correlated with biochemical and biophysical details capable of generating a coherent picture of the salient structural features and interactions that modulate and determine biomolecular recognition, specificity, and hydrolysis, provides us with very powerful tools to decipher, step-by-step, complex biological phenomena, thus permitting a profound understanding of the basic underlying role of molecular architectural diversity in efficiently performing essential, distinct, chemical reactions that are central to life.

The enormous number of protein and DNA sequences currently deposited in the data banks (e.g., http://www.expasy.org, http://www.uniprot.org) indicate that the one-dimensional representation of protein sequences generally contains a trace of the fingerprint of evolution, and often, only a faint residual of the ancestral protein is retained in the protein linear amino acid sequence. However, upon closer examination, the application of this concept exposes its fundamental limitations, and hence, its utility is strictly limited since (a) proteins that perform the same function or catalyze similar reactions often share only very low-sequence identity, (b) proteins that are about 20 % identical in their primary sequences may still catalyze distinct reactions and modulate different functions, and (c) point mutations in the active sites or cofactor-binding sites often produce proteins that catalyze different reactions or in extreme cases result in enzymatically inactive proteins.

A fundamental conceptual bridge linking the linear protein sequence and its primary biological function is encoded in the three-dimensional fold or, in other words, in the exact positions of the atoms of the protein in three dimensions. Central to this concept is the fact that the three-dimensional (3D) structure of a protein is

more highly conserved during evolution (Bajaj and Blundell 1984; Finkelstein and Ptitsyn 1987) than the linear amino acid sequence of the protein, and consequently, the shape of a protein, the spatial distribution of its atoms, and the surface charge, solvent accessibility, glycosylation, and solvent structure are key factors that determine its biological function. In general, proteins that possess the same three-dimensional structures also perform similar functions and catalyze analogous reactions (Thornton et al. 1991). Basically, only a small subset of amino acids perfectly positioned and conserved in three dimensions, rather than the linear conservation of the primary sequence, forms the core of the protein's biological function (Hasson et al. 1998; Kasuya and Thornton 1999) which is also often dependent on the exact positioning and interaction of the protein with solvent molecules that facilitate charge/proton transfer. Thus, protein structures are more conserved than their sequences (Lesk and Chothia 1980; Chothia and Lesk 1986), and the degree to which an amino acid is evolutionarily conserved in a certain position reflects its structural and functional importance.

Snake venom proteins are present in a wide variety of sizes, amino acid sequences, and consequently three-dimensional structures, which reflects their diverse roles in nearly all envenomation steps. Protein crystallography, nuclear magnetic resonance spectroscopy, and, to a lesser extent, electron microscopy are powerful tools that have been applied very successfully to determine the three-dimensional structures of toxins at the atomic and molecular levels. Structural analysis of snake venom proteins is especially rewarding since they can be obtained with high purity and due to their intrinsic stability, readily form large, well-ordered crystals that are suitable for high-resolution X-ray diffraction analysis.

Snake venom proteins have been reviewed in a number of recent articles (Kang et al. 2011; Kini 2005; Georgieva et al. 2008; Rossetto and Montecucco 2008; Yamazaki and Morita 2007), and here, only the structural features of leading members of select protein families will be presented. Protein crystallography has experienced amazing growth as evidenced by the fact that the protein data bank (www.rcsb.org) contains the atomic coordinates of over 100,000 proteins and excellent recent reviews (Wlodawer et al. 2008, 2013) present the current state of structural biology. Structural data extends our horizon and has provided us with the impetus to decipher complex interactions that define enzyme specificity, mechanism, charge distribution, modes of inhibition, and dynamics and, more importantly, also paves the way for the design of novel inhibitors and drugs with potential clinical and medical applications.

Counterparts of many snake venom proteins are encountered in the mammalian repertoire, and structural comparisons between the snake venom and mammalian proteins indicate the presence of the same basic structural scaffold. The structures of key snake venom proteins and their salient features are compared and highlighted. The catalytic mechanisms of these proteins are described with relation to their structural aspects. Since the structures of a significant number of snake venom proteins are now available, structure-based sequence alignments can be exploited, and this presentation will be limited only to the cases where accurate, high-resolution structural data is available.

Serine Peptidases

Proteinases or peptidases catalyze the hydrolytic cleavage of peptide bonds (Rawlings et al. 2013), and proteinases that contain a highly reactive nucleophilic Ser and are assisted by His and Asp, thus forming the catalytic triad His, Ser, and Asp, are widely referred to as serine peptidases or serine endopeptidases (Hedstrom 2002a, b; Neurath 1984). These enzymes catalyze the cleavage of covalent peptide bonds in proteins and peptides and have been the focus of much research since they participate in a number of diverse, essential biological processes ranging from digestion and blood coagulation to immune response and inflammation (Hedstrom 2002a) by exhibiting high stereospecificity and enantiospecificity. These enzymes, widely encountered in snake venoms (de Oliveira et al. 2013; Serrano 2013; Ullah et al. 2013), probably originated as digestive enzymes and subsequently evolved by gene duplication and sequence modifications to serve specific functions (Birktoft and Blow 1972).

Snake venom serine proteinases (SVSPs)play essential roles in envenomation since they interfere with the functioning, maintenance, and regulation of diverse physiological processes of the prey, specifically the maintenance, regulation, activation, and inhibition of the blood coagulation cascade and the fibrinolytic system, and often additionally trigger blood platelet aggregation (Gempeler et al. 2001; Murakami and Arni 2005; Zhang et al. 1995). Based on their amino acid sequences and functional similarities, SVSPs are classified as belonging to the SA clan and the S1 family (MEROPS classification, http://merops.sanger.ac.uk). Despite the fact that they share significant sequence identity (50–70 %), SVSPs display high specificity by binding to and cleaving distinct macromolecular substrates and hence have been classified as activators of the fibrinolytic system, procoagulant, anticoagulant, and platelet-aggregating enzymes.

Sequence Alignments

Structure-based sequence alignment of selected serine peptidases (Fig. 1) serves to highlight the similarities and differences between these enzymes and indicates that the protein chains of the SVSPs contain 245 amino acids and are of approximately the same length. In comparison, the amino acid chain of human α-thrombin which contains insertions in many loop regions is significantly longer. SVSPs differ from the trypsin family of enzymes since they contain a disulfide-linked C-terminal extension (Murakami and Arni 2005; Parry et al. 1998). The position of the three amino acids, His57, Asp102, and Ser195 (sequence numbering based on chymotrypsinogen) (Harley and Shotton 1971), is strictly conserved (marked in red). Only α-thrombin and trypsin bind monovalent and divalent ions (Na^+ and Ca^{2+} ions, respectively, highlighted in light blue). Interestingly, the thrombin exosites I and II, utilized for binding heparin and fibrinogen, are not conserved in SVSPs. Asp189 (marked in dark blue and positioned at the base of the oxyanion hole and which determines specificity) is totally conserved. The glycosylation sites of SVSPs

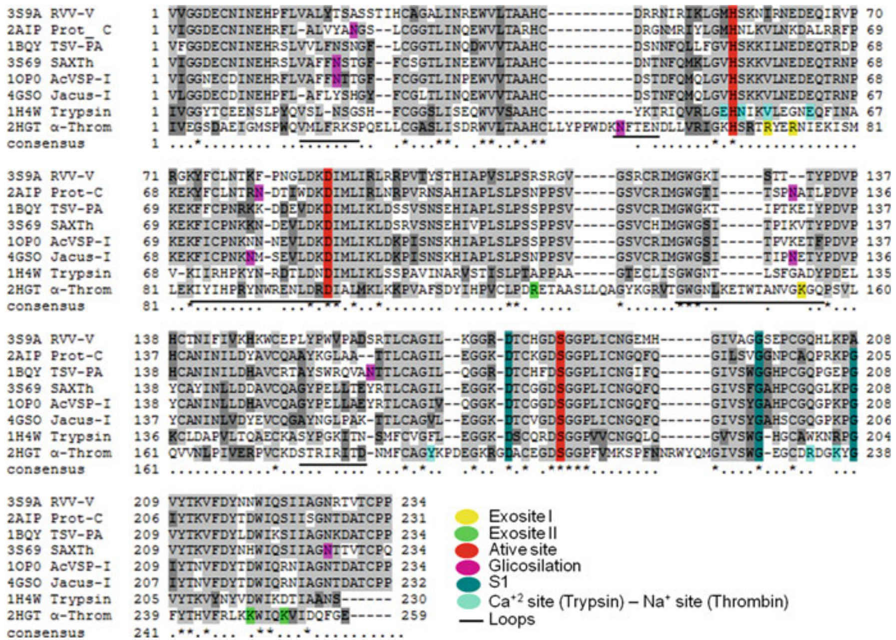

Fig. 1 Structure-based multiple sequence alignment of serine proteinases, whose crystal structures have been determined, performed using the program BoxShade (http://www.expasy.org). RVV-V (*Russel's viper venom*), Prot_C (*Agkistrodon contortrix contortrix*), TSV-PA (*Trimeresurus stejnegeri*), SAXTh (*Gloydius saxatilis*), AcVSP-I (*Agkistrodon acutus*), Jacus-I (*Bothrops jararacussu*), Trypsin (Human), and α-Thromb (Human). The Protein Data Bank four-letter ids are given on the *left*

(highlighted in pink) are significantly different. SVSPs are often referred to as thrombin-like enzymes; however, based on the above observations, it can be concluded that SVSPs are structurally more similar to trypsin than to α-thrombin.

Overall Structure

Like their mammalian counterparts, chymotrypsin and trypsin-like serine proteinases, SVSPs consist of about 245 amino acids distributed among the two domains (S and S´); each containing a six-stranded β-barrel and two short α-helices (residues, 165–173 and 235–244, sequence numbering based on chymotrypsinogen) (Harley and Shotton 1971) (Fig. 2a). The N-terminal S domains are stabilized by an intra-chain disulfide bridge (Cys42/Cys58) and two other disulfide bridges Cys22/Cys157 and Cys91/Cys245, the latter are unique to SVSPs (Murakami and Arni 2005; Parry et al. 1998). His57 and Asp102 which form part of the catalytic triad are located in this domain. The C-terminal S´ domain encompasses a six-stranded β-sheet and contains two α-helices, one inserted between strands 8 and 9 and the other located at the C-terminus preceding the extended C-terminal tail; a disulfide bridge interconnects

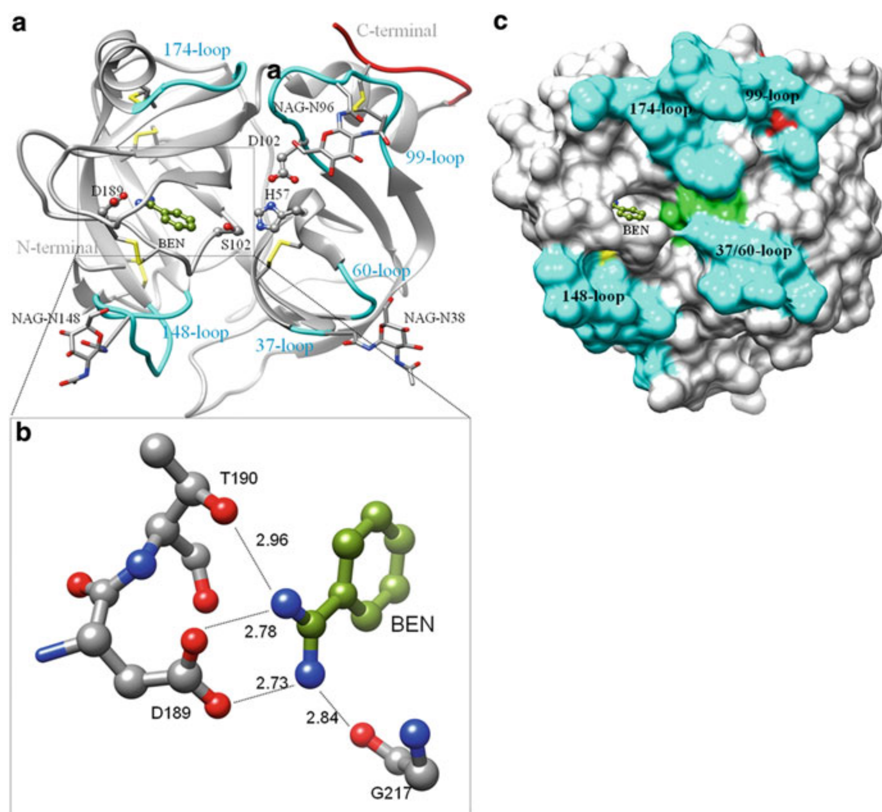

Fig. 2 (**a**) Ribbon representation of the crystal structure of ACC-C (*Agkistrodon contortrix contortrix*), oriented to indicate the N- and C-terminal lobes and highlighting the loops that are variable in SVSPs (*cyan*), the amino acids that form the catalytic triad (His57, Asp102, and Ser195) and determine specificity (Asp189) are atom color-coded (*white*, carbon; *red*, oxygen; and *blue*, nitrogen). The benzamidine molecule (BEN) is presented in *green*. The C-terminal extension that is unique to SVSPs is in *red*. The positions of the carbohydrate moieties are also indicated. (**b**) Zoom presenting details of the interaction of benzamidine. Thin lines and numbers indicate hydrogen bonds and hydrogen bond distances are in Å. (**c**) Space filling representation of ACC-C indicating the positions of the surface loops (*cyan*), the active site residue (*green*), benzamidine molecule (BEN), and the N-linked Asn (*red*). Visualization by UCSF – Chimera (Pettersen et al. 2004)

the tail with the N-terminal subdomain. This subdomain is further stabilized by three disulfide bridges (Cys136/Cys201, Cys168/Cys182, and Cys191/Cys220), and the highly reactive nucleophilic Ser195 is located in this domain. The catalytic triad (His57, Asp102, and Ser195) is located at the junction of both the barrels and is surrounded by the conserved 70-, 148-, and 218-loops and the nonconserved 37-, 60-, 99-, and 174-loops (Fig. 2a and b). As expected from the high sequence identity of SVSPs (about 60 %), superpositioning Cα carbon atoms of the structures results in r.m.s. deviations ranging from 0.6 to 0.7 Å, indicating a high degree of structural similarity primarily in the core region. As expected, due to the higher sequence

variation in the surface-loop regions, a greater degree of structural and charge variation is observed in the surface loops (Murakami and Arni 2005).

The principal structural difference between SVSPs and other members of the S1 family is that the SVSPs possess an extended C-terminal tail which is linked by an additional disulfide bridge and is considered important for stability and for allosteric regulation (Murakami and Arni 2005; Parry et al. 1998). Whereas the vitamin-K-dependent mammalian serine proteases require Na^+ ions for optimal catalytic activity and selectivity and for allosteric regulation, this site is not present in SVSPs suggesting a more simple and straightforward mechanism of substrate recognition and binding (Kraut 1977).

SVSPs often contain about 20 % carbohydrates, N-linked to Asn, principally glucosamine, neuraminic acid, and neutral hexoses (Murakami and Arni 2005). In the structures of ACC-C from *Agkistrodon contortrix contortrix* and AaV-SP-I and AaV-SP-II from *Agkistrodon acutus*, these carbohydrate moieties are strategically positioned around the entrance to the active site at the tips of the 37-, 99-, and 148-loops and probably modulate macromolecular selectivity. However, in other SVSPs (e.g., in *T. stejnegeri*-TSV-PA), Asn178 is located on the opposite face and apparently does not participate in recognition, selectivity, or binding of the substrate at the interfacial site.

Active Site

Looking down into the active site cleft (Fig. 2a), the peptide to be cleaved would extend from the north to the south in this cleft (Figs. 2a and c). The catalytic triad is perfectly positioned by the union of the N-terminal lobule containing His57 and Asp102 (Fig. 2a) and the C-terminal lobule containing Ser195. This region is surrounded by the conserved 70-, 148-, and 218-loops as well as the nonconserved 37-, 60-, 99-, and 174-loops (Fig. 2a and c). As in the mammalian counterparts, the catalytic residue, His57, possesses a nonoptimal Nδ1-H tautomeric conformation which is essential for catalysis. The catalytic triad is supported by an extensive hydrogen-bonding network formed between the Nδ1-H of His57 and Oδ1 of Asp102, as well as between the OH of Ser195 and the Nε2-H of His57. The hydrogen bond present between the latter pair is disrupted upon protonation of His57. Recent studies suggest that Ser214, which was once considered essential for catalysis, only plays a secondary role (Epstein and Abeles 1992; McGrath, et al. 1992). Hydrogen bonds formed between Oδ2 of Asp102 and the main-chain NHs of Ala56 and His57are structurally important to ensure the correct relative orientations of Asp102 and His57.

Oxyanion Hole and Subsites

A salient feature observed in the high-resolution structures of trypsin/chymotrypsin-like enzymes is the presence of a narrow channel referred to as an oxyanion hole formed by the backbone NHs of Gly193 and Ser195 (Birktoft and Blow 1972;

Birktoft et al. 1976) (Fig. 2b). These atoms contribute to the formation of a positively charged pocket that activates the carbonyl of the susceptible scissile peptide bond, and the residual positive charge additionally stabilizes the negatively charged oxyanion of the tetrahedral reaction intermediate. The oxyanion hole is in close proximity and is structurally linked to the catalytic triad and the Ile16–Asp194 salt bridge via Ser195. The serine proteinases are further characterized by the presence of a number of surface regions or patches referred to as subsites or secondary substrate binding sites that ensure the binding of the substrate and the perfect positioning of the susceptible scissile bond. The primary factors that determine specificity are the interactions at the S1/P1 and S1´/P1´ and also the S2/S2´ and S3/S3´ sites. The specificity of the mammalian chymotrypsin-like serine proteinases is primarily determined by the P1–S1 interaction. The S1 site or oxyanion is a hole located adjacent to the highly reactive Ser195 and is formed by a shallow pocket lined by the residues 189–192, 214–216, and 224–228. Residues 189, 216, and 226 determine specificity. In chymotrypsin, the oxyanion hole contains Ser189, Gly216, and Gly226 which determines its specificity for hydrophobic residues at the S1 subsite. In trypsin-like enzymes, Ser189 is substituted by Asp189, and hence, these enzymes display a marked preference for substrates containing Arg or Lys residues at the S1 subsite.

Due to the presence of Asp189, Thr190, and Gly217, as expected, benzamidine and benzamidine derivatives bind in the specificity pocket of *Agkistrodon contortrix contortrix* protein C activator (Murakami and Arni 2005) as illustrated in Fig. 2b.

Based on the above criteria, SVSPs can be classified as trypsin-like enzymes that possess highly conserved S1 subsites. However, since they display high selectivity towards macromolecular substrates, additional structural features need to be taken into consideration. Thus, the observed substrate specificity is not determined entirely by the interactions at the aforementioned subsites but likely involves more distant structural features.

The sequence alignments presented in Fig. 1 indicate that SVSPs are often glycosylated at different amino acid positions (Murakami and Arni 2005; Parry et al. 1998). In Protac (ACC-C), three carbohydrate moieties which are strategically positioned at the tips of the 37-, 99-, and 148-loops that surround the entrance to the active site cleft and extend outward could play a role in restricting access to the active site by macromolecular substrates, and it is speculated that they could play important roles in the modulation and expression of selectivity towards macromolecular substrates (Fig. 2b). Two snake venom serine proteinase isoforms from *Agkistrodon acutus*, AaV-SP-I and AaV-SP-II, also possess an N-linked carbohydrate group (Asn35) that likely interferes with the binding of macromolecular inhibitors and substrates (Zhu et al. 2003). In the case of TSV-PA, the enzyme has a unique glycosylation site at the Asn178 residue located on the opposite face and apparently does not play a role in the binding of macromolecular substrates at the interfacial site (Parry et al. 1998). Another key structural element implicated in the functional differentiation in SVSPs is the electrostatic surface potential, as calculated by PDB2PQR server for charges and radii assignments (Dolinsky et al. 2007) and APBS software for solving the Poisson–Boltzmann equation (Baker et al. 2001).

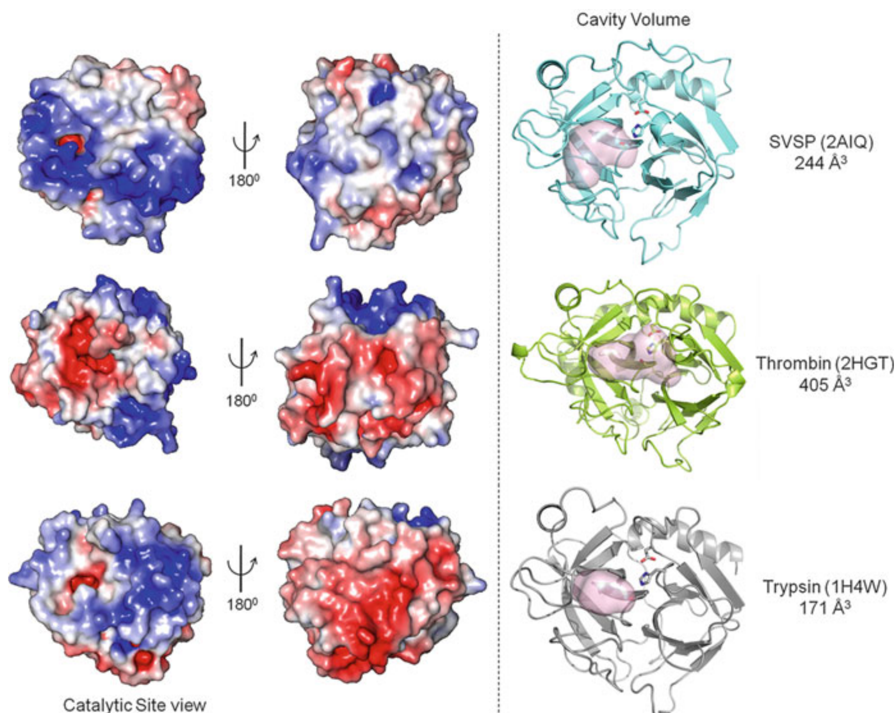

Fig. 3 *Left panel*. Electrostatic surface potential representation when viewed looking into the active site and when rotated 180°. *Right panel*, active site cavity volume of SVSP (PDB ID:2AIQ), human α-thrombin (PDB ID: 2HGT), and trypsin (PDB ID 1H4W). Visualization by The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC

It has been suggested (Murakami and Arni 2005) that the charge around the interfacial surface of Protac mimics the thrombin–thrombomodulin complex presenting high electrostatic affinity for the Asp⁄Glu propeptide of protein C (Fig. 3). Comparisons of the surface charges and active site cavity volumes of ACC-C (*Agkistrodon contortrix contortrix*), human α-thrombin, and trypsin (Fig. 3) indicate significant differences. The interface that contains the active sites is more negatively charged in thrombin and trypsin than in the SVSP (ACC-C). The active site cavity volume (Fig. 3, right panel) is 405 $Å^3$ in thrombin, 171 $Å^3$ in trypsin, and 244 $Å^3$ in the SVSP (*Agkistrodon contortrix contortrix* protein C activator).

Mechanism of Catalysis

Based on both biochemical and structural data of enzyme substrate and enzyme transition state substrate analogues, the catalytic mechanism of hydrolysis by serine peptidases has been very well characterized (Birktoft et al. 1976), and the steps involved in the acid–base catalytic mechanism can be inferred. In the first step,

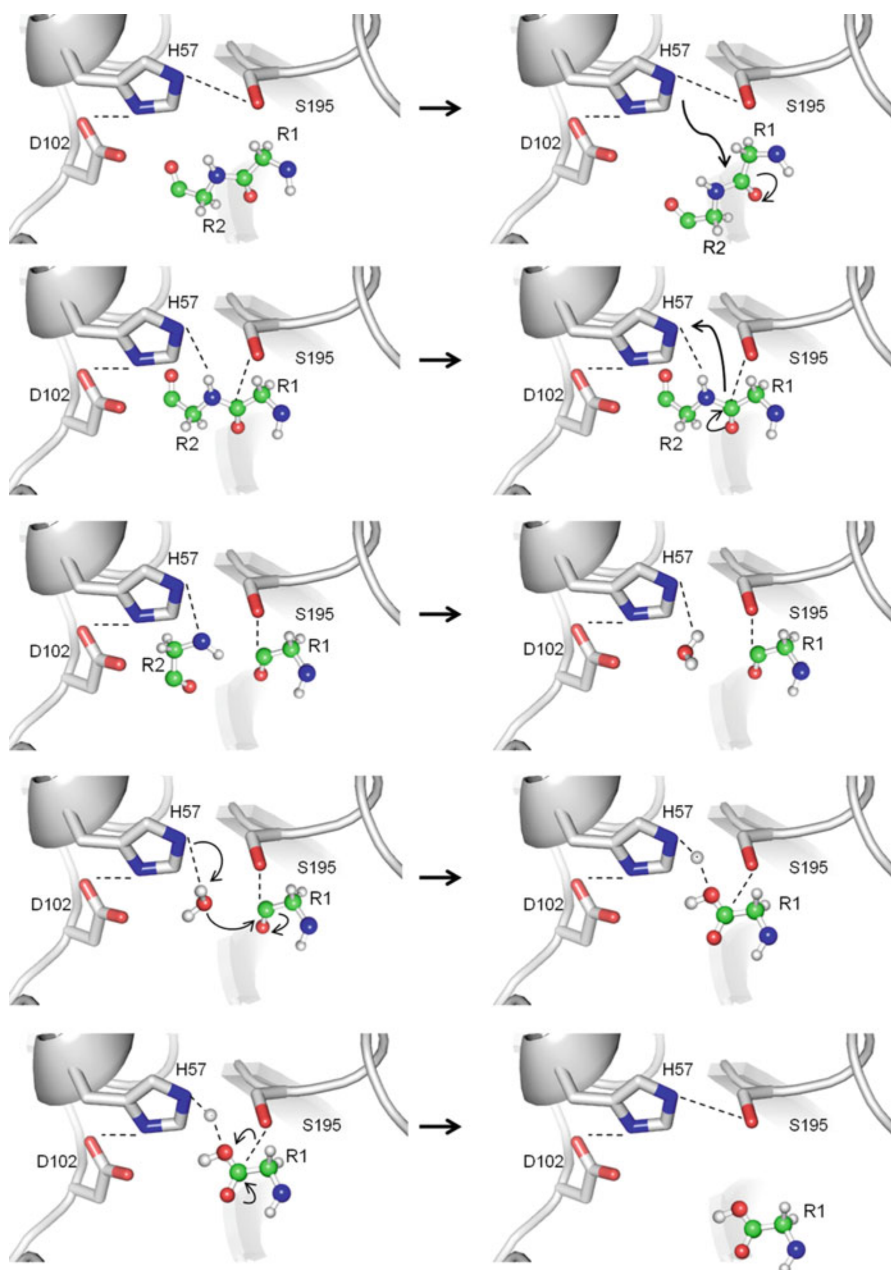

Fig. 4 The steps in the binding and hydrolysis of peptides by serine peptidases (see text for details)

Ser195 initiates the attack on the carboxyl group of the peptide. This reaction is subsequently assisted by His57 which, in turn, plays the role of a general base to form the tetrahedral intermediate. The transition state intermediate is stabilized by interactions formed with the main-chain NHs of the amino acids forming the oxyanion hole. Following the collapse of the tetrahedral intermediate and the expulsion of the leaving group, His57-H^+ plays the role of a general acid and the acyl–enzyme intermediate is formed. In the second step of the reaction, His57 deprotonates a water molecule which then interacts with the acyl–enzyme complex to yield a second tetrahedral intermediate. The collapse of this second intermediate results in the liberation of the carboxylic acid product.

Zymogen Activation

Mammalian serine proteinases participating in digestion and the blood coagulation cascade are synthesized as inactive zymogens and activation requires the cleavage of the N-terminal peptide and additional cleavages in the regions 142–152, 184–193, and 216–223 (Bode and Huber 1978). This autocatalytic cleavage and subsequent removal of the N-terminal peptide results in the formation of a salt bridge between the newly formed N-terminus and Asp194 and causes dramatic structural changes in both the S1 subsite and the oxyanion hole (Huber and Bode 1978; Bode et al. 1978).

Since neither the activity of SVSP zymogens nor their structures have been determined, only the molecular mechanism involved in the maturation process can be inferred. It is presumed that in the SVSPs, as in the case of trypsin, the S1 subsite and oxyanion hole are only formed upon cleavage and removal of this peptide since the N-terminal portion is conserved in snake and mammalian enzymes. Thus, as in the other serine proteinases, the loss of proteinase activity at high pH probably results from the deprotonation of the N-terminus and the disruption of the salt bridge, shifting the conformational equilibrium to resemble the inactive zymogen-like conformation (Hedstrom et al. 1996).

Snake Venom Metalloproteinases

Based on their primary structures and the configuration of their catalytic sites, zinc proteases are subdivided into the gluzincin, metzincin, inuzincin, carboxypeptidase, and DD carboxypeptidase subgroups (Hooper 1994). The metzincin subgroup is further divided into serralysins, astacins, matrixins, and adamalysins (Stöcker et al. 1995; Bode et al. 1996).

The metzincin superfamily of metalloproteinases which contain a conserved Met residue in a β-turn downstream of the zinc-binding motif includes four protein families, and the snake venom metalloproteinases (SVMPs) are leading members of

the reprolysin family. These enzymes, probably the most widely distributed venom proteinases, are encountered in both crotalid and viperid venoms and often constitute over 50 % of the total protein in Viperidae venoms (Calvete et al. 2007; Takeda et al. 2012). SVSPs are primarily hemorrhagic but fibrin(ogen)olytic (Retzios and Markland 1988); inhibition of blood platelet aggregation (Kamiguti et al. 1996; Moura da Silva et al. 2008) and other activities have been reported. Snake venom metalloproteinases (SVMPs) are synthesized as inactive precursors in the cytoplasm of secretory cells and are converted into active enzymes by proteolysis of a peptide bond and subsequent liberation of the short, N-terminal propeptide. Central to proteolysis is a metal ion, specifically a Zinc ion, which is coordinated by three His residues and one or two solvent molecules.

Domain Organization and Sequence Homology

Domain Organization

SVMPs range in size from 20 to about 100 kDa, and due to their diversity, a number of classification criteria currently exist. Both from a structural and domain organizational points of view, these enzymes display sequence and domain similarities to the ADAMs (A Disintegrin And Metalloproteinase) family of metalloproteinase proteins and can be grouped into four principal classes (Fig. 5). Apart from the metalloproteinase domain, these enzymes often contain other regulatory domains (Fig. 5). SVMPs belonging to the P-I class are the simplest and only contain a single domain, the zinc-dependent catalytic domain. Members of the P-II class additionally contain a second domain, the small, highly flexible disintegrin domain. The three-domain P-III class contains the aforementioned P-II domains and a cysteine-rich domain. The heterotetrameric P-IV class proteinases are the most complex and consist of the domains of the P-III enzymes linked by a disulfide bridge to a lectin-like domain (Fig. 5). The ADAMs contain additional domains such as the EGF (epidermal growth factor) and transmembrane (TM) domains (Fig. 5).

SVMPS contain a short 18-amino-acid hydrophobic signal peptide. The metalloproteinase or catalytic domains of SVMPs whose structures have been determined contain approximately 215 amino acids (Fig. 6) and indicate a high degree of identity with few, very short insertions. The zinc-binding motif HEBxHxBGBxHD, where B represents a bulky hydrophobic residue and X indicates any residue, is highly conserved (Fig. 6).

The metalloproteinase domain is linked to the disintegrin domain via a 13–15-amino-acid spacer peptide (Fig. 5).

The disintegrin and disintegrin-like domains show a fair degree of variation in the lengths of the polypeptide chain and the loop regions and hence are classified as short (49–51 amino acids), medium (~70 amino acids), and long (~84 amino acids) and contain 4, 6, or 7 disulfide bridges, respectively. The RGD sequence is contained in a short loop that is stabilized by disulfide bridges.

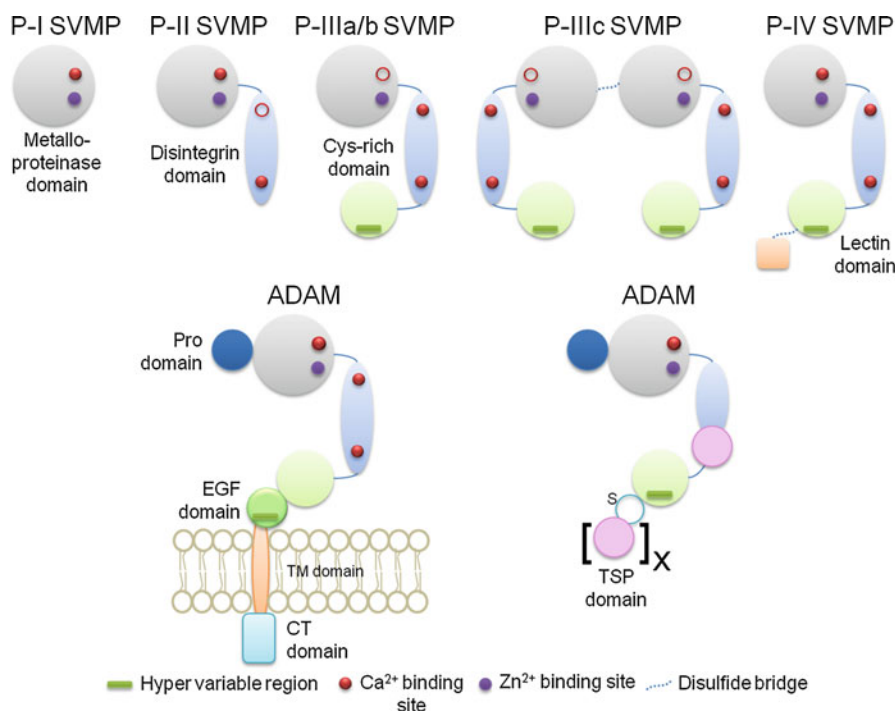

Fig. 5 Domain organization of SVMPs and ADAMs

As the name suggests, the cysteine-rich domain which is about 112 amino acids is rich in cysteines. Since structural information concerning the cystine-rich and C-type lectin domains is limited or absent, these domains will not be discussed in detail.

Crystal Structure

As expected, due to the high identity, the three-dimensional structures of the metalloproteinase domains are very similar (Fig. 8a). The structure can be described as ellipsoidal, α/β, two-domain structure; the shallow, catalytic cleft is created by the junction of the domains. The major (M, amino acid residues 1–152, sequences refer to *Bothrops asper*, BaP 1) domain consists of four α-helices (A–D) and a five-stranded β-sheet. Strands I, II, III, and V are situated parallel to each other, and strand IV is positioned in an antiparallel orientation relative to the others. The minor, S (residues 153–202) domain is formed by a α-helix and several loops. Three disulfide bridges stabilize the structure, and the bridge Cys 117–Cys 197 links the M and S domains, and Cys 159–Cys 181 and Cys 157–Cys 164 are located within the minor subdomain. Whereas adamalysin II and atrolysin C contain only two disulfide bridges, BaP1 contains a third disulfide bridge

```
1DTH       1 -QQNLPQRYIELVVVADHRVFMKYNSDLNTIRTRVHEIVNFINGFYRSLNIHVSLTDLEIWSNEDQINIQSASSDTLNAF 79
4AIG       1 ---NLPQRYIELVVVADRRVFMKYNSDLNIIRTRVHEIVNIINEFYRSLNIRVSLTDLEIWSGQDFITIQSSSSNTLNSF 77
1IAG       1 --ENLPQRYIELVVVADRRVFMKYNSDLNIIRTRVHEIVNIINKFYRSLNIRVSLTDLEIWSGQDFITIQSSSSNTLNSF 78
1ND1       1 --ERFSPRYIELAVVADHGIFTKYNSNLNTIRTRVHEMLNTVNGFYRSVDVHAPLANLEVWSKQDLIKVQKDSSKTLKSF 78
1QUA       1 ---PAPQTSIELFLIVDHSMYAKYNSNSSKITTTLKARVNIMNAIYSSLNLVITLSGIEMWSAADLITVQSSSRNTLKLF 77
1WNI       1 --QRFPQRYIELAIVVDHGMYKKYNQNSDKIKVRVHQMVNHINEMYRPLNIAISLNRLQIWSKKDLITVKSASNVTLESF 78
1KUG       1 EQQRFPQRYIELAIVVDHGMYTKYSSNFKKIRKRVHQMVSNINEMCRPLNIAITLALLDVWSEKDFITVQADAPTTAGLF 80
consensus  1   .......***....*.....**.. .. *.......... .*...........*.....**  * *.... .. *...*

1DTH      80 AEWRETDLLNRKSHDNAQLLTAIELDEETLGLAPLGTMCDPKLSIGIVQDHSPINLLMGVTMAHELGHNLGMEHDGKD-C 158
4AIG      78 GEWRERVLLTRKRHDNAQLLTAINFEGKIIGKAYTSSMCNPRSSVGIVKDHSPINLLVAVTMAHELGHNLGMEHDGKD-C 156
1IAG      79 GEWRERVLLIWKRHDNAQLLTAINFEGKIIGKAYTSSMCNPRSSVGIVKDHSPINLLVAVTMAHELGHNLGMEHDGKD-C 157
1ND1      79 GEWRERDLLPRISHDHAQLLTAVVFDGNTIGRAYTGGMCDPRHSVGVVRDHSKNNLWVAVTMAHELGHNLGIDHDTGS-C 157
1QUA      78 ASWRETDLLKRTSNDNAQLLTATNFNGNTVGLAYLKTMCNSKYSVGLIQDHSAIPLLMAVTMAHELGHNLGMNHDGAG-C 156
1WNI      79 GNWRETVLLKQQNNDCAHLLTATNLNDNTIGLAYKKGMCNPKLSVGLVQDYSPNVFMVAVTMTHELGHNLGMEHDDKDKC 158
1KUG      81 GDWRERVLLKKKNHDHAQLLTDTNFARNTIGWAYVGRMCDEKYSVAVVKDHSSKVFMVAVTMTHELGHNLGMEHDDKDKC 160
consensus 81 ..***   **  ....*.*.***.........*.*....**  ..  *.....*.*.......***.********..**...  *

1DTH     159 LRGASLCIMRPGLTKGRSYEFSDDSMHYYERFLKQYKPQCILNKP 203
4AIG     157 LRGASLCIMRPGLTPGRSYEFSDDSMGYYQKFLNQYKPQCILNKP 201
1IAG     158 LRGASLCIMRPGLTPGRSYEFSDDSMGYYQKFLNQYKPQCILNKP 202
1ND1     158 SCGAKSCIMASVLSKVLSYEFSDCSQNQYETYLTNHNPQCILNKP 202
1QUA     157 SCAT--CIMAPVLSSGPAKSFSDCSKHDYQSFLTIHKPQCLLN-- 197
1WNI     159 KCEA--CIMSDVISDKPSKLFSDCSKNDYQTFLTKYNPQCILNAP 199
1KUG     161 KCTT--CIMSAVISDKQSKLFSDCSKDYYQTFLTNDNPQCILNAP 199
consensus 161 . ....***.. .. .....*** *. .*. .*....***.**..
```

Calcium (Cys – via carbonyl oxygen)
Zinc catalytic
Active site

Fig. 6 Multiple sequence alignment of SVMP metalloproteinase domains. PDB id four-character codes are given on the *left*. Cys residues are in *yellow* and members of the two-disulfide family are the upper three sequences (1DTH, 4AIG, and 1IAG) and the three-disulfide family are the following four sequences (1ND1, 1QUA, 1WNI, and 1KUG). The active site Glu residue and the Met are boxed in *black*. The three zinc-binding residues are in *blue*, whereas the amino acids that bind calcium are in *dark yellow*. The zinc-binding motif is HEBxHxBGBxHD where B = bulky hydrophobic residue and X indicates any residue

```
1TEJ Ecarinatus  1 ---------NSVNPCCDPQTCKPIEGKHCISGPCCENCYFLRSGTICQRARGDGNNDYCTGITPDCPR-NRYNV 64
2PJF Rhodostomin 1 --GKECDCSSPENPCCDAATCKLRPGAQCGEGLCCEQCKFSRAGKICRIPRGDMPDDRCTGQSADCPRYH---- 68
1L3X Salmosin    1 EAGEECDCGSPGNPCCDAATCKLRQGAQCAEGLCCDQCRFMKEGTICRRARGDDLDDYCNGISAGCPR-NPFHA 73
1J2L Trimestatin 1 --GEECDCGSPSNPCCDAATCKLRPGAQCADGLCCDQCRFKKKRTICRIARGDFPDDRCTGQSADCPRWNGL-- 70
1FVL Flavoridin  1 --GEECDCGSPSNPCCDAATCKLRPGAQCADGLCCDQCRFKKKRTICRIARGDFPDDRCTGLSNDCPRWNDL-- 70
consensus        1   ..........*****..***...*..*..*.**..*.*.....**...***...*.*.*....***.. .
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Fig. 7 Multiple sequence alignment of the snake venom disintegrin domains. Cys residues are in *yellow* and the disulfide bridges are indicated by *lines*. The RGD sequence is boxed in *green*. Four-character code on the left indicates PDB id

(Cys 159–Cys 181) (Watanabe et al. 2003). The N- and C-termini are both located on the same side of the molecule and N-terminal amino acid is often a pyroglutamate (Watanabe et al. 2003).

Central to the active site is the well-characterized zinc-binding motif HEXXHXXGXXH (where X is any amino acid) and a methionine-turn (Fig. 4a) located in a shallow depression which also contains the three-catalytic histidines (His142, His146, and His152). This met-turn positions His152 such that it can also coordinate the Zn^{2+} ion and the side chain of Met166 serves as the hydrophobic base for the active site groups. The zinc ion is coordinated tetrahedrally by the Nε2 nitrogen atoms of the three histidines and one water molecule is encountered bound to Glu143 (Fig. 8a, c) in the crystal structure of BaP1 (Watanabe et al. 2003), whereas

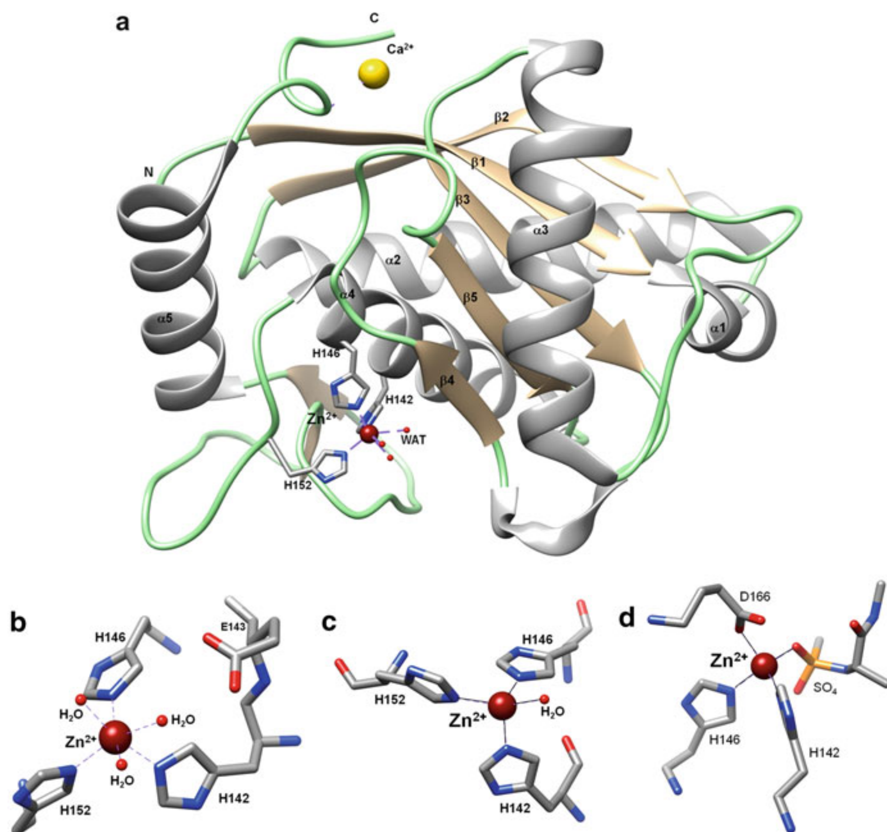

Fig. 8 (**a**) Ribbon representation of the crystal structure of the metalloproteinase domain from SVMP. (**b**), (**c**), and (**d**) present details of the hydrogen bonds formed with the zinc ion

it is octahedrally coordinated by additional solvent molecules in the crystal structure of BmooMPα-I (Akao et al. 2010) (Fig. 8b). Figure 8c indicates the interactions when BaP1 is complexed with a peptidomimetic inhibitor (Lingott et al. 2009).

Catalytic Mechanism

The catalytic mechanism for bacterial metalloproteinase thermolysin has been proposed, and based on the structural similarities, it can be inferred that (a) the Zn^{2+} ion orchestrates all steps of catalysis from peptide hydrolysis, stabilization of the reaction intermediates to release. In the initial step, Zn^{2+} is tetrahedrally coordinated by the aforementioned His142, His146, and His152 and a solvent water molecule. During catalysis, Zn^{2+} is pentahedrally coordinated with the participation of the substrate carbonyl oxygen atom (Fig. 9).

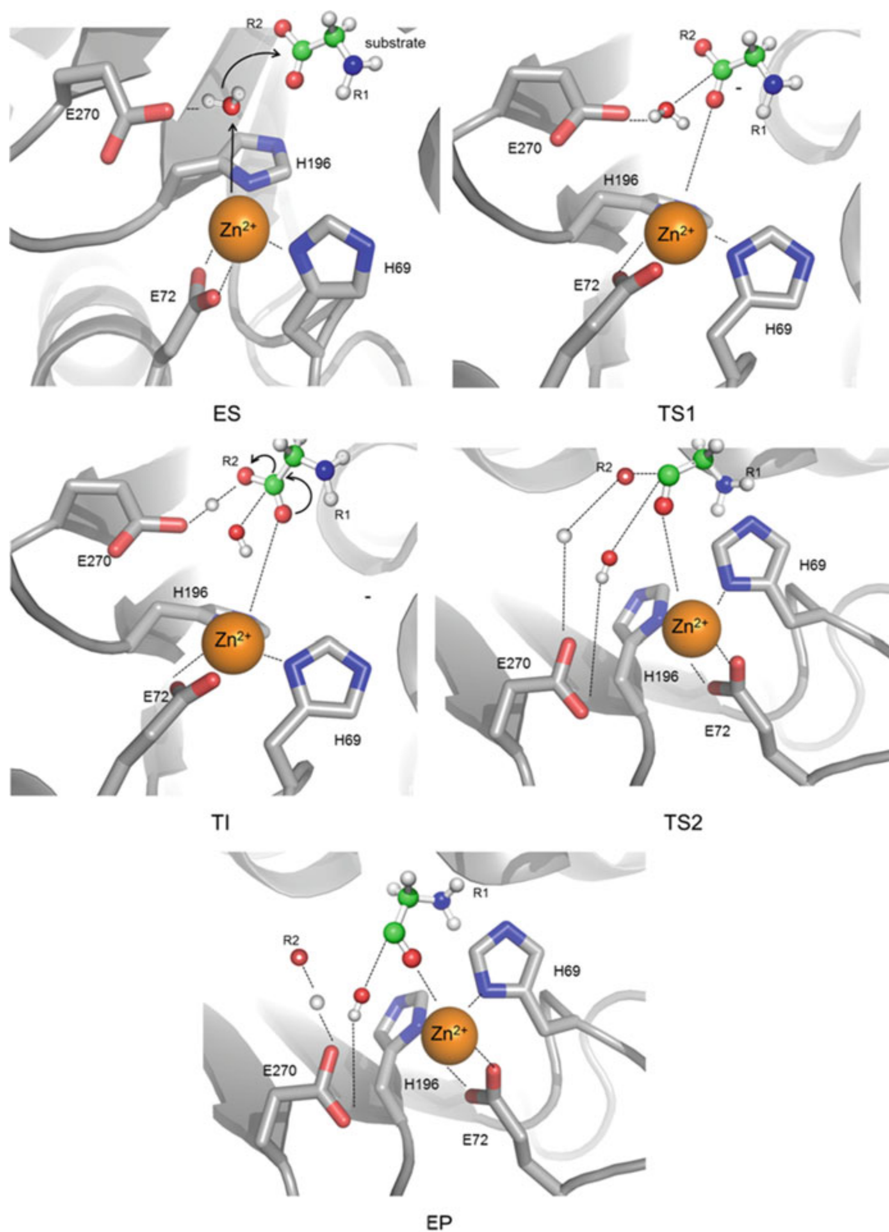

Fig. 9 Steps in the catalysis catalytic mechanism of Zn^{2+} metalloproteinases. ES enzyme + substrate, TS1 first transition state, TI tetrahedral intermediate, TS2 second transition state, and EP enzyme product complex

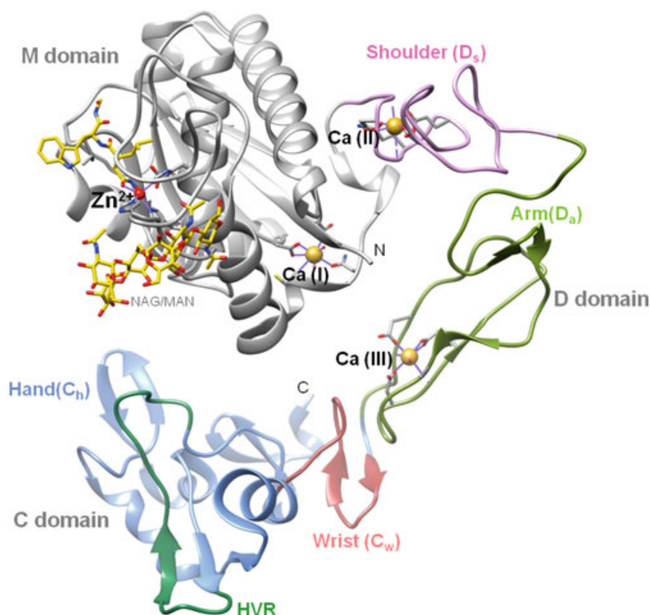

Fig. 10 The domain structure of SVMPs. NAG/NAM = carbohydrate moiety

Glu143 polarizes the catalytic water molecule by abstracting a proton, and this polarized water molecule, in turn, initiates a nucleophilic attack on the carbonyl carbon of the susceptible scissile peptide substrate bond. Glu143 transfers the proton abstracted from a water molecule to the amide leaving group. Site-directed mutagenesis demonstrates that the Glu143Asp substitution negates activity.

The hydrophobic S1´ site is conserved in many SVMPs although its depth might vary and in the structure of Bap1, it is lined with Phe178, Val138, Ala141, Tyr176, and Ile165.

The calcium ion located on the surface is implicated in the stabilization of the structure and is coordinated by the carbonyl oxygen atom of Glu9, carboxylate atoms of Asp93, carbonyl oxygen atom of Cys197, and the carboxamide oxygen of Asn200 and a solvent water molecule. It has been suggested (Gomis-Ruth et al. 1994) that this calcium ion likely plays a role in stabilizing the structures of multi-domain SVMPs.

As mentioned earlier, SVMPS often contain additional domains such as the disintegrin and cysteine-rich domain that are linked to the metalloproteinase domain by short peptides (Fig. 10).

Disintegrin Domain

Disintegrins are small, cystine-rich proteins that vary in mass from 4 to 14 kDa and bind to transmembrane proteins via an RGD motif located at the tip of a protruding loop (Figs. 7 and 11). The RGD-containing disintegrins are potent inhibitors of the platelet fibrinogen receptors, $\alpha_{iib}\beta_3$ integrins, whereas the non-RGD disintegrins do not possess this activity. Based on the length of the polypeptide chain and the disulfide bonding patter, the disintegrins have been classified into the following five groups (Calvete et al. 2003; Calvete 2013): group 1, 41–51 residues and four disulfide bonds; group 2, about 70 amino acids and six disulfide bridges; group 3, about 80 amino acids and seven disulfide bridges; and group 4, contains about 100 amino acids and 8 disulfide bridges and a C-terminal cysteine-rich domain cross-linked by six disulfide bridges (Calvete et al. 2003). The class 4 disintegrins are homodimeric and heterodimeric disintegrins with 67 amino acids and 10 disulfide bridges. The PII and PIII SVMPs contain the group 3 and group 4 disintegrins, respectively.

The NMR structures indicate that the loop regions, especially the C-terminus, are very flexible in solution although they contain six disulfide bridges. The disintegrin secondary structure is characterized by β-turns and short antiparallel β-sheets held together by six conserved disulfide bridges. A disulfide bridge determines the orientation of the long, irregular, hairpin loop that contains the RGD sequence at its tip. Due to the presence of a Gly, the side chains of Arg and Asp

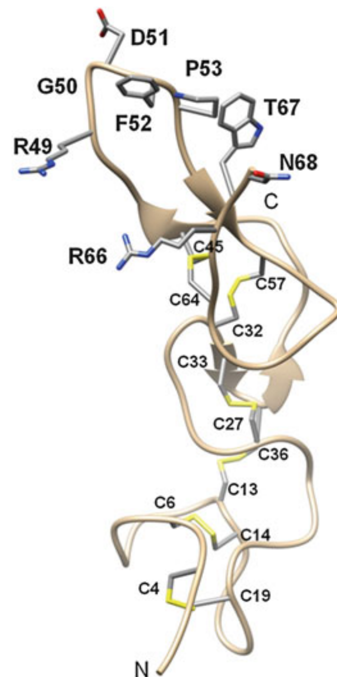

Fig. 11 Ribbon representation of the structure of a disintegrin (Trimestatin, *Trimeresurus flavoviridis*, Fujii et al. 2003)

point in different directions. Homodimeric disintegrins are stabilized by four intra-chain disulfide bonds and two N-terminal interchain disulfide bridges.

Phospholipases A_2

Phospholipases A_2 (PLA_2; phosphatide sn-2 acylhydrolase, EC 3.1.14) specifically catalyze the hydrolysis of the ester bond at the sn-2 position (sequentially numbered position 2) of glycerophospholipids generating fatty acids and lysophospholipids; their catalytic activity results in the release of arachidonic acid, a precursor of eicosanoids, which is implicated in triggering inflammatory reactions (Kudo et al. 1993). These enzymes are very widely distributed, display enhanced activity towards lipids in lamellar and micellar aggregates both in membranes and at other lipid–water interfaces (Jain et al. 1995; Ramirez and Jain 1991), and have been extremely well studied from the structural point of view, and over a hundred crystal structures of the class I and class II enzymes both with and without substrates, substrate analogues, inhibitors, fatty acid analogues, and other molecules have been determined, and a significant number of excellent, detailed reviews are currently available so this discussion will only present the salient structural features that are necessary to understand the protein fold and the steric and charge characteristics of the catalytic and calcium-binding sites.

Relevant to the study of snake venoms are the small, highly homologous, calcium-dependent secreted ($sPLA_2$s) PLA_2s with molecular masses ranging from 12 to 14 kDa (119 to 143 amino acids) that are divided into three classes (classes I, II, and III), based on their amino acid sequence and disulfide bonding patterns (Renetseder et al. 1985). The class I enzymes are encountered in *Elapidae* and *Hydrophiidae* snake venoms and mammalian pancreas; the class II PLA_2s are encountered in *Crotalidae* and *Viperidae* venoms and mammalian non-pancreatic tissues. The class III enzymes are almost exclusively found in lizard and bee (*Apis mellifera*) venoms. The interest in these enzymes stems from the fact that apart from their primary catalytic function, snake venom PLA_2s often display additional pharmacological activities such as hemorrhagic, myotoxic, hemolytic, edema-inducing, hypotensive, presynaptic, and postsynaptic neurotoxicity activities (Georgieva et al. 2008). Considering the vast amount of biochemical and structural data available, it is certainly very tempting to attempt to correlate biological activity with structural features, and indeed, a number of attempts have been made to delineate the sequence and structural elements or regions responsible for the specific activities cited earlier (Perbandt et al. 2003; Georgieva et al. 2004; Georgieva et al. 2012).

Primary Structure

The protein sequence databases contain a large number of partial and complete PLA_2 amino acid sequences, so the sequence comparisons in this article will be limited to a few selected examples of class I and II PLA_2s, whose crystal structures

have also been determined. The number scheme adopted is based on the homology numbering scheme with reference to the sequence of the bovine pancreatic PLA_2 (Dufton and Hider 1983; Renetseder et al. 1985).

The two structural criteria used to classify these enzymes as belonging to either class I or II are:

1. The positions of the seven disulfide bridges, class I PLA2s have a disulfide bridge Cys11–Cys77, whereas class II PLA2s have a disulfide bridge Cys51–Cys133. The other six disulfide bridges are generally conserved in the two classes.
2. The elapid loop, a two- or three-amino-acid insertion and the five-amino-acid insertion in the pancreatic loopof mammalian pancreatic PLA2s are features of class I PLA2s. On the other hand, the C-terminus of class II enzymes has 5–7 additional amino acids and the aforementioned Cys51–Cys133 disulfide bridge links this extension to the main body of the protein.

Whereas the class I and II enzymes share high sequence identity, the class III enzymes show greater sequence diversity, and since the class III enzymes are not encountered in snake venoms, it will not be further discussed except to point out that although the structures are radically different, their catalytic sites are similar.

Secondary Structure

Since a number of manuscripts have presented the results of the sequence alignments of PLA_2s and attempts have been made to correlate sequence information with biological activity, here only the alignment as a means of understanding the structure has been presented. The amino acids His47 and Asp99 that participate in catalysis are highly conserved (Fig. 12). However, Asp49, a key amino acid essential for calcium ion binding, is often replaced by Lys, Ser, or Arg. Thus, these natural mutants are unable to bind calcium and hence are catalytically inactive (Arni and Ward 1996).

Tertiary Structure

The predominant structural feature of the class I/II enzymes is a motif or platform formed by the relative positions of the two long, antiparallel helices (helices 2 and 3, residues 37–54 and 90–109) α-helices (Arni and Ward 1996). Two disulfide bridges ensure the maintenance of the helix axial distance of about 10 Å. An analysis of the hydrophobicity of the amino acids in these two helices does not indicate the existence of a clear amphipathic character. However, in general, the hydrophilic amino acids are exposed to the solvent, whereas the hydrophobic amino

```
1YXH        1 SNRPMPLNIYQFKNMIQCTVPSRSWWDFADYGCYCGRGGSGTPVDDLDRCCQVHDNCYNQ 60
1ZL7        1 -------SLWQFGKMINYVMGESGVLQYLSYGCYCGLGGQGQPTDATDRCCFVHDCCYGK 53
2QHE        1 -------SVVELGKMIIQETGKSPFPSYTSYGCFCGGGERGPPLDATDRCCLAHSCCYDT 53
1MG6        1 -------SLFELGKMIWQETGKNPVKNYGLYGCNCGVGGRGEPLDATDRCCFVHKCCYKK 53
2PH4        1 -------SLIELTKMVFQETGKNPVTYYTLYGCNCGVGRRGKPKDATDRCCFVHRCCYKK 53
consensus   1        .......*. ..... .. ... ***.**.*..* *.*..****..*..**..

1YXH       61 AQEITGCRPKWKTYTYECSQGTLTCKGRNNACAATVCDCDRLAAICF-AGAPYNDNNYNI 119
1ZL7       54 ---VTGCNPKIDSYTYSKKNGDVVC-GGDNPCKKQICECDRVATTCFRDNKDTYDIKYWF 109
2QHE       54 ---LPDCSPKTDRYKYKRENGEIIC-ENSTSCKKRICECDKAVAVCLRKNLNTYNKKYTY 109
1MG6       54 ---LTDCDSKKDRYSYKWKNKAIVC-GKNQPCMQEMCECDKAFAICLRENLDTYNKSFRY 109
2PH4       54 ---LTGCDPKKDRYSYSWENKAIVC-GEKNPCLKELCECDKAVAICLRKNLGTYDKNYRF 109
consensus  61    ...*..*...*.* . .....* .....*....*.**.. ..*.......... ...

1YXH      120 DLKARCQ------ 126
1ZL7      110 YGAKNCQEKSEPC 122
2QHE      110 YPNFWCKGDIEKC 122
1MG6      110 HLKPSCKKTSEQC 122
2PH4      110 TMKFLCD-KPEKC 122
consensus 121 .... *  .....
```

Active site
Calcium binding
Ser-49, Lys-49, Arg-49

Fig. 12 Structure-based sequence alignment of PLA_2s amino acids forming the active site, calcium-binding site, and the position of Ser/Lys/Arg49 are indicated in *red*, *cyan*, and *pink*, respectively. Note: Although His47 and Asp99 are conserved in the analytically inactive enzymes, they are not marked in *red*. The four-character PDB ids are given on the *left*, 1YXH (*Naja naja sagittifera*), 1ZL7 (*Bothrops jararacussu*), 2QHE (*Echis carinatus sochureki*), 1MG6 (*Agkistrodon acutus*), and 2PH4 (*Zhaoermia mangshanensis*)

acids are sequestered from the solvent. This rigid platform includes the amino acids forming the catalytic network and calcium ion-binding sites, His48, Asp49, Tyr52, and Asp99. This structural motif is highly conserved both within and between the class I/II PLA2s. The short antiparallel β-sheet, referred to as the β-wing, is rigidly held in position by a disulfide bridge in class I enzymes. Since this disulfide bridge is absent in the class II enzymes, this region is flexible and can adopt multiple conformations. The extended loop following helix 2 is referred to as the "elapid" or "pancreatic" loop (Fig. 13). This loop is much shorter in the class II enzymes.

Although the class I and II enzymes show some structural differences (Fig. 13a, b), upon superpositioning the atomic coordinates, it is observed that the active sites are analogous (Fig. 14).

Calcium is an essential cofactor (Teshima et al. 1989) and is coordinated by the carboxylate oxygen atoms of Asp49 (Fig. 15a) and three main-chain carbonyl oxygen atoms from the residues forming the calcium-binding loop (region 25–33). Two structurally conserved solvent water molecules complete the coordination sphere of the Ca^{2+} ion by forming a pentagonal bipyramid. As expected, this calcium-binding loop is perfectly positioned in relation to the catalytic site by a disulfide bridge (Cys27–Cys44) that links the second helix to the calcium-binding loop (Fig. 15a).

Calcium binding in this region is only possible when position 49 is occupied by Asp. Substitutions of Asp49 by Glu, Ala, Asn, Gln, Lys, Ser, or Arg which are encountered naturally in snake venoms result in inactive enzymes (Arni and Ward 1996).

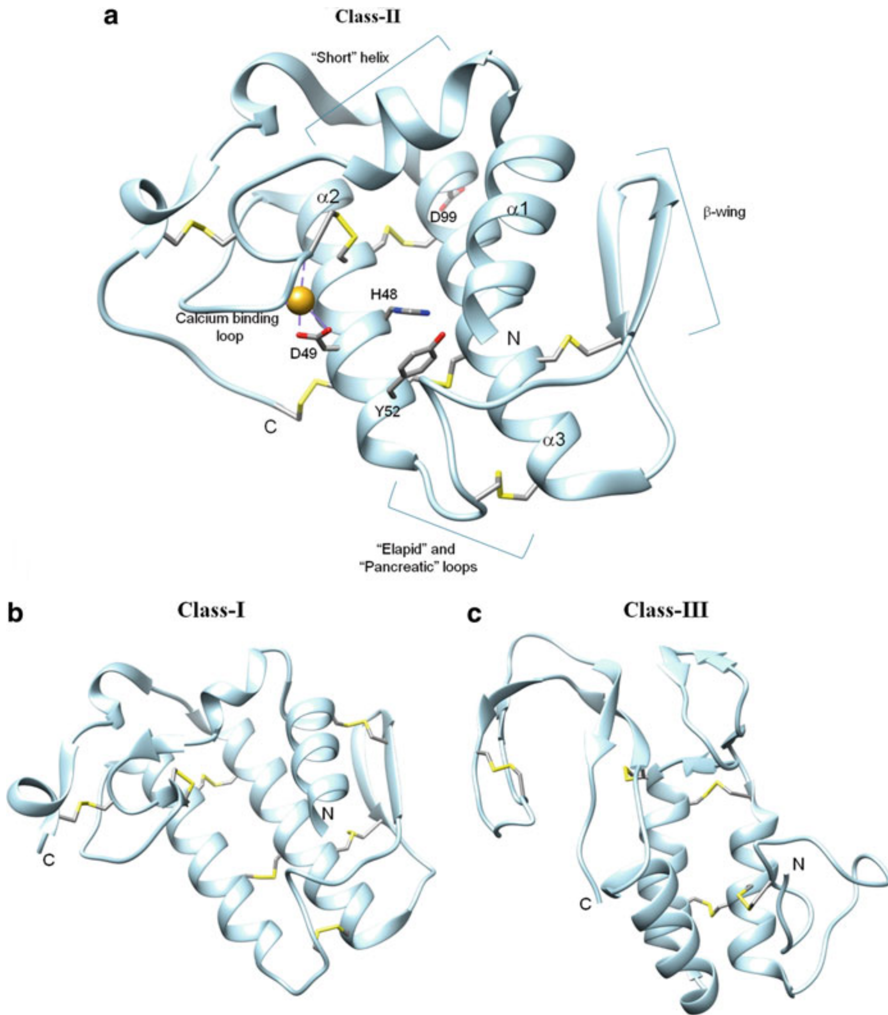

Fig. 13 Ribbon representation of the main structural features of (**a**) class II, (**b**) class I, and (**c**) class III PLA_2s. The positions of the disulfide bridges are indicated. In (**a**), the main features are labeled and amino acids involved in catalysis and Ca^{2+} binding are included

The Hydrophobic Channel

A hydrophobic channel is necessary to conduct the lipid molecule to the catalytic site. The amino acids in the short N-terminal amphiphilic helix (Helix 1) are highly conserved and stabilized by a disulfide bridge (class I) or hydrogen bonds (class II) form part of the protein core (Arni and Ward 1996). The inner surface of this helix is

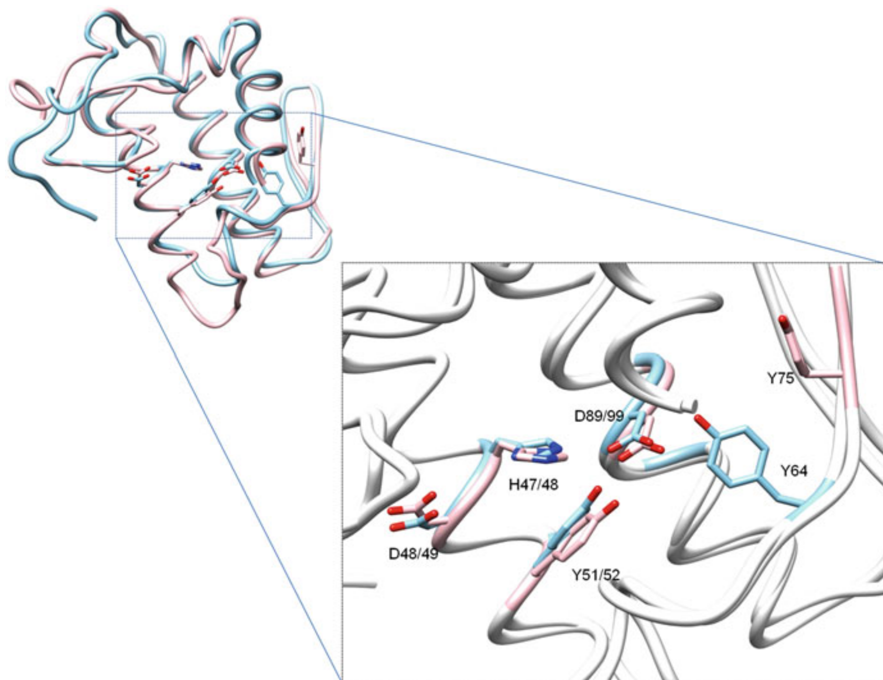

Fig. 14 Superpositioning of the atomic coordinates of class I (*pink*, PDB id 1YXH) and class II (*blue*, PDB id 1ZL7)

lined by hydrophobic amino acids that serve to line a sector of the channel and other amino acids from the single helical turn and the β-wing region complete the hydrophobic channel.

Catalytic Mechanism

The catalytic mechanism of the PLA_2s bears a strong resemblance to the mechanism involved in serine proteinases (Fig. 16). In this mechanism, the dyad + ion His48/Asp99/Ca^{2+} is the central component that triggers or initiates catalysis. The Ca^{2+} ion coordinated by a solvent water molecule polarizes the sn-2 carbonyl oxygen; His48 increases the nucleophilicity of the catalytic water via a second bridging water molecule.

Conclusion and Future Directions

Structural information obtained primarily by applying crystallographic techniques to understand the general shape, surface charge and binding pockets have been presented. An attempt has been made to correlate these results with

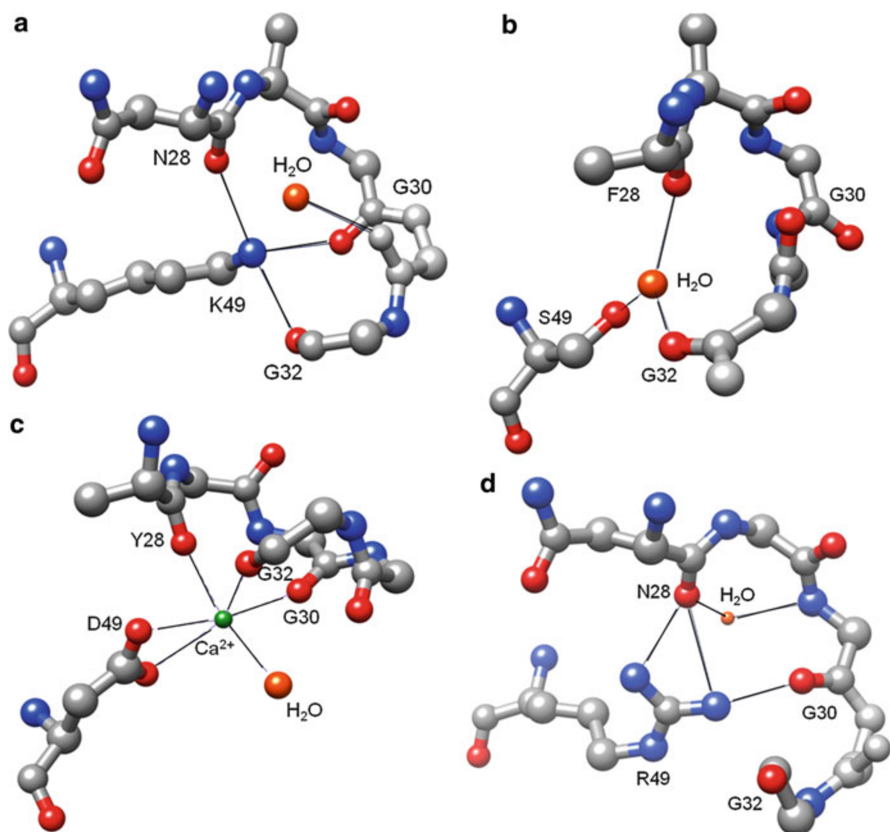

Fig. 15 Conformations of the calcium-binding loops when (**c**) Asp49 (PDB id 1ZL7) is substituted by (**a**) Lys (PDB id 1MG6), (**b**) Ser (PDB id 2QHE), and (**d**) Arg (PDB id 2PH4)

those obtained by other biophysical and biochemical techniques to understand enzyme specificity and mechanisms. Another powerful, albeit lesser used, technique to study biomolecular structure is nuclear magnetic resonance spectroscopy. The structure of the small (42 amino acids), highly basic (pI 10.3) peptide crotamine (Rádis-Baptista and Kerkis 2011) isolated from the venom of *Crotalus durissus terrificus* has been determined by both in solution (Fadel et al. 2005; Nicastro et al. 2003) and in the crystalline states (Coronado et al. 2012, 2013). Superpositioning the atomic coordinates obtained by these techniques (Fig. 17) indicates that the results obtained are similar and they complement each other since NMR techniques provide us with details of the flexibility of the molecule in solution, whereas crystallographic

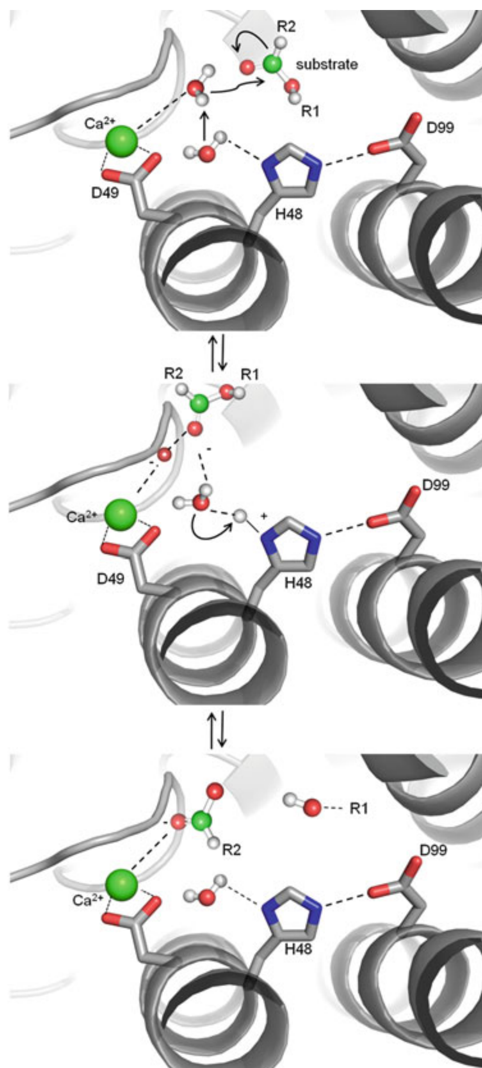

Fig. 16 The catalytic mechanism of PLA_2s. See text for details

techniques provide more detailed information regarding the solvent structure and the binding of ions.

The application of novel NMR spectroscopy techniques to study the interactions of macromolecules with peptides, substrates, and other small molecules combined with high-resolution crystallographic techniques should provide us with deeper insights regarding the mode of action of snake venom proteins.

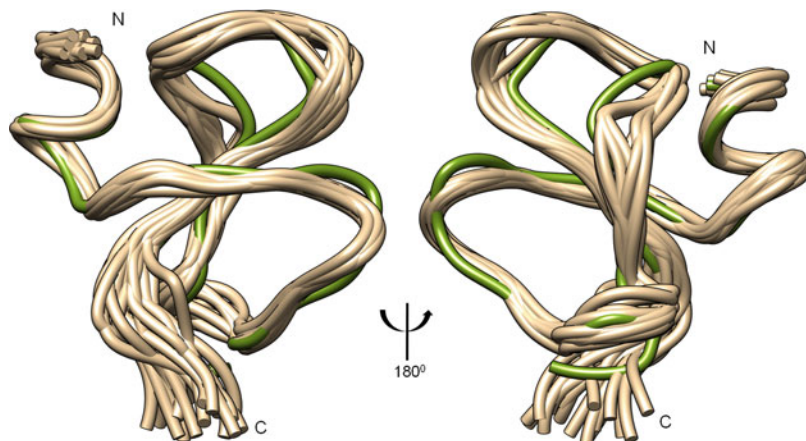

Fig. 17 Results of the superpositioning of the Cα atoms of crotamine (*Crotalus durissus terrificus*) obtained by applying NMR (beige, PDB id 1Z99) and crystallographic techniques (*green*, PDB id 4GV5)

Cross-References

► Biological Activities and Assays of the Snake Venom Metalloproteinases (SVMPs)
► Structure-Function Relationship in Heterodimeric Neurotoxin PLA2s from Viperidae Snakes Inhabiting Europe, South America, and Asia
► Structure-Function Relationship of Modular Domains of P-III Class Snake Venom Metalloproteinases

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Structure-Function Relationship in Heterodimeric Neurotoxin PLA2s from Viperidae Snakes Inhabiting Europe, South America, and Asia

12

Functional Importance of the Nontoxic Components

Dessislava Georgieva, Raghuvir K. Arni, and Christian Betzel

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Abstract

Snake venom heterodimeric non-covalent phospholipase A_2 (PLA_2) complexes ($ncHdPLA_2s$) are neurotoxins encountered in the venoms of Viperinae and

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Crotalinae snakes. In contrast to their monomeric counterparts, they have a sophisticated mechanism of action in order to avoid nonspecific binding to "wrong" targets and to increase the efficiency of the pharmacological attack. ncHdPLA$_2$s consist of a toxic and enzymatically active basic PLA$_2$ and an acidic and catalytically inactive PLA$_2$ protein. The main function of the acidic subunit is to direct the toxic component to the "correct" targets on the cell membrane and in this way to avoid binding to phospholipids, which are not important for the pharmacological effect of the toxin. The nontoxic component of ncHdPLA$_2$s is multifunctional. It can modulate the toxicity and catalytic activity of PLA$_2$.

The structure and function of ncHdPLA2s from the venoms of snakes inhabiting Europe, South America, and Asia are similar. There exists a high identity at the levels of primary and three-dimensional structures of ncHdPLA$_2$s from snakes inhabiting widely separated regions of the world. Although a substantial progress has been made during the last years in understanding the structure and biological action of ncHdPLA$_2$s, a number of questions still remain to be answered.

Introduction

Heterodimeric non-covalent phospholipase A$_2$ (PLA$_2$) complexes (ncHdPLA2s) have been isolated from Crotalinae and Viperinae snake venoms. These neurotoxins have a more complicated mechanism of pharmacological action in comparison to their monomeric counterparts. ncHdPLA2s consist of a basic toxic group IIA PLA$_2$ and an acidic nontoxic and enzymatically inactive PLA$_2$-like protein which probably results from accelerated evolution of the basic component for the acquisition of diverse physiological functions (Betzel et al. 1999 and references therein). There is evidence that the subunits of heterodimeric PLA$_2$s crotoxin and vipoxin, from the venoms of *Crotalus durissus terrificus* and *Vipera ammodytes meridionalis*, respectively, dissociate when they interact with biological membranes or micellar substrates (Bon and Jeng 1979; Georgieva et al. 2004a). The role of the second component of the complex is to avoid nonspecific binding of the toxic enzyme, and it is likely that the toxic PLA$_2$ binds to the target on the cell surface, whereas the nontoxic subunit remains in the solution. The structure of the nontoxic subunit of Viperinae heterodimeric neurotoxins differs from their Crotalinae counterparts. In the first case the acidic component is a PLA$_2$-like non-neurotoxic polypeptide which is catalytically inactive or has very low activity. In some cases the active site His48 is substituted by other amino acid residues (Betzel et al. 1999 and references therein). This substitution prevents the hydrolysis of substrates. However, the acidic component of Crotalinae HdPLA$_2$s is composed of three polypeptides covalently linked by disulfide bridges (Aird et al. 1990). The heterodimeric PLA$_2$s exert their neurotoxicity at presynaptic or postsynaptic levels.

The acidic subunits are multifunctional and differ in their function: apart from targeting the toxic component to specific membrane receptors, they potentiate or inhibit PLA$_2$ toxicity and can modulate its catalytic activity and stabilize the other subunit. In spite of sequence differences, the three-dimensional structures of the acidic and basic subunits of heterodimers from Viperinae snakes are very similar.

The members of the Crotalinae and Viperinae ncHdPLA$_2$s differ mainly in the structure of the acidic subunit. Comparison of ncHdPLA$_2$s from snakes inhabiting Europe, South America, and Asia showed unexpected structural similarity. The high sequence and structural similarities of ncHdPLA$_2$s from snakes inhabiting widely separated regions of the world, *Vipera ammodytes meridionalis* encountered in the Balkan peninsula of Europe and *Vipera russelli formosensis*, inhabiting the isolated island Taiwan, Asia, are surprising (Perbandt et al. 2003).

The topic of this chapter is structure-function relationships of snake venom non-covalent heterodimeric PLA$_2$ complexes for which there is sufficient structural data to correlate with their pharmacological activities. For the description of the structures, mainly the structural data of ncHdPLA$_2$ has been used and their components analyzed to high resolution applying synchrotron radiation at DESY, Hamburg. In particular, structure-function relationships of the unique complex vipoxin from the venom of *Vipera ammodytes meridionalis* and its isoform called viperotoxin F will be discussed. Vipoxin is an example of an evolutionary modulation of toxic and enzymatic functions into nontoxic and catalytically inactive protein inhibitor of PLA$_2$. Vipoxin is not an isolated case, and closely related homologs have been reported in widely separated regions.

Non-Covalent Heterodimeric Phospholipase A$_2$ Complexes from Venomous Snakes Inhabiting Europe

Vipera ammodytes is the most venomous snake in Europe where it is widely distributed. Five subspecies are known: *V. a. ammodytes* (East and Central Europe), *V. a. meridionalis* (South East of Bulgaria, Greece, incl. Corfu and islands), *V. a. montandoni* (Bulgaria, South Romania), *V. a. transcaucasiana* (Georgia, Turkish Thrace), and *V. a. gregorwallneri* (Austria, the former Yugoslavia) (Mallow et al. 2003). The major neurotoxic component of the *Vipera a. meridionalis* venom is the heterodimeric PLA$_2$ vipoxin (Aleksiev and Shipolini 1971). Later on, a similar heterodimeric toxin was identified in the venom of *Vipera a. ammodytes* (Georgieva et al. 2008). Vipoxin causes paralysis of the respiration, myotoxicity, hemorrhage, myonecrosis, and other pharmacological effects.

Vipera aspis is another venomous viper species found in South West Europe. According to the Integrated Taxonomic Information System, five subspecies are known: *V. a. aspis* (France), *V. a. zinnikeri* (Southwestern France, Andorra), *V. a. atra* (Switzerland), *V. a. hugyi* (Italy), and *V. a. francisciredi* (Italy). ncHdPLA$_2$s were isolated from the venoms of snakes of the first two subspecies (Komori et al. 1996; Jan et al. 2002; Guillemin et al. 2003).

Vipoxin: An Example of Modulation of the Toxic Function

Vipoxin consists of a neurotoxic and enzymatically active basic PLA$_2$ and a nontoxic, catalytically inactive PLA$_2$-like protein (Aleksiev and Tchorbanov 1976).

The separated PLA_2 subunit is unstable in the absence of the nontoxic component (Aleksiev and Tchorbanov 1976). At the same time, a threefold to fivefold increase of the neurotoxicity and a 60 % increase of the phospholipolytic activity of this subunit were observed after their separation (Aleksiev and Tchorbanov 1976). These properties demonstrate that the acidic PLA_2-like protein in ncHdPLA_2s can modulate the toxic and catalytic functions of the enzymatic component of the complex and can stabilize it. In this way the nontoxic PLA_2 isoform plays the role of a natural inhibitor of the enzyme. Also, the acidic component of vipoxin changes the target of the pharmacological attack when it is in a complex with the toxic PLA_2. Thus, the heterodimer shows a postsynaptic activity while the isolated PLA_2 acts on presynaptic membranes (Tchorbanov et al. 1978). The amino acid sequences of the two subunits of vipoxin show that they are closely related proteins, with the same length of the polypeptide chains of 122 residues and 62 % sequence identity but with opposite charges (Mancheva et al. 1987). Most probably, the acidic component of the neurotoxic complex is a product of the evolution of the toxic enzyme and acquires other functions for which catalytic activity is not necessary.

Explanation of the Vipoxin PLA_2 Reduced Catalytic Activity and Toxicity Using the High-Resolution (1.4 Å) X-ray Structure of the Complex

Vipoxin was successfully crystallized (Betzel et al. 1993), and its 3-D structure was solved and refined to 1.4 Å resolution (Banumathi et al. 2001). This is the first crystal structure of a non-covalent heterodimeric PLA_2 complex (Fig. 1). The whole complex has a shape of an oblate ellipsoid. The structure is similar to that of homodimeric PLA_2s, which is not surprising having in mind the high sequence identity between the basic toxic component and the acidic PLA_2-like protein. Both subunits have almost identical secondary structures consisting of three α-helices, two short helical segments, and one antiparallel β-sheet (Fig. 1). The 3-D structure of vipoxin revealed the "catalytic network" at the active site of PLA_2 including His47; Asp89; a water molecule, which serves as a nucleophile during the catalysis; Tyr51; and Tyr64 (His48, Asp99, Tyr52, and Tyr73, amino acid sequence numbering is based on the scheme proposed by Renetseder et al. 1988). The structures of the calcium-binding site and the hydrophobic channel of the substrate-binding site are similar to those of other group II PLA_2 molecules. The stability of the complex is very important for the physiological action of the neurotoxin. The vipoxin model revealed the structural basis of stability. As it was mentioned before, the toxic component is unstable in the absence of the second subunit and loses its enzymatic activity and toxicity. In a complex with the acidic PLA_2-like protein, it preserves the catalytic and pharmacological activities for years. The model demonstrates that the complex is stabilized by electrostatic and hydrophobic interactions as well as by the salt bridge between the carboxylic group of Asp48 of the PLA_2 component and the ε-NH2 of Lys60 of the acidic subunit (Asp49 and Lys69). The model explains the reduced enzymatic activity and neurotoxicity with the partial shielding

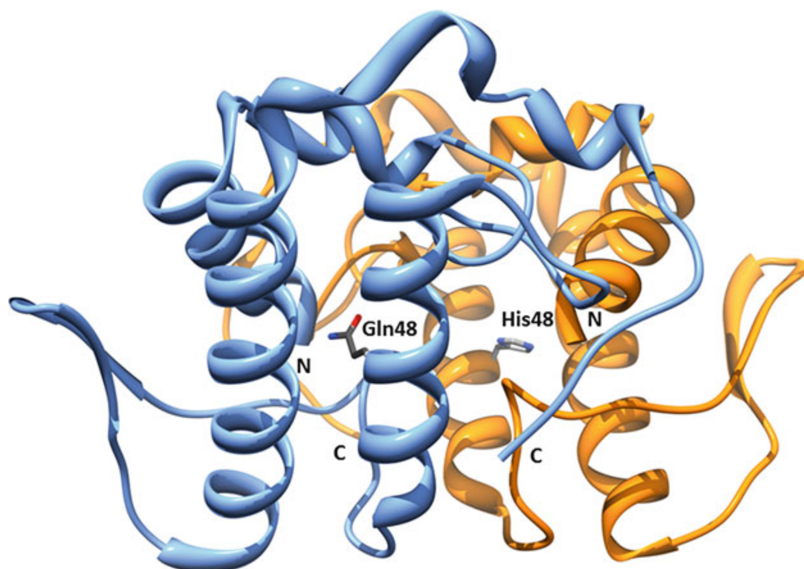

Fig. 1 Three-dimensional structure of vipoxin at 1.4 Å (PDB code 1 jlt). The basic, toxic, and catalytically active subunit is colored in *orange*. The acidic and nontoxic subunit is colored in *blue*. The substitution in position 48 in the acidic chain is shown

of two important sites in the PLA_2 molecule exerted by the acidic subunit which partially blocks the entrance to the active site and the segment 109–114 (residues 119–125) of PLA_2 which is supposed to be involved in the toxicity (Banumathi et al. 2001). In the presence of micellar structures, vipoxin dissociates to subunits (Georgieva et al. 2004a), and in this way, the two important sites in the catalytically and pharmacologically active PLA_2 are liberated.

A New Mechanism of PLA_2 Inhibition Revealed by the X-ray Structure of the Complex Between the Vipoxin Toxic Subunit and Elaidoylamide

The interaction of PLA_2 with fatty acid derivatives is of functional importance because fatty acids are components of the biological membranes where they are bound to phospholipids and cholesterol esters or exist as free compounds. The crystal structure of the complex between the vipoxin PLA_2 and its inhibitor elaidoylamide revealed a new mode of inhibition: the hydrocarbon chain of one molecule of elaidoylamide is bound simultaneously to the substrate-binding sites of two PLA_2 molecules (Georgieva et al. 2004b). In this way the amide of trans-9-octadecenoic acid blocks the access of substrates to the active site. The inhibition of PLA_2s is also of medical importance because these enzymes are involved in inflammatory processes in living organisms and in a number of diseases (Betzel et al. 2006 and references therein). High levels of PLA_2s in physiological liquids

can be used as a biomarker of pathological processes (Betzel et al. 2006). It can be supposed that the amide of a natural fatty acid is tolerant to living organisms and suitable for the design of novel anti-inflammatory drugs.

Anion-Binding Sites in the Vipoxin Acidic Subunit Revealed by the X-ray Structure at 1.9 Å Resolution

It was supposed that one of the major roles of the acidic subunits in ncHdPLA$_2$ is to target the toxic component to the "correct" for the pharmacological action substrate on biological membranes and to avoid nonspecific binding to other phospholipids (Bon and Jeng 1979; Georgieva et al. 2004a). Phospholipases A$_2$ act at the lipid-water interface (Dijkstra et al. 1981), and the binding to the negatively charged cell surface is an important step during catalysis. It was proposed that the acidic subunit of the ncHdPLA$_2$ crotoxin may participate in the formation of a transient complex with a membrane acceptor as a first step of catalysis (Delot and Bon 1993). In order to identify possible anion-binding sites within the acidic subunit of vipoxin which can interact with negatively charged groups of the membrane during the initial formation of a complex between the heterodimeric neurotoxin and the acceptor, the structure of the acidic subunit of vipoxin in a complex with sulfate ions which mimic the negatively charged groups of anionic membranes was solved at 1.9 Å resolution (Georgieva et al. 2004c). The crystallographic structure showed the formation of homodimers of PLA$_2$-like protein and revealed two anion-binding sites per subunit, one of them, site 1, being the same for both components (Fig. 2). This site is located in a cavity on the protein surface, and the anion is ligated by the side chains of Arg35 and Tyr109, as well as the main chain N-atoms of Arg35, Cys115, Thr116, and Glu117 (Arg36, Tyr120, Cys115, Thr116, and Glu117). The same region in the pharmacologically active PLA$_2$ component of vipoxin is involved in neurotoxicity (Banumathi et al. 2001). The second site is also completely exposed on the protein surface. The sites can interact with substrate molecules.

Vaspin from *Vipera aspis aspis* and the Heterodimeric Toxin from *Vipera a. zinnikeri* (PLA$_2$-I)

Heterodimeric neurotoxic PLA$_2$s were also isolated from the venoms of *Vipera aspis zinnikeri* and *Vipera aspis aspis*, venomous snakes widespread in Southeastern France (Komori et al. 1996; Jan et al. 2002; Guillemin et al. 2003). The two chains of the first toxin (vaspin) are closely related proteins with 67 % sequence identity (Guillemin et al. 2003). The vaspin A chain is 100 % identical to its counterpart from the complex PLA$_2$-I, the heterodimer from the *Vipera a. zinnikeri* venom. The acidic polypeptide has no catalytic activity. Probably, the nonenzymatic component of vaspin evolved from an ancestor common to ammodytin I$_2$ from *Vipera ammodytes ammodytes* (Jan et al. 2002). The deduced from the nucleotide sequence amino acid sequence of the vaspin B chain is identical to that of the PLA$_2$-I basic subunit

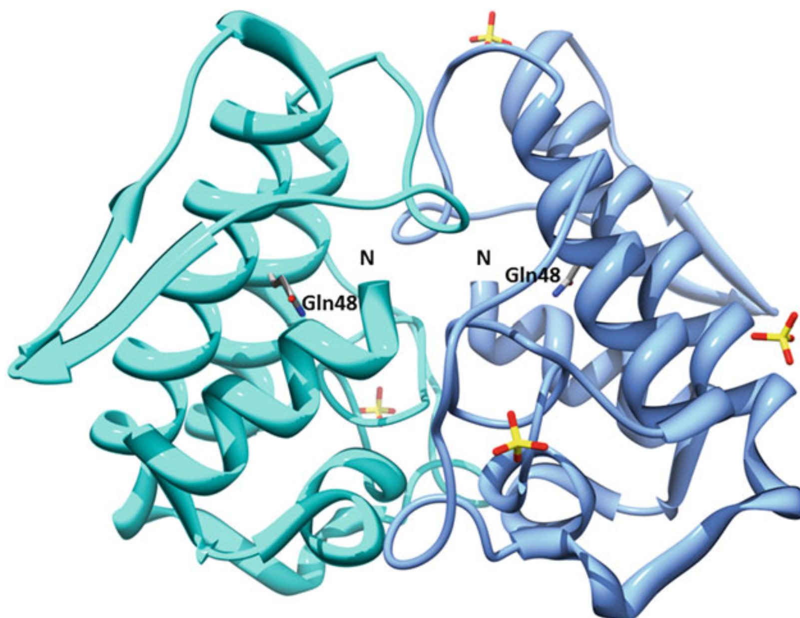

Fig. 2 Three-dimensional structure of the vipoxin acidic subunit in complex with sulfate ions (PDB code 1Q5T). The N- and C-terminus of the polypeptide chains and the anion-binding sites are shown

(Guillemin et al. 2003) but differs by three residues from the sequence published before (Komori et al. 1996). The subunits of both ncHdPLA$_2$s are structurally similar to the respective components of vipoxin. The sequences of the acidic subunits of vaspin and PLA$_2$-I differ only by two residues from the respective component of vipoxin. Comparison of the basic subunits showed two substitutions in the vipoxin/vaspin couple and four substitutions in the case of vipoxin/PLA$_2$-I. It was suggested that the ncHdPLA$_2$ from the *Vipera a. aspis* venom is a postsynaptic neurotoxin (Jan et al. 2002). Characterization of genes encoding viperid venom PLA$_2$s suggested that a new population of *V. a. aspis* may have resulted from interbreeding between this snake and *Vipera ammodytes ammodytes* (Guillemin et al. 2003).

Non-Covalent Heterodimeric Phospholipase A$_2$ Complexes from Venomous Snakes Inhabiting South America

The Viperidae snake family includes about 260 species subdivided into four subfamilies: Crotalinae, Viperinae, Azemiopinae, and Causinae (McDiarmid et al. 1999). More than half of them, 18 genera and 151 species, belong to the subfamily Crotalinae (pit vipers), distributed in the Old and New World (Crotalinae, Integrated Taxonomic Information System 2006). Pit vipers are the only viperids found in the Americas. The genus *Crotalus* is recognized only in the Americas from southern Canada to northern Argentina (McDiarmid et al. 1999).

Crotoxin: The First Animal Toxin Isolated and Purified 74 Years Ago

The heterodimeric neurotoxin crotoxin from the venom of *Crotalus durissus terrificus* is the first snake venom protein crystallized 74 years ago (in 1938) by Slotta and Fraenkel-Conrat (Aird et al. 1985 and references therein). It is the most intensively studied ncHdPLA$_2$. Crotoxin is a complex of a basic and weakly toxic PLA$_2$ component (crotactin, CB) and is acidic, nontoxic, and devoid of catalytic activity subunit (crotapotin, CA). The acidic component consists of three polypeptides generated by proteolytic degradation of a PLA$_2$-like precursor and linked by disulfide bridges (Bouchier et al. 1991). Crotoxin is a presynaptic β-neurotoxin (Bon 1997). It binds to a single class of sites in Torpedo presynaptic membranes (Delot and Bon 1993). The presynaptic action of crotoxin blocks the transmission across the neuromuscular junctions of the breathing muscles, which can lead to rapid death. However, postsynaptic effects of the toxin from *Crotalus d. terrificus* were also demonstrated on electroplaque from *Electrophorus electricus* and acetylcholine receptor-rich membranes of *Torpedo marmorata* (Bon et al. 1979). In the presence of synaptic membranes, the neurotoxic complex dissociates to subunits (Bon and Jeng 1979). Postsynaptic toxins prevent the binding of acetylcholine to its receptor (Changeux et al. 1970). A 48 kDa crotoxin-binding protein, crotoxin acceptor, was isolated from membranes of the Torpedo electric organ (Faure et al. 2003). CA increases the lethal potency not only of the basic PLA2 but also modulates the pharmacological activity of agkistrodotoxin and ammodytoxin from the venoms of *Agkistrodon blomhoffii brevicaudus* and *Vipera ammodytes ammodytes*, respectively (Choumet et al. 1993). The effect of CA on the basic subunit is opposite to that of the acidic component of vipoxin from the venom of the European snake *Vipera ammodytes meridionalis*, which decreases the neurotoxicity of the basic PLA$_2$ (Aleksiev and Tchorbanov 1976).

Crotoxin exerts neurotoxic, cytotoxic, myotoxic, edema-inducing, liposomal-disrupting, and anticoagulant effects (Bon 1997; Soares et al. 2001) and modulates immune and inflammatory responses as well (Zambelli et al. 2008). These activities of the toxin cause life-threatening effects after envenomation as neurological disorders and renal failure (Monteiro et al. 2001). However, some activities of crotoxin as the inhibition of tumor growth (Cura et al. 2002) can find application in medicine. Administration of crotoxin to cancer patients had an analgesic effect mediated by an action on the central nervous system (Zhang et al. 2006).

Diversity of Crotoxin Isoforms

Crotalinae snakes synthesize a large diversity of crotoxin isoforms, and 16 of them have been isolated (Bon 1997; Faure et al. 1993). The subunit isoforms differ in a small number of amino acid residues, and the crotoxin-like neurotoxins represent variable complexes of associated isoforms of the two types of subunits. Practically, the venoms of South American rattlesnakes contain a mixture of crotoxin isoforms representing combinations of variants of each subunit (Faure and Bon 1988). Faure et al. (1994) showed that the diversity of crotoxin isoforms is a result of posttranslational modifications occurring on a precursor. The isoforms differ in their

pharmacological and biochemical properties. In this respect two groups of the *Crotalus* HdPLA$_2$s exist: (a) complexes of high toxicity and low enzymatic activity and (b) crotoxins which are pharmacologically less potent and enzymatically more active (Faure et al. 1993). The more toxic isoforms are more stable and vice versa, and the less toxic HdPLA$_2$ are less stable. These differences suggest that the stability of the heterodimeric complex is important for the pharmacological action. Antibodies directed to the C-terminal part of ammodytoxin A from the venom of *Vipera ammodytes ammodytes* neutralize the toxicity of the PLA$_2$ subunit of crotoxin indicating that this part of the polypeptide chain is probably involved in the pharmacological activity (Čurin-Šerbec et al. 1994). However, investigations of the interactions of the two neurotoxins with their neuronal targets showed that crotoxin and ammodytoxin bind to different specific sites at presynaptic membranes from the electric organ of *Torpedo marmorata* (Križaj et al. 1997).

Structural Investigations on Crotoxin and Its Subunits: Structure-Function Relationships

There were many trials to obtain suitable for high-resolution X-ray studies crystals of crotoxin, but they were unsuccessful till 2011 when Faure et al. (2011) solved the crystal structure of the complex (CA2CBb). One of the reasons for the difficulties in crystallization could be the presence in the venom of a mixture of crotoxin isoforms, which is difficult to separate due to minimal sequence differences. The four CA isoforms and the four CH isoforms associate randomly producing different variants of heterodimers (Faure et al. 1988). The problem was solved after purification of a homogeneous isoform of the toxin (Faure et al. 2011, Faure and Saul 2012). Santos et al. (2007) described crystallization and preliminary X-ray investigations of crotoxin and its subunits. The crystal structure of a tetrameric complex formed by two dimers of the crotoxin PLA$_2$ isoforms (CB1 and CB2) was solved to 2.3 Å resolution (Marchi-Salvador et al. 2008). Analysis of the structural data showed that the oligomerization of the toxic PLA$_2$ may increase the neurotoxicity through a creation of new binding sites for the synaptic membrane. Also, the dimeric or tetrameric structures are more stable, and, as it was mentioned before, the more stable isoforms are more toxic. These assemblies exist in solution where the toxin realizes its biological action (Marchi-Salvador et al. 2008). Also, the substructures involved in the neurotoxicity, as C- and N-terminal portions of the PLA$_2$, the β-wing, and the calcium-binding loop, are exposed on the surface of the tetramer and dimer, i.e., they are accessible for interactions with the target sites of synaptic membranes. SAXS studies on the acidic subunit of crotoxin at pH 1.5 revealed a molecular shape of an oblate ellipsoid of revolution (Abrego et al. 1993).

Catalytic and Pharmacological Activities

Investigations to identify functional groups of crotoxin B with pharmacological activities showed that modification of histidyl or lysyl residues reduces the myotoxicity and the lethality (Soares et al. 2001). The correlation between the loss of catalytic ability after chemical modification of these residues and the strong reduction of lethal, myotoxic, and anticoagulant activities is explained by the

authors mentioned above as an indication that the enzymatic activity is also responsible for some pharmacological effects. The role of the PLA_2 catalytic activity, i.e., the phospholipid hydrolysis, in the epileptogenic properties of crotoxin was investigated by Dorandeu et al. (2002). However, the irreversible inactivation of the toxin by manoalide, leading to a neurotoxicity loss, did not permit to determine the role of the catalytic activity in toxicity. It is accepted that the phospholipid hydrolysis may participate in the toxicity exerted by PLA_2s (Dorandeu et al. 2002). At present, the significance of the catalytic activity for the toxicity is a topic in controversial debates, and more studies are necessary to confirm such a hypothesis.

The crystal structure of crotoxin (Faure et al. 2011) revealed differences between the catalytic site of the PLA_2 subunit and the corresponding region in the acidic component. The CB subunit possesses the conserved canonical PLA_2 catalytic and Ca^{2+}-binding sites. However, the CA component has no calcium-binding loop. The structure reveals residues important for the stability, toxicity, and reduced activity of the neurotoxic complex. The interactions of Trp31 and Trp70 from CBb with Asp99 and Asp89 of the β-chain of CA2 are important for the stability of the heterodimer (Faure et al. 2011).

The binding interface between the two subunits is important for the better understanding the mechanism of inhibition of the catalytic and anticoagulant activity of the basic PLA_2 by the acidic subunit. The CA component of crotoxin occludes the interfacial binding surface of the toxic enzyme precluding the interaction of functionally important residues with phospholipids (Faure et al. 2011; Faure and Saul 2012). It was supposed that the exposed region around Trp80 of CBb in the heterodimer is a binding site of crotoxin to its receptor (Faure et al. 2011).

Mojave Toxin

Mojave toxin (Mtx) is an $ncHdPLA_2$ complex isolated from the venom of *Crotalus scutulatus scutulatus* (Gopalakrishnakone et al. 1980). It is a presynaptic toxin exerting neurotoxicity/respiratory paralysis, myonecrosis, and hemorrhage (Gopalakrishnakone et al. 1980). Structurally and functionally Mtx is similar to crotoxin. The complete amino acid sequences of the basic (Aird et al. 1990) and acidic (Bieber et al. 1990) subunits were determined. The last component consists of three individual peptide chains held together by disulfide bonds derived from proteolytic processing of precursor (Aird et al. 1985). The basic subunit is weakly toxic and enzymatically active (John et al. 1994), as its counterpart from crotoxin. Both subunits show a high structural similarity to the respective components of the crotoxin complex. However, the Mtx acidic subunit differs from the respective subunit of vipoxin, an $ncHdPLA_2$ complex from the European snake *Vipera ammodytes meridionalis* (Aleksiev and Tchorbanov 1976). The genomic sequences encoding the two subunits of Mtx revealed a high degree of nucleotide identity (John et al. 1994). Most probably, the genes coding for the two components of the complex arose from a common ancestor.

Isoforms of the Mojave toxin were found in the venom of *Crotalus helleri* (Southern Pacific rattlesnake). The sequence identities of the acidic and basic subunits of the isoform to the components of the crotoxin complex are 85 % and 97 %, respectively (French et al. 2004). The crotoxin inhibitor from the *Crotalus* serum induces dissociation of the Mtx complex and binds to the basic PLA_2 subunit, but does not react with the acidic component (Faure et al. 2000).

Non-Covalent Heterodimeric Phospholipase A_2 Complexes from Venomous Snakes Inhabiting Asia: Viperotoxin F from *Vipera russelli formosensis*

Two PLA_2s, RV-4 and RV-7, were isolated from the venom of the Taiwan viper (*Vipera russelli formosensis*) (Wang et al. 1992). The two oppositely charged proteins, with 65 % sequence identity, form a complex named viperotoxin F (Perbandt et al. 2003). Like in vipoxin, one of the subunits, RV-4, is a neurotoxic PLA_2, and the other, RV-7, is acidic and nontoxic. The two subunits of viperotoxin F show 92 % sequence identity to their counterparts in vipoxin. This is surprising because the two snakes inhabit widely separated regions of the world, Europe and Asia, and there is no possibility for interbreeding. *Vipera a. formosensis* inhabits Taiwan which is an isolated island. The amino acid sequences of the toxin from *Vipera r. formosensis* and vipoxin are very similar, but the crystal structures show conformational differences in the regions responsible for the toxicity and anticoagulant activity (Perbandt et al. 2003).

Explanation of the Viperotoxin F Complex (RV-4/RV-7) Stability

The crystal structure of viperotoxin F was solved and refined at 1.9 Å (Perbandt et al. 2003) in order to compare its structural and functional properties with those of vipoxin. The neurotoxic complex from the *Vipera russelli formosensis* venom is stabilized through ionic, hydrophobic, and hydrogen-bonding interactions as it was observed in the crystallographic model of vipoxin (Banumathi et al. 2001). Again, in the absence of calcium, a salt bridge between Asp48 of RV-4 and Lys60 (Asp49 and Lys69) of RV-7 plays an important role for the stabilization of the heterodimer. The structure of viperotoxin F revealed substantial changes in the interfacial adsorption surfaces (IASs) of the two subunits which is the major reason for the loss of hydrolytic activity and toxicity of RV-7. Secreted PLA2s catalyze the substrate hydrolysis at a lipid-water interface. The IAS of RV-4 contains hydrophobic and basic residues, which are important for the adsorption of the enzyme on the negatively charged membrane surface. The model shows that most of these residues, including four basic amino acids, are substituted in RV-7, some of them with acidic residues. As a result, the positive charge of IAS in the catalytically active subunit is changed to a negative one in the acidic component. This is probably the major reason for the very low activity of RV-7 towards micellar and monomeric substrates.

The structure of viperotoxin F allows a prediction of the lack or very low anticoagulant activity of both subunits. The anticoagulant activity of group II basic PLA_2s is connected with the region composed by residues 53–77 and especially with a cationic surface within this site formed by four positively charged residues. This region binds anionic phospholipids when the enzyme exerts anticoagulant effects (Kini and Evans 1989; Mounier et al. 1998). Only two basic residues (Arg55 and Lys60) are preserved in the putative anticoagulant site of RV-4 (Arg56 and Lys69) and one positively charged functional group (Lys60) in the respective region of RV-7, which changes the polarity of these sites. Also, the glutamic acid in position 53, which is important for the anticoagulant activity (Carredano et al. 1998), is substituted by glycine in both subunits of viperotoxin F. Comparison of the viperotoxin F subunits revealed that one of the reasons for the lack of neurotoxicity in RV-7 could be the significant change of surface electrostatic charges in the regions interacting with presynaptic membranes. Thus, the β-wing (residues 64–78, or residues 74–85) and the C-terminal parts (residues 109–114 or residues 119–125) of the two components of the complex have opposite charges, positive in RV-4 and negative in RV-7. It was shown that a mutant of ammodytoxin A, with a changed charge in the C-terminal region from positive to negative, is 30-fold less lethal and its binding affinity for neuronal receptors decreased by a factor of five (Prijatelj et al. 2000). However, the neurotoxicity of the mutated toxin was not totally abolished.

The putative anticoagulant site in the vipoxin PLA_2 contains positive charges, suitable for binding phospholipids, and anticoagulant effects can be expected. The structure of the respective region in RV-4 is not suitable for such interactions. The different targets of the physiological attack, postsynaptic membranes for vipoxin and presynaptic membranes for viperotoxin F, can be explained by the observed changes in conformation and the location of electrostatic charges.

Other Snake Venoms: Non-Covalent Heterodimeric Phospholipase A_2 Complexes

A heterodimeric neurotoxin consisting of a basic and weakly toxic PLA_2 non-covalently associated with an acidic protein, devoid of enzymatic activity, was isolated from the venom of the Field's hornet viper *Pseudocerastes fieldi* (Tsai et al. 1983). The toxin causes neuromuscular blockade of a chick biventer cervicis by acting on presynaptic sites. The non-catalytic component potentiates the toxicity of the basic subunit (Francis et al. 1995).

An unusual two-component toxin was also found in the venom of *Vipera palaestinae* (Križaj et al. 1996). The components, an acidic PLA_2 and a basic protein, are not toxic when separated. However, their equimolar mixture is lethal as a consequence of the synergistic action of the two proteins (Križaj et al. 1996). Sequence analysis of the complete *V. palaestinae* PLA_2 genes showed that a positive Darwinian selection is limited to the third exon, in contrast to other viperid PLA_2 genes (Kordiš et al. 1998).

The crystal structure of a PLA_2 heterodimer from the *Naja naja sagittifera* venom was determined at 2.3 Å (Jabeen et al. 2006). The toxin is a complex between a group I PLA_2 and a PLA_2-like protein containing two extra cysteines. The conformations of the calcium-binding loops of the two subunits are different, and a zinc ion plays an important role for the stabilization of the dimer (Jabeen et al. 2006).

Functional Importance of the Nontoxic Components of Non-Covalent Heterodimeric Phospholipase A_2s

The acidic and nontoxic subunits of the ncHdPLA_2s show similarities but also some differences in their function as components of neurotoxic complexes. Thus, it was supposed that the PLA_2-like proteins of the three heterodimeric toxins, crotoxin, vipoxin, and viperotoxin F, target the toxic and enzymatically active subunit to the "correct" substrate on the membrane surface during the pharmacological attack (Georgieva et al. 2004a; Bon et al. 1979; Wang et al. 1992). Thus, nonspecific binding of the neurotoxic PLA_2 to phospholipids, which are not important for the neurotoxicity, is avoided. Probably, the mechanism proposed for crotoxin (Delot and Bon 1993; Bon et al. 1979) is also valid for the other snake venom heterodimeric PLA_2 complexes: the interactions with the synaptic membranes started with the formation of a ternary complex between the acidic subunit, the toxic PLA2, and the membrane acceptor. After dissociation of the complex, the toxic enzyme binds to a specific membrane receptor, whereas the nontoxic component remains in solution. The PLA_2-like proteins inhibit the enzymatic activities of the three heterodimeric neurotoxins (Aleksiev and Tchorbanov 1976; Wang et al. 1992; Rübsamen et al. 1971). In the same time, the acidic subunits, with a high sequence homology, differ in their catalytic activities and can exert distinct effects on the toxicity in the different ncHdPLA_2s. RV-7 of viperotoxin F possesses a low enzymatic activity (Wang et al. 1992) preserving the active site His48 which participates in the catalytic triad together with Asp99 and water molecule. The respective counterparts of this subunit in crotoxin, vipoxin, and in the neurotoxic complex from the *Vipera a. zinnikeri* venom are catalytically inactive, in the last two cases His48 being substituted by Gln48 (Komori et al. 1996; Mancheva et al. 1987). The acidic polypeptide chains of crotoxin, viperotoxin F, and of the ncHdPLA_2 from the venom of *Vipera a. zinnikeri* potentiate the neurotoxicity of the basic PLA_2 (Komori et al. 1996; Choumet et al. 1993; Wang et al. 1992), whereas the PLA_2-like protein of the vipoxin complex considerably decreases the neurotoxicity of the PLA_2 subunit (Aleksiev and Tchorbanov 1976). Also, this component of the vipoxin complex plays an important role for the structural stabilization of the toxic enzyme and for the long-term preservation of toxicity (Aleksiev and Tchorbanov 1976). Crotoxin and viperotoxin F are presynaptic toxins, while vipoxin and vaspin are postsynaptic (Jan et al. 2002; Aleksiev and Tchorbanov 1976; Aird et al. 1985; Wang et al. 1992).

Comparison of the Primary Structures of Non-Covalent Heterodimeric Phospholipase A_2s from Venomous Snakes Inhabiting Europe, South America, and Asia

Figure 3 shows comparison of the amino acid sequences of ncHdPLA$_2$ acidic and basic subunits from the venoms of Viperinae (*Vipera ammodytes meridionalis* and *Vipera russelli formosensis*) and Crotalinae (*Crotalus durissus terrificus* and *Crotalus scutulatus scutulatus*) snakes inhabiting three continents, Europe, South America, and Asia. The extremely high homology of the primary structures of

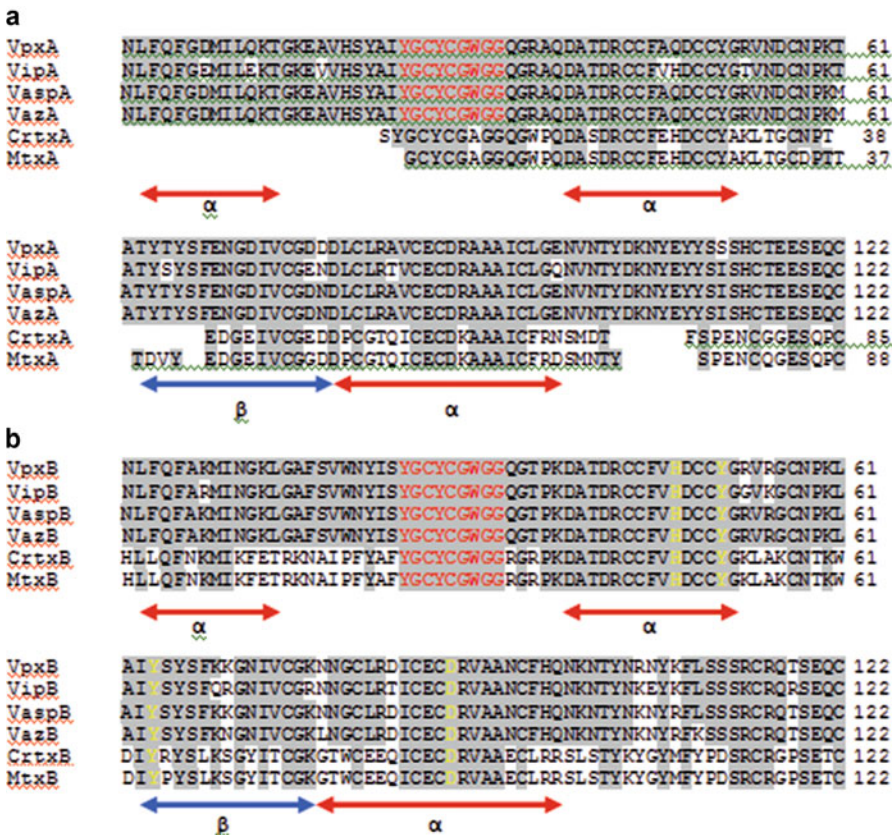

Fig. 3 Comparison of the amino acid sequences of the acidic (**a**) and basic (**b**) subunits of vipoxin (VpxA and VpxB, respectively; gi 16974941 and gi 16974940), viperotoxin F (VipA and Vip B, respectively; gi 64453), vaspin (VaspA and VaspB, respectively; Jan et al. 2002), PLA2-I neurotoxin from *Vipera aspis zinnikeri* (VazA and VazB, respectively, gi 1709547 and gi 1709548), crotoxin (CrtxA and CrtxB, respectively, Bouchier et al. 1991), and Mojave toxin (MtxA and MtxB, respectively; John et al. 1994). α-Helices and β-structures are labelled. The active site residues His47, Tyr51, Tyr64, and Asp89 are colored in *yellow*, and the calcium-binding loop (residues 24–32) in the basic subunits is colored in *red*. Putative calcium-binding sites in the acidic subunits are also colored in *red*. α-Helices are indicated by *red arrows* and β-structures by *blue arrows*

heterodimeric neurotoxins produced by snakes inhabiting widely separated regions of the world is impressive. All the basic subunits have the same length of the polypeptide chains, 122 residues, and show in average 57 % sequence identity characteristic for closely related proteins. The identity between the toxic components of vipoxin and viperotoxin F is even 92 %. The respective chains of crotoxin and Mojave toxin show no sequence differences. The acidic chains of vipoxin, viperotoxin F, vaspin, and the toxin from *Vipera a. zinnikeri* have the same length and show an average identity of 82 %. However, the acidic components of the ncHdPLA$_2$ have shorter chains (Fig. 3). A comparison of the crotoxin and vipoxin sequences revealed a preservation of the core PLA$_2$-like structure of the crotoxin subunit A. Analysis of cDNAs encoding crotapotin showed that the subunit A of crotoxin is generated from a PLA$_2$-like precursor by the removal of only three peptides (Bouchier et al. 1991). The disulfide bridges are preserved, and the core of the molecule is unchanged. This is evident from the organization of the respective cDNA encoding a signal peptide identical to that found for the precursor of the basic PLA$_2$ subunit, followed by a sequence homologous for PLA2s and including three regions corresponding to the three peptides of the CA subunit (Bouchier et al. 1991). It is evident also that the 22 N-terminal residues and two other smaller segments of the vipoxin acidic chain are not present in the polypeptide chain of the crotoxin CA subunit (Fig. 3). This means a lack of the N-terminal α-helix, typical for the group II PLA$_2$ (residues 2–14 according to the numeration of the vipoxin acidic subunit), which is a part of the substrate-binding channel. This fact can explain the lack of the catalytic efficiency of crotapotin. The important ligand of the anion-binding site in the vipoxin acidic subunit, Arg42 (sequence numbering based on the homology to the acidic chain), is preserved in CA as Arg20. A comparison of the sequences of the crotoxin subunit CA and the basic vipoxin PLA$_2$ demonstrated no homology in the C-terminal part of the polypeptide chain which is responsible for the neurotoxicity. This explains the missing toxicity of crotapotin. A comparison of the remaining sequences of the crotoxin and vipoxin acidic chains shows 66 % homology, which indicates closely related proteins. The sequences of important secondary structure elements as the two α-helices (res. 39–53 and res. 79–99, numbering based on sequence of the vipoxin acidic chain) and the β-wing (res. 64–78, numbering based on the vipoxin acidic chain) are preserved in crotapotin. The degree of sequence homology for the two α-helices is 80 % and 62 %, respectively; the homology for the β-wing regions is 87 %. The calcium-binding site is also preserved in the crotoxin subunit CA with only one substitution, Trp30-Ala8. Crotapotin binds one Ca^{2+} per mole (Radvanyi et al. 1989), which means that the calcium-binding loop is functioning.

A comparison of the Mojave toxin acidic subunit sequence with those of vipoxin or of the closely related viperotoxin F leads to analogical conclusions.

A comparison of the three-dimensional structures of the basic and acidic subunits from the same neurotoxic heterodimeric complexes or of the subunits from different ncHdPLA$_2$s shows preservation of the secondary structure elements. The Cα positions of the two polypeptide chains of vipoxin were superimposed with an r.m.s. difference of 1.5 Å and a maximum displacement of 5.4 Å (Banumathi et al. 2001).

The basic subunits of vipoxin and viperotoxin F were superimposed with Cα r.m.s. deviations of 0.35 Å and a maximum displacement of 2.69 Å, while the respective values for the acidic polypeptide chains were 0.47 and 1.82 Å (Perbandt et al. 2003).

Neurotoxic Action of Non-Covalent Heterodimeric Phospholipase A_2s

Neurotoxicity is the main pharmacological and the most dangerous effect of ncHdPLA_2. The neurotoxic action of these complexes will be considered in the case of crotoxin. The heterodimeric phospholipase A_2 complexes are active at both, pre- and postsynaptic levels. Most of them are β-neurotoxins, like crotoxin, Mojave toxin, and viperotoxin F (Bon 1997; Gopalakrishnakone et al. 1980; Wang et al. 1992) which bind to the presynaptic membranes blocking the release of acetylcholine and the transmission across the neuromuscular junctions of the breathing muscles (Changeux et al. 1970). They exert lethal effects through paralysis of the respiration. α-Neurotoxins act at postsynaptic level preventing the binding of acetylcholine to its receptor (Bon 1997). Vipoxin and vaspin are examples of postsynaptic toxins (Jan et al. 2002; Aleksiev and Tchorbanov 1976). Postsynaptic effects were observed also after experiments with crotoxin (Bon et al. 1979). The postsynaptic action was examined on isolated electroplaque from *Electrophorus electricus* and on acetylcholine receptor-rich membranes from *Torpedo marmorata* (Bon et al. 1979). The toxin affects the response of these preparations to cholinergic agonists. Crotoxin stabilizes the acetylcholine receptor in an inactive form. The authors mentioned above considered the possibility that the postsynaptic action of the toxin is connected with the PLA_2 activity. When applied alone, the isolated PLA_2 component of the complex is absorbed by nonspecific sites, while in the presence of the acidic subunit, it binds to specific sites in the membranes (Bon et al. 1979). In this way the nontoxic subunit prevents unspecific binding of the toxic one. Bon et al. (1979) supposed that the low-affinity sites are membrane phospholipids and the high-affinity sites are membrane receptor proteins.

β-neurotoxins were classified into three subclasses: (a) single chain neurotoxins, (b) covalent complexes of PLA_2 enzyme and a subunit homologous to Kunitz-type protease inhibitor (β-bungarotoxins), and (c) non-covalent heterodimeric PLA_2-like crotoxin (Choumet et al. 1992). There are differences in the mechanism of action of the β-neurotoxins from each subclass. For example, β-bungarotoxins bind to a voltage-sensitive potassium channel through the nonenzymatic subunit while crotoxin dissociates in the presence of membranes, the toxic PLA_2 binds to its receptor, and the enzymatically inactive component remains in solution (Choumet et al. 1992 and references therein). β-neurotoxins bind to specific protein acceptors. Three steps in the mechanism of their action were identified: an inhibition of the acetylcholine release, enhancement of its secretion, and irreversible blocking of the neurotransmission (Faure et al. 2003 and references therein). The pathway

of the crotoxin interaction with membranes involves the formation of a ternary complex between the subunits of the complex and the acceptor before the release of the nontoxic chain in the solution (Delot and Bon 1993). The degree of toxicity and the stability of the crotoxin complex are important for the neurotoxicity: more toxic isoforms are more efficient than the weakly toxic complexes, and a stronger association between the subunits leads to faster blockade of the neuromuscular transition and to a higher lethality (Faure et al. 1993).

Conclusion and Future Directions

A number of neurotoxic ncHdPLA$_2$s composed of basic and acidic subunits have been characterized from the venoms of Crotalinae and Viperinae snakes. The diversity of isoforms is due to frequent duplication events. The basic components are toxic and induce a number of pharmacological effects such as neurotoxicity, myotoxicity, cardiotoxicity, antiplatelet, convulsive, hemorrhagic, and hemolytic effects. Unlike their basic counterparts, the acidic subunits are neither toxic nor enzymatically active (or possess very low catalytic activity). The presence of non-neurotoxic and catalytically inactive polypeptide chains and their effects on the enzymatic activity and toxicity of the pharmacologically active PLA$_2$ raised several questions, which have not been answered so far. The main question is: what is the necessity of the second component? Only to avoid nonspecific binding to other targets on the plasma membrane? However, many toxic snake venom PLA$_2$s are effective in a monomeric form. Why in some cases (crotoxin, viperotoxin F, and PLA$_2$-I) the acidic chain potentiates the toxicity but in others (vipoxin) it reduces the pharmacological activity? Very often the nonenzymatic subunit of HdPLA$_2$s is defined as a “nontoxic component” of the complex. Usually, this means that it is not neurotoxic, and no data about other possible pharmacological activities are available. In many cases the investigations on this “PLA2-like protein” are limited to neurological effects. It is important to clarify if the nonenzymatic component influences or shares other types of activity, besides neurotoxicity, and further studies are required to better understand its functional role. The strong neurotoxicity of an equimolar mixture of two different PLA$_2$s, which are not toxic at all when separated, is intriguing, and the structural reasons for this phenomenon are still unclear. The presence of almost identical HdPLA2s in the venoms of snakes geographically separated in different parts of the world remains a puzzle as well. These snakes are not in contact, and interbreeding is not possible.

Cross-References

► Snake Venom Phospholipase A$_2$: Evolution and Diversity

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Snake Venom Phospholipase A_2: Evolution and Diversity

13

Inn-Ho Tsai

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Abstract

Snake venom phospholipases A_2 (vPLA$_2$s) most likely originated from more than one body gene and have undergone multiple convergent and divergent evolutionary events in the snakes' adaptation and survival. The evolution of vPLA$_2$s is inextricably linked to snake phylogeography, ecology, and natural history. It has been shown that both mammalian secretory PLA$_2$s and snake vPLA$_2$s exist as multiple isoforms. The vPLA$_2$ genes undergo duplication, accelerated evolution, and positive Darwinian selection, and so the structures, functions, and expression levels of the isoforms vary greatly. The successful applications of advanced liquid chromatography and mass spectrometry have promoted vPLA$_2$ isolation, characterization, and related research significantly. Major pharmacological effects of vPLA$_2$ isoforms have been found to be either antiplatelet, anticoagulant, myotoxic, neurotoxic, edematous, hypotensive, or any combination of the above. Some vPLA$_2$s have reduced or lost their catalytic activities to exert special target-binding, chaperoning, or membrane-disrupting functions. Structural changes at their interface recognition sites and specific mutations at the active sites affect both catalytic-dependent and non-catalytic-dependent toxicities

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of vPLA$_2$s. The *N*-terminal and the *C*-terminal regions of vPLA$_2$ usually contain special features highlighting the molecular evolution of each vPLA$_2$ subtype. Moreover, synergisms between vPLA$_2$s and other venom components may result in prominent hemorrhagic, neurotoxic, hypotensive, edematous, or antibacterial effects after envenoming. How vPLA$_2$s and snake venom have diversified and evolved to fulfill the role of an efficient and powerful arsenal toward potential preys and enemies is demonstrated through the ample updated examples.

Introduction

Both venom and front fangs are key evolutionary innovations in the diversification of advanced snakes (Colubroidea). The evolution of the venom toxins predates the evolution of Viperidae and Elapidae, and snake venom appears to emerge at the base of the phylogenetic tree of advanced snakes and probably developed over a period of more than 60 million years (Fry et al. 2008, 2012). The 14-kDa secretory phospholipases A$_2$ (sPLA$_2$s; EC 3.1.1.4) are present in the body fluid of all vertebrates and are one of the major toxin families in snake venoms. These enzymes catalyze the hydrolysis of the sn-2 fatty acyl bond of glycerophospholipids and release lysophospholipids and free fatty acids. The reaction products may further participate in a diversity of toxic effects or affect the local physiological conditions (Neidlinger et al. 2006; Rigoni et al. 2007). Like several other venom toxin families, the molecular evolution of the venom phospholipase A$_2$ (vPLA$_2$) families evolve via a process by which several genes encoding for body secretory proteins are duplicated, recombined, mutated, and selectively expressed in the venom glands. Functionally important vPLA$_2$ subtypes are reinforced through accelerated evolution and selection (Ikeda et al. 2010; Lynch 2007; Ogawa et al. 1996). The vPLA$_2$ genes of Elapidae are derived from snake body group I (GI-A/B) sPLA$_2$ genes, while those of Viperidae are from snake body group II (GII-A/E) sPLA$_2$ genes. Some of the vPLA$_2$ genes may be replaced by others and become useless pseudogenes. The expression of vPLA$_2$s can be regulated dynamically and affected by the prey-ecology of the snakes (Gibbs and Rossiter 2008).

Because of their abundance, small molecular weight, and ease of isolation, many vPLA$_2$s have been purified and studied extensively. The complementary DNAs (cDNAs) encoding vPLA$_2$s usually share highly conserved untranslated regions and signal peptides, so their cDNAs can be sequenced and amplified based on the sequences of known homologous vPLA$_2$ genes; the amino acid sequences can then be deduced from sequencing the cDNA clones or obtained from transcriptome analyses. Notably, vPLA$_2$s containing six or seven disulfide bridges are rather thermally stable and soluble under acidic conditions and in the presence of water-miscible organic solvent at room temperature, so it is not difficult to purify them using reversed-phase high-performance liquid chromatography without denaturation after the solvent removal. Liquid chromatography combined with mass spectrometry has greatly facilitated the proteomic analyses of many vPLA$_2$s from a wide array of venom species. Ample sequence data of the vPLA$_2$s have

demonstrated great intrageneric and intraspecific diversities and geographic variations of this toxin family. Recent discovery on the molecular diversities of $sPLA_2$s in humans and other mammals (Dennis et al. 2011) also shed light on the evolutionary variations of $vPLA_2$s. A comprehensive book related to the molecular biology, biochemistry, and mechanism of $vPLA_2$s has been published about two decades ago (Kini 1997). The present review aimed to update and discuss some interesting discoveries and insights related with the evolution, variations, and functional subtypes of $vPLA_2$s from front-fanged venomous snakes.

Snake Venom Phospholipase A_2, Evolution and Diversity

The $vPLA_2$s are Derived from Body Secretory PLA_2s

Ten members or groups of the $sPLA_2$ family (groups IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XII) have been identified in mammals and other vertebrates; these are numbered and grouped according to their pattern of disulfide bonds and in order of their discovery (Dennis et al. 2011). In humans and mice, the genes for groups GIIA, GIIC, GIID, GIIE, and GIIF are linked within the same chromosome and probably derived from gene duplication, while genes encoding GIB and GX map to another two chromosomes. Some mammalian GII-$sPLA_2$ genes are prone to undergo duplications. For example, five duplications of bovine GIID genes lead to various isoforms while a single gene copy of GIID is present in the human and rodent genomes (Golik et al. 2006). The bovine GIID isoforms are expressed in the mammary gland and possibly participate in the innate immune response. The vertebrate $sPLA_2$s have been found to be implicated in an array of physiological and pathological activities with specific tissue distribution and under dynamic regulations. For example, human and mouse GIIA-PLA_2s are highly basic and efficient bactericidal enzymes, whereas GIB-PLA_2 is a digestive enzyme for lipid micelles.

All $sPLA_2$s in groups GI, GII, and GX have very similar tertiary structures that contain three long α-helices, two β-sheets referred to as β-wings, and a conserved Ca^{2+}-binding loop. Although their sequence identity level is low and *C*-terminal region extension may be varied, they share a common protein fold and feature the same catalytic His48/Asp93 dyad. GIA and GIB $sPLA_2$s with 118–124 residues are found to be selectively expressed in pancreas and venom glands of elapid snakes and some non-front-fanged snakes, while GII-$sPLA_2$s with 121–123 residues are expressed in venom glands of viperid and crotalid snakes. Most of the $vPLA_2$s contain six or seven conserved disulfide bridges; however, GI-$vPLA_2$s differ from GII-$vPLA_2$s by substituting the Cys11-Cys77 for another disulfide bond and lacking the 5–7 amino acid residues C-terminal extension in GII-$vPLA_2$s. GIA-$vPLA_2$s are in general more reactive toward membrane substrates than GIB-$vPLA_2$s; an extra "pancreatic loop" at positions 62–66 of GIB distinguishes it from GIA. Remarkably, the mammalian GIIA and GIIE are structurally similar to each other, and it was suggested that the GIIA-$sPLA_2$s might be derived from duplication of GIIE-$sPLA_2$s (Yamaguchi et al. 2014). Interestingly, the amino acid

sequences of some $vPLA_2$s are more similar to that of human GIIE than to that of human GIIA. Recently, GIIE-PLA_2 messengers have been detected in the maxillary venom glands of *Dispholidus typus* and *Leioheterodon madagascarensis* (Fry et al. 2012) and in the venom glands of *Protobothrops flavoviridis* (Yamaguchi et al. 2014). However, the corresponding proteins have not been detected in any snake venom.

Catalytic Mechanism and Active Site of $vPLA_2$s

The 3D structures of many $vPLA_2$s are available in protein data banks, and the reaction mechanisms of not only $vPLA_2$s but also the model pancreatic GIB $sPLA_2$s have been studied extensively (Dennis et al. 2011; Kini 1997). Most $sPLA_2$ enzymes act on cell membranes or a water-lipid interface. They display a characteristic increase in activity when a substrate is switched from monomeric to higher-ordered lipid aggregates, which is known as interfacial activation (Cho et al. 1988). The catalytic site of $sPLA_2$s resembles that of serine proteases but with Ca^{2+}-linked water molecules playing the nucleophilic role, and both the hydrolase families use imidazole of His residue (linked with an Asp carboxyl group) as general base to facilitate the proton transfer and tetrahedron formation during the transition state of the hydrolysis. Thus, three conserve active-site residues or regions (His48, Asp49, and Ca^{2+}-binding loop), and the conserved Cys residues highlight the sequence conservation of the $vPLA_2$ superfamily. The *N*-terminal regions 1–13 of $vPLA_2$s form an alpha-helix, and residues 2, 6, and 7 and other regions (e.g., residues 19–22, 31, 63–68, and 106–112) are involved in the water-lipid interface interactions. The catalytic Ca^{2+} is coordinated by the β-carboxyl group of Asp^{49} and backbone carbonyl groups of Tyr28, Gly30, and Gly32 in the Ca^{2+}-binding loop ($Y^{25}GCYCGXGG^{33}$). Mutations at this loop in a $vPLA_2$ usually signify inactivation or reduction of its catalytic activity. It was also shown that micelle binding was accompanied by the conformational change of the *N*-terminal region of GII-PLA_2 (Qin et al. 2005), while the *N*-terminal region of GI-PLA_2s contained the disulfide 11–77 and adopted more fixed conformations upon binding the phospholipid micelles.

Additionally, the mutations at key catalytic residues (His48 and Asp49) or the charge and hydrophobicity of the substrate and interface recognition sites (e.g., Leu2, Phe5, Trp31) serve as clues to explain variations of the catalytic power and specificity of $vPLA_2$s. As long as the catalytic activities are not required for some of the $vPLA_2$ functions, their catalytic sites or interfacial binding residues could be mutated without affecting the protein folding or gross conformation, e.g., the Gln48, Asn49, or Pro31 substitutions may reduce the $vPLA_2$ catalytic activities (Tsai 2007). It is interesting to note that some vertebrate GIID-$sPLA_2$s also contain similar inactivating mutations in their Ca^{2+}-binding loops; e.g., the GIID-PLA_2s of *Alligator mississippiensis* (accession No. XP_006275522) contains the Asn49 mutation, while those from elephant (*Loxodonta africana*), polar bear (*Ursus maritimus*), monkey (*Macaca fascicularis* XP_005544618), and baboon (*Papio anubis* XP_003891301) contain Pro31 substitutions.

Phylogenetic Analyses of Snake Species and their $vPLA_2s$

The evolutions of $vPLA_2s$ are inextricably linked to snake phylogeography, natural history, ecology, and diets (Gibbs and Rossiter 2008; Gubenšek and Kordiš 1997; Lynch 2007). Using the intron nucleotide sequence of the GII-$vPLA_2s$ as the dataset generated a molecular phylogeny tree according to viperid taxonomy, but using the protein sequences of GII-$vPLA_2s$ resulted in the clustering of different paralogous subtypes in the tree, supporting that the accelerated evolution has occurred in the protein-coding regions of the GII-$vPLA_2s$ genes to generate different functional subtypes (Gubenšek and Kordiš 1997; Ogawa et al. 1996). In contrast, the GI-$vPLA_2s$ tree was consistent with the phylogeographic relationships of the elapid species (Tsai 1997). Thus far, the amino acid sequences of about 300 $vPLA_2s$ have been solved and deposited in databanks. Comprehensive phylogeny trees based on all the GII-$vPLA_2$ sequences further confirmed that there are two Viperinae clusters and four or five Crotalinae clusters of $vPLA_2s$; each of the clusters appears to represent a distinct functional or structural subtype (Lynch 2007; Malhotra et al. 2013; Mebs et al. 2006). Three-dimensional structural models of the four subtypes of crotalid $vPLA_2s$ have been built to show different functional epitopes and variations at the surface and interface residues of each subtype (Malhotra et al. 2013). Moreover, molecular phylogenetic analyses on GII-$vPLA_2$ could be very useful for function prediction or subtyping of novel $vPLA_2s$, e.g., phylogenetic analysis and sequence alignment have aided in the identification and functional assay of a new PLA_2 neurotoxin (Yang et al. 2015).

Like human GI- and GIIA/B-$sPLA_2s$, most of the $vPLA_2s$ have Asn, Ser as the *N*-terminal residue, while a few Crotalid GII-$vPLA_2s$ have His^1, Asp^1, and Gly^1 (Tsai et al. 2004b). It was noticed that the four Ser^1-PLA_2s of *Vipera a. ammodytes* venoms contain a transposon in one of the introns in contrast to the corresponding intron of the Asn^1-PLA_2s in the same venom (Gubenšek and Kordiš 1997), suggesting possibly different evolutionary history between the Viperinae Ser^1 and the Asn^1 $vPLA_2$ subtypes. Ammodytoxins from *V. a. ammodytes* venom contain Ser^1 while caudoxin from *Bitis caudalis* venom contains Asn^1; the low sequence homology between both Viperinae neurotoxins perhaps implies a convergent evolution of their neurotoxicity. Among the crotalid $vPLA_2$ isoforms, the amino acid residues 6, 49, and the pI value empirically serve as convenient references for the classification of functional subtypes or paralogues. Five or six subtypes have been proposed for the pit viper $vPLA_2s$, i.e., the K49, N6D49, acidic R6, basic R6, and E6D49-PLA_2 types (Tsai et al. 2004a). The separated evolution of these subtypes was supported by phylogenetic analyses based on more than 60 selected pit viper $vPLA_2$ sequences, and the E6-type was found to be rooted within the basic R6-type (Mebs et al. 2006). The crotalid $vPLA_2s$ with Glu, Asn, Arg, or Gly/Trp at position 6 (i.e., the E6, N6, R6, or K49 subtypes) represent the acidic antiplatelet, the neurtotoxic or myotoxic, the basic anticoagulant, and the noncatalytic membrane-disrupting subtypes of the $vPLA_2$ toxins, respectively (Tsai 1997; Tsai et al. 2004a). This simplified rule possibly is not surprising given the fact that residue 6 of GII-$vPLA_2$ is one of the most important interface recognition sites and the residues

1–7 and 19–22 contain important structural determinants for their functions (Qin et al. 2005; Tsai et al. 2011b). However, exceptions to this rule of relating residues 6 to the functional subtypes do exist; e.g., the monomeric E6-vPLA$_2$s isolated from North American *Agkistrodon* venoms are myotoxic and basic (Komori et al. 2002) in contrast to the dimeric and acidic E6-vPLA$_2$s in other pit viper venom; while most of the basic Arg6-vPLA$_2$s of Asian pit vipers are anticoagulants with low enzymatic activities (Tsai et al. 2004b; Wang et al. 2005), one of the Arg6-vPLA$_2$s isolated from *Protobothrops mucrosquamatus* and *Protobothrops jerdonii* venom is acidic, catalytically active, and plays antiplatelet roles (Tsai et al. 2004a).

Expression Level and Diversity of Isoforms

The expression level of vPLA$_2$ isoforms varied greatly according to the species, population and ecology, and age of the snake, among other factors. High vPLA$_2$ contents (48–73 % of total venom proteins) have been reported for a wide variety of species including *Bungarus fasciatus* (Tsai et al. 2007a), *Pseudachis australis* (Takasaki et al. 1990), *Micrurus nigrocinctus* (Fernández et al. 2011), *Daboia russelii* and *Daboia siamensis* (Tsai et al. 2007b), *Zhaoermia mangshanensis* (Mebs et al. 2006), and southern population of *Crotalus duressis terrificus* (Boldrini-França et al. 2010). In contrast, vPLA$_2$ enzymes could be below 3 ~ 5 % of the total venom proteins, or absent in the venom of *Dendroaspis, Causus*, certain Australian elapids (Jackson et al. 2013), and some *Crotalus horridus horridus* populations (Wang et al. 2010). The numbers of vPLA$_2$ isoforms that could be isolated from most venom species are usually 1–4 but could be 8 or more in the venom of certain elapids, e.g., *B. fasciatus* (Tsai et al. 2007a) and *P. australis* (Takasaki et al. 1990). Moreover, contents and proportions of the vPLA$_2$ isoforms usually vary intragenerically and intraspecifically, according to the age, natural environment, or prey ecology of the snake (Gibbs and Rossiter 2008; Tsai et al. 2004b). A strong relationship between venom composition and snake feeding adaptations may explain for the low vPLA$_2$ isoform number of sea snake venom, and the evolution trend appears to be adopted by different taxa in response to either loss or gain of neurotoxicity (Calvete et al. 2012).

The sequences of vPLA$_2$s from the same snake genus could be more similar than those from a different genus, as usually expected. However, species under the same genus can be rather diversified genetically, e.g., the less toxic vPLA$_2$s in *Bungarus fasciatus* venom are very distinct from the vPLA$_2$s in *Bungarus multicinctus* (with high content of potent β-bungarotoxins) (Tsai et al. 2007a). Furthermore, venoms of the same or closely related species could show dichotomy or bimorphism in their venom profiles and pharmacological effects. For example, the European *Vipera* and the Asian *Daboia* are phylogenetically related genera, and their venoms contain either of the two vPLA$_2$s subtypes (Gao et al. 2009; Jan et al. 2007). The neurotoxic vPLA$_2$s in the venom of *D. siamensis*, Pakistan *D. russelii* (Tsai et al. 2007b), *Vipera a. montandoni*, *Vipera aspis,* and *Vipera nikolskii* (Gao et al. 2009) are 1:1-heterodimeric complexes, while those in *D. russelii* (Sri Lanka and southern India),

V. a. ammodytes, *Vipera berus,* and *V. u. renardi* (Tsai et al. 2011a) are two or three similar and monomeric isoforms. Paradoxical molecular evolution of $vPLA_2$s has also been observed in some rattlesnake species. The entire $vPLA_2$ family was completely replaced in the *C. h. horridus* neurotoxic-type venom as compared to the hemorrhagic-type venom of the same species, possibly through intergeneric hybridization of rattlesnakes (Rokyta et al. 2015). Moreover, the venoms of neonates or juveniles of some *Crotalus* are more neurotoxic than those of their adults and contain distinct $vPLA_2$s; this ontological variation possibly reflects a phenomenon of paedomorphism (Mackessy et al. 2003). Remarkably, the numbers of $vPLA_2$ isoforms in the transcriptome (including those encoded by the pseudogenes) can be higher than those detected in the proteome or isolated from the venom, and their expression can be regulated epigenetically by special venom gland microRNA (Durban et al. 2013) and specific transcriptional factors (Nakamura et al. 2014).

The $vPLA_2$ isoform profile appears to be characteristic for each venom species, in spite of the fact that variations in the proportion of $vPLA_2$s are frequently observed. Important geographic variations in $vPLA_2$ isoforms have been unveiled by proteomic analyses of the venom from individual specimens or different populations of the same species, e.g., king cobra (Chang et al. 2013), Taipan (Chaisakul et al. 2014), *D. russelii* (Tsai et al. 2007b), *Trimeresurus stejnegeri* (Tsai et al. 2004b), and rattlesnakes (Boldrini-França et al. 2010; Tsai et al. 2003). It is essential to know the locality and number of snakes from which the venom has been collected when studying the geographic variations of a venom species. Occasionally, controversial or contradictory results of venom characterization were published in referred journals using unreliable venom collected from unidentified localities and even contaminated or mixed with venom from other species. Researchers should be responsible and cautious not to use unreliable venom samples or publish the results. Under such circumstances, possible venom contamination could be detected or verified by analyzing the accurate mass and *N*-terminal sequence or peptide mass fingerprint of each purified $vPLA_2$ in the sample since most snake venoms contain specific $vPLA_2$s. Because mRNAs encoding the expressed $vPLA_2$ isoforms are more abundant and their cDNAs are more frequently cloned or sequenced than those encoding the nonexpressed $vPLA_2$s, the $vPLA_2$ proteomic results should match the transcriptomic results, i.e., all the $vPLA_2$s that are present in a venom species should have the corresponding cDNAs cloned from its venom glands. This could serve as a further criterion to check whether the venom used is authentic and not contaminated.

Evolution of Different Pharmacological Effects

The lipolytic activities of $vPLA_2$s are difficult to assay in vivo, and only the in vitro models have been used to test or examine their pharmacological effects. Most of the acidic $vPLA_2$s induce or inhibit platelet aggregation, but their action mechanisms have not been well elucidated. Not only the $vPLA_2$ “effective doses” varied greatly, the effects could be either dependent or independent of the enzymatic

activities (Kini 1997). The antiplatelet responsible residues of pit viper acidic $vPLA_2$s were found to contain acidic and aromatic residues at positions 6, 20–22, and 114–116 by mutagenesis study (Liu et al. 2001). Recently, it was reported that an acidic $vPLA_2$ from *Naja naja* ($NnPLA_2$-I) could inhibit thrombin with a K_i value of 9.3 nM while $NnPLA_2$-I also dose-dependently inhibits the aggregation of platelet-rich plasma (Dutta et al. 2015). Earlier, an African cobra $vPLA_2$ was found to inhibit thrombin (Osipov et al. 2010). Therefore, the acidic $vPLA_2$s in general may contribute to thrombocytopenia and hemorrhage effects.

Although most $vPLA_2$s with catalytic activity could elicit weak anticoagulant and transient hypotensive effects, strongly anticoagulant $vPLA_2$s usually have high pI values and bind to coagulation factor Xa (Kini 2005), and they may show low or no lipolytic activities. The responsible residues and mechanism of several potent anticoagulant $vPLA_2$s have been explored by site-directed mutagenesis (Kini 2005; Prijatelj et al. 2006; Tsai et al. 2011b; Zhong et al. 2002). Remarkably, the anticoagulant responsible sites of the GI-$vPLA_2$ GII-$vPLA_2$ could be different, suggesting convergent evolution of the functions. In addition, some basic $vPLA_2$s can insert into the membrane outer layer and change the conformation and permeability of cell membranes (Qin et al. 2005). These highly basic $vPLA_2$s (pI >8.7) usually show antibacterial activities by penetration of the cell membrane, which could be independent of the catalytic activities (Mora-Obando et al. 2014).

Some moderately basic $vPLA_2$s with catalytic activities have evolved into neurotoxins or myotoxins, and high contents of neurotoxic $vPLA_2$ usually signify a high lethal potency of the envenoming. The difference and similarity in the reaction mechanism between the myotoxic and neurotoxic $vPLA_2$s have been reviewed (Montecucco et al. 2008; Šribar et al. 2014). Monomeric PLA_2 neurotoxins are present in the venom of highly poisonous Australian *Notechis* and *Pseudechis* and a few sea snakes. More potent and multimeric neurotoxic PLA_2s are present in the venom of kraits (e.g., β-bungarotoxins) and Australian elapids (e.g., taipoxin and texilotoxin). Recently, PLA_2 subunits with sequences homologous to all the three chains of taipoxin were identified in *Austrelaps superbus* venom (Marcon et al. 2013) and in the transcriptome of *Acanthophis wellsi* and *Suta fasciata*; supporting that the oligomeric PLA_2 neurotoxins were likely widespread in Australian elapid venoms (Jackson et al. 2013). Monomeric PLA_2 neurotoxins are present in many Viperidae, including *B. caudalis*, *Vipera, Daboia* (Tsai et al. 2007b), *Protobothrops,* and *Gloydius* (Chen et al. 2004; Yang et al. 2015). The heterodimeric PLA_2 neurotoxins (e.g., crotoxin and vipoxin) are only present in the venom of several genera of pit vipers (Chen et al. 2004; Yang et al. 2015) and true vipers (Šribar et al. 2014). Usually, a basic PLA_2 subunit that is enzymatically active is the key subunit in the multimeric toxins while other subunits are chaperons to increase the presynaptic specificity of the key subunit.

Monomeric and heterodimeric myo/neurotoxic Asn6-PLA_2s appear to be selectively expressed in the venom of Asian and American pit vipers. Crotoxin-like toxins have been identified in some rattlesnakes (*Crotalus* and *Sistrurus*) and *Bothriechis nigroviridis* (Lomonte et al. 2015) but not in most other rattlesnakes and *Bothriechis*. The recent discovery of Gintexin (a crotoxin-like neurotoxin) from

the venom of Chinese *Gloydius intermedius* revealed that the neurotoxic rattlesnakes are probably the descendants of an ancient sister species of *G. intermedius* that migrated to the New World about 30 million years ago (Wüster et al. 2008; Yang et al. 2015). In the absence of acidic subunits, the isolated basic subunits of crotoxin or similar neurotoxins showed higher anticoagulant and myotoxic effects than the native crotoxin (Yang et al. 2015). In contrast, monomeric and less potent neutotoxic PLA_2s, structurally similar to the crotoxin-B2 subunit, are present in the venom of *Gloydius brevicaudus and Gloydius saxilis* (Chen et al. 2004). Other monomeric and myotoxic Asn6-PLA_2s have been isolated from the venom of *Protobothrops* species and *Crotalus v. viridis* (Tsai et al. 2003) and *Bothriechis schlegelii* (Chen et al. 2004) and were also cloned from *Ovophis monticola* (Malhotra et al. 2013) and *Deinagkistrodon acutus* venom glands (Wang et al. 1996).

Increasing examples reveal that $vPLA_2$s are the most versatile enzyme in snake venom and the same enzyme molecule may play multiple roles (Gutiérrez and Lomonte 2013). At least in vitro or in animal models, certain $vPLA_2$ toxins show dual or multiple toxicities, e.g., ammodytoxin (Prijatelj et al. 2006) and trimucrotoxin (Tsai et al. 2011b) are both neurotoxic and strongly anticoagulant, and the major $vPLA_2$s of *Daboia and Vipera* are both neurotoxic and hypotensive (Chi 2001). Likewise, the venoms of Australian taipans and eastern brown snakes also adopt the evolutionary strategy of paralysis and hypotension, and their $vPLA_2$s are responsible for the vascular muscle relaxation and cardiovascular collapse in the victim (Chaisakul et al. 2014). Interestingly, a special $vPLA_2$s was recently reported to interact with nicotinic acetylcholine receptors rather than binding to presynaptic sites (Vulfius et al. 2014).

Synergism Between $vPLA_2$ and Other Venom Toxins

As expected, the venom components evolved as a whole and cooperated with one another in certain ways. Many examples demonstrate the synergism between different $vPLA_2$ isoforms or between $vPLA_2$s and other toxin families in the same venom, and more details remain to be explored. The potent presynaptic neurotoxins, β-bungarotoxins, synergize with the postsynaptic α-bungarotoxin and κ-bungarotoxin (i.e., three-finger toxins) and result in respiratory failure for lethal envenoming of kraits. Likewise, taipoxin (Chaisakul et al. 2014), texilotoxin, and the presynaptic neurotoxins of *A. superbus* venom (Marcon et al. 2013) may synergize with the postsynaptic α-neurotoxins (three-finger toxins) present in the venoms. The acidic $vPLA_2$s of cobra venom synergize with the abundant cardiotoxins (i.e., direct lytic factors) to damage cell membranes and result in hyperkalacemia (high plasma $[K^{+1}]$) and heart failure. The synergism between crotoxin and crotamine, a myotoxic and permeability-enhancing peptide (Rádis-Baptista and Kerkis 2011), may facilitate the internalization (Šribar et al. 2014) of crotoxin-B subunits and thus their toxicity to the neurons. It would be interesting to investigate whether the abundant crotamine in *C. v. viridis* venom also synergizes with its myotoxic Asn6-$vPLA_2$s (Tsai et al. 2003) for the envenoming. Additionally, it has been noted that kallikrein-like serine proteases

and bradykinin potentiation peptides and natriuretic peptides are more abundant in viperid venoms that contain neurotoxic $vPLA_2$s (e.g., *P. mucrosquamatus, G. intermedius, G. brevicaudus*, and some *Daboia* and *Vipera* venom) than in those containing mainly hemorrhagic toxins, suggesting possible coevolution of hypotensive and paralyzing effects of the venom components.

For hemorrhagic venoms, antiplatelet acidic $vPLA_2$s may synergize with disintegrins (that bind to the fibrinogen receptor or other platelet receptors) and various C-lectin-like binding proteins to induce thrombocytopenia. The highly catalytic $vPLA_2$s may also release lysophospholipids; this product could affect platelet aggregation and membrane permeability. In various yellowish snake venoms, *L*-amino acid oxidase can generate H_2O_2 and thus elicit strong antiplatelet effects and thus may also synergize with acidic $vPLA_2$s in the same venom. *Bothrops asper* venom is rich in myotoxic $vPLA_2$s as well as potent nucleotidase so the adenosine triphosphate (ATP) released from damaged muscle cells is converted into adenosine diphosphate (ADP) and monophosphate (AMP) and adenosine; the in situ generated adenosine could result in hypotension and anticoagulant effects (Caccin et al. 2013). It was recently confirmed by in vitro and in vivo study that the *B. asper* venom Asp49-myotoxin synergized with its Lys49-PLA_2 to ameliorate myotoxicity upon envenoming (Mora-Obando et al. 2014). This synergism may also occur in the envenoming by the American *Agkistrodon* since their venoms are abundant in both myotoxic Asp49-PLA_2 and Lys49-PLA_2 (Komori et al. 2002). Remarkably, some isolated acidic $vPLA_2$ isoforms showed rather low or ambiguous toxicities; whether they exert special effects in the presence of other venom components remains to be clarified.

Reduction or Loss of Lipolytic Activity as an Evolutionary Strategy

When a $vPLA_2$ does not depend on reaction products for its function, its catalytic activities could be lost through mutation at key residues at the active site, such as Gln4, Phe5, Tyr28, Lys31 or Trp31, His48, and Asp49. Many GI-$vPLA_2$s with much reduced catalytic activities have been found to contain Pro31 substitution, e.g., the major $vPLA_2$ isoforms of *B. fasciatus* and certain $vPLA_2$ isoforms of *Laticauda* and *P. australis* (Tsai 2007). These Pro31 mutants have reduced Ca^{2+}-binding ability and catalytic activity (Tsai et al. 2007a) and can be present in a higher amount than other $vPLA_2$s of the same venom and contribute to cytotoxic, myotoxic, and cardiotoxic effects (Liu et al. 1992). Many Viperidae and Crotalinae venoms also contain nonenzymatic $vPLA_2$s with distorted Ca^{2+}-binding loop or active site conformation. Besides the more commonly present Lys49-PLA_2 homologues in both Old World and New World pit vipers, basic GII-$vPLA_2$s with Arg49 or Asn49 substitutions have been found in some venom species of *Protobothrops* and *Trimeresurus* pit vipers (Mebs et al. 2006; Tsai et al. 2004b; Wei et al. 2010), respectively. Ser49-PLA_2s have been isolated from the venoms of *Echis* species, *V. a. ammodytes*, and *V. u. renardi* (Tsai et al. 2011a). Among the elapid venoms studied, a *B. fasciatus* $vPLA_2$ with unique Ala49 substitution (Liu et al. 1992) and a

Laticauda colubrina vPLA$_2$ isoform with His48Asn mutation (Takasaki et al. 1988) have been characterized. These basic proteins could be abundant in the venom and damage cell membranes through a noncatalytic mechanism and result in local edema, inflammation, and myonecrosis. It has been noted that the myotoxic effects of enzymatically active Asp49 vPLA$_2$s are systematic and that their mechanisms for sarcolemma damage differ from those of the locally effective Lys49 vPLA$_2$s, while both myotoxin types induce Ca^{2+} entry and release of ATP (Fernández et al. 2013).

Post-Translational Modifications of vPLA$_2$s

The compact and well-soluble vPLA$_2$ molecules in general do not undergo post-translational modification, except the disulfide bond formation that occurs during their biosynthesis and folding in endoplasmic reticulum. This fact allows a convenient and direct match of the protein mass of an isolated vPLA$_2$ to that calculated from its deduced protein sequence assuming complete disulfide pairing. Accurate pairing of the 12–14 Cys residues results in a compact 3D structure and the high thermal stability of vPLA$_2$s in an acidic buffer or in the presence of 40–60 % of acetonitrile or alcohol. The absence of Cys61-Cys99 in the Asp49-vPLA$_2$s of *Bitis gabonica* and *Bitis nacicornis* venoms and in the Lys49-vPLA$_2$s of *Trimeresurus puniceus* and *Trimeresurus borneensis* venoms does not affect protein folding but reduces the vPLA$_2$ thermal stability (Wang et al. 2005). Additionally, some exposed methionine residues of vPLA$_2$ can be partially oxidized in air to less hydrophobic Met-sulfoxide, especially when the venom contains abundant *L*-amino acid oxidase that may produce H_2O_2 by oxidizing free amino acids. The oxidation of methionine residue in a vPLA$_2$ usually results in its reduced toxicity and enzymatic activity (Tsai et al. 2000).

Tropidolaemus wagleri holds a unique taxonomic position among pit viper species (Wüster et al. 2008), and its acidic E6-PLA$_2$ was found to be fully glycosylated at residue 13 with a small complex type of *N*-glycan (Tsai et al. 2012). The bee venom sPLA$_2$, taipoxin γ-subunit, and the texilotoxin C-subunit are also glycosylated but at different positions of Asn residues. However, deglycosylation of the bee and the *T. wagleri* E6-vPLA$_2$s by specific glycosidase did not affect their enzymatic activities significantly (Tsai et al. 2012). The truncated acidic subunits of Crotoxin A and Gintexin A are derived from unique acidic PLA$_2$ precursors by endopeptidases to remove three interface recognition regions of the PLA$_2$s (Yang et al. 2015), and modification of the *N*-terminal Gln of one of the crotoxin-A fragment to a pyroglutamine residue is achieved by a Gln-cyclase in the venom.

Fatty acylation of the side chains of Lys7 and Lys10 in the basic Asp49-PLA$_2$ of *Agkistrodon p. piscivorus* occurred in vitro when the enzyme was incubated with a high concentration of activated esters or pseudosubstrates (Cho et al. 1988; Shen et al. 1994). The acylation of vPLA$_2$ at specific Lys side chains was based on an autocatalytic mechanism and could promote enzyme dimerization and increase

their activities toward densely packed liquid-crystalline phospholipid bilayers. However, there is no evidence to support that vPLA$_2$ can be acylated in vivo as part of its reaction mechanism and regulation.

Conclusion and Future Direction

In recent decades, toxinologists have made significant contributions to deciphering the structure, toxicity and selectivity, and biodiversity of venom proteins and aid in the treatment of envenomed patients. The present work highlights some of the most relevant contributions in the study of vPLA$_2$ evolution and diversity. Apparently, a vast amount of sequence data for vPLA$_2$s has been generated by the omic technology, and the information helps to clarify how venomous snakes and their venom evolved. Hopefully, the fascinating molecular diversity of vPLA$_2$ family can raise interest and concern in the study of new snake venom species and their ecology as well as the conservation of venomous snakes.

There is more to be discovered about the specificity, structure, and action mechanisms of vPLA$_2$s at molecular and cellular level. The genomics and expression regulation of vPLA$_2$s, the impact of vPLA$_2$s in overall pathophysiology of envenoming, and how their variations correlated with prey-ecology and snake adaptation remain to be elucidated. Receptors and binding proteins for vPLA$_2$s have been identified in mammals, suggesting that functions and specificities of vPLA$_2$s could be regulated through specific protein-protein interactions and membrane interactions. The potential for vPLA$_2$s in therapeutic and diagnostic applications, alone or combined with other molecules, also remains to be tested and formulated using assays and model systems related to humans.

Cross-References

► Squamate Reptile Genomics and Evolution
► Structure-Function Relationship in Heterodimeric Neurotoxin PLA2s from Viperidae Snakes Inhabiting Europe, South America, and Asia

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Automated Mass Fingerprinting of Venoms in Nanogram Range: Review of Technology

14

Pierre E. Bougis

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Abstract

Mass spectrometry (MS) is at present well recognized as a method of choice for the study of any venoms. As a most basic venomic analysis, venom mass fingerprinting (VMF) which depicts the venom's all-inclusive masses may be very informative when comparisons among close or distant venomous species have to be pictured, as well as intraspecific venom variations. This chapter reports, as an example, the technological requirements and pitfalls using the state-of-the-art nano-ultrahigh-performance liquid chromatography (UHPLC) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS in order to achieve in 2-h at the best and from tens of venom nanograms only a full-automated workflow. Lastly, having the opportunity of acquiring VMF in such a fast and easy way could be extremely valuable for carrying out phylogenetic studies based on mass clades.

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Introduction

Applying mass spectrometry (MS) to toxinology has been a large beneficial step forward (Escoubas 2006). Venom mass fingerprinting (VMF), i.e., the all-inclusive masses present in a venom, can be seen as the most basic approach to decipher venom contents on the unique basis of masses. Venoms are usually extremely diverse, and to be informative, VMF should be comprehensive as much as possible. In this way, comparisons among close or distant venomous species can be pictured, as well as intraspecific variations. Besides, obtaining VMF in an easy way may also be useful to carrying out phylogenetic studies based on mass clades. Two challenges are taken into consideration here. The first one is the possibility to generate a VMF in a fully automated way. The second one is acquiring a VMF from a very tiny amount of venom. This prospect will definitely open the way to study venoms collected from infrequent and small venomous animals producing less than microgram quantities of venom. This issue has been addressed taking in consideration the most recent advances in both chromatographic techniques and mass spectrometry. This chapter relates the technological requirements and pitfalls taken from the very few examples so far available.

Increasing the Efficiency of High-Performance Liquid Chromatography (UHPLC)

The technological transition at the late 1980s between the low flow gel filtration chromatography on Sephadex (Porath and Flodin 1959) and the high-performance liquid chromatography (HPLC; originally coined as high-pressure LC) (Molnár and Horváth 1977) has been pivotal for the study of venoms and toxins (Bougis et al. 1986; Kaneda and Hayashi 1983). Since then, this technology has been used widely to analyze venoms. Usual HPLC column loading capacity stands on the mg scale and the flowing rate on mL/min. Indeed, most of the venomic studies at present have been performed accordingly. To gain increase in HPLC efficiency, the rational has been to decrease the particle diameter (sub-2 μm) of the column stationary phase. This technological step forward has been the focus of the nano-ultrahigh-performance liquid chromatography (nano-UHPLC) (Jorgenson 2010; Varma et al. 2010). This new analytical technique has several key advantages including speed of analysis, higher resolution, and sensitivity with lower solvent consumption. However and consequently to the low particle size, pressure has to be increased nominally from 400 to 1,000 bar. Indeed, nano-UHPLC is a master choice if tiny amounts of material are only available, as venoms are often. Eugster and colleagues have recently and deeply analyzed peak capacity optimization in order to obtain high peptide profiling in complex mixtures as cone snail venoms (Eugster et al. 2012). This study provides an interesting decisional tree to sum up the strategies for profiling of complex mixtures of small peptides, taking into account their possible degradation at the high temperature that is somewhat mandatory for UHPLC. The best compromise between analysis time and peak capacity is to

achieve for a 5–95 % ACN gradient over 90–280 min, using a 2.1 mm I.D. column maintained at 90 °C. An alternative to particle-packed columns is monoliths, which is considered as a chromatographic stationary phase providing lower column back pressure and increasing substantially the sensitivity and which is particularly well adapted to the nano-chromatographic scale (Svec and Fréchet 1996; Wu et al. 2008). However, the fact that it might be a poor recovery of large proteins (more than 25 kDa) has to be noticed (Vuignier et al. 2013). Nevertheless, knowing that toxins are in majority less than 15 kDa, this constraint is not so essential.

Chromatographic Coupling to Mass Spectrometry (MS)

Mass spectrometry (MS) has evolved as a key tool for proteomic studies. MS measures the mass-to-charge ratio (m/z) of any ions in gas phase. A mass spectrometer consists of an ion source, a mass analyzer to separate ions on the basis of their individual m/z values, and a detector for ion numbering purpose. In proteomics, two types of soft ionization source are commonly used, the electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) sources. The usual mass analyzer formats are quadrupole (Q) and time of flight (TOF), which when combined to the source give the most popular MS setups, ESI-Q-TOF and MALDI-TOF/TOF, both having fragmentation capabilities (MS/MS) for peptide identification, if needed. Also, sensitivity is nearly pico- to femtomole when using a MALDI source. Furthermore, because a MALDI source primarily generates almost mono-charged ions, it is the most suitable to be used, avoiding the multiple spectrum deconvolution steps that are mandatory when using an ESI source, which is well known to generate a high-level of multi-charged ions.

Even if venoms may be directly spotted on MALDI-TOF sample holder to be further analyzed as unique one-shot spectrum, or alternatively investigated by direct infusion nano-ESI (Souza et al. 2008), the best format is the chromatographic coupling. Two main setups have been used in venomic studies using a liquid chromatography coupling to a mass spectrometer (Pimenta et al. 2001): an online setup that is a direct physical coupling of the HPLC column flow trough to an ESI-MS and an off-line setup that refers to handmade deposits of the collected column eluent onto the MALDI-TOF sample holder. Thereby, online LC/ESI/MS and off-line LC/MALDI-TOF/MS were used to obtain scorpion VMF (Barona et al. 2006; Borges and Rojas-Runjaic 2007; Bringans et al. 2008; Chen et al. 2005; Dyason et al. 2002; Goudet et al. 2002; Newton et al. 2007; Pimenta et al. 2001, 2003; Rates et al. 2008; Schwartz et al. 2007; Verano-Braga et al. 2013), as well as for spiders (Escoubas et al. 1997; Guette et al. 2006; Palagi et al. 2013; Trachsel et al. 2012), bees (Favreau et al. 2006; Matysiak et al. 2011), snakes (Fry et al. 2002; Sanz et al. 2008), sea cones (Dutertre et al. 2013; Kauferstein et al. 2011; Krishnamurthy et al. 1996), and sea anemones (Rodríguez et al. 2012), as few examples. However, these example studies have been performed using mostly conventional HPLC scales with multiple chromatographic steps.

The coupling of UHPLC with MS has some constraints. When using ESI in a direct coupling setup, an optimum mobile phase flow rate (usually 50–300 μL/min) has to be selected in order to achieve acceptable ionization yields and sensitivity. This is why 2.1 mm I.D. column and 200 μL/min optimal flow rate are most often reported. Consequently, these limits in flow rate and column I.D. do not permit to lower as much as desired the venom quantity loaded. To circumvent this restraint, the best choice is to use a MALDI-TOF-MS off-line setup. Column of 250 mm long and 100 μm I.D. can be operated at a flow rate of 1–2 μL/min under a pressure of 400 bar. Handmade deposit of the column eluent is evidently not appropriate in this case. The use of a dedicated device, a spotter, allows first the mixing of the column eluent with a matrix (usually α − cyano-4-hydroxycinnamic acid, HCCA) and then the spotting process itself in the desired XY format. In this instance, there is a manual task of analysis of each individual spot (one MALDI spectrum by each spotted fraction; usually one every 20 s) that may be extremely time-consuming and clearly not appropriate for optimum VMF workflows (i.e., few tens of nanogram of venom analyzed and in about 2 h). A fuzzy logic-based automatic acquisition (Suckau et al. 1998) of MALDI spectra is a prerequisite at this stage. Most of the MALDI-TOF suppliers actually offer such an option.

Data Sorting and Managing

This is the step of the overall automated data acquisition process that is the most difficult to gather. Indeed, several iterative steps have to be performed in order to end up with a unique and comprehensive list of masses. Due to the use of a continuous elution/spotting method, potentially mass overlaps between adjacent spots may exist and have to be eliminated. Doubly charged and dimer ions have to be identified and removed as well. A specially designed Excel macro may do all the process in once. The presence of ion adducts, not common but mainly sodium and potassium, may also be of concern here. The goal is to end up with a comprehensive and complete list of masses depicting as much as possible the reality of the venom composition, taking into consideration that a compromise has to be made with the total time of the analysis (2 h at best).

A Workflow Example

Recently, such an automated workflow has been reported in detail (Martin-Eauclaire et al. 2013). Its main stages are as follows. Nano-UHPLC analysis was achieved using a Dionex UltiMate® 3000 RSLCnano system (Dionex-Thermo Fisher scientific, San Jose, USA). The elution profile was monitored the wavelength of 214 nm. HPLC grade elution solvents were water plus 0.05 % TFA (A) and 80/20 (v/v) acetonitrile/water plus 0.04 % TFA (B). Pre-column (200 μm × 5 mm) and column (100 μm × 250 mm) were C18-reversed phase PepSwift Monolithic Nano PS-DVB from Dionex. Peptides were column desorbed with a linear gradient of

4–40 % of B in A over a period of 40 min and at a flow rate of 1.5 μL/min. The column pressure was about 400 bars and its temperature kept at 60 °C. This high temperature is absolutely mandatory to lower the fluidic pressure on a nano-column. As the toxin thermal stability might be questioned at high temperatures, especially concerning deamidation of Asn and Asp-Gly cleavage, controls completed over a period of 2 h showed that no hitches of this kind append. MALDI-TOF analyses were performed on an Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker, Karlsruhe, Germany) operated in the positive linear mode. Direct online spotting of the column effluent on a Bruker MTP 384 polished steel plate was performed using an LC Packing Probot. The sampling rate was 3 spots/min adding a crystallization matrix 0.5 μL (v/v) of filtered and twice-diluted saturated α-cyano-4-hydroxycinnamic acid (CHCA) solution made in 50/50 (v/v) acetonitrile/water plus 0.1 % TFA. It is well known that the nature of the matrix plays an important role in the ion yield efficiency in protein desorption/ionization from crystalline matrices. The matrix choice may have a large effect on recorded mass spectrum (Liu and Schey 2005; Padliya and Wood 2004). This specific point has to be carefully evaluated both in terms of nature and of final concentration that has to be used at best in order to keep the signal-to-noise ratio at the minimum level.

MALDI-TOF spectra (usual *m*/*z* range: 800–16,000) were automatically recorded with the AUTOXECUTE fuzzy logic feedback control system. Each spectrum was the accumulation of 1,000 laser shots (S/N threshold 10) in 50 shot-steps across each spot. Laser intensity varied between 40 % and 80 % at the frequency rate of 200 Hz and following a hexagonal raster. External multipoint peptide calibrations were included. Spectra were batch processed with FLEXANALYSIS 2.4 and exported in a unique Excel workbook of numerous sheets (one for each spots), this for each MALDI-TOF sample holder acquisition process corresponding to one nano-UHPLC. Therein, each worksheet within the workbook contains all the *m*/*z* peaks detected for one spot. A specially designed Excel macro (PMF Merger v1.00) assembled the data and removed redundancy as the following. Firstly, doubly charged or dimer ions in each peak list are annotated, and the retention time is added as a new column. After removal of doubly charged and dimer ions, all peak lists are combined in a unique peak list filtering out intensity below a user-defined intensity threshold. This step leads in a "merge worksheet." Secondly, peaks are sorted by mass, and a sliding window of mass allows one to aggregate peaks that might belong to the same molecule detected on contiguous spots. As soon as the gap between two consecutive masses is greater than a user-defined threshold, a new cluster is started. To avoid aggregation of peaks of the same *m*/*z* that may appear at rather different time (i.e., a phenomenon of chromatographic drag), the same process is repeated with time variable: peaks are sorted by time, and as soon as the gap in time is greater than a user-defined threshold, the mass cluster is closed and a new cluster is added. The most intense peak is kept as the representative of the cluster. This step leads in a combined worksheet. Lastly, summarized results are presented in a final and unique worksheet. All user-defined parameters (*m*/*z* error, spotting time start, time between each spot, minimum intensity threshold, mass aggregation threshold, time aggregation threshold) are entered at the beginning of the Excel macro running.

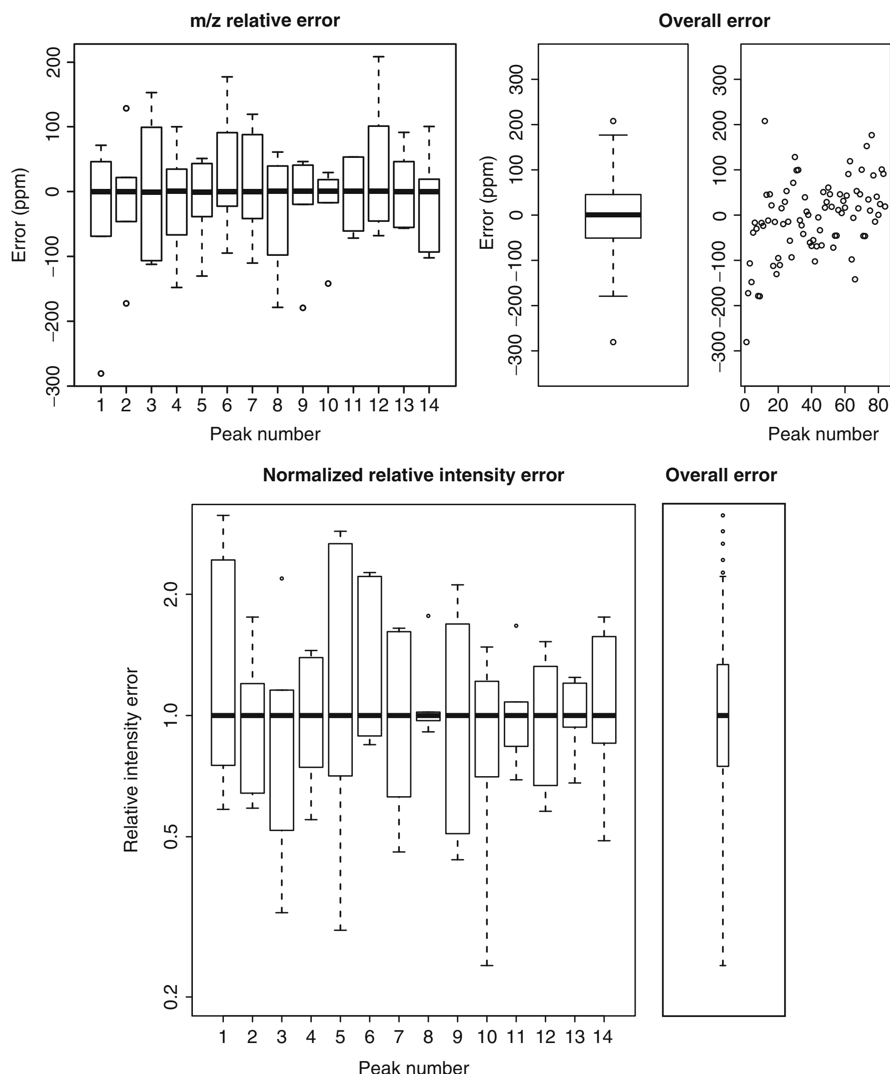

Fig. 1 Reproducibility of the full analytical workflow as observed errors on *m*/*z* and intensity values. Errors were evaluated on 14 identical masses taken from 6 consecutive UHPLC runs of *Androctonus australis* venom (55 ng) (Taken from (Martin-Eauclaire et al. 2013) with Copyright Permission)

Martin-Eauclaire and colleagues also assessed another important point at this stage that is the degree of robustness of the mass list obtained by injecting the same sample several times in the raw and comparing the data acquired in terms of *m*/*z* value and peak intensity. As shown in Fig. 1, a very low dispersion (*m*/*z* overall relative error, Q1 = −51 ppm and Q3 = 45 ppm) for mass values was established, which is particularly crucial for VMF comparison. Concerning peak intensities, the dispersion (normalized overall relative intensity error, Q1 = 0.75 and Q3 = 1.34)

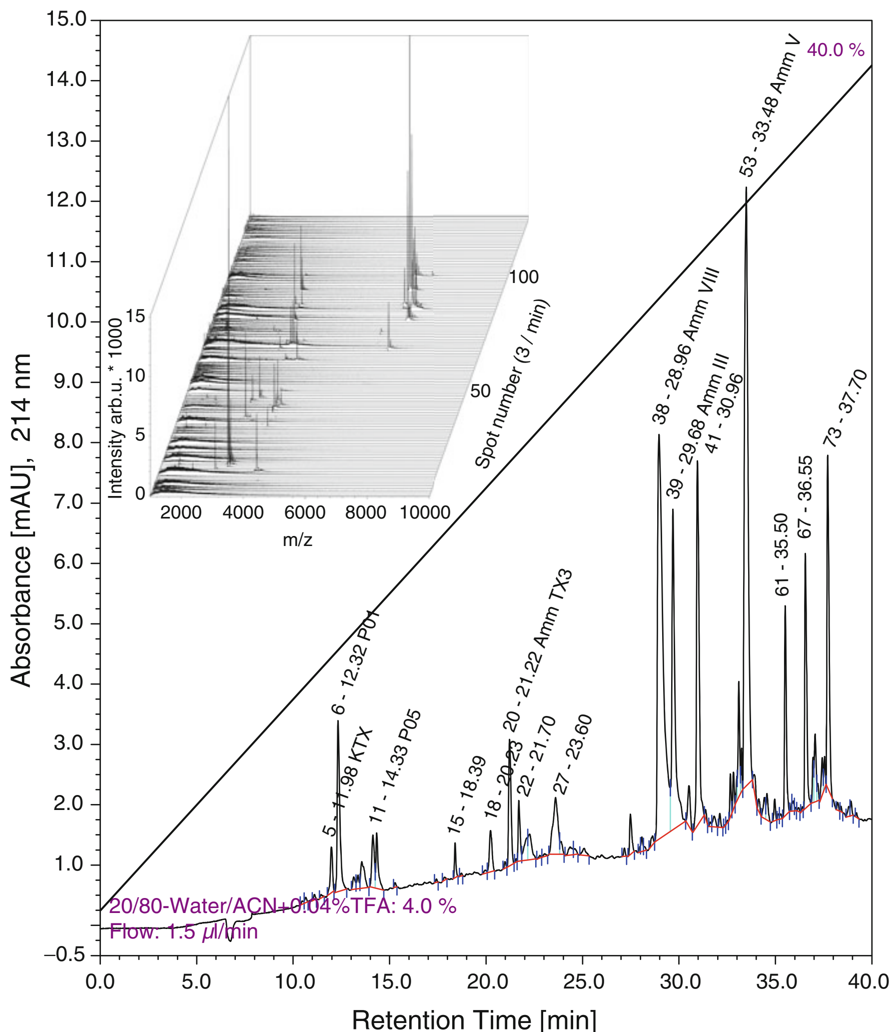

Fig. 2 Nano-UHPLC chromatogram of the *Androctonus mauretanicus mauretanicus* venom (55 ng, from a venom pool obtained under manual stimulations). Inset is the 3D drawing of the 120 MALDI-TOF spectra (*m*/*z* 1,000–10,000 vs. intensity, matrix used CHCA with a sampling rate of 3 spots/min) (Taken from (Martin-Eauclaire et al. 2013) with Copyright Permission)

was much broader and not really surprising since MALDI-TOF-MS is known as a weakly quantitative technique.

Applying this workflow to the analysis of diverse scorpion venoms, Martin-Eauclaire and colleagues depicted several other important facts. The nano-column loading capacity is less than 500 ng, the optimum using range being actually 50–100 ng. At the lower limit of 5 ng, the signal-to-noise ratio increased significantly and does not permit to extract any robust data set. Displayed on Fig. 2 is the

chromatogram they obtained injecting 55 ng of a venom pool obtained under manual stimulation and that should be considered as close as possible to the natural venom gland secretion of the species *Androctonus mauretanicus mauretanicus*.

Using a Dionex UltiMate® 3000, there is also the possibility to collect the nano-UHPLC column effluent rather than spotting it on the MALDI-TOF sample holder. This also allows performing some biological testing such as pin-sharp immunological detection (at least 0.01 ng) of a specific toxin family using high-tittered specific antiserum.

Conclusion and Future Direction

Starting from tens of venom nanograms, here it has been shown that using the state-of-the-art chromatographic technique nano-UHPLC, once coupled to a fully automated MALDI-TOF/MS data acquisition and processing system, is a realistic and easy way to acquire VMF in a short period of time. This is undoubtedly a step forward considering that most of the venomic data published until now have been obtained using multistep protocols from milligram venom quantities. Future developments may include the molecular identification of the unassigned masses. In this respect, VMF and high-resolution MS/MS data could be well merged with genomic information. Lastly, VMF acquired in a fast and easygoing way could be extremely valuable for carrying out phylogenetic studies based on mass clades.

Cross-References

► Snake Venom Peptidomics
► Snake Venom Phospholipase A_2: Evolution and Diversity

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Snake Venom Peptidomics

15

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Abstract

Even though the beginning of the studies of snake venom peptides is strongly correlated to the first discoveries of bradykinin-potentiating peptides, in the mid-twentieth century, snake venom peptidomics is still in its infancy. Over the last decade, the development of mass spectrometry, transcriptomics, and bioinformatics and the application of these tools to the study of snake venoms have unveiled the proteomes of several species and produced considerable knowledge of diverse aspects of snake biology. Though the studies of snake venomics are growing rapidly, snake venom peptidomics is still an emerging research field due to specificities of snake venoms and of peptidomic analysis.

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In this chapter, the main findings of snake venom peptidomics research are highlighted, and the strategies of venom preparation and mass spectrometry (MS) analysis, de novo sequencing of MS spectra, and the integration of "omics" strategies for exploring peptidomes are presented and discussed. The understanding of snake venom peptidomes could help to design specific protocols to avoid undesirable proteolysis of venom components, hence, improving the preparation of immunization mixtures used for antivenom production. As more sophisticated methods of analysis and high-throughput approaches are used, a number of new peptides will be discovered, and more importantly, the biological meaning of snake venom peptidomes will be revealed.

Introduction

Snake venoms are refined biological products elaborated throughout millions of years of evolution. The richness of biological activities presented by snake venom components motivated several research groups to isolate and characterize toxins over the last five decades. From these studies, new drugs have been developed, toxins with potential to become models of new therapeutical agents have been found, and important biochemical and physiological mechanisms in which they act in vertebrates have been revealed. A landmark in snake venom peptide research was the discovery of the bradykinin-potentiating factor (BPF) from *Bothrops jararaca* venom by Sérgio Ferreira and Mauricio Rocha e Silva (Ferreira 1965; Ferreira and Rocha e Silva 1965) and, subsequently, the isolation and characterization of the bradykinin-potentiating peptides (BPPs) (Ferreira et al. 1970a, b; Ondetti et al. 1971). The discovery of the BPPs paved the way to the development of the antihypertensive drug captopril and other related inhibitors of the angiotensin-converting enzyme, which saved and keeps saving millions of lives (McCleary and Kini 2013). Other examples of important biologically active peptides present in snake venoms include the poly-His-poly-Gly peptides (pHpG) (Favreau et al. 2007), the C-type natriuretic peptide (CNP) (Higuchi et al. 1999), sarafotoxins (Kloog et al. 1988), and crotalphine (Konno et al. 2008).

Snake venom peptides have been investigated by traditional biochemical methods of isolation and analysis of purified components. These approaches are still in use and continue to reveal new peptides and important biological activities; however, they are not sufficient to elucidate the complex composition of snake venom proteomes and peptidomes. From the 2000s, proteomic tools such as tandem mass spectrometry (MS), transcriptomics, and bioinformatics started to be used in snake venom research, revealing that snake venoms are composed of hundreds of proteins and peptides belonging to around a dozen toxin families (Valente et al. 2009; Calvete 2013; Juárez et al. 2004; Tashima et al. 2008). Although proteomics of snake venoms (venomics) progressed significantly in the last decade, the large-scale study of venom peptidomes did not progress at the same rate. In fact, there are big challenges in the investigations of peptidomes with proteomics tools.

In bottom-up proteomics, proteins are digested with some enzyme, in general trypsin, to produce peptides with defined amino acids at the C- or N-terminal position, depending on the enzyme specificity. Based on this information, the bioinformatic tools compare peptide MS spectra with the theoretical ones generated in silico by the specific enzyme from the sequences in the database. This C- or N-terminal specificity is not previously known in peptidomics; therefore, all possibilities need to be searched, increasing significantly database size and search time (Elias and Gygi 2007) and reducing the number of correct assignments of peptide-spectrum matches (Borges et al. 2013; Yen et al. 2006). Moreover, the lack of comprehensive databases and accelerated evolution of snake venom toxins are limitations to snake venom peptidomics (Calvete 2013; Ohno et al. 1998; Zelanis and Tashima 2014). Due to accelerated evolution of toxins, cross-correlation of toxins among species is not simple (Zelanis and Tashima 2014), and ideally, each species under investigation should have its own sequenced genome or transcriptome database for comparison with MS spectra. This is not always possible, and therefore, an essential methodology for snake venom peptidomics is de novo sequencing of MS spectra and homology search by tools as BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or MS-BLAST (http://genetics.bwh.harvard.edu/msblast) for peptide family classification. De novo sequencing of MS spectra is a valuable tool and has disclosed novel peptides and new classes of peptides from snake venoms (Favreau et al. 2007; Konno et al. 2008; Valente et al. 2009; Ianzer et al. 2004; Pimenta et al. 2007; Tashima et al. 2012; Zelanis et al. 2010), but it is a specialized and laborious task (Calvete 2013).

Nevertheless, snake venom peptidomics research is growing due to the importance to understand the roles of these biologically active peptides in the envenomation process and to the interest in prospecting new molecules with potential for pharmacological application. In this chapter, the main findings of snake venom peptidomics research are highlighted, and the strategies of venom preparation and MS analysis, de novo sequencing of MS spectra, and the integration of "omics" strategies for exploring peptidomes are presented and discussed.

Bradykinin-Potentiating Peptide (BPP) Precursors: Heterogeneity and Identification Through "Omics" Approaches

The landmark report of Sérgio Ferreira and Mauricio Rocha e Silva (Ferreira 1965; Ferreira and Rocha e Silva 1965) on the discovery of bradykinin-potentiating factors in the venom of the South American pit viper *Bothrops jararaca* (Serpentes, Viperidae) opened a new and exciting field in cardiovascular pathophysiology research, which eventually led to the development of captopril, an active site directed inhibitor of the angiotensin-converting enzyme. However, it was only thirty years later that the molecular details of the precursor of BPPs would begin to be understood. In 1997, Murayama and coworkers (Murayama et al. 1997) isolated a 1.8 kb cDNA clone from a *Bothrops jararaca* venom gland cDNA library that encodes the precursor of BPPs. In situ hybridization studies revealed the

expression of BPP precursor mRNAs in distinct tissues (other than venom gland) such as the brain and spleen (Murayama et al. 1997; Hayashi et al. 2003). Interesting to note was the fact that the BPP's precursor revealed to be a "molecular package" that comprises the sequences of seven BPPs aligned in tandem after a hydrophobic signal peptide sequence, followed by a putative intervening sequence and a C-type natriuretic peptide (CNP) at C-terminus (Murayama et al. 1997). This modular "all-in-one" architecture is also found in other peptide hormone precursors such as pro-opiomelanocortin in which a single precursor gives rise to at least ten distinct peptide hormones (Raffin-Sanson et al. 2003).

On the last decade, "omics" methodologies have started being systematically used in toxinology, revealing molecular details of a number of venom toxins as well as their precursors (Calvete 2013; Zelanis and Tashima 2014). Transcriptomic studies have tremendously improved the identification of several toxin classes including BPP/CNP precursors in several snake species (Calvete 2013). In this context, recent reports have shown that besides the BPP and CNP sequences, the BPP precursor has also other interesting features: pHpG sequences and the tripeptide sequence Z-K-W (where Z stands for pyroglutamic acid, a cyclic form of glutamate or glutamine, generated by the activity of glutaminyl cyclases) – both of them are believed to play a role in the venom gland homeostasis by interacting with catalytic zinc atom of mature snake venom metalloproteinase enzymes and, therefore, inhibiting their activities in the lumen of the venom gland (Favreau et al. 2007; Marques-Porto et al. 2008). Indeed, a recent study showed that despite the marked individual variability in venom composition, the presence of the tripeptide Z-K-W was ubiquitous among 21 *B. jararaca* siblings (Dias et al. 2013).

In addition, the discovery of "new" BPPs (i.e., processed at sites different from that of the canonical form) pointed out to the existence of distinct proteolytic processing sites within the precursor protein structure. Recent reports revealed new BPP structures in the venoms of South American pit vipers *B. jararaca*, *B. fonsecai*, and *B. cotiara*, with amino acid extension at either N- or C-terminus (Tashima et al. 2012; Zelanis et al. 2010). An example is shown in Fig. 1 (corresponding to Uniprot entry Q6LEM5; the BPP/CNP precursor from *B. jararaca*), where at least 13 processed products can be identified. Even though a number of distinct processing sites have been proposed for the precursor of BPPs, to date, there is no experimental evidence of the identity of the enzyme responsible for this activity; though, since the majority of BPP primary structures reported so far are flanked by basic amino acid residues (Arg or Lys), it is believed that the proteolytic processing might be related to the activity of proprotein convertases with serine proteinase activity, such as furin (Murayama et al. 1997; Lipkind et al. 1995; Wu et al. 2003). In fact, Wu and coworkers (Wu et al. 2003) demonstrated the critical role of furin for the processing of human pro-C-type natriuretic peptide.

A number of "omics" studies have shown the ubiquitous presence of BPPs and/or natriuretic peptides in the venom (or in the venom gland, as a precursor) of snakes from Colubridae, Viperidae, and Elapidae families (Higuchi et al. 1999; Ching et al. 2006; Cidade et al. 2006; Corrêa-Netto et al. 2011; Durban et al. 2013; Lomonte et al. 2014). However, even within the same species the content of

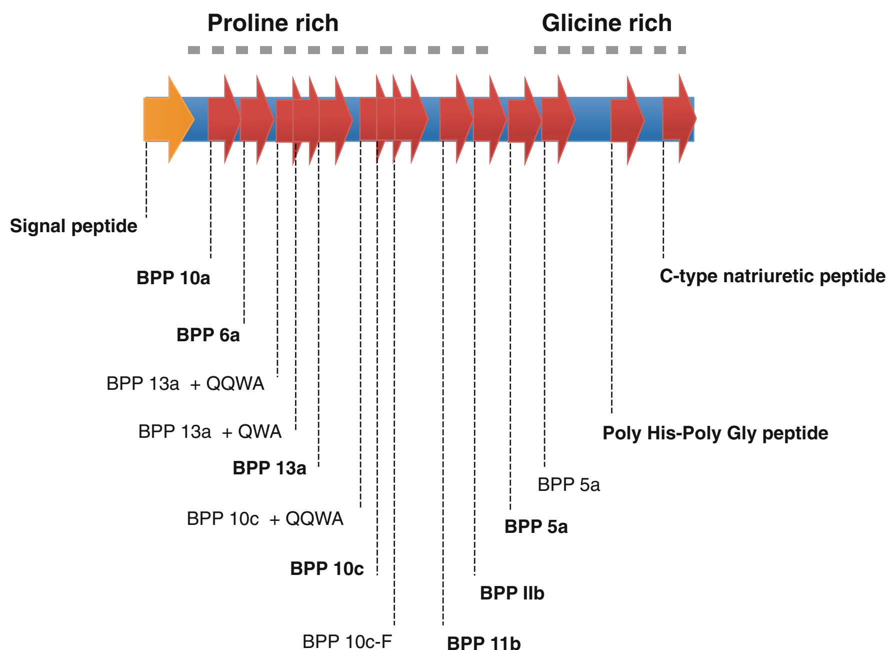

Fig. 1 Schematic structure of the bradykinin-potentiating peptide/C-type natriuretic precursor (Uniprot entry # Q6LEM5) from *Bothrops jararaca* snake venom

BPP precursor can vary expressively depending, for example, on the snake's ontogeny. The analysis of the venom gland transcriptome of newborn and adult *B. jararaca* specimens revealed a higher content of BPPs in adult venom gland – the content of BPP precursors more than doubles in adulthood (from ~10 % in newborn venom gland to ~23 % in adult venom gland) (Zelanis et al. 2012). However, in a previous report, Zelanis and colleagues (Zelanis et al. 2010) found no ontogenetic differences in the identified BPPs in newborn and adult venoms, suggesting that these peptides are important for the snake to deal with prey irrespective of the animal age and size. This apparent discrepancy may be related to the fact that there might exist different BPP precursors in both venoms whose primary structures remain to be elucidated and, therefore, could not be assigned after database search of mass spectrometry data.

According to the report of Zelanis and colleagues (Zelanis et al. 2012), in adulthood, the BPP content seems not to vary expressively in *B. jararaca* venom. Indeed, Pimenta and colleagues (Pimenta et al. 2007) found four new BPPs, identified as cleaved BPPs lacking the C-terminal Gln-Ile-Pro-Pro (Q-I-P-P) sequence, only in female *B. jararaca* venoms. Since the C-terminal of BPPs is crucial for the interaction with the angiotensin-converting enzyme, the synthetic replicate BPPs (lacking the Q-I-P-P at the C-terminus) were less potent than the full-length BPPs in potentiating the bradykinin hypotensive effect. This finding was the first report on gender-related variability in venom peptide content of a snake species.

Despite the heterogeneity displayed in their primary structures, BPPs share common structural features on both N- and C-termini: the cyclization of amino terminal residues into pyroglutamic acid (Z) and the X-X-P-P at C-terminus (where X-X is commonly – but not exclusively – Q and I). These structural features were used in mass spectrometric analysis by Menin and colleagues (Menin et al. 2008) in the search for BPPs in *Bothrops moojeni* snake venom. This high-throughput approach was successfully implemented because the fragmentation of BPPs by collision-induced dissociation during electrospray tandem mass spectrometry analysis (ESI-MS/MS) produces an intense signal at *m*/*z* 213.1 corresponding to the y-type ion of the terminal P–P fragment. Moreover, signals at *m*/*z* 226.1 and 240.1 correspond to the b-type ions of the N-terminus pGlu–Asn and pGlu–Lys, respectively. Therefore, using the mass spectrometer in the precursor ion scan mode, the authors could efficiently identify over 20 BPP-related peptides in *B. moojeni* venom. This rapid, yet simple, method is a very good example that illustrates the progress that has been made in snake venom peptidomic studies with the use of targeted analysis.

The Peptide Content of Snake Venoms

Unlike ordinary snake venom proteins, the only toxin genes that can be predicted to be ultimately expressed as peptides are those encoding for bradykinin-potentiating peptides, which can be identified by their modular precursor structure, comprising BPPs arranged in tandem together with pHpG regions and a C-type natriuretic peptide at the C-terminus (Murayama et al. 1997). Thus, the estimation of the peptide content within the venom of a given species is not a trivial task, since the extension of posttranslational modifications cannot be accurately predicted beforehand (i.e., based solely on in silico analysis). Therefore, the venom of a single specimen can display a distinct set (or subset) of (new) peptides according to, for example, the proteolytic processing events that might take place after protein synthesis.

In this context, the use of mass spectrometry-based approaches has greatly improved the knowledge of snake venom peptidomes as well as the identification of novel posttranslational modifications. Wermelinger and colleagues (Wermelinger et al. 2005) showed the use of mass spectrometry to perform toxin mass fingerprinting of venoms from different species belonging to *Bothrops* and *Crotalus* genera. Since venom integrity is an important feature for antivenom production, snake venom peptidome fingerprinting has a direct application in quality control analysis of immunogens.

From the point of view of the biological meaning, posttranslational processing of toxin precursors into smaller molecules (peptides) enables dynamism to the venom proteome without changing the protein itself (or its corresponding gene), an important feature which allows the diversification of the molecular targets of snake venom toxins toward distinct prey types. In fact, the so-called cryptides (i.e., bioactive peptides generated after proteolysis of ordinary proteins rather than the classical processing pathways) have been implicated in several biological processes, such as

antigen presentation, neuronal signaling, and inflammatory response (Pimenta and Lebrun 2007; Samir and Link 2011); however, few attention has been paid to this area in snake venom studies. It is virtually impossible to develop a targeted experimental approach without having at least a priori information on which peptide(s) should be present in the sample. Therefore, data mining of high-throughput data, such as those derived from mass spectrometric analysis is a promising approach.

Importance of Venom Preparation in Peptidomic Studies

Venom handling is an important step from the start of peptidomics analysis. The search for natural peptides with specific biological activities in snake venoms prompted researchers to avoid the use of toxin inhibitors in order to preserve the venoms in their original state as much as possible. In general, milked venoms are only centrifuged at low temperatures, then frozen, and lyophilized for long-term storage (WHO 2010). In principle, snake venoms possess natural mechanisms for the inhibition of proteolytic activity, as the acidic pH is far from the optimal basic range of snake venom serine proteases (SVSP) and snake venom metaloproteinases (SVMP) (Odell et al. 1998; Sousa et al. 2001) and with the presence of citrate to chelate metal ions and small peptidic inhibitors of SVMP (Odell et al. 1998; Robeva et al. 1991).

However, the process of manipulation and preparation of venom solutions in laboratory changes the original homeostasis and may activate proteolytic enzymes as SVSP and SVMP, which may act as autolytic enzymes. Such behavior was observed by Sousa et al. (2001) and Tashima et al. (2012) under different perspectives. Sousa et al. (2001) observed the decrease of activities given by *Bothrops jararaca* venom proteins due to proteolysis, while Tashima et al. (2012) verified the increased detection of venom peptides with the absence of proteolytic enzyme inhibitors in venom preparations of the Brazilian pit vipers *Bothrops jararaca*, *Bothrops cotiara*, and *Bothrops fonsecai*. Besides the novel sequences found as peptides from BPP and pHpG families, numerous L-amino acid oxidase (LAAO) and some SVMP and SVSP fragments were identified. Increasing inhibition of SVMP with EDTA and SVSP with PMSF in the venom solutions resulted in decrease of LAAO and SVMP and SVSP fragments detected in the venoms, suggesting a role mainly of SVMPs in the artificial proteolytic generation of venom toxin peptides (Tashima et al. 2012). To answer whether or not these peptides have a function in envenomation requires further investigations, but the point is that the peptidome complexity increases with the generation of these additional non-endogenous peptides.

On the one hand, in venom research, it is important to maintain the activities of venom components with the absence of toxin inhibitors, but on the other hand, the absence of inhibitors may cause the generation of artificial toxin fragments, which may puzzle snake venom peptidomic analysis. It is not uncommon to have limited amounts of venoms for venomics research, which may restrict the experimental design and the number of analysis. However, ideally, in peptidomic studies, the proteolytic enzymes present in the venoms should be identified and analysis performed in both conditions, with and without inhibition in order to separate the

real endogenous molecules from the autolytically generated ones. In cases where this is not possible due to quantity restrictions, venoms should be used as fresh as possible, and the peptidic fraction promptly separated from proteins.

De Novo Sequencing of Venom Peptides from MS/MS Spectra

Before the advent of soft ionization methods for MS, the most practical way for sequencing proteins or peptides was by the Edman degradation method (or N-terminal sequencing) in which the N-terminal amino acids are reacted with phenyl isothiocyanate, successively removed by a chemical route and identified by high performance liquid chromatography (HPLC) (Edman and Begg 1967). With the development of soft ionization methods such as matrix-assisted laser desorption/ ionization (MALDI) and electrospray ionization (ESI) and the refinement of techniques for fragmentation in MS analysis, MS/MS spectra are now used for sequencing new peptides and even entire proteins (Seidler et al. 2010). Without a genomic or transcriptomic database, peptide sequence is obtained by direct de novo sequencing of the ion series obtained by fragmentation of the precursor ion in an MS/MS spectrum (Standing 2003). The sequences of the first BPPs were determined by a combination of amino acid content analysis, enzymatic digestion, and Edman degradation (Ferreira et al. 1970a; Ondetti et al. 1971); however, several other snake venom peptides had their structures determined by MS analysis and de novo sequencing (Konno et al. 2008; Ianzer et al. 2004; Pimenta et al. 2007; Tashima et al. 2012; Menin et al. 2008).

For primary structure determination of an unknown peptide by de novo sequencing, it is necessary to obtain the complete fragmentation spectra by MS/MS. In a typical MS analysis, the ratio mass over charge (m/z) of a charged peptide is firstly measured in the mass analyzer. Afterward the precursor ion of this peptide is selected and fragmented, and the m/z of the fragments is determined. Fragmentation by collision-induced dissociation (CID), in which the ionized molecule is accelerated to collide with an inert gas, is the most frequently used in snake venom peptidomics. In CID, the main fragmentations are originated from disruption of the amide bonds, producing ions of the b- and y-series, according to the usual peptide fragmentation nomenclature (Kinter and Sherman 2000). The b- and y-series are complementary parts of the molecule; b-ions belong to the N-terminal side of the peptide, while the y-ions are from the C-terminal side. According to the mobile proton hypothesis, protons can migrate from the ionization site along the peptide chain and produce subpopulations of ions for each of the different amide bonds, directing fragmentation reactions (Kinter and Sherman 2000). Each subpopulation of ionized fragment produces a peak in the MS/MS spectrum, and mass differences between consecutive peaks from ions of the same series allow determination of amino acid identity and position in the peptide sequence. An important type of low mass ions observed in CID fragmentation, the immonium ions, is internal fragments of single amino acids which can be used as markers for the presence of amino acids and assist de novo sequencing (see amino acid nominal mass references in Table 1).

Table 1 Amino acid residues nominal masses and m/z values for the b_1, $y_{1,}$ and immonium ions

Amino acid		Residue mass (Da)	b_1 ion	y_1 ion	Immonium
Gly	G	57	58	76	30
Ala	A	71	72	90	44
Ser	S	87	88	106	60
Pro	P	97	98	116	70
Val	V	99	100	118	72
Thr	T	101	102	120	74
Cys	C	103	104	122	76
Pyr	Z	–	112	–	84
Ile	I	113	114	132	86
Leu	L	113	114	132	86
Asn	N	114	115	133	87
Asp	D	115	116	134	88
Gln	Q	128	129	147	101
Lys	K	128	129	147	101
Glu	E	129	130	148	102
Met	M	131	132	150	104
His	H	137	138	156	110
Met-ox	M-ox	147	148	166	120
Phe	F	147	148	166	120
Arg	R	156	157	175	129
Cys-cm	C-cm	160	161	179	133
Tyr	Y	163	164	182	136
Cys-am	C-am	174	175	193	147
Trp	W	186	187	205	159

C-cm Carboxymethylcysteine, *C-am* Acrylamide-modified cysteine, *M-ox* Oxidized metionine

Many snake venom peptides are characterized by the pyroglutamic acid (Z) in the N-terminal and high proline content, as the BPPs (Tashima et al. 2012). The b_1-ion for Z has a signal at m/z 112.1. The C-terminus of BPPs frequently ends with the amino acid sequences PP, AP, PAP, and PPAP, showing typically strong MS signals at m/z 213.1 (y_2), 187.1 (y_2), 284.2 (y_3), and 381.2 (y_4), respectively (Tashima et al. 2012; Menin et al. 2008). For example, the peptide BPP-10e (ZNWPSPKVPP) from the venom of *Bothrops cotiara* has a monoisotopic mass of 1131.6 Da (Tashima et al. 2012). The doubly positively charged precursor ion generated by ESI has an m/z of 566.8. Fragmentation by CID produces complementary signals of the b- and y-series, allowing the complete determination of primary structures by de novo sequencing (Figs. 2 and 3). Fragments of the bond N-terminal to proline produce intense peaks in the spectrum, as the y_2 PP (Fig. 2), due to preferential cleavage at this site (Zelanis et al. 2010; Fernandez Ocaña et al. 2005). Consequently, proline-rich peptide spectra from CID are difficult to interpret because signals from other fragments may be low abundant or absent (Fernandez Ocaña et al. 2005).

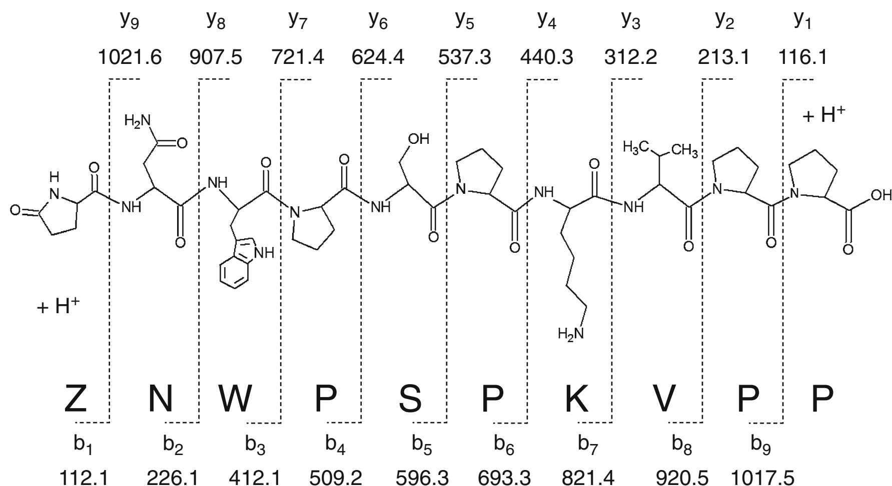

Fig. 2 Amino acid sequence, chemical structure, sites of CID fragmentation and the theoretical b- and y-series of fragment ions from the *Bothrops cotiara* peptide BPP-10e

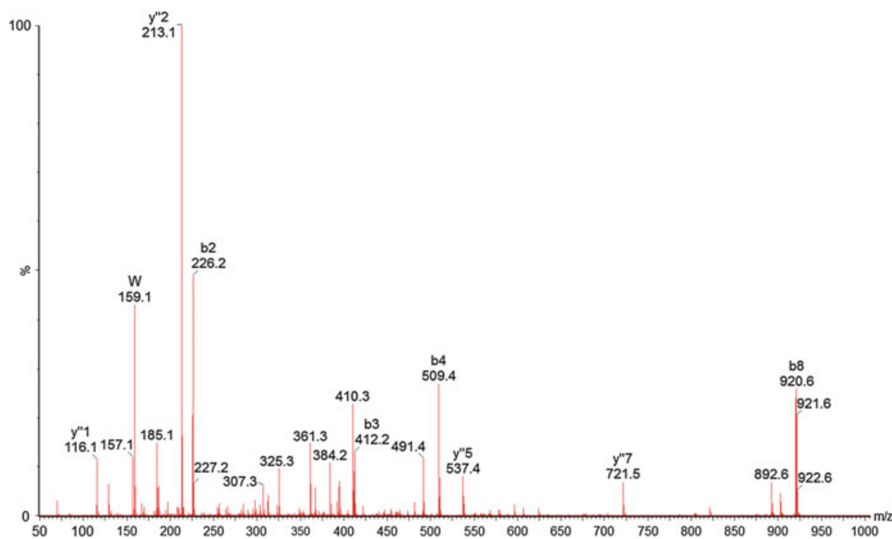

Fig. 3 MS/MS spectrum of the doubly charged precursor ion of BPP-10e (m/z 566.8^{+2}) and the complementary b- and y-series elucidated by de novo sequencing

Strategies combining different ionization and fragmentation methods may be suitable for obtaining complementary sequencing information of snake venom peptides (Tashima et al. 2012; Menin et al. 2008). Precursor ions generated by MALDI are preferentially singly charged, while the ones generated by ESI are mainly multiply charged ions. Fragmentation patterns from singly charged MALDI

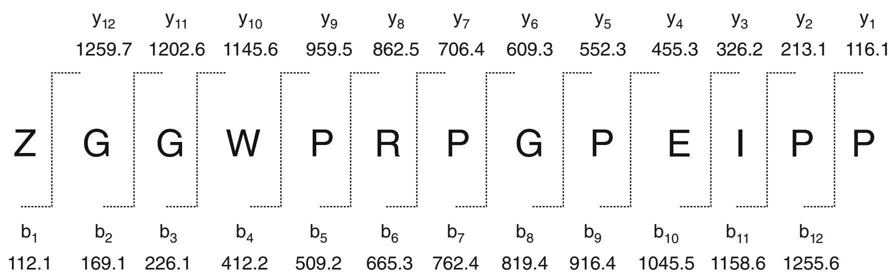

Fig. 4 Amino acid sequence, sites of CID fragmentation and the theoretical b- and y-series of fragment ions from the *Bothrops jararaca* peptide BPP-13a

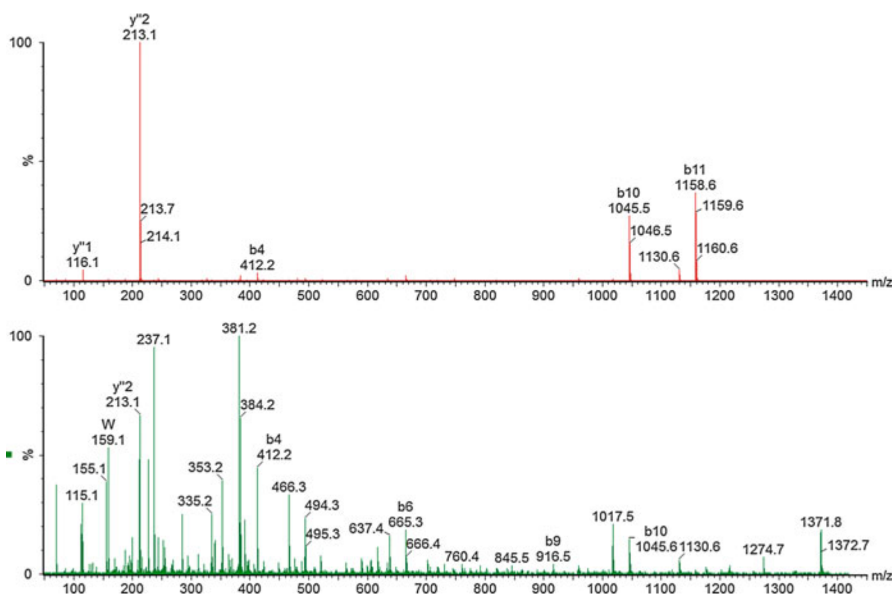

Fig. 5 MS/MS spectra of the *Bothrops jararaca* peptide BPP-13a. Upper panel: ESI-Q-TOF MS/MS spectrum from the doubly charged precursor ion at m/z 685.8^{+2}; lower panel: MALDI-Q-TOF MS/MS spectrum from of the singly charged precursor ion at m/z 1370.7^{+1}

ions are more heterogeneous mixtures of y-, b-, and a-ions and internal fragments, while multiply charged ions from ESI are mainly y-ions with b-ions in the lower mass range (Wattenberg et al. 2002). The CID MS/MS spectra from the peptide BPP-13a (Fig. 4) by ESI and MALDI present different ions and intensity patterns (Fig. 5). The high intensity of the C-terminal PP (y_2) in the ESI spectrum illustrates the difficulty of sequencing proline-rich peptides by de novo sequencing. Differences in the subpopulations of ion fragments obtained from MALDI spectrum provide additional information for the interpretation of the peptide sequence, illustrating that these combined techniques may compose useful approaches in snake venom peptidomics.

Conclusion and Future Directions

The study of snake venom peptides has revealed a new and exciting field of snake venomics. In order to fully understand the complexity of snake venom peptidomes, several factors should be taken into account, such as sample handling procedures, the composition of toxins within the venom gland (i.e., the snake venom proteome), and the processing pathways that might act during venom protein synthesis. Since snake venoms are a rich source of proteolytic enzymes, one should keep in mind the potential role of proteolytic enzymes in the generation of (new) peptides, therefore, increasing venom complexity, both in terms of structural diversity and biological action. Furthermore, given the irreversible nature of protease signaling (i.e., the signal is transmitted through the cleavage(s) of protein substrates resulting in their activation, inactivation, or modulation of function), cleavage site specificity studies could be highly benefited from the study of snake venom peptidomics, since they preserve in their primary structures important clues that have the potential to aid the study of (unconventional) proteolytic processing pathways of protein precursors. Likewise, the understanding of artifactual venom peptidomes (i.e., those generated by sample handling rather than through natural processing pathways within the venom gland) could help to design specific protocols in order to prevent undesirable venom proteolysis during the preparation of immunization mixtures used for antivenom production, an important feature in immunogen quality control.

Even though the beginning of the studies of snake venom peptides is strongly correlated to the first discoveries of bradykinin-potentiating peptides, in the mid-twentieth century, snake venom peptidomics is still in its infancy, and as more sophisticated methods of analysis and high-throughput approaches are used, a number of new peptides will be discovered, and more importantly, the biological meaning of snake venom peptidomes will be revealed.

Cross-References

► Automated Mass Fingerprinting of Venoms in Nanogram Range: Review of Technology
► Snake Venom Proteopeptidomics: What Lies Behind the Curtain

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Snake Venom Proteopeptidomics: What Lies Behind the Curtain

16

Richard Hemmi Valente, Carolina Alves Nicolau, Jonas Perales, and Ana Gisele da Costa Neves-Ferreira

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Abstract

The study of snake venoms has led to the discovery/development of a number of pharmaceutical drugs, diagnostic kits, and research molecular tools in the last 50 years. Furthermore, a deep knowledge of snake venom protein composition is necessary for better understanding the envenomation physiopathology and to contribute to the broadening in the clinical efficacy and range of antivenom therapy. During the past 15 years, several groups have applied proteomic techniques for the determination (to different extents) of peptide and protein compositions of snake venoms from almost 200 species (mostly from Viperidae and Elapidae families). The present review details the different methodological approaches used so far, stressing that none of them has generated a comprehensive snake venom proteopeptidome, ideally defined as the quali-quantitative

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representation of all proteoforms (including different peptide forms) present in any given snake venom. Due to the technological advancements in high-throughput genomics and transcriptomics allied to state-of-the-art *bottom-up* proteomics (as well as the possibility of *top-down* venomics), it is proposed that the time has come to actively pursue the proteopeptidome "dream." Several issues that should be addressed to achieve this goal are discussed. Finally, it is expected that in the next decade, snake venomics shall surpass the protein family-level-based identification and attain a proteoform-level-based one.

Introduction

In nature, snake venom inoculation mainly represents an adaptive foraging strategy (Casewell et al. 2013) used to paralyze and/or kill the prey and assist in its digestion (Chippaux et al. 1991). However, when related to human accidental envenoming, venoms could also be viewed as a defensive strategy. An estimate on global snakebite envenomation reports a yearly incidence ranging from 421,000 to 1,841,000 cases (Kasturiratne et al. 2008). The most effective treatment for snakebites relies on the administration of antivenom derived from hyperimmune equine serum (Chippaux and Goyffon 1998). Recently, due to the hindered availability and accessibility to antivenoms in highly populated low-income countries, snakebite envenomation has been classified as a neglected health condition by the World Health Organization (Gutierrez 2012). The lack of treatment leads to high mortality and morbidity rates (Gutierrez et al. 2007). Furthermore, even when antivenom therapy is available, the neutralization of local effects has been shown to be only partially effective by existing antivenoms, which can lead to chronic disability, including amputations (Gutierrez et al. 2006; Theakston et al. 2003). Therefore, there is still room for improvement in antiophidic therapy which has not significantly changed since its implementation, at the end of the nineteenth century, by Albert Calmette (1894). In order to achieve that goal, a deep understanding of snake venom proteic composition is required, and several groups are applying proteomic technology in venomics and antivenomics studies hoping to eventually contribute to a broadening in the clinical efficacy and range of antivenom therapy as well as get a better grasp at the envenomation physiopathology (Calvete 2011).

On the other hand, despite the dreadful effects of snake envenomation, the isolation and characterization of several enzymatic and nonenzymatic proteins from different snake venoms led to the discovery/development of a number of pharmaceutical drugs, diagnostic kits, and research molecular tools. Four snake venom-derived drugs were FDA approved and are in the market being used for treatment of hypertension, acute coronary syndromes, and perioperative bleeding. Three others are in clinical trials with indications for congestive heart failure, human immunodeficiency virus, and multiple sclerosis. Finally, there are ongoing preclinical studies using snake venom-derived molecules to be used in chronic pain treatment, wound healing, congestive heart failure, cancer, blood loss during vascular trauma, and non-compressible hemorrhage (Fox and Serrano 2007; King 2011).

Several diagnostic test kits were developed for the field of hemostasis, based on Viperidae snake venom enzymes. They can be used to assess deficiency or abnormalities in fibrinogen and determine protein C, protein S, factor V, factor X, and von Willebrand factor levels (Fox and Serrano 2007).

Snake venom components have also been used as molecular tools in understanding normal physiological processes. There is an excellent review recently published (McCleary and Kini 2013) that highlights the following examples: (a) snake venom nerve growth factor helping to explain cell growth regulation, (b) dendrotoxins assigning specific ion channels to different neurons and synapses, (c) sarafotoxins for studying vasoconstriction, and (d) cobra venom factor for determining the role of the complement system in host defense and immune response.

With the advent of proteomic technology, a window of opportunity to discover new biologically active molecules in snake venoms as well as to improve antiophidic therapy arose. In 2008, Fox and Serrano published a review on snake venom proteomics describing the different methodological workflows used that far, the relationship (or the lack of) between snake venom proteomic and transcriptomic data, as well as the possible applications of snake venom proteomics (Fox and Serrano 2008). An updated review on snake venomics was published by Calvete in 2013. Although this work listed the majority of snake venom proteomics (snake venomics) articles published up to that year, its focus was less on methodology and more on the establishment of evolutionary and ecological trends based on proteomics data. Furthermore, it demonstrated the use of antivenomics generated data in upgrading antivenom production strategies (Calvete 2013).

The present review will mainly focus on methodological details of snake venomics approaches used up to now, reminding the reader that none of them reported a comprehensive **snake venom proteopeptidome**, ideally defined as the quali-quantitative representation of all proteoforms (proteins and peptides) (Smith et al. 2013) present in a snake venom. The term "proteoform" is to be "used to designate all of the different molecular forms in which the protein product of a single gene can be found, including changes due to genetic variations, alternatively spliced RNA transcripts and posttranslational modifications" (Smith et al. 2013). A quantitative proteopeptidome will ultimately rely on obtaining its qualitative counterpart. Finally, some insights into possibilities and limitations on obtaining results representing a snake venom proteopeptidome will be discussed.

Snake Venom Proteomics ("Snake Venomics")

Initial Expectations and Updated Figures

From the 1950s to the 1990s, the majority of studies on snake venoms used biochemical techniques for isolation and characterization (structural and functional) of their individual components. Resorting to a variety of techniques such as 1D-PAGE (one-dimensional polyacrylamide gel electrophoresis), liquid

chromatography, Edman sequencing, and enzymatic and in vivo biological assays, it was possible to (1) determine that snake venoms are represented by approximately 90 % of their dry weight by polypeptides (Chippaux et al. 1991; Markland 1998) and (2) ascribe the effects of snakebite envenoming to peptides and proteins (Fox and Serrano 2008). However, the lack of snake genome information, allied to the low resolutive power of the techniques used until then, made the answers to the following questions difficult to be reached: (a) What are the complete protein and peptide profiles/identities in snake venom and what are the differences between venom compositions from different snake species and within the same species? (b) What are the modifications that these proteins could suffer during and/or after the translation process? (c) Could these post/co-translational modifications influence the protein's functionality and, if so, how?

With the emergence of proteomics in the early 1990s (Wilkins et al. 1996) and its application to snake venom analysis, later called "snake venomics" (Calvete et al. 2007b; Juarez et al. 2004), some of these questions have started to be answered while, naturally, new ones arose.

During the last 15 years, there has been a great effort from the proteomics community to characterize the venoms of several snakes. The methodological approaches used varied (and evolved) so that some venoms were more in-depth characterized than others. Tables 1 and 2 list those snakes for which there has been at least some level of larger-scale proteic/peptidic analysis done for their venoms. Up to when this manuscript was submitted, 5 families of venomous snakes distributed across 72 genera and 184 species (44 of those at the subspecies level) were analyzed by proteomics. By far, the Viperidae and Elapidae families, which are the most important from a medical point of view, were also the most studied, with 95 and 63 species, respectively. Colubridae, Lamprophiidae, and Homalopsidae families had 21, 4, and 1 species studied, respectively.

The Early Days of Snake Venom Proteomics

One of the first works that attempted a broad-range analysis of snake venom contents was the study on venom glands from the sea snake *Laticauda colubrina* and the terrestrial *Daboia russelli*. Some known snake venom proteins, such as neurotoxins (in *L. colubrina*), hemorrhagic and coagulation-related factors (in *D. russelli*), and PLA_2 (phospholipase A_2) (in both snakes), as well as gland-associated proteins, were found. The methodological strategy used a combination of 2D-PAGE (two-dimensional PAGE) for protein separation coupled to amino acid composition analysis and Edman *N*-terminal sequencing for identification (Rioux et al. 1998). This kind of approach was very labor and time intensive and did not yield the necessary sensitivity. But the use of mass spectrometry to the analysis and identification of peptides and proteins was already in place (Kuster and Mann 1998), and the first group to apply it to snake venom studies was Jay Fox's lab (Kamiguti et al. 2000). In this early work, using 1D-PAGE followed by band excision, trypsin digestion,

Table 1 Snake families,[a] genera[b] and species (subspecies) for which snake venom proteomics data (superficial to in-depth) were generated in the past 15 years

Colubridae	
Ahaetulla prasina	Fry et al. (2003)
Boiga cynodon	Fry et al. (2003)
Boiga dendrophila dendrophila	Fry et al. (2003)
Boiga dendrophila gemmicincta	Fry et al. (2003)
Boiga drapiezii	Fry et al. (2003)
Boiga irregularis	Fry et al. (2003)
Boiga nigriceps	Fry et al. (2003)
Boiga trigonata	Fry et al. (2003)
Cerberus rynchops	Fry et al. (2003), OmPraba et al. (2010)
Coelognathus radiatus	Fry et al. (2003)
Coluber rhodorachis	Fry et al. (2003)
Dispholidus typus	Fox et al. (2002), Fry et al. (2003)
Gonyosoma oxycephalum	Fry et al. (2003)
Heterodon nasicus	Fry et al. (2003)
Philodryas olfersii	Ching et al. (2006)
Philodryas patagoniensis	Fry et al. (2003)
Ptyas carinatus	Fry et al. (2003)
Rhabdophis tigrinus	Fry et al. (2003)
Telescopus dhara	Fry et al. (2003)
Thamnodynastes strigatus	Ching et al. (2012)
Trimorphodon biscutatus	Fry et al. (2003)
Elapidae	
Acantophis antarticus	Birrell et al. (2007)
Aipysurus duboisii	Fry et al. (2003)
Aipysurus foliosquama	Fry et al. (2003)
Aipysurus fuscus	Fry et al. (2003)
Aspidelaps lubricus	Fry et al. (2003)
Austrelaps ramsayi	Birrell et al. (2007)
Austrelaps superbus	Birrell et al. (2007)
Bungarus fasciatus	Nawarak et al. (2003)
Bungarus multicinctus	Nawarak et al. (2003)
Bungarus sindanus	Ali et al. (2013)
Crytophis nigrescens	Fry et al. (2003)
Demansia papuensis	Fry et al. (2003)
Demansia vestigiata	St Pierre et al. (2007)
Dendroaspis polylepis	Fry et al. (2003)
Drysdalia coronoides	Chatrath et al. (2011)
Echiopsis curta	Fry et al. (2003)
Enhydrina schistosa	Fry et al. (2003)
Glyphodon tristis	Fry et al. (2003)
Hoplocephalus stephensii	Birrell et al. (2007), Earl et al. (2006)

(*continued*)

Table 1 (continued)

Hydrophis cyanocinctus[c]	Calvete et al. (2012a)
Lapemis curtus	Fry et al. (2003)
Laticaudac olubrina	Rioux et al. (1998)
Laticauda schistorhyncus	Fry et al. (2003)
Micropechis ikaheka	Fry et al. (2003)
Micrurus altirostris[c]	Correa-Netto et al. (2011)
Micrurus corallinus[c]	Correa-Netto et al. (2011)
Micrurus frontalis	Ciscotto et al. (2011)
Micrurus ibiboboca	Ciscotto et al. (2011)
Micrurus lemniscatus	Ciscotto et al. (2011)
Micrurus mipartitus[c]	Rey-Suarez et al. (2011)
Micrurus nigrocinctus[c]	Fernandez et al. (2011)
Micrurus spixii	Ciscotto et al. (2011)
Micrurus surinamensis	Olamendi-Portugal et al. (2008)
Naja haje legionis	Malih et al. (2013)
Naja katiensis[c]	Petras et al. (2011)
Naja mossambica[c]	Petras et al. (2011)
Naja naja	Ali et al. (2013)
Naja naja atra	Li et al. (2004), Nawarak et al. (2003)
Naja naja haje	Nawarak et al. (2003)
Naja naja kaouthia	Kulkeaw et al. (2007), Nawarak et al. (2003, 2004)
Naja naja siamensis	Nawarak et al. (2003)
Naja nigricolis[c]	Petras et al. (2011)
Naja nubiae[c]	Petras et al. (2011)
Naja pallida[c]	Petras et al. (2011)
Notechis ater niger	Birrell et al. (2007)
Notechis ater serventyi	Birrell et al. (2007)
Notechis scutatus	Birrell et al. (2007)
Ophiophagus hannah	Nawarak et al. (2003)
Oxyuranus microlepidotus	Birrell et al. (2007), Earl et al. (2006)
Oxyuranus scutellatus[c]	Birrell et al. (2007), Earl et al. (2006), Herrera et al. (2012)
Pelamis platurus	Fry et al. (2003)
Pseudechis australis	Birrell et al. (2007), Earl et al. (2006), Herrera et al. (2012)
Pseudechis colletti	Birrell et al. (2007)
Pseudechis guttatus	Birrell et al. (2007)
Pseudechis porphyriacus	Birrell et al. (2007)
Pseudonaja affinis	Birrell et al. (2007)
Pseudonaja inframacula	Birrell et al. (2007)
Pseudonaja nuchalis	Birrell et al. (2007)
Pseudonaja textilis	Birrell et al. (2006)
Rhinoplocephalus nigrescens	Birrell et al. (2007), Earl et al. (2006)

(*continued*)

Table 1 (continued)

Suta suta	Fry et al. (2003)
Toxicocalamus longissimus[c]	Calvete et al. (2012a)
Tropidechis carinatus	Birrell et al. (2007)
Homalopsidae	
Enhydris chinensis	Fry et al. (2003)
Lamprophiidae	
Atractaspis microlepidota	Fry et al. (2003)
Leioheterodon madagascariensis	Fry et al. (2003)
Psammophis mossambicus	Fry et al. (2003)
Viperidae	
Agkistrodon bilineatus bilineatus[c]	Lomonte et al. (2013)
Agkistrodon bilineatus howardgloydi[c]	Lomonte et al. (2013)
Agkistrodon contortrix contortrix[c]	Lomonte et al. (2013)
Agkistrodon contortrix laticinctus[c]	Lomonte et al. (2013)
Agkistrodon contortrix mokasen[c]	Lomonte et al. (2013)
Agkistrodon contortrix phaeogaster[c]	Lomonte et al. (2013)
Agkistrodon contortrix pictigaster[c]	Lomonte et al. (2013)
Agkistrodon piscivorus conanti[c]	Lomonte et al. (2013)
Agkistrodon piscivorus leucostoma[c]	Lomonte et al. (2013)
Agkistrodon piscivorus piscivorus[c]	Lomonte et al. (2013)
Agkistrodon taylori[c]	Lomonte et al. (2013)
Atropoides mexicanus[c]	Angulo et al. (2008)
Atropoides picadoi[c]	Angulo et al. (2008)
Azemiops feae	Fry et al. (2003)
Bitis arietans[c]	Fasoli et al. (2010), Juarez et al. (2006a)
Bitis caudalis[c]	Calvete et al. (2007a)
Bitis gabonica gabonica[c]	Calvete et al. (2007c)
Bitis gabonica rhinoceros[c]	Calvete et al. (2007a)
Bitis nasicornis[c]	Calvete et al. (2007a)
Bothriechis lateralis[c]	Lomonte et al. (2008)
Bothriechis nigroviridis[c]	Fernandez et al. (2010)
Bothriechis schlegelii[c]	Lomonte et al. (2008)
Bothriechis supraciliaris[c]	Lomonte et al. (2012b)
Bothrops alternatus	Ohler et al. (2010), Sousa et al. (2013)
Bothrops asper[c]	Alape-Giron et al. (2008)
Bothrops atrox[c]	Calvete et al. (2011), Guercio et al. (2006), Kohlhoff et al. (2012), Nunez et al. (2009), Rocha et al. (2009), Sousa et al. (2013)
Bothrops ayerbi[c]	Mora-Obando et al. (2013)
Bothrops barnetti[c]	Kohlhoff et al. (2012)
Bothrops bilineata	Paes Leme et al. (2009)
Bothrops caribbaeus[c]	Gutierrez et al. (2008)

(*continued*)

Table 1 (continued)

Bothrops colombiensis[c]	Calvete et al. (2009a)
Bothrops cotiara[c]	Paes Leme et al. (2009), Sousa et al. (2013), Tashima et al. (2008)
Bothrops erythromelas	Paes Leme et al. (2009)
Bothrops fonsecai[c]	Tashima et al. (2008)
Bothrops insularis	Paes Leme et al. (2009), Rocha et al. (2009), Valente et al. (2009)
Bothrops jararaca	Fox et al. (2002, 2006), Paes Leme et al. (2009), Rocha et al. (2009), Serrano et al. (2005), Sousa et al. (2013), Zelanis et al. (2010, 2011, 2012b)
Bothrops jararacussu	Correa-Netto et al. (2010), Paes Leme et al. (2009), Sousa et al. (2013)
Bothrops lanceolatus[c]	Gutierrez et al. (2008)
Bothrops moojeni	Paes Leme et al. (2009)
Bothrops neuwiedi	Paes Leme et al. (2009), Sousa et al. (2013)
Bothrops pauloensis[c]	Rodrigues et al. (2012)
Bothrops pictus[c]	Kohlhoff et al. (2012)
Bothrops pirajai[c]	Bernardes et al. (2013)
Causus rhombeatus	Fry et al. (2003)
Cerastes cerastes[c]	Bazaa et al. (2005), Fahmi et al. (2012)
Cerastes vipera[c]	Bazaa et al. (2005)
Cerrophidion sasai[c]	Lomonte et al. (2012a)
Crotalus adamanteus	Margres et al. (2013)
Crotalus atrox[c]	Calvete et al. (2009b), Fox et al. (2002, 2006), Rocha et al. (2009), Serrano et al. (2005)
Crotalus durissus cascavella[c]	Boldrini-Franca et al. (2010)
Crotalus durissus collilineatus[c]	Boldrini-Franca et al. (2010)
Crotalus durissus cumanensi[c]	Calvete et al. (2010)
Crotalus durissus durissus[c]	Calvete et al. (2010)
Crotalus durissus ruruima[c]	Calvete et al. (2010)
Crotalus durissus terrificus[c]	Calvete et al. (2010), Georgieva et al. (2010)
Crotalus oreganus helleri	Jurado et al. (2007)
Crotalus scutulatus scutulatus[c]	Massey et al. (2012)
Crotalus simus simus[c]	Calvete et al. (2010), Durban et al. (2013)
Crotalus tigris[c]	Calvete et al. (2012b)
Daboia russelli formosensis	Nawarak et al. (2003)
Daboia russelli russelli	Nawarak et al. (2003), Rioux et al. (1998)
Daboia russelli siamensis	Risch et al. (2009)
Deinagkistrodon acutus	Nawarak et al. (2004)
Echis ocellatus[c]	Juarez et al. (2006b), Wagstaff et al. (2009)
Gloydius brevicaudus	Gao et al. (2013)
Gloydius halys	Li et al. (2004)
Gloydius shedaoensis	Liu et al. (2011)
Lachesis acrochorda[c]	Madrigal et al. (2012)

(*continued*)

Table 1 (continued)

Lachesis melanocephala[c]	Madrigal et al. (2012)
Lachesis muta muta[c]	Sanz et al. (2008b)
Lachesis muta rhombeata[c]	Pla et al. (2013)
Lachesis stenophrys[c]	Madrigal et al. (2012), Sanz et al. (2008b)
Macrovipera lebetina[c]	Bazaa et al. (2005), Makran et al. (2012)
Macrovipera lebetina obtusa[c]	Sanz et al. (2008a)
Macrovipera mauritanica[c]	Makran et al. (2012)
Ovophis okinavensis	Aird et al. (2013)
Porthidium nasutum[c]	Lomonte et al. (2012a)
Porthidium ophryomegas[c]	Lomonte et al. (2012a)
Proatheris superciliaris	Fry et al. (2003)
Protobothrops flavoviridis	Aird et al. (2013)
Protobothrops mucrosquamatus[c]	Nawarak et al. (2003), Villalta et al. (2012)
Sistrurus catenatus catenatus[c]	Gibbs et al. (2009), Sanz et al. (2006)
Sistrurus catenatus edwardsii[c]	Sanz et al. (2006)
Sistrurus catenatus tergeminus[c]	Sanz et al. (2006)
Sistrurus milarius barbouri[c]	Gibbs et al. (2011), Juarez et al. (2004), Sanz et al. (2006), Yanes et al. (2007)
Sistrurus milarius milarius[c]	Gibbs et al. (2009, 2013)
Sistrurus milarius streckeri[c]	Gibbs et al. (2013)
Tropidolaemus wagleri	Fry et al. (2003)
Vipera ammodytes ammodytes	Georgieva et al. (2008)
Vipera ammodytes meridionalis	Georgieva et al. (2008)
Vipera raddei[c]	Sanz et al. (2008a)
Viridovipera stejnegeri[c]	Nawarak et al. (2003), Villalta et al. (2012)

[a]Family taxonomy according to (Pyron et al. 2013)
[b]*Bothrops* taxonomy according to (Carrasco et al. 2012)
[c]Species venom analyzed by Calvete's snake venomics approach

and LC-ESI-MS/MS (liquid chromatography hyphenated to electrospray ionization tandem mass spectrometry), they reported the unexpected (at the time) presence of a class P-III/P-IV metalloproteinase in the venom of the colubrid *Dispholidus typus* (Kamiguti et al. 2000).

Another pioneering work used liquid chromatography separation coupled to mass spectrometry to analyze the whole venom of 42 snake species distributed along the Colubridae (19 spp.), Elapidae (15 spp.), Homalopsidae (1 sp.), Lamprophiidae (3 spp.), and Viperidae (4 spp.) families. The data generated distribution patterns (retention time versus molecular mass) that could be used to reach a series of conclusions related to clinical management and, most of all, to new evolutionary and taxonomical trends. Unfortunately, the proteins were not identified since the possibility of *top-down* proteomics (to be discussed later in this text) was not available at the time (Fry et al. 2003).

Table 2 Snake families[a], genera[b], and species (subspecies) for which snake venom peptidomics data (superficial to in-depth) were generated in the past 15 years

Elapidae	
Micrurus surinamensis	Olamendi-Portugal et al. (2008)
Naja naja atra	Li et al. (2004)
Lamprophiidae	
Atractaspis irregularis	Quinton et al. (2005)
Viperidae	
Atheris chlorechis	Favreau et al. (2007)
Atheris nitschei	Favreau et al. (2007)
Atheris squamigera	Favreau et al. (2007)
Bothriechis supraciliaris	Lomonte et al. (2012b)
Bothrops alternatus	Wermelinger et al. (2005)
Bothrops cotiara	Tashima et al. (2012)
Bothrops fonsecai	Tashima et al. (2012)
Bothrops insularis	Valente et al. (2009), Wermelinger et al. (2005)
Bothrops jararaca	Ianzer et al. (2004), Pimenta et al. (2007), Tashima et al. (2012), Wermelinger et al. (2005), Zelanis et al. (2010)
Bothrops jararacussu	Munawar et al. (2011), Wermelinger et al. (2005)
Bothrops moojeni	Menin et al. (2008)
Bothrops neuwiedi	Wermelinger et al. (2005)
Crotalus adamanteus	Wermelinger et al. (2005)
Crotalus durissus terrificus	Wermelinger et al. (2005)
Crotalus viridis	Wermelinger et al. (2005)
Gloydius halys	Li et al. (2004)
Vipera ammodytes meridionalis	Munawar et al. (2011)

[a]Family taxonomy according to (Pyron et al. 2013)
[b]*Bothrops* taxonomy according to (Carrasco et al. 2012)

Bottom-Up Proteomics

In proteomics, there are currently three strategies in place for the identification of proteins present in a sample: *bottom-up*, *middle-down*, and *top-down* (Han et al. 2008). The most used approach is the *bottom-up*, where fractions of the sample or the whole sample are submitted to enzymatic digestion (usually with trypsin) and the generated peptides are analyzed (sequenced) by mass spectrometry, leading to the identity of the proteins originally present in the sample (*shotgun* proteomics). In the *top-down* approach, which is a technique still under development, the intact proteins are previously separated and directly analyzed by mass spectrometry, where they will be fragmented and sequenced. When this strategy is not possible, the *middle-down* approach is employed. It represents a compromise between the other two strategies, where the protein is enzymatically digested into longer peptides and then identified by mass spectrometry. There are still no articles in the literature reporting *middle-* or *top-down* strategies usage in snake venomics.

Venom Fractionation Previously to Enzymatic Digestion and MS Analysis

One of the initial reports coupling electrophoretic separation to mass spectrometry identification of snake venom components was published by Fox and coworkers in 2002. This work used both 1D-PAGE (SDS-PAGE) linked to identification by LC-ESI-MS/MS with de novo sequencing and 2D-PAGE coupled to LC-ESI-MS with PMF (peptide mass fingerprinting) identification. Although some proteins were identified by de novo sequencing, the lack of a genome sequence database rendered the PMF approach highly ineffective, as expected (Fox et al. 2002). More recently, the 1D-PAGE approach was used for a preliminary characterization of *Gloydius shedaoensis* venom proteome (Liu et al. 2011).

In 2003, a study analyzed the venom of ten snake species from the Elapidae (*Naja naja atra*, *N. n. kaouthia*, *Bungarus multicinctus*, *B. fasciatus*, and *Ophiophagus hannah*) and Viperidae (*Vipera russelli siamensis*, *V. r. formosensis*, *Deinagkistrodon acutus*, *Trimeresurus mucrosquamatus*, and *T. stejnegeri*) families using a variety of separation methods [(SDS-PAGE, *lab-on-a-chip*, 2D-PAGE, and RPC (reversed-phase chromatography)]. This work generated an important contribution about some intraspecies as well as interfamily differences by demonstrating that it was possible to ascribe a unique separation pattern to a venom species. Fourteen of the most intense *spots* across all ten venoms were identified by Edman sequencing as known snake venom toxins. The authors considered other 133 *spots* of the 2D-PAGE identified by PMF against the NCBI (National Center for Biotechnology Information) and SWISS-PROT databases, being that most of the identifications were not related to snake venoms (Nawarak et al. 2003). One possible explanation is the unreliability of PMF identification against such big databases, especially given that no snake genome/transcriptome was present in the databases, leading most probably to a series of false-positive results (Stead et al. 2006).

In 2004, a comprehensive analysis in snake venomics was performed by Li and collaborators (Li et al. 2004). They used four different methodological strategies to compare the venoms of *Naja naja atra* and *Agkistrodon halys*. The first three strategies included (1) direct venom trypsin digestion in solution, (2) SDS-PAGE venom separation followed by *in-gel* trypsin digestion, and (3) <10 kDa pool from SEC (size/molecular exclusion chromatography) of venom followed by trypsin digestion. For these strategies, tryptic digests were analyzed by LC-ESI-MS/MS on a low-resolution mass spectrometer and proteins identified by PSM (peptide-spectrum match). The fourth strategy consisted of submitting >10 kDa pool, from venom SEC, to 2D-PAGE followed by *spot in-gel* digestion with trypsin and analysis by MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry); in this case, proteins were identified by PMF. The authors reported the identification of 124 and 74 proteins/peptides for the Elapidae and Viperidae snakes, respectively. However, it is worth noting that their results did not differentiate the naturally occurring peptides in these venoms since they submitted the low molecular mass pooled fraction to trypsin digestion, before analysis.

For the analysis of several Viperidae and one Elapidae venoms, different research groups applied 2D-PAGE separation followed by *spot* content tryptic digestion and identification by nanoLC-nanoESI-MS/MS (Guercio et al. 2006; Juarez et al. 2004; Jurado et al. 2007; Risch et al. 2009; Serrano et al. 2005). Although quite time-consuming (approximately 1 h per *spot* analyzed), this strategy generated an effective way of assigning a protein family to each sample, either by PSM or de novo sequencing analyses. Nowadays, the use of high-resolution hybrid mass spectrometers is a common practice for this methodological workflow, generating high-confidence identifications (Georgieva et al. 2010. 2011; Ohler et al. 2010).

Another common workflow consists of venom separation by 2D-PAGE, followed by *spot* excision and trypsin digestion and analysis by MALDI-TOF/TOF MS (MALDI *tandem* MS). The generated data can be analyzed by PMF, PSM, and de novo sequencing, and this strategy has been widely used by the group of Martin Lavin to study the venom of several elapidic Australian snakes (Birrell et al. 2006, 2007; Earl et al. 2006; St Pierre et al. 2007). It is worth mentioning that, nowadays, the identification of snake venom components only by PMF is considered deprecated. However, some works demonstrated that, for those snakes whose venom gland transcriptomes are known, PMF can represent a wise cost-benefit approach (Ching et al. 2006; Valente et al. 2009).

Actually, the most widespread snake venom proteomics strategy is a slightly modified version of the method implemented by Juárez, Sanz, and Calvete for the study of *Sistrurus barbouri* venom (Juarez et al. 2004). The current workflow includes an initial venom RPC fractionation in the low-milligram range, followed by characterization of each chromatographic peak by a tetrapartite analysis (*N*-terminal Edman sequencing, SDS-PAGE, intact molecular mass, and cysteine content determinations by mass spectrometry). Protein fractions that are not immediately identified, either for being heterogeneous or having their *N*-termini blocked, are *in-gel* digested from the SDS-PAGE and submitted to PMF and selective de novo sequencing for identification (Bazaa et al. 2005; Calvete et al. 2007b). One advantage of this method is its quantitative nature regarding toxin families, where one can estimate their weight% (and eventually mol%) related to whole venom composition. This approach, that has been developed and implemented by Juan Calvete's group, can be considered the canonical methodological pipeline for obtention of quali-quantitative snake venomics data and has been extensively reviewed (Calvete 2011; Calvete et al. 2007b). From the 184 species listed in Table 1, the venoms from 79 spp. (43 %) have been analyzed by the Calvete approach, being 12 from the Elapidae and 67 from the Viperidae families. According to Dr. Calvete, the "long-term goal is a detailed analysis of viperid venoms" (Calvete et al. 2007c). Moreover, his interest is not on discovery-driven proteomics *per se* but on using snake venomics to formulate (and even test) different biological hypotheses related to snake ecology and evolution, as well as finding potential clinical applications regarding antivenom therapy (Calvete 2013).

Whole Venom Enzymatic Digestion and MS Analysis

Another strategy for the analysis of snake venom proteomes precludes the initial protein fractionation step(s) and is commonly referred in the proteomics community as the *shotgun* approach (Zhang et al. 2013). The venom sample is directly submitted to enzymatic digestion (usually with trypsin), and the resulting peptide "soup" is submitted to nanoLC-nanoESI or nanoLC-MALDI-MS/MS. This has been done for the venoms of *Crotalus atrox* and *Bothrops jararaca* (Fox et al. 2006) and later on for those from one Colubridae (OmPraba et al. 2010) and one Elapidae (Chatrath et al. 2011) representatives. Recently, it has been successfully applied to indicate little connection between taxonomic position and venom composition in some bothropic species (Sousa et al. 2013); interestingly the same conclusion was reached by others, using the Calvete venomics approach, for a clade of rattlesnakes (Gibbs et al. 2013). In summary, the *shotgun* workflow used in these papers was far less laborious and generated a solid global view of the venom contents. However, it should be noted that this strategy, as it stands, is not comprehensive enough for an in-depth proteomic analysis. Recently, some authors have used a digestion protocol with multiple enzymes (trypsin, chymotrypsin, endoproteinase Glu-C) along with MS/MS analyses using high-resolution instrument. Furthermore, PSM analyses for peptide/protein identifications were done against specific databases, constructed with high-throughput transcriptomics-generated sequences, for the study of *Ovophis okinavensis* and *Protobothrops flavoviridis*. They were able to obtain average protein sequence coverage of *ca*. 60 % and good correlation between transcriptomics and proteomics data (Aird et al. 2013), indicating that there is plenty of room for improving *shotgun*-generated snake venomics data.

Mixed Strategies

Other pipelines have been used over the years that consisted of a combination of the methods (and their variations) above described. For instance, in 2007, the venom composition of *Naja kaouthia* was investigated by 2D-PAGE separation linked to LC-ESI-MS/MS identification and by two-dimensional [SCXC (strong cation-exchange chromatography)/RPC] *shotgun* workflows (Kulkeaw et al. 2007). A similar approach but with one-dimensional *shotgun* was used for the venoms of *Bungarus sindanus* and *Naja naja* (Ali et al. 2013). Others have enriched their 2D-PAGE LC-ESI-MS/MS dataset with SAXC (strong anion-exchange chromatography) venom fractionation coupled to Edman sequencing identification, when analyzing venoms from *Vipera ammodytes ammodytes* and *V. a. meridionalis* (Georgieva et al. 2008). For the relatively low-complexity *Micrurus surinamensis* venom, a peptidomic and proteomic analysis was attempted. Venom was initially separated in two fractions (peptides < 10 kDa < proteins). The peptidic fraction was analyzed by mass profiling (LC-MALDI-TOF) and peptide sequencing (LC-ESI-MS/MS and Edman chemistry). The proteic fraction was analyzed by 2D-PAGE separation linked to LC-ESI-MS/MS identification (Olamendi-Portugal et al. 2008). Another work used a multimethod strategy aiming for the elucidation

of *Bothrops insularis* venom composition using (a) 2D-PAGE with *spot* identification by PMF and/or PSM, (b) whole venom LC-ESI-Q/TOF MS, (c) MALDI-TOF/TOF MS with de novo sequencing, and (d) <10 kDa fraction, trypsinization, and LC-MS/MS with PSM analysis (Valente et al. 2009). For *B. jararacussu*, only 2D-PAGE with *spot* identification by PMF and/or PSM was done (Correa-Netto et al. 2010). For the analysis of *Micrurus frontalis*, *M. ibiboboca*, and *M. lemniscatus* venoms, fractions from two-dimensional chromatography were submitted to mass profiling and identification by Edman sequencing; moreover, venom components were also identified by PSM after 2D-PAGE MALDI-TOF/TOF MS (Ciscotto et al. 2011). A modified version of the Calvete approach has been published for the venomics analysis of *Naja haje legionis*. Basically, SEC was done before the RPC separation, and all fractions were analyzed by tricine-SDS-PAGE, instead of conventional SDS-PAGE (Malih et al. 2013). Recently, a very promising workflow was published for *Crotalus adamanteus* venom (Margres et al. 2013). The authors employed (a) a shotgun approach coupled to MS^E [data-independent high-resolution tandem mass spectrometry (Plumb et al. 2006)]; (b) SEC, trypsin digestion, and LC-ESI-MS/MS; and (c) RPC, trypsin digestion, LC-ESI-MS/MS, and Edman sequencing. Furthermore, their PSM searches were done against a database built with high-throughput transcriptomics data for that snake species. With an average sequence coverage (for the MS^E pipeline) of *ca.* 55 % and *ca.* 39 % for the LC-ESI-MS/MS experiments, they were able to distinguish between different protein family members at much higher rates than any other work published so far.

Comparative Quantification Analysis

Very little has been done in terms of quantifiable analysis using established state-of-the-art proteomics quantification methods. Difference gel electrophoresis (Unlu et al. 1997) was applied to compare *Pseudonaja textilis* venoms from different geographical areas (Birrell et al. 2006). Also, isobaric tag labeling (Ross et al. 2004) was used to quantify venom toxin composition of newborn and adult *Bothrops jararaca* (Zelanis et al. 2011) and *Gloydius brevicaudus* (Gao et al. 2013) snakes.

Subproteomics

Combinatorial Peptide Ligand Libraries (CPLL: *ProteoMiner™*)

One strategy that has been used to detect and analyze low-abundance proteins in venomics is based on the so-called protein equalizer technology. The principle is that by allowing the sample to interact with beads containing an immobilized combinatorial library of hexapeptides, one can reduce the concentration of highly abundant proteins and enhance the abundance of low-concentration ones, leading to the "equalization" of components' abundance (Righetti et al. 2006). In venomics, after CPLL reaction, the "equalized" sample was submitted to 2D-PAGE separation followed by

spot identification by LC-ESI-MS/MS. The first work used two different CPLL versions (unmodified and carboxylated) to study the venom of *Crotalus atrox* (Calvete et al. 2009b), whereas another work used the unmodified CPLL at three different pH values (3.8, 7.2, and 10.0) to probe the "hidden proteome" of *Bitis arietans* (Fasoli et al. 2010). Although there are no published studies defining the protein dynamic range in venoms, it is estimated that the Calvete venomics approach "detects proteins present in amounts greater than 0.05 % of the total venom proteins" (Calvete et al. 2009b). Both works using CPLL were successful at complementing data from common venomics approaches by detecting low-abundance components (<0.05 % total venom protein content) such as peroxiredoxin, glutaminyl cyclase (Calvete et al. 2009b), and 5′-nucleotidase (Fasoli et al. 2010).

Screening Using Immunostaining

A complementary methodology for venom proteome characterization is the immunostaining of nitrocellulose membranes to which 2D-PAGE-separated venom components were transferred. This technique has been used to characterize SVMPs (snake venom metalloproteinases), SVSPs (snake venom serine proteinases), and basic PLA_2 distribution in Viperidae (Serrano et al. 2005) as well as prothrombinase complex, heavy chain factor Xa-like proteinase, textilinin, and textilotoxin in Elapidae (Birrell et al. 2006; St Pierre et al. 2007).

Selective Enrichment with Specific Bait Compounds

In order to assess the presence of heparin-binding venom components in bothropic venoms (eight species), Solange Serrano's group used affinity chromatography (*Heparin-Sepharose™* column) followed by 1D-PAGE and LC-ESI-MS/MS identification of selected gel bands (Paes Leme et al. 2009). Another affinity chromatography-based workflow used immobilized DM43 [(snake venom metalloproteinase inhibitor) (Neves-Ferreira et al. 2002)] and 2D-PAGE with MALDI-TOF/TOF MS to compare metalloproteinase (and its fragments) composition from four Crotalinae venoms (Rocha et al. 2009). Interestingly, in another work, the DM43 affinity column approach was used for an in-depth analysis of the "metalloproteome" from the venom of the colubrid *Thamnodynastes strigatus*, confirming the major occurrence of svMMPs (snake venom matrix metalloproteinases) (Ching et al. 2012).

Posttranslational Modifications (PTMs)

Glycosylation

This is a common PTM in snake venoms. In *Bothrops jararaca* venom, for example, it has been extensively detected for its main toxin families, namely, SVMPs and SVSPs (Zelanis et al. 2010). The role of this PTM in snake venom proteins still needs further study, but it has already been associated with (a) increasing protein's scaffold stability, (b) preventing the interaction with possible natural inhibitors (Zhu et al. 2005), and (c) modulating toxin's enzymatic activity (Paes Leme et al. 2008).

The pioneering work on snake venom glycoproteomics used a panel of fluorescein isothiocyanate (FITC)-labeled lectins to establish a specific glycoprotein pattern for Elapidae (five species) and Viperidae (five species) venoms. Furthermore, the venom of *Naja naja kaouthia* was submitted to Con-A Sepharose fractionation followed by 2D-PAGE of bound and unbound fractions, but at the time, just a handful of *spots* were identified (Nawarak et al. 2004). The FITC-lectin pipeline was also used for the analysis of *Pseudonaja textilis* (Birrell et al. 2006) and other 18 Australian elapids (Birrell et al. 2007; Earl et al. 2006), while the Con-A Sepharose pipeline was used to compare the glycoproteomic profile of newborn and adult *Bothrops jararaca* venoms (Zelanis et al. 2010).

Another way of establishing a glycoprotein profile is the use of fluorescence-based periodate-oxidized carbohydrate group staining (*Pro-Q Emerald^{TM}*) following 1D- or 2D-PAGE separation. This technique has been used for Viperidae (Serrano et al. 2005; Zelanis et al. 2010) and Elapidae venoms (Birrell et al. 2006, 2007).

Recently, a broad *N*-glycosylation profiling was undertaken by the Serrano group for the venoms of newborn and adult *B. jararaca* snakes (Zelanis et al. 2012b). In order to identify the *N*-glycans and demonstrate the presence of some structural isomers, they used an MS^n (*tandem-in-time* where $n = 5$) strategy and were able to demonstrate that both venoms had similar *N*-glycan composition and that previous observed differences (Zelanis et al. 2010) could be due to different glycosylation site occupancy.

Other PTMs

The first snake venom proteomics work that described the occurrence of phosphorylation analyzed the venom of *Pseudonaja textilis* and used 2D-PAGE followed by fluorescence-based staining with *Pro-Q Diamond^{TM}* (Birrell et al. 2006). Subsequent work from this group, using the same approach to screen for phosphorylations in several Elapidae snakes, demonstrated that the detected phosphorylations were, in fact, from venom glycoproteins containing phosphorylated carbohydrates (Birrell et al. 2007).

One-dimensional and 2D-PAGE immunostaining, after incubation with monoclonal antibodies, yielded positive results for gamma-carboxylation of the light chain of factor Xa-like protein across several Elapidae venoms (Birrell et al. 2006, 2007; St Pierre et al. 2007), while no evidence for lysine acetylation was found when analyzing *Pseudonaja textilis* venom (Birrell et al. 2006).

Immunomics/Antivenomics

Another kind of subproteomic analysis is the search for immunogenic snake venom components recognized by a specific antivenom and, most importantly, the identification of those toxins that are not recognized by it. This kind of study generates clues towards antivenom clinical efficacy against the envenomation, relatively to the different snake species/genera tested, and has a number of clinical applications for the improvement of snake venom therapy (Calvete 2010; Gutierrez et al. 2009).

A pioneering work reported the immunomic study of *Naja kaouthia* venom and biotinylated anti-*N. kaouthia* venom hyperimmune equine serum. Venom was

submitted to 2D-PAGE in duplicate analysis. One of the gels was stained and its *spots* identified by LC-ESI-MS/MS. The "mirror" gel was electroblotted to a nitrocellulose membrane, incubated with the biotinylated serum, and immunostained with alkaline phosphatase-labeled streptavidin (Kulkeaw et al. 2007). The term "immunomics" was coined to this approach, and a similar strategy was used later for the immunomics determination of *Bothrops jararacussu* venom against three specific antisera (anti-*Bothrops jararaca*, anti-*Bothrops jararacussu*, and anti-*Crotalus durissus terrificus*) (Correa-Netto et al. 2010).

The term "antivenomics" is associated with the method developed by Lomonte and colleagues for studying the venoms of *Bothriechis lateralis* and *B. schlegelii* (Lomonte et al. 2008). In their words, antivenomics stands for "the identification of venom proteins bearing epitopes recognized by an antivenom using proteomic techniques." It basically consists of an immunoprecipitation (immunodepletion) reaction. Venom and antivenom-purified horse IgGs are incubated, following addition of rabbit anti-horse IgG (to maximize immunoprecipitation), and a new incubation period is allowed. The mixture is submitted to centrifugation, and the supernatant fraction (proteins not recognized and/or poorly recognized by antivenom IgGs) is submitted to analysis by the traditional Calvete venomics approach (described earlier in the text). A modified protocol does not use the anti-horse IgG addition. Instead, Protein G *Sepharose*^{TM} beads are added to bind to immunocomplexes and/or free horse IgG, followed by centrifugation and supernatant analysis by the venomics pipeline (Antunez et al. 2010). Recently, "second-generation" antivenomics approaches were introduced. They rely on immunoaffinity protocols, where intact IgG-based (Pla et al. 2012) or $F(ab')_2$ fragments-based (Fahmi et al. 2012; Makran et al. 2012) antivenoms are initially covalently immobilized to chromatographic beads. The venom of interest is submitted to an incubation with the beads, and the unbound fraction is submitted to the snake venomics pipeline. Several venoms have been subjected to antivenomics analyses, and the reader has a number of excellent reviews on the subject at his/her disposal in the literature (Calvete 2010, 2013; Gutierrez et al. 2009).

Snake Venom Peptidomics

The simplest method for peptide composition analysis was employed for the venom of *Atractaspis irregularis* and consisted of direct infusion of the sample for analysis by ESI-FTICR MS (electrospray ionization with Fourier transform ion cyclotron resonance mass spectrometry) and performance of de novo sequencing; this led to the identification of a number of sarafotoxins (Quinton et al. 2005). For *Bothrops jararaca* venom, some authors did mass profiling by MALDI-TOF and LC-ESI-MS as well as direct infusion ESI-MS/MS analysis leading to the confident de novo identification of some bradykinin-potentiating peptides (BPPs) (Pimenta et al. 2007). Mass profiling by MALDI-TOF MS and LC-ESI-MS was also used for the peptidomic analysis of *Micrurus surinamensis* although the toxins (neurotoxins and short- and long-chain three-fingered toxins) were identified by

shotgun (LC-ESI-MS/MS) and Edman sequencing (Olamendi-Portugal et al. 2008). Another simple approach included venom desalting on C_4 *ZipTip*^{TM} followed by analysis by MALDI-TOF/TOF MS and was used to screen bothropic (five species) and crotalic (three species) venoms, leading to the identification of several BPPs (Wermelinger et al. 2005).

In a different workflow, also used for *B. jararaca*, the first step was to obtain the low molecular mass venom components by SEC and to subject them to RPC. Fractions were directly infused to an ESI-Q/TOF MS for MS/MS sequencing leading to the identification of 18 B.P. (Ianzer et al. 2004). Low-mass "enrichment" SEC was also applied for *Naja naja atra* and *Agkistrodon halys* venom peptidomic analyses, although the fractions were further analyzed by one-dimensional *shotgun* approach, leading to the identification of cardiotoxins and neurotoxins (*Naja naja atra)* and high-mass toxin "degradation" products (*Agkistrodon halys*) (Li et al. 2004). For the analysis of *Vipera ammodytes meridionalis* and *Bothrops jararacussu*, the prefractionation involved three consecutive chromatographic steps: SEC, SAXC, and RPC; the final obtained fractions were analyzed by different MS workflows (ESI-TOF, ESI-FTICR, and MALDI-TOF) for mass mapping; digestion with trypsin for *shotgun* analysis was also employed. This complex approach led to the identification of only 18 peptides for the Elapidae snake (12 BPPs, 2 natriuretic peptides, 3 tripeptidic-metalloproteinase inhibitors, and 1 Kunitz-type inhibitor) and 36 peptides for the Viperidae snake (27 BPPs/C-type natriuretic peptides, 5 BPPs, 2 SVMPs, 1 short neurotoxin, and 1 SVMP inhibitor) (Munawar et al. 2011).

An interesting sample concentration methodology was firstly used for the analysis of three *Atheris* species venoms and consisted of a first step of C_{18} solid-phase extraction (SPE) with 60 % acetonitrile elution followed by 10 kDa cutoff ultrafiltration and analysis by LC-ESI-MS and MS/MS. This work led to the discovery of the poly-His and poly-Gly peptides, members of the so-called pHpG peptides family (Favreau et al. 2007). It is worth mentioning that this peptide family was also found during the snake venomics analysis (the Calvete approach) of *Bothriechis supraciliaris* (Lomonte et al. 2012b).

SPE enrichment was also the technique of choice for the development of a BPP large-scale screening approach that used *Bothrops moojeni* venom analysis as proof of principle. The method relied on scanning for characteristic fragment ions (*m*/*z* 213.1, 226.1 and 240.1), usually generated during BPP fragmentation using LC-ESI-MS/MS, and led to the identification of more than 20 BPPs, whereas 15 of them were unknown at the time (Menin et al. 2008).

An initial contribution of the Serrano group to snake venom peptidomics used SPE and compared the peptide composition of newborn and adult *Bothrops jararaca* venoms. On one hand they performed a traditional RPC separation followed by MALDI-TOF MS mass profiling, and on the other hand, they submitted venom to SPE with 30 % acetonitrile elution and analysis by LC-ESI-MS/MS (low-resolution instruments). This last approach identified only 10–12 B.P. in

each sample by PSM data analysis, but indicated the presence of several peptides of unknown identity (Zelanis et al. 2010). The latest contribution of this group to the field represents the state of the art of snake venom peptidomics. The venoms of *B. jararaca*, *B. cotiara*, and *B. fonsecai* were submitted to (a) SPE-RPC and fractions analyzed by MALDI-Q/TOF MS/MS or ESI-Q/TOF MS/MS with de novo sequencing data interpretation and (b) SPE followed by LC-ESI-Q/TOF MS/MS with PSM data interpretation. This workflow led to the identification of 331 peptides, an unprecedented number, including 17 new BPP/C-type natriuretic peptide sequences and three new pHpG members. Also, several LAAO (L-amino acid oxidase), SVMP, and SVSP peptides were found and considered by the authors to be part of a venom degradome, artificially induced by lyophilization (Tashima et al. 2012). Nevertheless, it is important to stress that more than 95 % of the venoms analyzed so far (both by proteomics and peptidomics) were in the lyophilized state, and none of the described methodological strategies had been able to detect and identify such a high number of peptide species, until this seminal work.

The Journey So Far and the Way Ahead for a Comprehensive Snake Venom Proteopeptidome

The previous sections have described the *plethora* of methodological approaches that have been used in the pursuit of determining the snake venom proteomes (mostly) or peptidomes of several species.

Regarding proteomics, although hundreds of *spots* could be visualized (especially for Viperidae) by 2D-PAGE and thousands of peptides sequenced by MS/MS, the unambiguous identification of unique proteoforms has seldomly been achieved. The data are usually summarized as pie charts, containing protein family-level identification assignments, numbering from 3 to 16 families. Transcriptomics and proteomics data do not correlate (for the majority of studies), and genomics information is still in its infancy. Even for the more abundant protein families, the sequence coverage is usually not high enough (95 % or more) to assign protein identifications to single transcripts or, putting it in another way, to distinguish between closely related primary structure proteoforms. Of course, this only holds true when there is no possibility of using a targeted-approach aiming at proteotypic peptides (Picotti and Aebersold 2012). Furthermore, snake venom proteoforms may be a result of the same transcript being subjected to different PTM events. As discussed earlier, snake venom “modificomics” studies, using modern mass spectrometric high-throughput approaches, are still lacking; the use of *middle-down* or *top-down* approaches could be fundamental to attack this problem. Peptidomics papers were much fewer and indicated a relatively low-complexity composition. The recent work by the Serrano group has challenged this view for some Viperidae. It is expected that the use of appropriate sample preparation, analysis by high-throughput/high-resolution MS instruments and data analysis with latest de novo

sequencing and homology search algorithms, will prove that snake venom peptidome complexity has been seriously underestimated. Finally, if researchers are able to overcome the qualitative challenges, it will be time to address the lack of quantitative data in snake venom proteopeptidomics. Next, specific topics that should be taken into consideration in order to achieve the proteopeptidomics goal will be discussed.

Optimistic Hints for Generating the First Comprehensive Snake Venom Proteopeptidome

Genomes and Transcriptomes

The need for a specific database, based on genomic/transcriptomic data, is a known bottleneck for a thorough proteomic characterization of any sample/organism, and the snake venom gland is no exception (Fox et al. 2006). For the last 15 years, several snakes had their venom gland transcriptomes determined (Calvete 2013). For some of them, transcriptomic and proteomic data were compared, and little correlation was found (Calvete 2011). Recently, the use of high-throughput transcriptome-generated (*RNA-Seq*) venom gland database in conjunction with venom LC-ESI-MS/MS data led to a significant correlation between these "omics" approaches (Aird et al. 2013). Another work also displayed good qualitative correlation between published *RNA-Seq* transcriptomics data (Rokyta et al. 2012) and their high-throughput data-independent (LC-ESI-MS^E) proteomics results (Margres et al. 2013). These authors discuss the possible technical limitations (in transcriptomics) that led to earlier observed discrepancies, especially in the cases where a protein family could be found in the proteomic but not in the transcriptomic dataset (Margres et al. 2013). Finally, an exciting paper has demonstrated that, at least during ontogenesis of *Crotalus simus simus*, miRNAs can modulate the snakes' venom gland transcriptome (Durban et al. 2013). This translational regulation could explain venom plasticity and eventual lack of correlation (especially quantitative) between transcriptomics and proteomics. On another note, the first draft of a venomous snake (*Ophiophagus hannah*) genome was recently published, and so far, all genes were represented in its transcriptome. Hopefully, others will come soon, such as the ongoing project for the genome determination of *Bothrops jararaca*, the most medically relevant snake in Brazil (http://www.bv.fapesp.br/pt/auxilios/56575/). In summary, the literature indicates that high-throughput transcriptomics databases are paramount for any snake venom proteopeptidomics project and that generating them is within reasonable technical and financial means to several research groups.

Finally, the comparison between *RNA-Seq* transcriptomic data and state-of-the art proteomics results can unveil unknown alternative splicing products (Sheynkman et al. 2013), as well as non-synonymous single nucleotide variants (Sheynkman et al. 2014), leading to a better understanding of snake venom diversity origin.

Venom Sample Pool: The Wider, the Better

One suggestion is that, whenever possible, the sample used for the proteopeptidome determination be a pool of hundreds of venom samples, accounting for individual, gender-specific, ontogenic, and geographical variations.

Bottom-Up, *Middle-Down*, and *Top-Down* Proteomics

The application of *top-down* proteomics to snake venom proteopeptidomics should become feasible in the near future, the main requirement being access to high-resolution hybrid mass spectrometers and a robust methodology for previous 2D- or 1D-LC directly coupled to the MS. This approach is still maturing but, in theory, would allow for the separation of the different proteoforms by chromatography followed by their direct intact mass measurement and fragmentation by ETD (electron-transfer dissociation) leading to complete sequence coverage and PTM content determination. One alternative, which is already feasible, is the *middle-down* approach. Here, the proteins are cleaved chemically or enzymatically into "bigger" fragments (e.g., 5–20 kDa) than the usual *bottom-up* approach but are still easily analyzed by current top-notch MS instrumentation using ETD fragmentation (Ahlf et al. 2013).

Meanwhile, 2D-PAGE is still the technique of choice for a fast and comprehensive picture of the number of proteoforms, related to a specific gene, present in the venom. This approach has severe limitations in terms of sequence coverage, and the achievement of the 100 %SC ("100 %" sequence coverage) objective, for appropriate proteoform assignment, is elusive. Nevertheless, it can serve as a reference map to indicate how well other approaches are performing in terms of detection and characterization of proteoforms *per* gene.

Improving the Comprehensiveness of *Bottom-Up* (*Shotgun*) Analysis

There are many options, in the proteomics literature, that could be considered in order to try to achieve 100 %SC, leading to the identification of the proteoforms composing a snake venom proteopeptidome. For practical reasons, all options will not be detailed, but key references are provided for some of the most important topics, such as (a) the use of multiple proteinases (Swaney et al. 2010) and different chemical reagents (Meyer et al. 2011) for venom processing before MS analysis, (b) the orthogonal separation of generated peptides by two-dimensional methods such as *OFFGEL*^{TM}-Low pH RPC or High pH RPC-Low pH RPC before MS analysis (Stein et al. 2013), (c) the use of data-dependent and/or data-independent *tandem* mass spectrometry in conjunction with different fragmentation methods such as CID (collision-induced dissociation) and ETD (Chapman et al. 2013), (d) the use of high-accuracy/resolution MS instruments to generate high-quality MS and MS/MS data, and (e) due to the complementary nature of MALDI and ESI (Meyer et al. 2011),

samples should be analyzed both by nanoLC-nanoESI-MS/MS and nanoLC-MALDI-MS/MS, whenever possible.

Data Analysis: The Need for High Resolution for Reliable Automated De Novo Sequencing

As previously discussed, having a specific database (genome- or transcriptome-derived) is the ideal requirement for maximizing peptide/protein identification when using PSM approaches, individually or in combination (Shteynberg et al. 2013). Nevertheless, with the advancement of bioinformatics tools and the generation of high-accuracy/resolution MS and MS/MS data, automated high-throughput de novo sequencing (database independent) is a reality (Ma and Johnson 2012) and should be of mandatory use for data analysis. De novo sequenced peptides can serve a number of purposes such as (a) "orthogonal" confirmation of peptide identifications done by PSM approaches, (b) expansion of sequence coverage for PSM-identified proteins, (c) identification of peptides with no match by PSM sequencing but that were present in the database used, and (d) identification of peptides not present in any database.

The Special Case of the "Natural" Snake Venom Peptidome

Recent published results by the Serrano group (Tashima et al. 2012), regarding the peptidome of three bothropic species, have revealed that a snake venom peptidome (the idealized universe of all naturally occurring peptides) can be much more complex than previously thought. Hence, an appropriately enriched peptide fraction should be generated and analyzed separately from the proteome fraction. Tashima and coworkers have done this using SPE and SPE-RPC-enriched preparations previously to MS analysis.

PTMs

By definition, the proteoforms of a gene product also include all posttranslationally modified forms of that product. Based on the literature (Olsen and Mann 2013), it is still premature to accomplish that goal for snake venom proteopeptidomics. On the other hand, it is time for more "fishing expeditions" looking for acetylations, glycosylations, phosphorylations, and ubiquitinations, which are PTMs with well-established high-throughput MS-based methodologies. In that sense, the work by Zelanis, Serrano, and Reinhold pioneered the field with the *N*-glycome profiling of *Bothrops jararaca* (Zelanis et al. 2012b).

Quantification

Without a doubt, the most challenging task shall be the mass spectrometry-based quantification applied to snake venoms. Due to the high number of proteoforms *per* gene product, sharing more than 95 % sequence identity, it will be imperative to achieve the 100 %SC goal first. Only then the *proteotypic/quantotipic* peptides can be determined and the relative or absolute quantifications performed for each proteoform, using an appropriate methodology (Picotti and Aebersold 2012).

Conclusion and Future Directions

Snakes from the genus *Bothrops* are responsible for more than 90 % of the reported cases of envenoming in Brazil. Among these bothropic species, *Bothrops jararaca* is responsible for the majority of accidents. Transcriptomics (Cidade et al. 2006; Zelanis et al. 2012a) data (although none generated from high-throughput approaches, yet) are available for *B. jararaca*, and its genome determination is on its way (see section "Genomes and Transcriptomes"). Consequently, Nicolau and coworkers (manuscript in preparation) have chosen this species for its proteopeptidome determination. The first step involved the separation of the whole venom with the *OFFGEL*™ technology using a nominal 3–10 pH gradient, high-resolution strip (24 fractions with 0.26 pH units per fraction, from pH 3.35 to pH 9.65) (Horth et al. 2006). After focusing, each of the 24 fractions was desalted with *ZipTip*™ C_{18} columns (60 % organic solvent elution) and analyzed by high-resolution data-dependent MS and HCD (higher-energy collisional dissociation) MS/MS mass spectrometry. This constituted the peptidomics portion of the method. For the proteomics approach, each of the 24 OFFGEL fractions were submitted to 7 kDa cutoff desalting columns, and the >7 kDa fractions were individually submitted to digestion with trypsin followed by data-dependent LC-ESI-MS/MS (high-resolution MS and low-resolution CID MS/MS). All peptidomics and proteomics data were analyzed by PSM (NCBI non-redundant "Serpentes" subset database) and de novo approaches. So far, the peptidomics data led to the identification of roughly one order of magnitude more peptides than the best report from the literature (Tashima et al. 2012). As for the proteomics data, representatives of 22 protein families could be identified totaling 127 proteins with at least 5 unique peptides and sequence coverages of 69 ± 18 %. Furthermore, several hundreds of high-confidence de novo generated sequences are still being searched against other databases using PepExplorer, a new homology search tool (Leprevost et al. 2014). These results are very promising, and based on this experimental platform, the authors intend to enlarge the body of data by employing some of the ideas discussed in the section "Optimistic Hints for Generating the First Comprehensive Snake Venom Proteopeptidome" (multiple proteinases for enzymatic digestion, data-independent analysis etc.). Finally, with the recent publication of an ultrahigh-pressure fast SEC

method that uses MS-compatible mobile phase (Chen and Ge 2013), the *OFFGEL*™ fractions can be directly submitted to *top-down* analysis. Furthermore, if one uses post-column split, only a small portion of the sample is injected in the MS, while the rest can be submitted to enzymatic digestion and analyzed by *shotgun* proteomics, leading to a "mirrored" *top-down/bottom-up* proteopeptidome.

Snake venom characterization using proteomics has come a long way, since its beginning, approximately 15 years ago. Nowadays, any laboratory capable of performing well-established proteomic methodologies can describe any snake venom, both qualitatively and quantitatively, at a protein family level. For a long time, the proteomic community has been avoiding taking the next step (to describe any snake venom at its proteoform level) due to previous technical limitations, allied to the fact that it would be an extremely time-consuming endeavor. Some even say that the "100 % sequence coverage" goal, a necessary precondition for proteoform-level description, is a "modern form of surrealism in proteomics" (Meyer et al. 2011). Based on the free translation, by A. S. Kline, of André Breton's 1924 *Manifesto of Surrealism*, he believed, "in the future resolution of these two states, seemingly so contradictory, of dream and reality, in a kind of absolute reality, a surreality, so to speak." The time has come to put the latest method and equipment developments in proteomics (*reality*) up to the test of reconciling with the *dream* of snake venom proteomics at the proteoform level, leading to the *surreality* of proteopeptidomics. The task ahead is intimidating, and there are no assurances that the hard work will pay off. More importantly, the ideal situation would involve focusing the analyses on one snake species *per* time and forming a consortium of laboratories (as done for the king cobra genome) to generate an "integrated *omics* profiling" (Durban et al. 2013).

Cross-References

▶ Automated Mass Fingerprinting of Venoms in Nanogram Range: Review of Technology
▶ Shotgun Approaches for Venom Analysis
▶ Snake Venom Peptidomics
▶ Squamate Reptile Genomics and Evolution
▶ Venoms of Colubrids

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Shotgun Approaches for Venom Analysis

17

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Abstract

Shotgun proteomics relies on the identification, quantification, and characterization of proteins in complex samples. Recent advances in instrumentation allow for sensitive and comprehensive shotgun protein analysis in a high-throughput manner. Combination of shotgun techniques to novel analytical strategies opens interesting possibilities for the implementation of new approaches and methodologies in the frontiers of venom biology. Examples are (i) identification of proteins in low abundance, using combinatorial ligand peptide libraries; (ii) relative and absolute protein quantitation; and (iii) identification of posttranslational modifications. The full potential of shotgun analysis in venomics is yet to be explored. Some of the pioneer works in the field will be reviewed.

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Introduction

Venoms are complex mixtures of proteins, peptides, and other molecules that display diverse biological activities in different organisms. First overviews of venom complexity, mostly studying snakes, were performed at the end of the twentieth century with emerging "omics" technologies based in two-dimensional (2D) electrophoresis conjugated to mass spectrometry (MS) and Edman degradation (Li et al. 2004; Nawarak et al. 2003). Widespread utilization of 2D technology for mapping venom proteins was due to its reproducibility, accessibility, and relatively high resolving power, enabling the detection of dozens to hundreds of components in a single gel.

Later on, a pipeline termed "snake venomics" introduced by Calvete's group (Calvete et al. 2007) consisted in fractionation of crude venom by reverse-phase LC and subsequent characterization combining N-terminal sequencing, SDS-PAGE, and mass spectrometric determination of the molecular masses and cysteine/cystine (SH and S-S) content. Using this approach venoms from more than 80 snakes have been characterized (Calvete 2014). More recently, a complementary approach based on solid-phase combinatorial peptide ligand library (CPLL) has been applied to this pipeline, which was useful to reveal low-abundance proteins within a mixture (Calvete et al. 2009).

Development of faster and more sensitive mass spectrometers allied with improvements in nanoflow high-performance liquid chromatographic (nLC-MS/MS) and computational power allowed characterization of complex mixtures of peptides coming from different proteins in the same analysis. On that basis, shotgun proteomics was set to directly analyze complex protein mixtures, rapidly generating a global profile of proteins within a mixture. It has been intensively applied to proteome profiling, protein quantitation, posttranslational modifications, and protein − protein interaction [for review see (Gelpí 2008, 2009; Yates 2013)].

Shotgun proteomics relies on the MS analysis of peptides obtained from complex samples after tryptic digestion, i.e., bottom-up approach. Complex mixtures of peptides are separated by liquid chromatography (LC) prior to MS analysis in direct interface to a mass spectrometer (LC-MS/MS). Originally, multidimensional protein identification technology (MudPIT) was established as one of the most important and powerful methods for peptide separation. MudPIT combines strong cation-exchange chromatography (SCX) followed by reverse-phase chromatography (RP), on line, with a mass spectrometer analyzer. Peptides are loaded and separated in the SCX column by a series of increasing salt steps. Each eluted step is individually transferred to the RP column, usually C18, and the eluted peptides are directly analyzed by MS (Link et al. 1999).

Reverse-phase chromatography and nano-liquid chromatography (nano-LC) are alternatives to MudPIT for peptide separation. RP is widely used for peptide separation in proteomics. Peptides in the mobile phase interacting with the stationary phase are gradually eluted with increasing concentrations of organic solvents. Nano-LC comes as a sophistication of this technique; it is performed in longer columns, using smaller particle size, and under controlled temperature allowing for greater

chromatographic resolution and larger dynamic range of peptide identification (Hebert et al. 2014; Thakur et al. 2011).

In both cases, peptides are ionized using electrospray (ESI), which is advantageous over matrix-assisted desorption/ionization (MALDI), as it produces multiply charged ions. This reduces the m/z of larger molecules and extends the mass range of the analyzer. Several setups of mass analyzers can be used for shotgun proteomics including scanning or ion beam type, ToF (time of flight) and quadrupole, or trapping, IT (ion trap), orbitrap, and FT-ICR (Fourier transform ion cyclotron resonance). Inside these instruments peptide fragmentation is performed to generate complementary ion pieces to cover protein sequence and enable identification [reviewed in (Nogueira and Domont 2014)]. Despite the excellent results obtained with use of shotgun proteomics in different biological areas, application of shotgun to venom analysis is still in its infancy. Developments in the field, technical challenges and perspectives, will be introduced.

Shotgun in Venomics

Advantages in the use of shotgun approaches for venom studies are obvious. First, parallelization and shortage of analysis time allows investigation of multiple samples. In this way, one can study, with high sample-to-sample reproducibility, several venom samples in which animal sex, age, and environmental differences could be compared on equal basis. Second, high sensitivity and dynamic range of today's equipment setups present the capacity to identify over 4,000 proteins from a complex mixture in a single run and proteome profile an organism in one hour analysis (Hebert et al. 2014; Thakur et al. 2011). Third, background coming from human keratins and trypsin is reduced compared to in-gel protocols because of lesser sample manipulation. And fourth, shotgun is compatible with peptide fractionation/ enrichment and quantification methods.

Early contributions from shotgun approaches to venomics relied on the combination of liquid chromatography coupled to mass spectrometry (LC-MS/MS). The application of shotgun to *Acanthophis* (death adder) venoms revealed a novel panel of venom molecules not contemplated in previous studies (Fry et al. 2002). Following this trend, Li and colleagues (Li et al. 2004) utilized multiple approaches in an attempt to fully characterize the proteome of *Naja naja atra* and *Agkistrodon halys* venoms, including in-solution digestion followed by HPLC coupled with an ion-trap MS. The first snake venom work completely based on shotgun proteomics and using high-resolution FT-ICR MS was published in 2006 by Fox and colleagues. They unraveled the proteome of *Bothrops jararaca* and *Crotalus atrox* identifying hundreds of proteins belonging to 12 and 9 protein families, respectively (Fox et al. 2006).

Shotgun proteomics has been successfully employed in recent publications. Rokyta and co-workers used information from venom gland transcriptome of *Crotalus adamanteus* as a database to catalog its venom system (Margres et al. 2014). Using nanospray LC/MSE they were able to identify 52 of the 78 unique putative toxin transcript clusters, including 44 of the 50 most highly

expressed transcripts. In another study high-throughput profiling of snake venom gland transcriptomes and proteomes of *Ovophis okinavensis* and *Protobothrops flavoviridis* were achieved using shotgun proteomics (Aird et al. 2013). In this study 100 % of transcripts that occurred at higher than contaminant levels had their corresponding peptides identified. Analytical strategy included digestion with three enzymes: trypsin, chymotrypsin, and Glu-C. Remarkably, shotgun proteomics was used in annotation of king cobra (*Ophiophagus hannah*) genome along with transcriptome data (Vonk et al. 2013). Vonk and co-workers were able to annotate open reading frames of 12 venom toxin gene families after genome assembly. Transcriptome analysis revealed 20 toxin families from which 14 protein families were identified using shotgun proteomics.

Application of MudPIT approach interfaced with MS for venome analysis was reported for *Naja kaouthia* (Kulkeaw et al. 2007). This approach allowed identification of 61 proteins classified in 12 groups. Authors compare shotgun analysis with identification of 24 visible spots in two-dimensional electrophoresis (2-DE) and highlight the limitations of 2-DE-based MS related to sample complexity, lower recovery of membrane proteins, and problems to separate highly acidic or basic proteins (Wolters et al. 2001). MudPIT approach in combination with multi-tissue transcriptomic analysis was utilized together for the first time by Haney and colleagues to explore the venome of Western black widow spider *Latrodectus hesperus*. Combining these two powerful techniques, the authors were able to identify 61 proteins including latrotoxins, inhibitor cystine knot (IKC) toxins, cysteine-rich secretory proteins (CRISPs), hyaluronidases, chitinases, serine proteases, metalloproteinases, leucine-rich repeat proteins, and latrodectins (Haney et al. 2014).

Compatibility of shotgun proteomics to multiple fractionation methods opens interesting analytical possibilities. The use of combinatorial peptide ligand library (CPLL), for instance, allows identification of low-abundance proteins in complex samples (Righetti et al. 2011). Van Vaerenbergh and co-workers report the use of combinatorial peptide ligand library to disclose honeybee venom proteome using sample pretreatment to enrich for minor components followed by LC-MS/MS analysis. This strategy revealed an unexpectedly rich venom composition: 102 proteins and peptides were found; 83 were newly described in bee venom samples (Van Vaerenbergh et al. 2014).

Frontier Methodologies in Shotgun Venomics

Protein Identification

Peptide spectrum match (PSM) is the gold standard for protein identification by MS. This method relies on comparing experimental MS/MS mass spectra obtained by peptide fragmentation using collision-induced dissociation (CID), higher-energy collisional dissociation (HCD), electron-transfer dissociation (ETD), or others methods, to theoretical spectra in silico generated from a database containing

protein sequences. In general, the identification process follows from the sequence whose theoretical spectrum yields the highest matching score according to some probabilistic (e.g., false-discovery rate – FDR) or empirical (e.g., Washburn criterion) function. Computational tools, which apply PSM, are SEQUEST, ProLuCID, Mascot, MaxQuant, and others. Given database dependency, PSM is very efficient and sensitive for proteins coming from organisms with a large number of protein sequences deposited in databases. A drawback of this method is that discrepancies between the experimental data and predicted peptide sequences can avoid protein identification. Mismatches between theoretical peptide fragmentation and experimental mass spectrum can be due to unexpected posttranslational modification, amino acid substitution, or unusual fragmentation or merely because the sequence of the peptide is not in the database.

In general, the field of venom proteins faces the problem of working with un-sequenced organisms and, consequently, with fewer protein sequences in databases and limited representation of proteoforms. Thus, identification of proteins coming from venom samples must surpass incompleteness of database information. This scenario has been progressively changing over the last years with several projects involving the sequence of snake genomes (Castoe et al. 2013; Vonk et al. 2013), the use of transcriptomes (Valente et al. 2009), and more recently, the next-generation sequence (Calvete 2014). These works permitted more complete characterization of the genes expressed in active venom glands, improving the possibility of protein identification by MS.

Considering that there are numerous homologous proteins in each venom that share significant extents of identical sequence information, different strategies based in de novo sequencing combined to sequence similarity search have been developed for a more efficient identification of proteins coming from un-sequenced organisms by MS. De novo sequencing consists in the reconstruction of the original peptide sequence, manually or automatically, not taking in account protein sequence databases. Automatic interpretation of MS/MS spectra using algorithms, such as Lutefisk, Sherenga, Peaks, and PepNovo, results in a list of de novo candidate sequences. Those sequences are searched against multiple protein databases for cross-species identification using sequence alignment algorithms that perform sequence similarity search, such as MS-BLAST, FASTS, and ProBLAST. Sequence similarity search is error tolerant; this way, multiple mismatches between compared sequences are allowed, which increases cross-species identification, compared to conventional strategies. Computational simulations suggested that MS-BLAST allows an efficient cross-species identification of peptides down to 50 % of the sequence identity. Since sequence similarity search employs peptide sequence candidates rather than raw MS/MS as in the conventional database search, it can be considered an orthogonal strategy for the identification of proteins. It can be used for error-tolerant search on top of conventional searches or validate statistically borderline hits obtained by conventional database searches.

Duan and co-workers combined venom gland transcriptome and 2-DE-shotgun approach to study the venom of the Chinese orb-weaving spider *Araneus ventricosus*. Using PSM and manual de novo sequencing followed by sequence

similarity search to identify proteins and toxins, they were able to find more than 86 % of toxins on the EST database. A total of 130 of 150 nonredundant toxins sequences, including twelve sequences with a posttranslational modification correspondent to methyl esterification of glutamic acid, were unambiguously identified only by manual de novo sequencing (Duan et al. 2013). Guercio et al. have applied an automated pipeline combining MASCOT conventional search with de novo sequencing using BioAnalyst QS software and MS-BLAST-based sequence similarity search to the characterization of *Bothrops atrox* venom (Guercio et al. 2006). 2D spots containing proteins from three different stages of maturation: juveniles, subadults, and adults were submitted to this pipeline. The identification of these proteins substantiated the conclusion that snake venom is subjected to ontogenetic variations. Recently, Tashima et al. utilized an approach for efficient de novo sequencing of peptides previously uncharacterized belonging to bradykinin-potentiating peptides, poly-histidine-poly-glycine peptides, and L-amino acid oxidase fragments in the venom of two rare snake species, *Bothrops cotiara* and *B. fonsecai* and the Brazilian pit viper *B. Jararaca* (Tashima et al. 2012).

Bandeira et al. developed a sophistication of this method using the overlap of peptides coming from digestion with different proteases, which was named shotgun protein sequencing. Those overlapping fragments were used to generate accurate de novo reconstructions of various proteins in western diamondback rattlesnake venom (Bandeira et al. 2007). Recent approaches combine multiple protease digestion with triplet fragmentation method, CID, HCD, and ETD, to extract protein sequence directly from MS2 spectra leading to up to 99 % sequence accuracy (Guthals et al. 2013).

To date, the aforementioned methods for protein identification by MS were applied in different venom studies contributing to identification of countless number of new toxins. Nevertheless, a large diversity of new organisms and new toxin mixtures are still to be explored to achieve a more complete database of toxin sequences coming from various "noncanonical" organisms. The widespread implementation of next-generation sequencing platforms and facilities can propel the database repertoire for un-sequenced organisms. This will increase identification using conventional search methods. In this scenario, de novo sequencing and sequence similarity search consolidate as robust orthogonal identification methods, which will be relevant to new posttranslational modifications identification, amino acid switches, and unexpected proteolysis. On top of that, they emerge as database-independent tool for protein sequencing.

Posttranslational Modification and Venomics

Posttranslational modifications (PTMs) are covalent processing events in proteins mainly by attachment of chemical moieties and also by proteolytic processing (Portes-Junior et al. 2014). These modifications are abundant during all cellular events and may determine protein conformation, function, localization, turnover, and interaction. Over the last two decades, MS proved to be a suitable tool for

PTM identification. Advantages of MS rely upon its very high sensitivity, ability to identify sites and discover novel PTMs, capability to identify modified peptides from complex protein mixtures, and ability to quantify PTM site occupancy. Most studied PTMs are phosphorylation, glycosylation, acetylation, and ubiquitination (Jensen 2006). Additionally other modifications are found in proteins from venoms like proteolysis, hydroxylation, and carboxylation (Buczek et al. 2005).

PTM analysis is challenging mainly because they are located at specific amino acid residues in proteins, usually present in substoichiometric levels and, some of them, dynamically regulated, such as, phosphorylation and acetylation. In addition, some PTMs are labile during MS and MS/MS. Furthermore, some of the modifications increase hydrophobicity, which complicates sample processing, may affect the cleavage efficiency of proteases, such as trypsin, and reduce ionization and detection efficiency in MS. For all these reasons, it is necessary to employ complementary approaches to have success in PTM analysis, comprising the use of high-resolution mass spectrometers, combination of multiple protease treatments and shotgun proteomic approach, enrichment of modified proteins and/or peptides prior to MS, application of specific MS methods, and the use of particular bioinformatics tools.

Strategies employed to enrich modified peptides are specific to each kind of PTM. For phosphorylated peptides, the main strategies are immunoprecipitation with anti-phosphoserine (pS), anti-phosphothreonine (pT), or anti-phosphotyrosine (pY); affinity chromatography using immobilized metal affinity chromatography (IMAC), titanium dioxide (TiO_2), or SIMAC, which is a sequential elution of IMAC combining TiO_2; chemical derivatization where phosphate groups are β-eliminated and subsequently undergo Michael addition; and affinity purification of modified peptides. In the case of glycopeptide enrichment, the key strategies are lectin affinity chromatography, hydrophilic interaction liquid chromatography (HILIC), hydrazine chemistry, and TiO_2 for sialylated peptides. For other modifications, the enrichment methods are based mainly in immunoaffinity experiments applying specific antibodies. These strategies to enrich modified peptides can be used in tandem, reaching better specificity (for a review see (Olsen and Mann 2013).

PTM characterization is a current topic in venom analysis. In the last decades, PTM strategies have been focused mainly in proteins isolated by reverse-phase liquid chromatography from different venoms. Another common strategy is critically dependent on two-dimensional electrophoresis (2-DE) for venom analysis and staining with specific PTM dyes, for example, ProQ Diamond for phosphoproteins and ProQ Emerald for glycoproteins (Birrell et al. 2006, 2007). Shotgun proteomic and PTM characterization strategies employed to venomics are still in their infancy, and only few cases are found in the literature, such as Verano-Braga and co-workers' (Verano-Braga et al. 2013) in which a large-scale venomic analysis was performed in combination with phospho- and glycopeptide enrichments for PTM identification and localization of scorpion toxins, from *Tityus serrulatus*. In a pipeline combining transcriptome and shotgun approach to study multidomain toxins in centipedes, Undheim and colleagues identified posttranslational processing localization of the mature toxins at centipedes' venom gland (Undheim et al. 2014).

Venom proteins and peptides undergo a plethora of PTM variations that are important for their action, and, for this reason, each modification is fine-tune adjusted, as reported by Resende and co-workers (Resende et al. 2013). The authors identified phosphorylation sites in two major toxins, icarapin and melittin, from Africanized and European honeybee venoms. Pharmacological tests demonstrated that melittin phosphorylated at 18Ser was less toxic compared to the native peptide revealing toxicity alteration by PTM (Resende et al. 2013). The use of shotgun proteomics allied to strategies for PTM enrichment, such as TiO_2 and IMAC for phosphorylation as well as lectin affinity and hydrophilic chromatography for glycosylation, can project venomics to a new level, allowing identification of new PTMs, site occupancy, and modification stoichiometry. Besides, new advances in proteome analysis, e.g., middle- and top-down strategies, are able to identify different modification in venom toxins, making possible characterization of cross talk among PTMs and their roles in toxin action and function.

Quantitative Proteomics

Protein quantitation in shotgun proteomics may provide precise information about complex sample composition. In venomics this information is vital to compare venoms as well as to link protein composition with pharmacological or pathological effects, ultimately setting the ground for new treatments and development of antivenoms [for details, see the review (Nogueira and Domont 2014)].

Two different types of protein quantitation can be performed in shotgun proteomics experiments: relative protein quantitation, most commonly applied, based on protein ratio between two or more samples, or absolute protein quantitation that determines protein concentrations or copy numbers present in each sample. Relative quantitation is basically performed using label-based or label-free methods. The first is based on labeling proteins with stable isotopes in vivo (metabolically) or in vitro (chemically or enzymatic) with molecules composed of light and heavy isotopes of ^{2}H, ^{13}C, ^{15}N, and ^{18}O or isobaric tags. On the other hand, label-free approaches use normalized ion intensity of identical peptides, extracted ion chromatogram, or normalized spectral counts of each protein to compare protein abundance in distinct samples.

Absolute quantitation is based in selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) methods. It requires the use of standard, isotopically labeled synthetic proteotypic peptides, to target selected peptides combined with internal standards.

ICAT (isotope-coded affinity tag) was introduced in 1999 by Steven Gygi and colleagues. It is based on the labeling of sulfhydryl lateral chain of cysteine residues using light and heavy versions of the reagent (Gygi et al. 1999). Many snake venom proteins are extremely cysteine rich, making them excellent targets for this approach. Advantage of the ICAT approach is that enrichment of peptides containing cysteine reduces complexity of the sample. On the other hand, it also reduces the number of identified peptides per protein and cannot detect proteins/peptides that do not contain cysteine residues. Other disadvantages are shift on

retention time of light- and heavy-labeled peptides and increase in difficulty to analyze MS2 spectra because many observed fragments come from the marker instead of the polypeptide chain.

A variant of ICAT original method termed SoPIL (polymer-based isotope labeling) uses soluble polymers instead of solid phase to avoid heterogeneous reaction conditions and nonlinear kinetics. This approach was utilized to compare type A and B venom of *Crotalus scutulatus scutulatus* and the venom of two regionally distinctive snakes *Crotalus oreganus helleri* and *Bothrops colombiensis*. Both comparisons were able to quantify and identify ca. 100 unique peptides representing over 30 venom proteins. Quantitative data were in accord with pharmacological/biological tests and supported the theory that there are intraspecific variation in venoms of some species and interspecies from different geographical locations. This was the first quantitative proteomics analysis of snake venom based on stable isotope labeling (Galan et al. 2008).

Isobaric mass tags are widely utilized nowadays in shotgun proteomics. Isobaric mass tags available are iTRAQ (isobaric tags for relative and absolute quantification) and TMT (tandem mass tags). Both tags rely on protein chemistry targeting primary amines, N-terminal and ε-amino and groups, to label peptides. Each plex reagent has the same mass achieved by a combination of ^{13}C, ^{15}N, and ^{18}O in the reporter and balance groups. Reporter subunit is responsible for peptide quantitation; when fragmented from the iTRAQ reagent, distinct reporter ion masses are recorded in the m/z spectrum lower than 150 Da. A clear-cut difference to other approaches is the possibility of multiplexing with iTRAQ, 4 or 8-plex, and TMT, 2, 6, or 10-plex. Derived peptides have similar retention times in LC and appear as a single isobaric peak in MS without increase sample complexity at this level.

iTRAQ was used for the first time in a quantitative analysis of venom proteins by Zelanis and co-workers. Venom of newborns and adults of *Bothrops jararaca* were analyzed to ontogenetic variation. Isobaric label approach was able to identify and quantify 29 venom proteins at different live stages. Thus, snake venom metalloproteinases (SVMP) were detected as more abundant toxin family in the newborn venom; snake venom serine proteinases (SVSP) were found as major toxin family in the adult venom; cysteine-rich secretory proteins (CRISP) and snake venom vascular endothelial growth factor (svVEGF) are more abundant in newborns and L-amino acid oxidases (LAAO) in adults. No clear differential expression was detected in phospholipases A2 (PLA2) toxin family. These differences suggest a shift of the main components upon transition from newborn to maturity (Zelanis et al. 2011).

Label-free quantification workflows have become extremely popular in shotgun experiments, and as the title suggests, this method quantifies proteins/peptides without any additional labeling step. Quantification is already present in collected LC-MS/MS data, an attractive simple feature for its simplicity, cost, and reproducibility, deleting steps, reducing sample complexity, and allowing a larger number of samples for comparison than multiplex experiments. However, it is not as accurate as isotope or isobaric label, and quantification quality is strongly dependent on reproducibility of the LC-MS/MS data and on bioinformatics tools for processing. There are two main concepts applied for sample comparison using label-free: the first uses the comparison

of peak area (extracted ion chromatogram) generated by mass spectrometric signal intensity for any given peptide, and the second is based on spectra counts, the number of acquired spectra matching to a peptide/protein.

Using label-free quantification based on data-dependent acquisition (DDA) and extracted ion chromatogram, Resende and colleagues compared the venom proteome of Africanized honeybees (AHB) with that of two European subspecies, *Apis mellifera ligustica* and *A. m. carnica*. From 51 identified proteins, only 11 significantly changed among all samples, and nine of these are enriched in AHB venom. Melittin was the most abundant toxin in all venoms; second major toxin phospholipase A2 and mast cell degranulating peptide are more abundant in AHB species. Melittin polymorphism in each venom sample was also quantified, and an alanine residue in the variable position (15Ala) was the most abundant for all bee species. Considering these results in terms of neutralization of toxic effects, antivenoms specific for AHB should be effective in the treatment of toxic reactions from other European honeybee subspecies (Resende et al. 2013).

Peptide or spectral count approach relies on the empirical observation that most abundant proteins in a sample present more tandem MS spectra and peptide spectrum matches (PSM) than less abundant proteins. Quantitation is performed correlating the number of PSM and sequence coverage to the amount of a protein in a sample, a proportion that exists in data-dependent acquisition experiments.

Using only the number of spectra-matched peptides to infer the relative protein abundance Tayo and colleagues performed a qualitative and quantitative work in the differences of conotoxin components presented in the proximal, central, and distal sections of the *Conus textile* venom duct. King-Kong 2 toxin was found as the most abundant peptide in the entire venom duct followed by conotoxins TxO4 and TxO6. Most of the identified *C. textile* toxins were differentially expressed in the venom duct, like TxMKLT-0223 toxin only identified at the central region or King-Kong 2 present in all parts but more abundant in proximal region. Based in these results, authors suggested that specialization for conotoxin biosynthesis occurs in the different parts of the venom duct (Tayo et al. 2010).

In another paper, venoms from six different species of Bothrops complex were analyzed by shotgun proteomics in a comparative and phylogenetic study. The normalized mean of each protein family spectral counts was utilized to measure the amount of toxin families in venoms. Quantitative and qualitative differences were observed in venom composition of the Bothrops complex, mostly for *B. jararacussu* venom. However there was no apparent significant relationship between phylogeny of the snakes and venom composition (Sousa et al. 2013).

Employing emPAI index (exponentially modified protein abundance index), Li and co-workers compared quantitatively and qualitatively manually extracted glandular venom and venom extracted through the use of electrical stimulation from Italian honeybees (*Apis mellifera ligustica*). Twenty proteins were identified in the samples, and 9 venom toxins showed higher abundance in electrically extracted venom than in gland venom. Further 5 toxins exhibit no significant difference between samples, and other 6 were only identified in one sample. In this fashion, this study demonstrated that venom extracted manually is different

from venom extracted using electrical stimulation, and these differences may be important in their use as pharmacological agents (Li et al. 2013).

Overall, distinct shotgun approaches for quantification were utilized to evaluate toxin/protein content in animal venoms. However, multiplex quantification and cross-species quantification remain a challenge in the field. Furthermore, application of absolute quantitation is still to be explored.

Conclusion and Future Directions

Literature search demonstrates that the study of venoms is still mostly performed using classical proteomics approaches rather than up-to-date rationales and instrumentation. To quickly advance in the field, studies using shotgun proteomics, an integrative action that employs modern mass spectrometers, rationales, approaches and techniques, must be implemented.

In spite of still being a difficult task to accomplish, venomes do not have the same high protein complexity of cell extracts, and the challenge to qualify and quantify their proteomes is a less difficult enterprise. The compelling necessity to understand and compare pathophysiological effects of venoms can be achieved using the modern arsenal of instruments and proteomics techniques. Individual and biologically related venomes can be characterized including posttranslational modifications; their proteins/ toxins identified and quantified. It is imperative, however, that proteomic scientists and cell biologists join efforts to disclose molecular/cellular targets of toxins, specificities of interaction and action, physicochemical and conformational parameters of these interactions, as well as the regulatory pathways involved. This knowledge will help to postulate new strategies to improve efficiency of immunotherapies for treating, principally, snake envenomation and local tissue degradation/ regeneration, a neglected disease according to the World Health Organization.

Cross-References

► Snake Venom Peptidomics
► Snake Venom Proteopeptidomics: What Lies Behind the Curtain

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Purinergic Mechanisms of Prey Acquisition by Venomous Organisms 18

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Abstract

Venomous organisms are equipped with "venom," which is injected through specialized devices (spines, fangs, stingers, hypostomes, spurs, harpoons) that help in the acquisition of prey and its digestion. The venom also serves as a defensive armament in protecting itself against the predators and aggressors. The diverse and synergistically acting cocktail of biologically active molecules of the venoms have been evolved with precise function to intervene in the biological systems of the prey/victims, thus helping in acquisition and its subsequent digestion. Although the potential role of many of the major principal

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constituents of venom in envenomation has been well documented, there is lack of information on the functional significance on the near ubiquitous distribution of purines and its endogenous (prey tissue) release-related enzymes in the venom of the venomous organisms. Purines acting via purinergic receptors are known to act as multitoxin and are known to be a central component of envenomation strategies, thus exemplifying the involvement of purinergic signaling in envenomation (immobilization and digestion). This work attempts to give an insight into the existence of purinergic signaling in envenomation strategies of venomous organisms. The work also explains how injecting exogenous (venom) nucleosides and releasing endogenous (prey tissue) nucleosides with venom enzymes are of complementary envenomation strategies. Further, it focuses on the recent experimental evidence in support of purinergic signaling in envenomation and highlights the importance of estimation of the physiological concentration of purines injected or released (in prey tissue) by action of venom enzymes for the establishment of purinergic signaling during envenomation.

Introduction

Venomous organisms such as snakes, scorpions, spiders, jellyfish, hymenopterans (ants, wasps, and bees), cone snails, sea anemones, lizards, centipedes, and the platypus are equipped with "venom," which is a modified saliva, that is injected through specialized devices (spines, fangs, stingers, hypostomes, spurs, harpoons). Venom is an evolutionary adaptation of an organism that helps to immobilize and kill and also plays a role in predigestion of the intended meal (prey). It also serves as a defensive armament in protecting itself against the predators and aggressors. Venom is known to consist of a diverse and synergistically acting cocktail of biologically active molecules that have been evolved to intervene in biological systems with precision, thus helping in prey acquisition and subsequently its digestion (Aird 2002; Dhananjaya and D'Souza 2010). The protein/peptide toxins (such as PLA_2s, proteases, hyaluronidases, acetylcholinesterases, myotoxins, three-finger toxins (3FTX), hemostatically active proteins/peptides, etc.), which are the principal components of venomous organisms are known to be involved in envenomations strategies by inducing both systemic and local effects that eventually help in prey acquisition. The composition of venoms of these organisms is known to vary based on their sex, diet, season, geographical locations, etc. (Alape-Giron et al. 2008; Chippaux et al. 1991; Daltry et al. 1996). This variation in composition is known to greatly influence the acquisition of prey and also in defending themselves from predators. Therefore it is known that this diversity in the venom constituents is known to have influence in the development of different strategies to capture the prey on which they feed and also on the various means to defend themselves from predators.

Envenomation

Envenomation is basically the subcutaneous or intramuscular injection of venom into the prey/human victims. It employs three well-integrated strategies, among which two of them are prey immobilization strategies and the other is the digestive one. The prey immobilization strategies include the "hypotensive" and "paralytic" strategies, which serve to limit the prey mobility (Aird 2002; Dhananjaya et al. 2010). Under this category, some of the species strike, release (venom), and then track their prey. Also, in few cases they seize and bulldog their prey in order to overcome prey resistance. The third strategy, that is, the digestive one, where in the venom constituents commences degradation of prey tissues internally, even before the prey has been engulfed. Normally, all three of the strategies operate simultaneously and venom constituents either individually or synergistically are known to frequently participate in more than one of the strategies (Aird 2010; Dhananjaya et al. 2010). Each of these strategies contains interchangeable elements, mechanisms, or sub-strategies for the prey accusation and its subsequent digestion. Different venomous species employ different combinations of mechanisms and no single species is known to employ them all (Aird 2002; Dhananjaya et al. 2010). Further, the pathophysiology of envenomation is known to be of a complex series of events that depend on the combined action of toxic and nontoxic components which induces both local and systemic manifestations (Girish and Kemparaju 2011). Although the potential role in envenomation of many of the major principal constituents (such as PLA_2s, proteases, hyaluronidases, acetylcholinesterases, myotoxins, three-finger toxins (3FTX), hemostatically active proteins/peptides, etc.) of venom have been well documented, there is lack of information on the functional significance on the near ubiquitous distribution of purines and its endogenous (prey tissue) release related enzymes of venoms in venomous organisms (Aird 2002; Dhananjaya et al. 2010). Of late, the free purines and endogenously liberated ones by the action of venom constituents, acting via purinergic receptors, are suggested to act as multifunctional toxins, excreting synchronous effects upon virtually all cell types (Aird 2002, 2005; Dhananjaya et al. 2010). It is suggested that these purines, acting as multitoxins, are simultaneously known to bring about prey immobilization via hypotension, prey immobilization via paralysis, and prey digestion (Aird 2002, 2005; Dhananjaya et al. 2010). Further, it is interesting to note that as these purine nucleosides have similar pharmacological roles in all vertebrates, they are useful against all prey classes. In addition, considering the near ubiquitously distributed purine receptors among various organisms are envenomed (Ralevic and Burnstock 1998; Burnstock 2006; Sawynok 2007; Aird 2005), the existence of purinergic mechanism in prey acquisition by venomous organism is evident. Therefore, the purinergic receptor-mediated physiological response that is elucidated by the action of purines in the prey organisms helps in prey acquisition, i.e., immobilization and its digestion (Dhananjaya et al. 2010). The existence of purinergic mechanism in prey acquisition (envenomation) may be

due to the free purines available in venoms or due to the endogenously (Prey tissue) liberated purines by the venom enzymes or due to the both of them (Aird 2002; Dhananjaya et al. 2010).

The Functional Significance of Purines in Envenomation

More than 50 years ago, the purine nucleosides (adenosine) were first discovered as constituents of puff adder (*Bitis arietans*) and the Eastern green mamba (*Dendroaspis angusticeps*) snake venom (Fischer and Dorfel 1954). It was suggested that adenosine might contribute to the hypotension elicited by these venom actions as Drury and Szent-Gyfrgyi (1929) had reported on the hypotensive effects of adenosine. Later on, purines like adenosine, guanosine, inosine, etc., were reported from various snake species like *Acanthopis antarcticus*, *Notechis scutatus*, *Dendroaspis angusticeps*, *Denisonia superba*, *Bitis arietans*, *Naja atra*, *Bungarus multicinctus*, *Maticora bivirgata*, and *Micrurus frontalis* (Aird 2010). Purines are also reported in spider, ant, and scorpion venoms (Aird 2002, 2010). Aird (2005) for the first time undertook the systematic quantitative investigations on purine nucleosides in 32 elapids and viperids snake venoms. Free purines, principally adenosine (ADO), inosine (INO), and guanosine (GUA), comprised as much as 8.7 % of the solid components of some of the venoms. Thus, concluding that the purines were far more abundant in some of the venoms than many proteinaceous toxins (Aird 2005), it was also concluded that the presence of nucleosides in venoms is not simply an incidental by-product of degradation of venom gland cell DNA and has a functional significance in envenomation, as thymidine (THY) was not detected in any of the venoms tested (Aird 2005, 2010; Dhananjaya et al. 2010).

Injecting exogenous (venom) nucleosides and releasing endogenous (prey tissue) nucleosides with venom enzymes are complementary envenomation strategies (Aird 2002, 2005, 2010; Dhananjaya et al. 2010). According to Dhananjaya et al. (2010), the components that are known to endogenously release purines are classified as nucleases (DNases, RNases, phosphodiesterases), nucleotidases (5′ nucleotidases, ADPases, and ATPases), and phosphatases (acid and alkaline phosphomonoesterases). When the data on the titers of venom enzymes responsible for purine liberation from prey tissue that includes 5′ nucleotides (5′ NUC), phosphodiesterase (PDE), and phosphomonoesterase (PME) (Aird 2002, 2005, 2010; Dhananjaya et al. 2010) were examined, it was found that, on an average, viperine and crotaline venoms have 3.0- and 3.7-fold more 5′ NUC, respectively, than the elapid venom. Further, elapids are known to have more PDE levels than either crotalines (8.3-fold) or viperines (13.5-fold); however, it was found that the PME levels were not so disparate. The crotalines were shown to have an average of 1.4-fold more PME than elapids and 2.2-fold more than viperines (Aird 2005). Further, it was interesting to note that where the free purines are found in higher quantities, the related endogenous (prey tissue) purine release-related enzyme levels were low and, subsequently, where free purines are found in lower quantities, the related endogenous

purine release-related enzyme levels were high in venoms (Aird 2005; Dhananjaya et al. 2010). Therefore, it is logical to assume that if a venom constituent is far widespread among different venoms, and also present in a significant quantity, it is functionally significant in envenomation. Although, purine nucleosides have been reported in venomous organisms, the functional significance of venom purines remained enigmatic until Aird (2002, 2010) and Dhananjaya et al. (2010) suggested that purines acting via purinergic receptors act as multifunctional toxins and contribute to prey immobilization via hypotension, prey immobilization via paralysis, and prey digestion (Aird 2002, 2005, 2010; Dhananjaya et al. 2010). The purines act as multitoxins by elucidating purine receptor-mediated physiological response in the prey and bring about prey immobilization and its digestion, thus suggesting the involvement of purinergic mechanism in prey acquisition (Dhananjaya et al. 2010).

Strategies in Endogenous (Prey Tissue) Generation of Purines (Particularly Adenosine)

As reported it is found that where free purines are found in lower quantities, the related endogenous purine release-related enzyme levels were high in venoms (Aird 2005; Dhananjaya et al. 2010), suggesting that the endogenous (prey tissue) generation of purines is particularly very important by the synergistic action of nucleases, nucleotidases, and phosphatases (Aird 2002, 2005; Dhananjaya et al. 2010). Among various nucleotides, adenosine generation is pharmacologically known to be important as it exhibits several snake envenomation-related symptoms (Ralevic and Burnstock 1998; Aird 2002; Burnstock 2006; Sawynok 2007; Tables 1 and 2). Generation of purines, particularly adenosine by synergistic actions of venom enzymes, can take place in different pathways. Enzymes like nucleotidase and PDE act immediately upon envenomation on the available ATP molecules to release adenosine as shown in Fig. 1. Also, DNases, RNases, and PDE liberate purine and pyrimidine nucleotides from the cell genome. However, the action of these enzymes is known to require cell necrosis brought about by proteases/hemorrhagins, phospholipases, myotoxins, cardiotoxins, and cytolytic peptides of the venom (Ownby et al. 1978; Bernheimer and Rudy 1986; Nunez et al. 2001; Ma et al. 2002). Once the cell is ruptured, the venom PDE and DNases/RNases act on preys DNA/RNA and release 5′ nucleotide monophosphates (NMPs), and subsequently, 5′ nucleotidases specifically or phosphatases nonspecifically acting on these 5′ NMPs liberate adenosine (Fig. 2). There is always a possibility that the released adenosine in vivo due to the action of adenosine deaminase of the prey/victim will be converted into inosine, which is also an important purine metabolite, responsible for inducing many pharmacological actions (Table 2). The liberated purines bring about physiological response in the prey by acting through purine (adenosine) receptors, contributing to prey immobilization and its digestion (Table 1).

Table 1 Pharmacological effects of Adenosine related to envenomation

Pharmacological effect	Mediation	References
Behavioral effects		
Sedative affects	Central neuronal A_1 receptors	Barraco et al. (1983)
Anxiolytic activity	Central neuronal A_1 receptors	Jain et al. (1995)
Anticonvulsant effect	Central neuronal A_1 receptors	Dunwiddie and Worth (1982)
Aggression inhibition	Central neuronal A_1 receptors	Palmour et al. (1989)
Alterations of cognitive		
Functioning	Central neuronal A_1 receptors	Winsky and Harvey (1986)
Locomotor depression	Central A_1 and A_2 receptors	Nikodijevic et al. (1991)
Nociceptive actions		
Analgesia	Adenosine A_1 receptor	Sawynok (1998)
Pain	Adenosine A_2 receptor	Sawynok (1998)
	Mast cell A_3 receptors	Sawynok et al. (1997)
Systemic effects		
Vasodilatation	Vascular A_{2A} receptors	Hargreaves et al. (1991)
	Vascular A_{2B} receptors	Sobrevia et al. (1997)
Cardiac block	Cardiac adenosine A_1 receptor	Olsson and Pearson (1990)
Vascular permeability	Mast cell A_3 receptors	Tilley et al. (2000)
Inhibition of neurotrans	Adenosine A_1 receptors	
Mitter release	in central and	Ralevic and Burnstock (1998)
	Peripheral neurons	Redman and Silinsky (1993)
Anti-platelet agrregation	A_1 and A_2 receptors	Seligmann et al. (1998)
Renal failure	Renal adenosine A_1 receptor	Castrop (2007)
Local effects		
Edema	Mast cell A_3 receptors	Ramkumar et al. (1993)

Table 2 Pharmacological effects of inosine related to envenomation

Pharmacological effect	Mediation	References
Inflammation	Mast cell A_3 receptors	Tilley et al. (2000)
Vascular permeability	Mast cell A_3 receptors	Tilley et al. (2000)

Envenomation-Related Symptoms Elucidated By Purines

As discussed earlier, the free purine in venoms or the endogenously (prey tissue) liberated ones, due to the action of venom enzymes, acting via purine receptors bring about envenomation symptoms that help in prey acquisition and its digestion. Although the physiological concentration of purines injected or generated for physiological response is not yet known, it is suggested that the concentrations of purine nucleosides (purines) found in some of the venoms are sufficient to promote localized and systemic effects such as vasodilatation and others (Aird 2002, 2005, 2010; Graham et al. 2005; Dhananjaya et al. 2010).

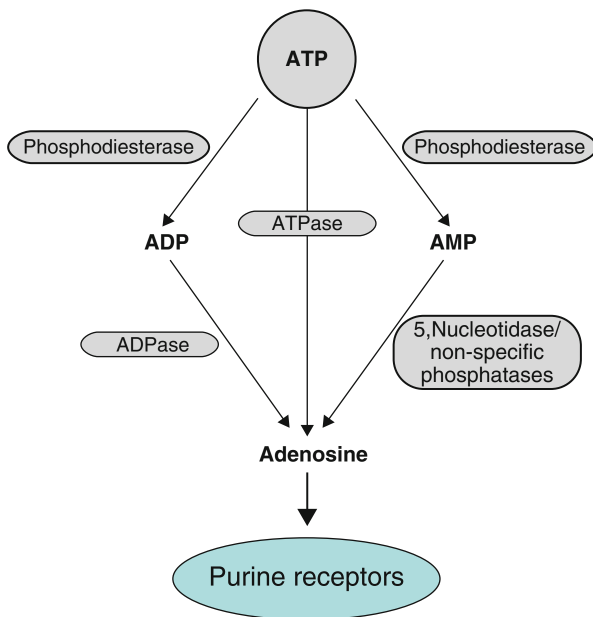

Fig. 1 Schematic representation of adenosine generation by venom enzymes from ATP hydrolysis and its action via purine receptors. Venom constituents are outlined with ovals and bold letters indicate end products released upon enzyme action

Some of the envenomation symptoms elucidated by purines are described in Tables 1 and 2 (for more details see Aird 2002; Dhananjaya et al. 2010). In regard to prey immobilization, adenosine is known to be involved by activating the neuronal adenosine A_1 receptors (Arrigoni et al. 2006; Aird 2010; Dhananjaya et al. 2010) and by suppressing acetylcholine release from motor neurons (Silinsky 2004) and excitatory neurotransmitters from central sites (Dunwiddie and Masino 2001). Adenosine is also known to exacerbate venom-induced hypotension by activating A_2 receptors in the vasculature (Lo et al. 1998). Inosine is known to potentiate coronary vasodilatory effects of adenosine (Ko et al. 1990). Adenosine and inosine are known to activate mast cell A_3 receptors, liberating vasoactive substances that increase vascular permeability (Ramkumar et al. 1993; Sawynok et al. 2000). The other pharmacological actions of adenosine that would benefit venomous organisms during prey capture that would limit prey flight and suppress aggressive actions include those acting at central A_1 receptors, adenosine-induced sedative effects (Barraco et al. 1983), anxiolytic activity (Jain et al. 1995), aggression inhibition (Navarro et al. 2000), alterations of cognitive functions (Winsky and Harvey 1986), and locomotor depression (Jain et al. 1995). It is also demonstrated that adenosine induces sleep when administered via either i.c.v. route (Radulovacki et al. 1985) or i.p. (Sarda et al. 1986). It is also noted that prey organisms preoccupied with pain are less likely to respond aggressively. Depending upon adenosine concentration, analgesia and hyperalgesia are mediated by adenosine A_1 and A_2 receptors (Aird 2010). In addition, activation of mast cell A_3 receptors produces pain via liberation of histamine and 5-HT (Ramkumar et al. 1993). Adenosine and inosine promote inflammation and plasma extravasation via activation of mast cell A_3 receptors

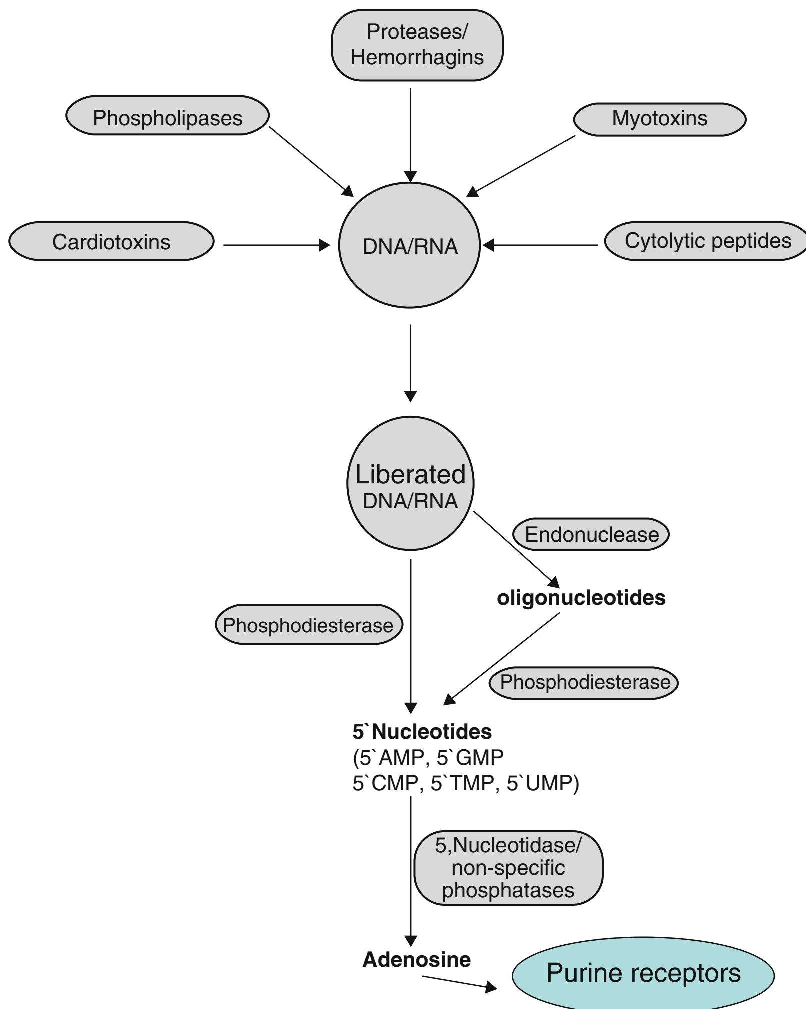

Fig. 2 The necrotic cell death brought about venom enzymes and the schematic representation of adenosine generation by venom enzymes from DNA/RNA hydrolysis. Venom constituents are outlined with ovals and bold letters indicate end products released upon enzyme action

(Ramkumar et al. 1993; Fan and Jamal 2006). Adenosine is known to inhibit platelet aggregation (Born et al. 1964). All of these activities are known to help in enhancing tissue access for other venom constituents to act and help in prey acquisition. In regard to digestion, it is well known that induction of apoptosis or fast necrotic cell death would contribute significantly for prey digestion. Ralevic and Burnstock (1998) suggested that apoptosis that occur during pathological conditions results in cell damage and release of high levels of purines (e.g., in envenomation).

Purine Nucleosides Elegance as Multifunctional Toxins

The diverse action accompanied with the precision with which the purine nucleosides acting via purinergic receptors bring about the pathological effects gives it an edge in acting as multifunctional toxins during envenomation (Aird 2002, 2005, 2010; Dhananjaya et al. 2010). In addition, as the purine nucleosides are near ubiquitous in distribution and as they serve as endogenous regulatory constituents in the prey, it is impossible for any prey organisms to develop resistance to them. The elegance of using purines as toxins is also apparent in their broad taxonomic specificity as it is found that purines have similar pharmacological role in all vertebrates (Burnstock 2006) and purine receptors are distributed across a wide range of prey organism envenomated (Aird 2010), thus exemplifying its usefulness as toxins against all prey classes. These all suggest that purinergic strategy of prey acquisition and digestion seems to be of central importance in envenomation.

Studies Indicating Purine Strategies in Envenomation

The first experimental report on the possible existence of purinergic signaling in venomous organisms was reported in colubrid venoms. It was reported that *Boiga dendrophila* venom induced concentration-dependent inhibition of electrically evoked twitches in the prostatic segment of rat vas deferens (Lumsden et al. 2004). This inhibitory effect of venom (100 μg/ml) was attenuated by adenosine A_2 receptor antagonists 8-phenyltheophylline and 8-cyclopentyl-1,3-dipropylxanthine. Further, it was demonstrated that the inhibitory effect of venom was eliminated by adenosine deaminase and was amplified by 10 μM dipyridamole, a PDD inhibitor and an antagonist of adenosine transporters. In addition, it was shown that venom in the range of 5–7.5 mg/kg i.v caused a hypotensive effect. These all results indicated that *Boiga dendrophila* venom contains adenosine, which brings about the physiological response observed in envenomation. A recent study of Caccin et al. (2013) demonstrated the evolutionary advantage of including both myotoxins and nucleotidases in the same venom in envenomation. It was observed that the purified myotoxin from the snake *Bothrops asper* induced rapid release of ATP, which was subsequently converted into ADP, AMP, and adenosine by powerful action of nucleotidases present in that venom. Therefore, this study for the first time provided experimental basis for the hypothesis that the in situ-generated adenosine plays an important role in envenomation via its hypotensive, paralyzing, and anticoagulant activities.

Conclusion and Future Directions

Although the potential envenomation role of many of the major principal constituents of the venom like PLA_2s, proteases, hyaluronidases, acetylcholinesterases, myotoxins, three-finger toxins (3FTX), hemostatically active proteins/peptides, etc. has been well

documented, there is lack of information on the functional significance on the near ubiquitous distribution of purines and its endogenous (prey tissue) release related enzymes of venoms in venomous organisms. The near ubiquitous presence of purine nucleosides and its release related enzymes in various venomous organisms suggests a functional significance of purines in envenomation strategies. Injecting exogenous (venom) nucleosides and releasing endogenous (prey tissue) nucleosides with venom enzymes are complementary envenomation strategies. Purines are known to act via purine receptors contributing to prey immobilization via hypotension, prey immobilization via paralysis, and prey digestion. It is found that purines have similar pharmacological role in all vertebrates and purine receptors are distributed across a wide range of prey organism envenomated. Further, as they serve as endogenous regulatory constituents, it is impossible for any prey organism to develop resistance, thus making them as an elegant multitoxin.

Although it is logical to assume that if venom constituents are widespread across various organisms in significant quantities, it is functionally significant. However, only few experimental studies have demonstrated the presence of purinergic signaling in envenomation. Considering the fact that this envenomation strategy is based on receptor activation, it becomes critical to estimate the physiological concentration of purines injected or released (in prey tissue) by action of venom enzymes during envenomation. Although, as till date, no in vivo studies are carried out to estimate the physiological concentration of purines present during envenomation. However, studies have suggested that the concentrations of purine nucleosides (purines) found in some of the venoms are sufficient to promote localized and systemic effects such as vasodilatation and others. Therefore, it becomes more interesting to estimate the concentration of free purines injected or purines generated by venom enzymes during envenomation and its relation to prey acquisition (immobilization) and digestion. Further, studies aimed at estimating physiological concentration of purines during ontogenic shifts might give an idea on the involvement of purinergic mechanism in envenomation during the growth of an organism, as the prey specificity is known to change from juvenile to adult. Also estimating the free purines and purine-related enzyme level in species of venoms that are geographically differently distributed, sex, and diet would throw light on purinergic signaling as an envenomation strategy in prey acquisition.

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Applications of Snake Toxins in Biomedicine

19

Francis S. Markland and Stephen D. Swenson

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Abstract

This chapter provides an overview of the application of snake toxins in biomedicine and the use of snake venom proteins that are employed in diagnostic testing procedures in clinical laboratories around the world. The chapter is subdivided into several different areas, and a summary of work ongoing in those areas is presented to provide the reader an overview of the importance of snake venom proteins and peptides in biomedicine. The subdivision of the chapter is as follows: sections "Use of Neurotoxic Snake Venom Proteins/Peptides in Biomedicine," "Use of Snake Venom Proteins/Peptides as Antihypertensive Agents," "Use of

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Snake Venom Proteins/Peptides in Cancer Therapy," "Uses of Snake Venom Proteins/Peptides in Cardiovascular Disease," and "Diagnostic Use of Snake Venom Proteins."

Included in section "Use of Neurotoxic Snake Venom Proteins/Peptides in Biomedicine" are investigations into the application of venom peptides for pain relief, preliminary studies on the use of detoxified cobra toxin for treatment of multiple sclerosis, and preliminary investigations on the application of cobra toxin for treatment of mesothelioma. Section "Use of Snake Venom Proteins/Peptides as Antihypertensive Agents" deals with the development of the drug captopril, an angiotensin-converting enzyme inhibitor, whose structure was based on a small peptide isolated from the Brazilian pit viper (*Bothrops jararaca*) venom. Section "Use of Snake Venom Proteins/Peptides in Cancer Therapy" primarily focuses on in vivo activities of a class of snake venom peptides known as disintegrins. The anticancer/antiangiogenic activity of the disintegrins is based on their interactions with a subset of integrins involved in cancer and angiogenic endothelial cell motility. A brief discussion is given for cilengitide, a cyclic pentapeptide whose structure is based on the venom disintegrin Arg-Gly-Asp motif. Not discussed in this section are a variety of other anticancer agents found in snake venom, but whose potential toxicity and side effects have not been explored in sufficient depth to merit being considered of interest for clinical application. In section "Uses of Snake Venom Proteins/Peptides in Cardiovascular Disease," the discussion is focused on the use of inhibitors of platelet integrin $\alpha IIb\beta 3$, the fibrinogen receptor, which is involved in platelet aggregation, and the development of these inhibitors based on snake venom disintegrins from which their structures evolved. Also discussed in this section are the snake venom metalloproteinases with fibrinolytic activity and the clinical development of one of these for the treatment of arterial occlusion and stroke. The section continues with an analysis of snake venom defibrinogenating enzymes and their use for a number of thrombotic disorders such as deep vein thrombosis and acute ischemic stroke. Finally, snake venom inhibitors of blood serine proteinases and their application in biomedicine are discussed. In section "Diagnostic Use of Snake Venom Proteins," the diagnostic use of a number of different venom components is discussed including the protein C activator, ProTac®, from pit viper venom; the prothrombinase-induced clotting time, which relies on the factor V activator from Russell's viper venom; the use of noscarin, a prothrombin activator from the Australian tiger snake venom, in the activated protein C resistance test to determine the presence of factor V Leiden in patients' blood samples; and several other diagnostic tests that employ venom fractions are also discussed.

Introduction

Although most people would say that snakes are harmful to man, the venom of these creatures contain many interesting molecules that when appropriately purified from the other components in the venom have quite interesting properties, which

have proved in the past and undoubtedly will in the coming years prove to be quite beneficial to mankind. Through millions of years of evolution, snakes have developed a significant repertoire of proteins and peptides for their defense and for immobilizing and digesting their prey. Despite its bad reputation, snake venom will continue to serve now and even more in the future as the source of interesting molecules with important applications for biomedicine. With the present revolution in biomedicine that is ongoing, including the development of sophisticated and extremely sensitive mass spectrometers that enable scientists to characterize very minor components in venom and then using recently developed molecular biological techniques to produce them recombinantly in large quantity, investigators are poised to gain a heretofore unattainable source of new knowledge about these molecules that may lead to important biomedical applications. Further, the high-throughput screening techniques now available also offer the opportunity to identify and extract therapeutically important molecules with great speed and efficiency. Yet at the same time the constriction of the range of a number of species of snakes due to encroachment by mans' urban spread is threatening to eliminate a resource, which could hold great promise for important pharmacological discovery. Therefore, it is important that the scientific community devote the resources to ensure the maintenance of this vast treasure, the snakes and their venom, so that researchers will be able to continue the search for novel components with applicability to biomedicine both now and into the foreseeable future.

In this chapter, there are some great success stories (captopril and eptifibatide) described but also a number of heartbreaking failures (alfimeprase and Exanta) as venom-derived products try to make their mark on an already crowded and financially depressed pharmaceutical marketplace. Nonetheless, there are important venom peptides/proteins waiting to be discovered, and with the ever-expanding repertoire of physical/chemical and biological tools at the disposal of the scientific community, new investigations and investigators have a great future to look forward to in the realm of biomedicine development.

Use of Neurotoxic Snake Venom Proteins/Peptides in Biomedicine

In view of the neurologically active components in the venom of many snake species, particularly members of the Elapidae family, there are a number of potential biomedical applications in which purified fractions containing these activities may be applied. This section will cover some of the potential biomedical applications in which these purified snake venom fractions may be used. The application of venom fractions for therapy of stroke, including the fibrinogen-depleting serine proteinases and the fibrinolytic snake venom metalloproteinases, will be covered in section "Uses of Snake Venom Proteins/Peptides in Cardiovascular Disease" of this chapter.

Pain Relief Peptides

An interesting neurotoxic peptide with analgesic properties was isolated from king cobra (*Ophiophagus hannah*) venom and is called hannalgesin (Pu et al. 1995). The preclinical and early clinical development of this agent for acute pain relief is being handled by a company in France. The peptide is administered sublingually and is a non-opioid, water-soluble 11-amino acid peptide derived from hannalgesin. Studies in preclinical models have demonstrated the drug to be safe and to have analgesic properties, and while the mechanism of action is not fully understood, preclinical studies have shown that it has a far stronger analgesic effect than morphine. It is believed to operate via a neural nitric oxide synthetase-dependent mechanism that results in the activation of the endogenous enkephalin system.

There are several other promising leads for new venom-derived painkillers. Researchers in France announced the discovery of two peptides from black mamba (*Dendroaspis polylepis*) venom that can block acid-sensing ion channels (ASICs). ASICs are expressed within both the peripheral nervous system and the central nervous system and play a key role in the pain pathway in humans. The two peptides are each composed of 57 amino acids and 8 cysteine residues and only differ by one amino acid residue; they are called mambalgins (Diochot et al. 2012). They are members of the three-finger toxin (TFT) family, a superfamily of nonenzymatic proteins found in all families of snakes, but are main venom components of snakes from the Elapidae family. TFT family members have a common structure of three beta-stranded loops extending like three outstretched fingers from a small, globular, central core that is cross-linked by four conserved disulfide bonds. In mice, the mambalgins showed potent analgesic effects, as powerful as morphine, with no toxicity observable. The peptides also induced less tolerance than morphine to repeated drug exposure and no respiratory distress. These peptides have the potential to help in the understanding of pain as well as to be developed as novel, powerful, naturally occurring peptides of potential therapeutic value as analgesics. They show potent activity against different ASIC channel subtypes, thereby providing potentially novel targets for therapeutic intervention. The mambalgins are being developed into a human pain therapeutic by the same French pharmaceutical company that is developing the hannalgesin-derived peptide.

Detoxified Cobratoxin for Multiple Sclerosis

Modified (detoxified) cobratoxin from the Thailand cobra venom is being developed for the treatment of adrenomyeloneuropathy (AMN) and multiple sclerosis (MS) (Reid 2007). AMN is a rare X-linked peroxisomal metabolic disorder causing deficient β-oxidation of long-chain fatty acids. It is characterized by the loss of the myelin sheath on nerve fibers within the brain (cerebral demyelination) and the progressive degeneration of the adrenal gland (adrenal atrophy). The neurological problems in AMN progress slowly over several decades and this disease is of interest because of its similarities to MS. The use of snake venom for the treatment

of MS has a long and very controversial history. However, the anecdotal reports on the effectiveness of snake venom in MS treatment may now have some scientific support with the studies on the neurotoxin from the Thailand cobra. Cobratoxin binds to nicotinic acetylcholine receptors at neuromuscular junctions and in neuronal synapses of the brain; it has several pharmacological properties that support its use for MS treatment. Further, the ability to treat the toxin with a chemical detoxification step has further advanced the possible application of cobratoxin for treatment of MS in the clinical setting with potentially only minor side effects. MS is a painful immune-mediated disease, possibly induced or worsened by viral infection. As part of the immune attack on the central nervous system, myelin (the fatty substance that surrounds and protects the nerve fibers in the central nervous system) is damaged, as are the nerve fibers themselves, producing the symptoms of MS. There is an accepted murine model for MS, which is experimental autoimmune encephalitis; however, there has been a poor correlation between experimental studies in this model and therapeutic response in humans.

It is of interest that cobra venom, after first being partially denature by heating, has been used for decades in China for cancer and rheumatoid arthritis treatment. In view of anecdotal reports of effectiveness of detoxified cobratoxin from Thai cobra venom in MS, a more recent study in a small group of patients with AMN was performed. However, this study could not repeat previously reported positive results (Mundy et al. 2003). The short-term treatment used in this study appears to be safe and well tolerated, but the study period may have been too short or too few patients were enlisted to demonstrate positive results. The authors recommended further evaluation of detoxified cobratoxin in studies with greater numbers of patient or for longer periods of time. In view of preclinical findings reported by Reid that detoxified cobratoxin has antiviral, neuromodulatory (analgesic), and immunosuppressive activities (Reid 2007) and because of the significant side effects with most present therapies for MS, there is significant rationale for the controlled clinical evaluation of the detoxified cobratoxin in MS patients.

Cobratoxin for Mesothelioma

Interestingly, the active α-cobratoxin (not detoxified) from the monocled cobra, *Naja kaouthia*, venom has been used for the treatment of mesothelioma in animal model systems (Catassi et al. 2008). Mesothelioma is a rare form of cancer that most frequently arises from the cells lining the pleural cavity or the peritoneal cavity. Pleural mesothelioma is the most common form, often presenting with symptoms in the chest area. Most people with malignant mesothelioma have worked on jobs where they breathed asbestos. α-Cobratoxin is a high-affinity antagonist of α7-nicotinic acetylcholine receptor (α7-nAChR) that induces a significant inhibitory effect on the growth of mesothelioma. However, in this tumor model study, α-cobratoxin treatment began 48 h after grafting mesothelioma cells, possibly before the cells were implanted and without vascular support. Thus, the possibility exists that resistant cells might be selected after implantation, suggesting

that additional experiments need to be performed to evaluate the antitumor effects of α-cobratoxin in a model in which pleural implantation has been well established (Catassi et al. 2008). Further studies are also needed to understand the complex α7-nAChR antagonist system in cancer cells and to evaluate α7-nAChR as a potential target in cancer therapy. Importantly, since an active neurotoxin is being used for therapy, potential off-target side effects and immunogenicity of the venom peptide need to be carefully examined in the preclinical studies in preparation for eventual clinical application as therapy for mesothelioma.

Use of Snake Venom Proteins/Peptides as Antihypertensive Agents

It has been known for many years that following envenomation by a number of species of venomous snakes, humans experience a profound hypotensive effect. The first real blockbuster drug to be derived from snake venom was captopril, whose structure was based on a small peptide from Brazilian pit viper (*Bothrops jararaca*) venom. Captopril is a very effective antihypertensive drug developed as a potent and specific inhibitor of angiotensin-converting enzyme (ACE), which is a zinc metalloproteinase that participates in the synthesis of a hypertensive peptide, angiotensin II, and in the degradation of a hypotensive peptide, bradykinin. The evolution of the *Bothrops jararaca* venom-derived ACE inhibitor that eventually made its way into clinical use as an antihypertensive agent is an excellent example of drug development with a venom-derived agent with a successful outcome.

The discovery of captopril is a fascinating story and is clearly recounted by the parties involved, David Cushman and Miguel Ondetti (Cushman and Ondetti 1999). John Vane was a consultant for the Squibb Institute for Medical Research in 1967 and he suggested that it might be interesting to look for inhibitors of ACE. The same John Vane in 1982 would be awarded the Nobel Prize in Physiology or Medicine, in part for his role in the introduction of ACE inhibitors. Cushman immediately volunteered and one of the first things he did was to develop a spectrophotometric assay for ACE, which was found in high concentrations in and purified from the vascular bed of rabbit lung. Soon thereafter he and Ondetti began a collaboration that led to the discovery of ACE inhibitors. Their studies on ACE suggested that this enzyme might act similarly to the exopeptidase carboxypeptidase A, a metalloproteinase whose crystal structure fortuitously had already been determined (it is now known that these enzymes belong to completely different classes). Their feeling was that ACE cleaved the penultimate peptide bond at the carboxy-terminus of the peptide chain, rather than the terminal peptide bond as carboxypeptidase A. Their first real breakthrough came when Vane suggested that they collaborate with a colleague named Sérgio Ferreira. In 1965 Ferreira in Brazil was the first to observe that factors that greatly enhanced the smooth muscle relaxing activity of bradykinin (bradykinin-potentiating peptides, BPPs) were present in *Bothrops jararaca* venom. This activity was also found in a number of other *Bothrops* species (Ferreira 1965). As it turned out the collaboration between Ferreira and the Squibb

group never occurred as Ferreira pursued the BPPs and the Squib group isolated ACE inhibitors. The venom BPP with the best in vivo activity as an ACE inhibitor was a nonapeptide given the generic name teprotide. Teprotide was made as a synthetic nonapeptide (5-oxo-L-prolyl-L-tryptophyl-L-prolyl-L-arginyl-L-prolyl-L-glutaminyl-L-isoleucyl-L-prolyl-L-proline), and it was shown to inhibit the conversion of angiotensin I to angiotensin II (a powerful hypertensive peptide) by inhibiting ACE and blocking the loss of a His-Leu dipeptide at the carboxy-terminus of angiotensin I. It soon became clear that this BPP was actually an ACE inhibitor and the blood pressure-lowering effect is due to the dual actions of the following: (i) potentiation of the vasodilator bradykinin by blocking its degradation and (ii) inhibition of the vasoconstrictor angiotensin II-mediated effects by blocking its formation from angiotensin I. Bradykinin is a nonapeptide that causes vasodilation resulting in hypotension; it is a proteolytic product derived by kallikrein cleavage of plasma high-molecular-weight kininogen and is degraded by ACE. Although teprotide normalized the blood pressure in clinical studies in severely hypertensive human patients, it can only be delivered by injection, which limits its potential application in the clinic. Based on studies using the carboxypeptidase A model, it was determined that the best ACE inhibitors had a Phe-Ala-Pro sequence at the carboxy-terminus. Also, the realization that an inhibitor of carboxypeptidase A, benzylsuccinic acid, might bind to the zinc atom of ACE by the carboxyl group of the succinic acid suggests to the Squibb group that the incorporation of a succinyl-amino acid into the ACE inhibitor might lead to enhanced binding of the inhibitor. Indeed, this was the case, and with some structural manipulations and the demonstration of oral activity in rats, one of the compounds (D-2-methylsuccinyl-Pro), an Ala-Pro analog, had optimal ACE-binding activity except for one group, the zinc-binding carboxyl group. When this was replaced by a sulfhydryl group, there was 1,000-fold enhanced inhibitory potency, and the structure of captopril was born (Fig. 1). Captopril gained FDA approval in 1981. This compound initiated the era of ACE inhibition in cardiovascular medicine and multicenter clinical studies soon followed. Interestingly, these studies pointed out some shortcomings of captopril, such as the need for multiple daily doses and the toxicity of the sulfhydryl group. Thus, further modifications and second-generation agents followed. Nonetheless, ACE inhibition is even now one of the preferred treatments for hypertension.

Although ACE inhibitors are an important class of antihypertensive agents, alternative approaches are being developed, such as B-type natriuretic peptide, which is a neurohormone secreted by the heart in response to elevated volume

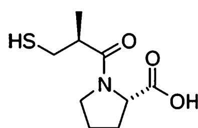

Fig. 1 The structure of captopril, (2*S*)-1-[(2*S*)-2-methyl-3-sulfanylpropanoyl] pyrrolidine-2-carboxylic acid, an angiotensin-converting enzyme inhibitor used for the treatment of hypertension

and pressure, leading to dilated blood vessels and sodium and water loss. A synthetic form of a natriuretic peptide from the green mamba *Dendroaspis angusticeps* may have therapeutic potential (Chen et al. 2002). Also, recent research has led to the discovery of a new target for BPPs from *B. jararaca* venom, the argininosuccinate synthetase enzyme in the kidney, and this target has been implicated in decreasing blood pressure (Guerreiro et al. 2009). This study revealed an ACE-independent pathway for the regulation of blood pressure and offered the opportunity for the development of a new class of antihypertensive agents. In this work affinity chromatography was used to identify the kidney cytosolic protein argininosuccinate synthetase as the major protein binding to a proline-rich decapeptide member of the BPPs from *B. jararaca* venom. The authors also showed that this interaction activates the catalytic activity of argininosuccinate synthetase dose-dependently. This enzyme is recognized as an important member of the citrulline–nitric oxide (NO) cycle that represents the potential limiting step in NO synthesis. The importance of this interaction was demonstrated by the finding that the decapeptide caused an increase in NO production in human umbilical vein endothelial cells in culture and arginine production in human embryonic kidney cells and an increase in arginine concentration in plasma of spontaneous hypertensive rats. Additionally, a specific inhibitor of the enzyme significantly reduced the antihypertensive activity of BPP in the rat model. The authors suggest that the activation of argininosuccinate synthetase may represent a new mechanism for the antihypertensive effect of the BPP and this enzyme may represent a novel target for therapy of hypertension (Camargo et al. 2012).

Use of Snake Venom Proteins/Peptides in Cancer Therapy

Disintegrins

Probably the most advanced venom-derived peptides/proteins for cancer therapy are the disintegrins, although none have undergone clinical trials for cancer therapy as of this writing. Anti-integrin therapy for cancer has received much attention lately due to the importance of the integrins in processes of great importance to cancer (Goodman and Picard 2012), which are basically threefold: angiogenesis, invasion, and tumor progression/metastasis.

Integrins are heterodimeric receptors that evolved to mediate complex cell–ECM interactions. Since they are exposed on the surface of tumor cells and angiogenic vasculature and are sensitive to pharmacological blockade, they are attractive targets for anti-integrin therapy. Integrin-mediated interactions regulate the ability of cells to mechanically sense their environment by integrating multiple signaling pathways initiated by extracellular cues with the cell's cytoskeleton. Integrins (including $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$) when activated are involved in several cancer-relevant pathways such as cell attachment, migration, invasion, cytoskeletal organization, angiogenesis, proliferation, and survival. The pharmacological blockade of integrin $\alpha v\beta 3$ has been demonstrated to significantly reduce

tumor angiogenesis in numerous cancer models, and several drug candidates are currently in clinical trials based on interaction with this integrin. This integrin can also serve as an effective target for therapy directed at processes critical to tumor progression (adhesion, invasion, and migration). Similarly, $\alpha v5$ and $\alpha 5\beta 1$ as well as a number of other integrins (notably $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 4\beta 1$, $\alpha 6\beta 4$, and $\alpha 9\beta 1$) have also been shown to play important roles in tumor progression and angiogenesis, and their pharmacological targeting by soluble ligands or monoclonal antibodies has led to reduced tumor microvessel density in various cancer models. Due to the pivotal role in cancer biology, these integrins could serve as effective targets for cancer therapy.

Disintegrins are small, disulfide-rich peptides (4–16 kDa), which were first discovered in 1983 (Ouyang and Huang 1983) and named in 1990. Many disintegrins contain an Arg-Gly-Asp (RGD) tripeptide motif in an 11-amino acid loop held in place at the base by two disulfide bonds; disintegrins bind specifically to integrins on the surface of normal and malignant cells (McLane et al. 2008).

RGD-containing disintegrins were originally characterized as platelet aggregation inhibitors due to their binding to integrin GPIIb–IIIa (the fibrinogen receptor also known as $\alpha IIb\beta 3$) on the platelet surface and inhibiting the ability of platelets to bind to the RGD site in fibrinogen (this binding leads to platelet aggregation). While many disintegrins display the RGD motif, this is not the case for all members of the family. There are a number of short, non-RGD-containing, disintegrins such as obtustatin (KTS) and jerdostatin (RTS) (Walsh and Marcinkiewicz 2011). Also, there are heterodimeric disintegrins such as EC6, which contains either the MLD or RGD sequences in its two chains, and EC3, which contains MLD and VGD sequences (Walsh and Marcinkiewicz 2011). More recently, mainly due to the superb work by Calvete and Marcinkiewicz and colleagues (Calvete et al. 2007; Walsh and Marcinkiewicz 2011), disintegrins have been categorized into two additional groups, the MLD disintegrins in which the RGD motif in the 11-amino acid loop is replaced by the MLD sequence and the K/RTS disintegrins in which the loop is only 9 amino acids and the RGD motif is replaced by the K/RTS sequence.

There have been a number of interesting studies with disintegrins as potential anticancer agents and several of these are discussed here. Marcinkiewicz and colleagues have been studying non-RGD disintegrins for a number of years and have made many interesting observations regarding the potential antiangiogenic and antitumor activity of members of the non-RGD disintegrin families. One such observation was the antiangiogenic effect of an MLD (and VGD) heterodimeric disintegrin VLO5; this disintegrin also inhibited glioma growth through interaction with integrin $\alpha 9\beta 1$ and induced apoptosis of the glioma cells. Additional studies revealed that this disintegrin was not toxic in an in vivo embryonic quail angiogenesis assay. The MLD motif has been shown to interact with integrins $\alpha 4\beta 1$, $\alpha 4\beta 7$, and $\alpha 9\beta 1$ (Walsh and Marcinkiewicz 2011), although there is differential integrin specificity depending on the amino acid adjacent to the MLD motif on its NH_2-terminal side. Additionally, it was shown by the same group that the K/RTS disintegrins which specifically target integrin $\alpha 1\beta 1$ (a collagen receptor) also have antitumor activity. It was shown that obtustatin, a 41-amino acid KTS disintegrin,

inhibits the growth of human melanoma in nude mice and that this effect is primarily related to an antiangiogenic effect, which in turn is due to the induction of apoptosis in the endothelial cells (Brown et al. 2008). Additionally, the Marcinkiewicz group showed that viperistatin, a KTS disintegrin, was partially active in blocking experimental hematogenous metastasis of human melanoma in a mouse model (Staniszewska et al. 2009). McLane and coworkers have also been studying the effect of disintegrins on human melanoma experimental metastasis in mouse models (Hailey et al. 2013). The snake venom disintegrin eristostatin, a 49-amino acid RGD disintegrin from the venom of the viper *Eristicophis macmahoni*, when co-injected via the tail vein into immunodeficient mice along with human melanoma cells, significantly inhibited lung colonization by the melanoma cells (Tian et al. 2007). These authors also showed that the effect by eristostatin was not due to an antiangiogenic action. It appeared from cytotoxicity studies that eristostatin makes the melanoma cells a better target for lysis by human natural killer cells. The binding of eristostatin to melanoma cells was only partially inhibited by an RGD-containing peptide, suggesting that there may be another non-integrin-binding site (Hailey et al. 2013).

One of the first reports on the antiangiogenic activity of disintegrins came from Sheu et al. (1997) who compared the effect of triflavin, the 7.5 kDa disintegrin from *Trimeresurus flavoviridis* venom, to that of an anti-αvβ3 monoclonal antibody on tumor necrosis factor alpha (TNFα)-induced angiogenesis in a chicken chorioallantoic membrane (CAM) assay. The authors found a much more pronounced antiangiogenic effect of the disintegrin at a significantly lower concentration than the antibody and speculated that triflavin might prove to be of therapeutic interest for the prevention of angiogenesis-induced diseases. Accutin is a small RGD-containing disintegrin (47 amino acids) from *Deinagkistrodon acutus* venom that has been studied by Huang and his group in Taiwan (Yeh et al. 1998). Accutin was also shown to effectively inhibit angiogenesis in the CAM model and induce HUVEC apoptosis, and its clinical potential as an antiangiogenic agent was discussed. These early studies defined the RGD disintegrins as potential antiangiogenic agents.

Kang and colleagues in Korea in 1999 published on the antiangiogenic activity of a recombinant RGD disintegrin (Kang et al. 1999), salmosin (from *Agkistrodon halys brevicaudus* venom), after expressing it in *E. coli*. Interestingly, the recombinant peptide not only blocked neovascularization in the CAM model, it also displayed antiproliferative effects in vitro. To assess whether salmosin affects metastatic tumor growth, Lewis lung carcinoma cells were injected into the tail vein of mice and lung tumor colonization was examined following daily i.v. delivery of 1.25 mg/kg salmosin; a dramatic inhibition of experimental metastases was observed. The authors further reported that when Lewis lung carcinoma cells were injected subcutaneously (s.c.) in mice and treated daily with s.c. injections of salmosin, tumor growth was notably reduced in about a week relative to that of the PBS control mice. This study was the first demonstration of the antiangiogenic activity of a recombinant disintegrin, and the findings further supported disintegrins as potential antitumor/antiangiogenic agents for therapeutic application.

The Korean group then developed an innovative approach to disintegrin delivery in biological system. The gene encoding salmosin was encapsulated into cationic liposomes (Kim et al. 2003). When delivered subcutaneously, the gene was expressed systemically and inhibited the growth of melanoma cells and suppressed pulmonary metastases in mice. This approach was suggested as an efficient mechanism to maintain the disintegrin at an effective therapeutic level.

An interesting study by the Taiwanese group on the effect of disintegrin therapy on bone metastasis in an experimental metastasis model using MDA-MB-231 human breast cancer cells provided some interesting results (Yang et al. 2005). Using rhodostomin, an RGD disintegrin from *Calloselasma rhodostoma* venom, the investigators showed that following injection of the breast cancer cells into the tibia of mice, in one tibia with simultaneous injection of the disintegrin, and in the other tibia tumor cells alone, there was dramatic inhibition of tumor growth on the side co-injected with disintegrin. This effect was attributed to the inhibition by the disintegrin of tumor cell adhesion, migration, and osteolytic activity in bone, and it was suggested that the anti-metastatic activity of disintegrin may be through the suppression of local tumor growth.

Finally, DisBa-01 is a disintegrin isolated from a cDNA library made with RNA from the venom gland of *Bothrops alternatus*; it is an 11.6 kDa recombinant monomeric disintegrin containing an RGD motif. The anti-metastatic activity of DisBa-01 was evaluated in a mouse model of lung colonization using a B16F10 melanoma subclone transfected with the luciferase gene; this allowed noninvasive bioluminescent imaging (Ramos et al. 2008). DisBa was shown to inhibit lung metastasis time- and dose-dependently in this experimental metastasis model. DisBa also dose-dependently inhibited in vivo angiogenesis using a Matrigel plug assay in nude mice.

Recently, a sequence-engineered RGD disintegrin (called vicrostatin or VCN) was produced in large quantity directly in the oxidative cytoplasm of bacteria (Minea et al. 2010). The recombinant expression system, for which a proprietary production method was developed, is capable of generating over 200 mg of purified active recombinant disintegrin from one liter of bacterial culture. VCN, which is a monomer, inhibits ADP-induced platelet aggregation dose-dependently with an $IC_{50} = 60$ nM, identical to that of contortrostatin (CN, the native homodimeric disintegrin isolated from southern copperhead venom and the precursor of VCN). Through multiple integrin ligation ($\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$), VCN targets both endothelial and cancer cells. Importantly, integrin $\alpha v\beta 3$ is expressed at low levels on epithelial cells and mature endothelial cells but is overexpressed on the endothelial cells of the tumor neovasculature and on tumor cells. Therefore, this integrin presents an attractive and tumor-specific therapeutic target for rapidly growing solid tumors. VCN potently collapses the actin cytoskeleton of HUVEC in the low nanomolar range (10–100 nM). VCN also has a direct effect on breast cancer cells inhibiting their in vitro motility.

A human breast cancer model (MDA-MB-231) had been established and validated in the mouse following injection of human breast cancer cells into the mammary fat pad. To develop a clinically relevant delivery method, VCN was

efficiently packaged in liposomes (LVCN) and evaluated in vivo in the MDA-MB-231 breast cancer model after intravenous (i.v., tail vein) injection (twice weekly, 100 μg per dose). The animal tumor model demonstrates that LVCN is well tolerated and its intravenous administration induced a significant inhibition of tumor growth (>75 %) and an increase in animal survival (Minea et al. 2010). Survival was significantly prolonged by LVCN alone: 80 % of mice treated with LVCN survived for >70 days while those receiving empty liposomes died on or before day 53. Although the combination of docetaxel with LVCN did not show any additive inhibition of tumor growth, animal survival indicated a clear benefit of the combination since at day 73, 100 % of animals treated with combination therapy were still alive. Antiangiogenic activity of LVCN was examined by CD31 immunohistochemistry and potent antitumor/antiangiogenic activity of VCN was demonstrated; this provides definitive proof of concept for efficacy in this triple-negative breast cancer mouse model. In a slight variation to this experimental protocol, the tumor was allowed to become more established before a short course of treatment (six doses) with either LVCN or avastin was initiated. To assess the impact of VCN on cell death, tumor cryostat sections from each group were stained with FITC-TUNEL and the impact of VCN treatment on tumor proliferation was assessed by Ki-67 staining. In this experimental setting, a large and significant difference in the amount of cell death (TUNEL staining) was observed between LVCN (~45 % cell death), avastin (~25 %), and PBS (~8 %) control groups. Although there were statistically significant differences in the amount of Ki-67 staining between the groups, the LVCN group showing the least amount of staining, these differences were much smaller than the ones observed for cell death. Some advantages of the liposomal formulation are the significant increase in the circulatory half-life of LVCN as compared to the naked protein, the lack of interaction with platelets or other blood elements in the circulation due to encapsulation, the accumulation of the liposomal formulation in the tumor bed by passing through the "leaky" angiogenic vasculature supplying the tumor (passive accumulation), and the apparent release in the tumor of the disintegrin from the liposomes leading to significant antitumor and antiangiogenic activity. Importantly, the liposome is not recognized by the immune system and does not provoke an immune response, thereby minimizing the risk of undesirable side effects.

The toxicity of a single, escalating, i.v. dose of VCN or LVCN (3–75 mg/kg) was evaluated in female Wistar rats. Animals were evaluated for signs of physical toxicity or stress over 14 days. Signs of toxicity were monitored via physical status, activity, and total body weight and, following sacrifice, gross and microscopic pathology and hematological analysis. There were no adverse effects seen in any of the treated animals and animals in all treatment groups thrived and gained weight indistinguishable from control. No differences in hematological parameters between treated and controls were observed, and microscopic examination of body organs revealed no inflammation, and no evidence of pathological changes or vascular changes. These toxicity studies, although very preliminary, reveal that VCN has limited or no toxicity in rats.

Other cancers have also been studied using vicrostatin including prostate, ovarian, and glioma, and the results have been most promising but, due to space limitations, will not be described here.

Cilengitide is a cyclized pentapeptide [Arg-Gly-Asp-DPhe-(NMeVal)] containing the Arg-Gly-Asp sequence found in disintegrins. This peptidomimetic binds with high affinity to integrins αvβ3 and αvβ5 and was designed to block integrin-mediated adhesion and migration. Cilengitide has shown excellent antitumor and antiangiogenic activity in glioblastoma multiforme (GBM), a devastating brain cancer (Reardon et al. 2008). GBM is the most common adult primary central nervous system tumor, and patients with GBM have a very grim prognosis. In phase I and II clinical trials in GBM, cilengitide was shown to be safe and well tolerated. Ongoing phase II and III trials are continuing to evaluate cilengitide as an antiangiogenic agent in combination with other chemotherapeutic agents and to further evaluate a subgroup of patients with a methylated O^6-methylguanine-DNA methyltransferase (MGMT) promoter who appeared to show the greatest benefit from the addition of cilengitide to the standard of care for GBM (Seystahl and Weller 2012). CENTRIC is a phase III randomized clinical trial using cilengitide in combination with temozolomide and radiotherapy in newly diagnosed glioblastoma patients who have methylated MGMT gene promoter status. Unfortunately, recent results (February 25, 2013) of the CENTRIC trial in more than 500 patients from 23 countries worldwide indicate cilengitide did not meet its primary end point of significantly increasing overall survival when added to the current standard chemoradiotherapy regimen (http://www.rttnews.com/2063322/merck-kgaa-late-stage-trial-of-cilengitide-fails-to-meet-primary-endpoint.aspx).

Other Snake Venom Anticancer Agents

In addition to the disintegrins, there are a number of other potential anticancer agents in snake venom, but most of them have not been tested in vivo using animal cancer models, and there is concern regarding tumor selectivity and specificity and potential toxicity in humans. Some of these venom-derived agents have been reviewed (Vyas et al. 2013), but will not be discussed here due to lack of space.

Uses of Snake Venom Proteins/Peptides in Cardiovascular Disease

Fibrinogen Receptor Antagonists for Antiplatelet Therapy

There have been two fibrinogen receptor antagonists designed based on the structure of disintegrins isolated from snake venom (Table 1). One of them is called eptifibatide (Integrilin), and it is a cyclic heptapeptide whose structure is based on the disintegrin found in the venom of the southeastern pygmy rattlesnake (*Sistrurus miliarius barbouri*). This disintegrin called barbourin has a KGD (Lys-Gly-Arg) sequence that is highly specific for GPIIb–IIIa (Scarborough et al. 1993). Eptifibatide, which

Table 1 Platelet aggregation inhibitors derived from snake venom components

Commercial name	Toxin origin	Indication	Status (clinical trials and use)	References
Integrilin	Eptifibatide; *Sistrurus miliarius barbouri*	Reduced the risk of acute cardiac ischemic events (death and/or myocardial infarction) in patients with unstable angina or non-ST-segment elevation (e.g., non-Q-wave) myocardial infarction	Approved	Scarborough et al. (1993)
Aggrastat	Tirofiban (based on the structure of echistatin) from *Echis carinatus*	Used in combination with heparin and aspirin in the management of patients with unstable angina or non-Q-wave myocardial infarction	Approved	Gan et al. (1988)

contains the KGD sequence in its heptapeptide structure, is approved for clinical use and is highly selective for its integrin target and rapidly dissociates from the integrin receptor after therapy is stopped. Eptifibatide has been used clinically following intravenous delivery to reduce the risk of acute cardiac ischemic events and to prevent thrombotic complications during balloon angioplasty and stenting. In clinical trials, the administration of eptifibatide to patients undergoing percutaneous coronary intervention (PCI) reduced thrombotic complications. In patients with STEMI, eptifibatide has been studied as an adjunct to fibrinolysis and PCI, and it was shown to improve epicardial flow and tissue reperfusion. STEMI is an acronym meaning “ST segment Elevation Myocardial Infarction,” which is a severe type of heart attack, and the changes observed in the electrocardiogram are a characteristic elevation in what is called the “ST segment.” The elevated ST segment indicates that a large part of the cardiac muscle is damaged. In patients with non-ST-segment elevation acute coronary syndromes, eptifibatide compared to placebo significantly reduced the primary end point of death and nonfatal myocardial infarction at 30 days. Eptifibatide is usually used with aspirin or clopidogrel and heparin.

The second disintegrin-derived fibrinogen receptor antagonist is tirofiban (Aggrastat). The structure of tirofiban was based on the RGD-containing disintegrin echistatin from *Echis carinatus* (saw-scaled viper) snake venom (Gan et al. 1988). Tirofiban is a small, synthetic, non-peptide tyrosine-like structure that acts as an RGD mimetic and is specific for the GPIIb–IIIa receptor (Starnes et al. 2011). Non-peptide antagonists such as tirofiban imitate the geometric and surface charge properties of the RGD sequence and occupy the RGD-binding pocket in GPIIb–IIIa to competitively inhibit the binding of fibrinogen and block platelet aggregation. Tirofiban delivered intravenously in combination with heparin and aspirin is approved for use

in the treatment of patients with unstable angina and acute myocardial infarction, to reduce the rate of refractory ischemic conditions, new heart attacks, and death (Starnes et al. 2011).

Both eptifibatide and tirofiban reversibly and competitively bind to the fibrinogen receptor and have short half-lives (2 h); due to the reversibility of their effect, the antithrombotic affects rapidly disappear after stopping treatment with coagulation parameters returning to normal within 8 h (Starnes et al. 2011).

Fibrinolytic Enzymes for Treatment of Arterial Occlusion and Stroke

Didisheim and Lewis (Didisheim and Lewis 1956) were the first to suggest that snake venoms contain fibrinolytic enzymes that could be useful for clinical application since they should not be inhibited by SERPINs (serine proteinase inhibitors) in mammalian blood. The most advanced enzyme from this group as far as its clinical application is fibrolase. This enzyme and its clinical application have been reviewed in detail previously (Markland and Swenson 2010). Fibrolase was originally purified from the southern copperhead (*Agkistrodon contortrix contortrix*) snake venom. Fibrolase is a 23-kDa fibrinolytic enzyme that acts directly on fibrinogen/fibrin primarily cleaving the Aα-chain of fibrinogen. It also cleaves the Bβ-chain, but at a slower rate, and has no effect on the γ-chain. The enzyme is a member of the reprolysin family (members of this family are metalloproteinases and most are snake venom proteases). Fibrolase acts directly on fibrin, which distinguishes it from the plasminogen activator-based thrombolytics such as tissue plasminogen activator (t-PA). Although fibrolase is not inactivated by blood SERPINs, it is rapidly inactivated by covalent binding to alpha-2 macroglobulin (α2 M) (Markland and Swenson 2010).

With the direct action of fibrolase on fibrin and its lack of inhibition by blood SERPINs, it appeared that fibrolase should be an effective clot-dissolving (thrombolytic) agent. The thrombolytic activity of fibrolase was examined in a canine reoccluding carotid arterial thrombosis model, and it was demonstrated that the enzyme had excellent lytic activity in this 100 % reoccluding arterial thrombosis model. A recombinant version of the enzyme was then obtained from Chiron Corporation (Emeryville, CA), and these results were validated with the recombinant enzyme (Markland and Swenson 2010). At this point the development of the fibrolase technology was put on hold. However, Chris Toombs at Amgen read the paper on in vivo efficacy of fibrolase a few years later and was intrigued by the findings and contacted the authors about taking a look at the enzyme. Studies on fibrolase at Amgen moved forward rapidly and the results were quite positive (Deitcher et al. 2006). Moreover, at Amgen fibrolase was slightly altered to stabilize the enzyme for storage during clinical trials. This altered form, which involved a minor change at the amino-terminus, is called alfimeprase (Markland and Swenson 2010; Table 2). Alfimeprase has an amino-terminal sequence of SFPQR–, whereas the amino-terminus of fibrolase is EQRFPQR–.

Animal studies demonstrated that compared to the plasminogen activators, the speed of lysis by alfimeprase when delivered locally (adjacent to the thrombus) is

Table 2 Fibrinolytic snake venom protein

Commercial name	Toxin origin	Indication	Status (clinical trials and use)	References
Alfimeprase	Recombinant protein based on fibrinolytic metalloproteinase from *Agkistrodon contortrix contortrix* venom	Peripheral arterial occlusions (PAO)	Failed phase III trials for both PAO and CVAD occlusions	Han et al. (2010), Markland and Swenson (2010)
		Central venous access device (CVAD) occlusion		

significantly faster and the potential for hemorrhagic problems is greatly decreased. Based on the rapid dissolution of carotid arterial thrombi in the canine studies and other compelling experimental and animal studies at Amgen, where alfimeprase was shown to lyse clots about six times more rapidly than the plasminogen activators (Deitcher et al. 2006), the clinical potential of fibrolase was then explored. Alfimeprase was produced recombinantly using the methylotrophic yeast species *Pichia pastoris*. A further distinct advantage for clinical application of alfimeprase, as compared to the plasminogen activators, is the ability of α2 M to instantaneously inhibit alfimeprase, limiting the potential for systemic bleeding complications. Interestingly, during the animal model studies, it was noted that after delivering the lytic agent, there was a transient hypotension, which could be prevented by treatment with bradykinin antagonists.

In 2002 Nuvelo (then known as Hyseq) obtained the rights to alfimeprase from Amgen and initiated clinical trials. In designing the clinical trials, advantage was taken of the rapid interaction of α2 M with alfimeprase, such that the thrombolytic agent was delivered directly to the site of the thrombus in the clinical setting. The rationale for this was that after clot lysis alfimeprase would enter the general circulation and be immediately inactivated by α2 M (Deitcher et al. 2006). By estimating the levels of α2 M in human blood, an upper level of alfimeprase that could be safely administered in patients was estimated. Therefore, clinical dosages in the first human trial of alfimeprase did not exceed 0.5 mg/kg. Despite these findings, transient bouts of hypotension were experienced at the highest doses in the peripheral arterial occlusion (PAO) trial (Deitcher et al. 2006). These cases spontaneously resolved or could be easily managed by supportive care.

Initially, a phase I clinical trial was designed to evaluate the safety profile, pharmacokinetics, and thrombolytic activity of alfimeprase in patients with chronic PAO. Treatment involves rapid restoration of arterial patency and blood flow and limb preservation. In the phase I trial, the primary end point was safety as assessed by adverse event rates. The phase I trial demonstrated that alfimeprase at doses as high as 0.5 mg/kg was generally safe in patients with chronic PAO. There were no bleeding complications and no systemic thrombolysis was noted. Also, stable fibrinogen and plasminogen concentrations indicated that alfimeprase action is

limited to the target thrombus. There were no deaths and none of the patients experienced adverse hemorrhagic events. Thus, this phase I clinical trial demonstrated that alfimeprase holds the potential to achieve dissolution of PAO with minimal risk of hemorrhage (Moise and Kashyap 2008). The phase II NAPA-1 (Novel Arterial Perfusion with Alfimeprase-1) trial was to evaluate safety as the primary objective and efficacy was a secondary objective. Alfimeprase lysed up to 76 % of the thrombi restored arterial flow up to 60 % within 4 h of dosing and 52–69 % of patients avoided surgical intervention. There were no intracerebral hemorrhages or deaths. There was transient hypotension at high dose, which could be easily managed, and α2 M dropped 40–60 % but recovered by day 14 (Deitcher et al. 2006). In the phase 3 trials, NAPA-2 and NAPA-3, alfimeprase was compared to placebo. The intent was to enroll ~600 patients worldwide and the primary end point was avoidance of open vascular surgery within 30 days of alfimeprase treatment. In January 2006 alfimeprase was granted fast track designation for the NAPA-3 trial for PAO. During the NAPA-2 and NAPA-3 studies, it was noticed that there was a correlation between the length of the thrombus and the success of lysis by alfimeprase: for short occlusion lengths, there was virtually no benefit, whereas for occlusion lengths of $\geq$40 cm, the 4 h flow restoration rate was 45.5 % for alfimeprase and only 16.7 % for placebo. This correlation also held when there was no early decline in circulating α2 M level, where the 4-h flow restoration rate for alfimeprase was 70 %, whereas for placebo, it was 37 %. These results suggested that when alfimeprase is trapped in longer thrombi and is not released into the general circulation, where it is inactivated by α2 M, the enzyme is degrading the thrombus, leading to more rapid flow restoration (Han et al. 2010). These findings suggested that there may have been some flaws in the side-hole catheter method used for delivery and that alternative delivery methodologies resulting in increased residence time in the clot should be explored. In summary, the phase III NAPA trials did not meet the primary end point, and there was no significant difference between the intrathrombus alfimeprase and intrathrombus placebo groups (Han et al. 2010). In both trials the majority of hypotensive episodes occurred within 15 min of alfimeprase administration without apparent clinical sequelae. In NAPA-3 catheter site bleeding was confirmed, but no surgical bleeding or major hemorrhage risk was observed. Finally, interim analysis of NAPA-3 showed favorable efficacy trends, but the sponsor decided the delivery method was not optimal and terminated the study.

The second clinical application of alfimeprase was central venous access device (CVAD) occlusion. There are almost five million catheters positioned annually in the United States to deliver chemotherapy, nutrients, antibiotics, and blood products, and up to 25 % of them become occluded. It was hypothesized that in patients with an occluded CVAD, there would be rapid clot dissolution following alfimeprase treatment. A phase II dose-ranging trial was initiated. Safety and efficacy of one or two instillations of three intraluminal doses of alfimeprase or alteplase were compared (alteplase is a version of recombinant t-PA developed by Genentech). Alfimeprase was shown to restore CVAD function in up to 60 % of treated patients in less than 30 min, with the majority of these successes occurring in 15 min or less with 3 mg of alfimeprase, and there was an acceptable and

favorable safety profile with no evidence of hypotension. This phase II trial demonstrated that all three alfimeprase doses were more successful than alteplase at 5, 15, and 30 min after treatment. Alfimeprase at 3.0 mg produced the highest patency rate of 60 % at 120 min after the first and 80 % after the second doses, and no major hemorrhagic or embolic events were reported (Deitcher and Toombs 2005). A phase III trial called SONOMA-2 (Speedy Opening of Non-functional and Occluded catheters with Mini-dose Alfimeprase-2) was then initiated. The trial compared efficacy and safety of 3 mg of alfimeprase versus placebo and was to include 300 patients with CVAD occlusion. The primary end point was the restoration of function to occluded catheters in 15 min. Unfortunately, it was soon determined that alfimeprase did not meet the end point of restoration of function of occluded central venous catheters in 15 min. Alfimeprase restored catheter function in 15 min, but with a p-value of 0.022, it did not meet the more stringent p-value of <0.00125 required of this trial. Thus, enrollment in the SONOMA-3 trial was suspended for 8 months. SONOMA-3 reopened as an open-label, single-arm trial of alfimeprase alone using a 10 mg dose at 5 mg/mL in up to 100 patients. The primary end point was safety; efficacy was also evaluated. Alfimeprase restored catheter function in ~50 % of patients in 15 min and ~60 % at 1 h, but Cathflo Activase® (alteplase or rt-PA) cleared ~80 % of occluded catheters in patients by 2–4 h. Alfimeprase was to restore catheter function with similar efficacy to Cathflo Activase®, but in a shorter time. In combination with the poor results in NAPA-3, Nuvelo abandoned the development of alfimeprase in March 2008 (http://www.bizjournals.com/sanjose/stories/2008/03/17/daily14.html?t=printable).

Prior to dropping the alfimeprase project, Nuvelo had initiated a phase II clinical trial in acute ischemic stroke in 2007 (http://www.fiercebiotech.com/node/13455/print). Fast track designation for alfimeprase was granted for this proof of concept study. The acute ischemic stroke trial with alfimeprase was called CARNEROS-1 (Catheter-directed Alfimeprase for Restoration of NEurologic function and Rapid Opening of arteries in Stroke). The primary efficacy end point was the recanalization of main occlusive lesion within 120 min of alfimeprase treatment; safety was to be assessed by a lack of symptomatic intracerebral hemorrhage within 24 h of drug administration. Due to the lack of enrollment in this trial as well as the failed phase III trials in CVAD and PAO, Nuvelo decided to terminate this study. Thus, in March 2008, Nuvelo discontinued the clinical development of alfimeprase and the program was shut down (http://www.fiercebiotech.com/node/20663/print).

Snake Venom Defibrinogenating Enzymes for Anticoagulation

The Italian Fontana was most likely the first person to recognize that snake venom has the ability to clot whole animal blood and to cause circulating blood to become incoagulable (Fontana 1787). About 100 years later studies with eastern diamondback rattlesnake (*Crotalus adamanteus*) venom showed that the venom destroyed the coagulability of animal blood and the globulin (protein) fraction was found to be responsible for this action (Weir-Mitchell and Reichert 1886).

In 1937 the venom of *C. adamanteus*, as well as other venoms, was shown to act directly on fibrinogen to coagulate plasma and this action was not dependent on calcium (Eagle 1937).

The incoagulability of animal blood following envenomation occurs with many different snake species. In particular, this activity was observed following envenomation by the Malayan pit viper *Calloselasma rhodostoma* (formerly known as *Agkistrodon rhodostoma*) (Reid et al. 1963) and the responsible enzyme was purified and called ancrod (Esnouf and Tunnah 1967). A similar enzyme was also isolated from the venom of *Bothrops atrox*, a pit viper found in Central and South America and called reptilase or batroxobin (Stocker and Egberg 1973). Paradoxically in vitro the venom causes blood to coagulate, whereas in vivo the venom acts as an anticoagulant by a defibrinogenating action. These snake venom serine proteinases are single-chain glycoproteins with molecular weights of about 35 kDa. They have some properties in common with mammalian thrombin and catalyze the conversion of fibrinogen to a fibrin clot in vitro. However, unlike thrombin, the snake venom enzymes are not inhibited by heparin, and they do not cause activation of blood coagulation factor XIII that cross-links and stabilizes thrombin-generated clots. The fibrin clot induced by the venom enzymes in vivo is not cross-linked and is removed rapidly from the circulation, at least in part by the reticuloendothelial system. It would appear that the mechanism whereby these enzymes produce a benign state of defibrinogenation involves the formation of an abnormal clot. This is in contrast to thrombin clots which are cross-linked by factor XIII and are quite stable. Within minutes of administration of the venom enzymes in vivo, there is a depression of the fibrinogen concentration in plasma. Fibrinogen can be maintained at the depressed level by repeated infusions (daily or twice daily) (Bell 1990). The low levels have been maintained in some patients for up to 7 weeks. Upon termination of venom enzyme infusion, there is a slow recovery of the fibrinogen levels. There is also an increase in the levels of fibrin(ogen) degradation products in the plasma upon venom-induced defibrinogenation. These degradation products appear to be derived by plasmin digestion of the non-cross-linked fibrin clot, which is highly susceptible to the action of the fibrinolytic enzyme. The net result is that there is a benign state of defibrinogenation. A clinical study (six patients) indicated that although there is a rise in fibrin(ogen) degradation products and depletion of plasminogen following ancrod infusion, there is no rise in tissue or urokinase-type plasminogen activator levels. It appeared that fibrinopeptide A was removed from fibrinogen to produce a soluble form of fibrin which was then removed from the circulation with no evidence of increase in plasminogen activator levels, but with significant activation and consumption of plasminogen and depletion of α2-antiplasmin. Additionally, the fibrin degradation products formed were identical to those formed by plasmin degradation of soluble, non-cross-linked fibrin, suggesting that plasminogen activation by the fibrinolytic system accounts for the degradation of fibrin. The role of the fibrinolytic system in the action of ancrod was demonstrated by the use of inhibitors of fibrinolysis in animals following ancrod infusion. This treatment led to significant clot formation in vivo and death.

Table 3 Medical uses of defibrinogenating snake venom proteins

Commercial name	Toxin origin	Indication	Status (clinical trials and use)	References
Defibrase	Batroxobin; *Bothrops atrox*	AMI	Phase II: rBat in trials for the prevention and treatment of surgical-related bleeding	Xu et al. (2007)
		Acute cerebral infarction		
		Angina		
		Central retinal venous thrombosis		
		DVT		
		Priapism		
		Peripheral arterial disease		
		Perioperative anticoagulation		
		Pulmonary embolism		
		Sickle cell crisis		
Reptilase® time	Reptilase; *Bothrops jararaca*	Diagnostic for dysfibrinogenemia	Approved	Stocker and Egberg (1973)
Viprinex	Ancrod; *Calloselasma rhodostoma*	Acute ischemic stroke	Failed phase III trial for ischemic stroke	Levy et al. (2009)
		Coronary artery bypass		
		Critical ischemia		
		DVT		
		Heparin-induced thrombocytopenia		

These venom defibrinogenating enzymes (Table 3) are referred to as venombins and are classified in three groups (A, B, or AB) depending on their ability to release fibrinopeptide A (FPA), fibrinopeptide B (FPB), or both. Ancrod and batroxobin are members of the venombin A group.

Ancrod and batroxobin have been administered intravenously (i.v.) to humans, primarily in Europe and other countries (not the United States) as defibrinogenating agents to prevent and treat thrombotic disorders for a number of clinical conditions including deep vein thrombosis, myocardial infarction, pulmonary embolus, central retinal vein occlusion, peripheral vascular disease, angina pectoris, glomerulonephritis, priapism, sickle cell crises, and renal transplant rejection (Markland and Swenson 2013). Ancrod and batroxobin have also been used in combination with thrombolytic agents in canine models of arterial thrombosis. Batroxobin acted to prevent coronary artery reocclusion, while ancrod enhanced the effect of thrombolytic agents in a carotid arterial thrombosis model, probably by depleting fibrinogen and preventing propagation of existing thrombi. Ancrod was also shown to decrease

cyclic flow variations and to cause thrombolysis in a coronary artery model in the canine. A beneficial effect of ancrod (and presumably batroxobin) infusion results from the decrease in blood viscosity due to fibrinogen depletion. This improves blood-flow characteristics and may aid therapeutic effectiveness (Markland and Swenson 2013).

Treatment of acute ischemic stroke with intravenous (i.v.) thrombolytic therapy was approved in the United States in 1996 by the Food and Drug Administration (FDA). Because of concern with increased bleeding risk following thrombolytic therapy, the widespread use of this treatment modality for acute ischemic stroke has been limited. Further, initiation of i.v. thrombolytic therapy more than 3 h after onset of symptoms is not recommended. However, the use of defibrinogenation therapy for acute ischemic stroke using ancrod has been examined in several trials and positive results were obtained in two out of three of these trials. Ancrod not only reduced fibrinogen levels, decreased blood viscosity, and created an anticoagulant state but also has an indirect fibrinolytic affect, as already noted. The effectiveness of ancrod in treating acute ischemic stroke has been evaluated, and although the initial results were very promising, ancrod failed phase III clinical trials and its use for ischemic stroke victims was stopped due to the lack of efficacy in December 2008 (Levy et al. 2009). Defibrinogenation with enzymes like ancrod still holds promise for a variety of thrombotic conditions, but efficacy in stroke patients has only been demonstrated in patients who commence treatment within 3 h of onset of symptoms. Interestingly, there seems to be an association of poor outcomes in stroke patients with higher plasma fibrinogen levels at stroke onset; this suggests that therapy aimed at lowering fibrinogen levels might be effective in acute ischemic stroke patients (del Zoppo et al. 2009); this is an area that needs further investigation. It should be noted that ancrod is still being used outside of the United States for several thrombotic conditions including deep vein thrombosis (DVT) and coronary artery bypass surgery.

Batroxobin was found to be effective in abolishing symptoms of DVT (pain and swelling of lower limbs) and achieving limb salvage in a study of a small group of patients with DVT. Batroxobin has also been recommended for treatment of patients with ischemic stroke and transient ischemic attack (TIA) who show hyperfibrinogenemia (Xu et al. 2007). Intravenous injection of batroxobin can effectively reduce the risk for stroke/TIA recurrence in patients with hyperfibrinogenemia. These findings suggest that batroxobin may be a safer and more effective treatment for ischemic stroke than ancrod. Recent reports indicate recombinant batroxobin (rBat) significantly reduced bleeding time and volume of blood loss in a phase IIa trial of surgical bleeding. This trial is part of the development of an antibleeding kit centered around rBat for use in emergency situations, such as traffic accidents and combat (http://seekingalpha.com/article/47598-sinobiomed-finds-recombinant-batroxobin-effective-in-trial). Additionally, batroxobin has been successfully used in a small clinical trial to compare aspirin alone to batroxobin in preventing restenosis and reocclusion in postsurgical diabetic patients; however, it did not provide an advantage in limb salvage or amputation-free rates (Wang et al. 2010).

If the venom defibrinogenating enzymes are used for a prolonged time or if there is a necessity for repeated usage, there will be a loss of enzyme activity due to the development of immune resistance by the patients. But since there is no immunological cross-reaction between ancrod and batroxobin, these enzymes should be able to replace one another in successive courses of long-term anticoagulant therapy without loss of activity.

Snake Venom Inhibitors of Serine Proteinases

Textilinin-1 is a Kunitz-type serine proteinase inhibitor isolated from the Australian brown snake (*Pseudonaja textilis*) venom (Willmott et al. 1995). It is a peptide of 6.7 kDa of known 3-D structure that acts as a potent and specific inhibitor of human plasmin. Its use as a systemic antibleeding agent in surgery, in place of aprotinin, is being considered. Aprotinin, another Kunitz-type serine proteinase inhibitor from bovine tissue, is a peptide of 6.5 kDa. It has been used to inhibit plasmin and to stop bleeding during surgical procedures. However, its use is associated with a number of complications or death, most likely due to inhibition of other serine proteinases or to the irreversibility of its reaction with plasmin, which could lead to adverse thrombotic events; its use as an antibleeding agent has been suspended in a number of countries. Preclinical trials are in progress with textilinin-1 as an experimental drug, and it has been shown to reduce loss of blood more quickly than aprotinin in an animal model (Flight et al. 2009). Textilinin-1 is believed to be a more effective and safer choice than aprotinin as an antibleeding agent.

A cobra venom-based anticoagulant, ximelagatran (Exanta), is the first direct thrombin inhibitor that can be administered orally. Ximelagatran is a prodrug form of melagatran (Fig. 2), which enables it to be rapidly absorbed in the small intestine. After absorption, ximelagatran is quickly bioconverted to its active form. Melagatran (glycine, *N*-[(1R)-2-[(2S)-2-[[[[4-(aminoiminomethyl)phenyl]-methyl] amino]carbonyl]-1-azetidinyl]-1-cyclohexyl-2-oxoethyl]-) is a small dipeptide

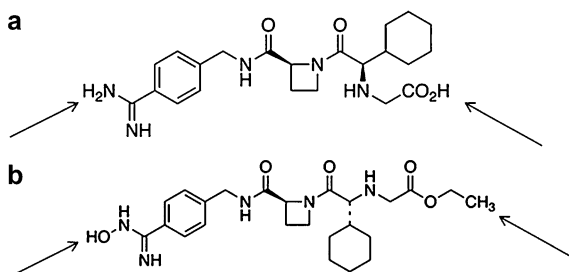

Fig. 2 Structures of melagatran (**a**) and ximelagatran (**b**): ximelagatran is a modified form of melagatran with the modifications being a replacement of the two terminal hydrogens (noted by *arrows* in **a**) with a hydroxide and an ethyl (noted by *arrows* in **b**). This alteration and conversion from the inactive prodrug ximelagatran to melagatran allow for activity as a powerful reversible thrombin inhibitor

mimetic which binds only to the active site of thrombin. The structure of the inhibitor is based on a peptide found in cobra venom with an amino acid sequence that mimics a sequence in fibrinopeptide A preceding the site that is cleaved by thrombin during the conversion of fibrinogen to fibrin (Motsch et al. 2006). Ximelagatran, in its active form (melagatran), functions as a potent, rapid, selective, direct, and competitive reversible inhibitor of human thrombin and has the potential to replace warfarin. As opposed to warfarin, which has a narrow therapeutic index and must be monitored constantly, ximelagatran has predictable pharmacokinetics and pharmacodynamics and is used with a fixed dose without coagulation monitoring. Three indications had been examined in phase III trials: the prevention of venous thromboembolism (VTE) in patients undergoing total knee replacement surgery, the prevention of stroke and other thromboembolic complications associated with atrial fibrillation, and the long-term secondary prevention of VTE after standard treatment of an episode of acute VTE. Oral ximelagatran and subcutaneous melagatran were licensed in Europe for VTE prevention in patients undergoing knee or hip replacement surgery. The use of ximelagatran, however, is associated with unexplained elevation of liver enzymes, which may occur in approximately 6 % of patients, and a higher incidence of withdrawals due to adverse events, including acute myocardial infarction. In view of these issues, in 2004, the FDA concluded that the benefit to risk ratio of ximelagatran was unfavorable for the proposed indications. The FDA reviewers said that the drug maker "had not adequately addressed the risks of liver toxicity, heart attack, or drug-induced bleeding associated with Exanta." In 2006 the manufacturer announced that it was halting ongoing ximelagatran trials and would withdraw pending applications for marketing approval for proposed use for orthopedic surgery and other indications in the United States, Europe, and elsewhere, and it would terminate ximelagatran development and discontinue its distribution (Boos and Lip 2006).

Diagnostic Use of Snake Venom Proteins

It has been known for many years that snake venom components induce important changes in proteins involved in the mammalian coagulation pathway, including procoagulant venom proteins that activate factor X or prothrombin or convert fibrinogen to fibrin. Some of these procoagulant proteins have been used to help clarify physiological mechanisms. Additionally, some venom proteins or peptides have activities that serve as useful diagnostic agents for clinical laboratory application (Table 4). This topic has been effectively reviewed elsewhere, and for a more complete description of this topic, the readers are referred to more definitive reviews (Marsh and Williams 2005; Schoni 2005). Among the venom proteins that serve as useful diagnostic agents is the protein C activator isolated from southern copperhead venom (*Agkistrodon contortrix contortrix*) (Stocker et al. 1987); this activity is also found in many other snake venoms. Activated protein C (APC) is a vitamin K-dependent serine protease that degrades coagulation factors VIIIa and Va and thus acts as an anticoagulant and assists in the regulation

Table 4 Diagnostic uses of some snake venom components

Commercial name	Toxin type; origin	Indication
Botrocetin	Platelet aggregation inducer; *Bothrops* species	Assay of vWF in plasma, aggregates formalin-fixed platelets
ProTac®	Proteinase (serine); *Agkistrodon contortrix*	Determination of protein C and protein S levels
Reptilase® time	Batroxobin (serine proteinase); *Bothrops jararaca*	Diagnostic for dysfibrinogenemia
RVV-V	RVV-V (serine proteinase); *Daboia russelli russelli*	Factor V determination, destabilizes and inactivates factor V
RVV-X	RVV-X (metalloproteinase); *Daboia russelli russelli*	Quantitative conversion of factor X to factor Xa

of the hemostatic process. The venom protein C activator rapidly converts human protein C into activated protein C and avoids the use of the physiological activators thrombin and thrombomodulin; this simplifies and reduces the cost of the protein C assay. Activity of protein C can be measured either by assessing its prolongation of the activated partial thromboplastin time (APTT) or by the use of a specific chromogenic substrate. The venom activator serves as a useful laboratory reagent and can be used for the determination of plasma levels of protein C and protein S; it is commercially available as ProTac®.

There are a number of other tests for clotting factors based on venom-derived components. Included is the prothrombinase-induced clotting time, which relies on the factor V activator, a serine proteinase, obtained from Russell's viper (*Daboia russelli russelli*) venom (RVV-V) (Schoni 2005). The test is used to monitor anticoagulants, such as unfractionated heparin, low-molecular-weight heparin, and thrombin and FXa inhibitors. Oral anticoagulants, such as warfarin, which act on vitamin K-dependent coagulation factors, are not detected. During incubation, the patient's factor V (FV) interacts with RVV-V contained in the activator reagent. Activated factor V (FVa), which is an essential cofactor in the prothrombinase complex, dramatically increases the rate of the conversion of prothrombin to thrombin through its interaction with activated FX (FXa). FXa and phospholipids, critical to the formation of the prothrombinase complex, are also provided with the activator reagent. When Ca^{2+} is added to start the reaction, the prothrombinase complex forms very rapidly and this complex forms thrombin immediately. Thrombin then converts fibrinogen to fibrin and this generates a measurable end point due to the formation of the fibrin gel. An almost linear dose–response and high sensitivity of the assay for unfractionated heparin, low-molecular-weight heparins, r-hirudin, and argatroban (a small-molecule direct thrombin inhibitor) was found (Calatzis et al. 2008). RVV-V has also been used in a test to determine the levels of FV in plasma since it selectively activates FV, and in the presence of FXa and phospholipid, Ca^{2+} ions convert prothrombin to thrombin, and the initial rate of thrombin formation can be assayed by a chromogenic thrombin substrate assay, which gives an indication of the amount of FV in

the plasma. RVV-V can also be used to prepare plasma devoid of FV that can then be used for FV determination.

Factor V (FV) Leiden is an autosomal dominant condition that results in a FV variant that cannot be degraded by activated protein C. This condition is perhaps the most important mutation of a coagulation factor that increases thrombotic risk with an incidence in Caucasians of from 5 % to 15 %. Several different assays employing snake venom components have been developed to assess this genetic defect (Marsh and Williams 2005). Since there may be other mutations that result in activated protein C resistance, DNA sequence analysis may be necessary to confirm the FV Leiden diagnosis. In the first step of the APC resistance (APC-R) factor V Leiden test, factor V contained in the patient's plasma sample is activated by RVV-V. FVa is readily inactivated by APC added with the APC reagent in normal patient plasma. However, in the absence of APC, there is no inactivation, and FVa in the plasma sample remains active to serve as a cofactor for prothrombin activation. In the second step, Noscarin is added to initiate thrombin generation. Noscarin is a prothrombin activator from the venom of the Australian tiger snake *Notechis scutatus scutatus* and is dependent on FVa as a cofactor. Since only the activated form of FV is inactivated by APC, the role of RVV-V in this test is to activate FV present in the patient's plasma and make it susceptible to cleavage by APC. Dependent on how much FVa is left in the plasma, the activity of Noscarin is strongly enhanced or not. Thus, in a normal patient, the APC clotting time is much higher than the clotting time in the absence of APC, with a ratio of about 2.5 or more. When the patients FVa is resistant to the inactivation by APC due to a homozygous factor V Leiden mutation, the added APC has no influence on FVa activity and the clotting times measured results in a ratio of about 1.0. With a heterozygous patient, the calculated ratio lies somewhere between these extremes.

Another diagnostic laboratory application involves the dilute Russell's viper venom time (dRVVT). This test is based on the ability of RVV to directly activate factor X and convert prothrombin to thrombin in the presence of factor V and phospholipid. In this test, rate-limiting concentrations of both RVV and phospholipid are used so the test is sensitive to the presence of lupus anticoagulants (LAs), which are antibodies (including those developed against phospholipids) produced in the autoimmune disease lupus (systemic lupus erythematosus) that interfere with the role of phospholipids in test tube clotting tests and result in a prolonged clotting time. Interestingly, there is a paradoxical coagulant effect in patients affected with lupus in that they exhibit arterial and venous thrombotic complications. There are a number of variations of this test, which are described in more detail elsewhere (Marsh and Williams 2005). More recently, a combination of snake venom prothrombin activators (oscutarin from taipan, *Oxyuranus scutellatus*, venom and ecarin from *Echis carinatus* venom) has proven successful in an assay for lupus where ongoing anticoagulation therapy makes it difficult to identify the lupus anticoagulant. The taipan/ecarin ratio is a test for a lupus anticoagulant (LA) based on the differential dependence of these two snake venoms on phospholipid to activate coagulation. The venom from the taipan contains a prothrombin activator that converts prothrombin to thrombin after the addition of phospholipid

and calcium, whereas ecarin activates prothrombin to form meizothrombin in the absence of any added reagents (Kini 2005). In the presence of a LA, the taipan time is prolonged due to its phospholipid dependence, but the ecarin time is not. The newest modification to this test employs a direct acting factor Xa (FXa) inactivator, rivaroxaban, for anticoagulant therapy, and it was shown that the taipan/ecarin ratio was not affected by this anticoagulant and that this test serves as a sensitive assay for the presence of the lupus anticoagulant (van Os et al. 2011).

Separately, the factor X activator from RVV (RVV-X) has been purified and used in a number of clotting measurements for the determination of FX and for distinguishing between factor VII and X deficiencies (Marsh and Williams 2005). RVV-X is used for factor X assay by converting FX into the active enzyme FXa, which is then directly determined by means of a clotting assay in the presence of FX-deficient plasma or using a suitable synthetic chromogenic substrate. A prolonged RVV-X clotting time indicates a deficiency in FX or FV.

Thrombin-like snake venom enzymes have been used as diagnostic agents, and one of the most frequently used diagnostic tests involving venom proteins employs the fibrinogen-clotting serine proteinase, batroxobin, from the venom of *Bothrops atrox*. The test is known as the Reptilase® time and is used in clinical lab diagnosis of bleeding and other coagulation disorders. Importantly, the clotting time of plasma after the addition of batroxobin (Reptilase® reagent) is not prolonged by heparin or hirudin, whereas the thrombin-clotting time is prolonged in the presence of these anticoagulants. Thus, the Reptilase® time is a simple alternative to the thrombin time to measure the fibrinogen/fibrin reaction in patient's samples that contain heparin or fibrinogen degradation products (Karapetian 2013). Batroxobin can also be used to investigate dysfibrinogenemias by measuring alterations in the Reptilase® time. Furthermore, batroxobin is used for the quantitative determination of plasma fibrinogen in which the clotting time is measured on plasma dilutions with different fibrinogen concentrations to obtain a standard graph, and this serves to convert the batroxobin clotting time determined in sample plasma into the fibrinogen concentration. Additionally, batroxobin can be used for defibrinogenation of plasma. The Reptilase® time assay is also useful in the assay of antithrombin III (ATIII). In this application plasma can be prepared free of fibrinogen without adding thrombin, which would react with ATIII and interfere with the assay (Marsh and Williams 2005).

As far as the use of venom-derived proteins in diagnostic tests with platelets, botrocetin is a potent platelet aggregator of citrated platelet-rich plasma, and its aggregatory effect is dependent on von Willebrand factor binding to platelet GPIbα; this action has been widely utilized for vWF assays (Marsh and Williams 2005). Botrocetin was isolated from the South American pit viper *Bothrops jararaca* (Read et al. 1978) but is present in the venom of many of the *Bothrops* species and has been described as an activator of von Willebrand factor-dependent platelet aggregation. Botrocetin induces von Willebrand factor (vWF)-dependent aggregation of native or formalin-fixed platelets. It appears to act via a two-step manner in which it first binds to vWF to form a complex (the active agglutinating agent). Then, the complex binds to GPIbα, and this serves as a bridging agent to form

platelet agglutinates. All three components, vWF, botrocetin, and GPIb, appear to be required for maintenance of stable platelet agglutinates. Botrocetin (~25 kDa nonreduced) has been identified as a heterodimer, and its sequence and 3-D structure have been determined, and specific binding sites within the A1 domain of von Willebrand factor have been identified. Botrocetin can agglutinate platelets without the requirement for platelet activation and GPIIb–IIIa activity. Therefore, its activity is not blocked by anti-αIIbβ3 antibodies or EDTA (Andrews et al. 2004).

Conclusions and Future Directions

Several revolutionary events have facilitated the application of venom proteins in biomedicine. First is the increased interest in natural molecules by the biotech industry and their willingness to explore these natural molecules or recombinant or modified versions thereof for potential biomedical application. Second is the continuing improvement in the resolving power of highly sophisticated technologies (mass spectrometry, HPLC, and next-generation sequencing), and the interest by investigators worldwide in snake venomics, based on both proteomes of the venom and transcriptomes of venom glands; both have contributed to a significant interest in snake venom proteins/peptides, even those in low abundance. A long-term research goal of venomics is novel drug discovery and obtaining an understanding of the potential biomedical applications provided by snake venom. To date, proteomic and transcriptomic studies have demonstrated significant potential to explore in great depth the molecular complexity of venoms. Significant progress in these areas has fueled tremendous interest from both academia and the biotech industry to devote the time and resources to characterize with ever-increasing rapidity the myriad components in venoms of multiple snake species with the hope of identifying molecules with novel and clinically relevant activities. JW Fox and SM Serrano in 2007 (Fox and Serrano 2007) stated that the research community is "approaching the golden age of natural product pharmaceuticals from venom libraries" and this statement seems to still be holding true 7 years later.

However, of concern to the continued increasing knowledge in the field of snake venomics is the threat of increasing urbanization of many of the worlds' great metropolitan centers and the corresponding impingement that this brings to the geographical range of many snake species. Environmentalists and scientists throughout the world need to ensure that this encroachment on the impacted snake species does not lead to their permanent eradication and the concomitant loss of the treasure trove of potentially important biomolecules in their venom that would be forever lost to society.

This chapter has pointed out the significant successes that have followed the isolation and characterization of snake venom proteins and peptides; these studies may ultimately lead to important and novel new biomolecules with significant impact on patient care. This was illustrated quite effectively by the story of the discovery and clinical application of captopril, which was derived from a peptide discovered in the venom of a South American pit viper and used clinically as an

antihypertensive agent based on its inhibition of angiotensin-converting enzyme. Similarly, another success story is represented by the inhibitors of platelet integrin αIIbβ3, whose structures were based on the R/KGD motif in snake venom disintegrins. These include Integrilin (eptifibatide), a cyclic heptapeptide based on the KGD sequence of barbourin, the disintegrin from the pygmy rattlesnake, and Aggrastat (tirofiban), the non-peptide RGD mimetic whose structure is based on the RGD disintegrin echistatin from the saw-scaled viper. These antiplatelet agents have been used to prevent thrombotic complications with various clinical applications.

On the other hand, there have been some devastating failures; most noteworthy of these are alfimeprase and Exanta. Alfimeprase, a fibrinolytic metalloproteinase slightly modified from the native southern copperhead venom enzyme fibrolase, sailed through phase I and II clinical trials and into the phase III trial for both peripheral arterial occlusion and central venous access device occlusion, but failed to meet the expected end points for rapidity and statistical significance and was ultimately abandoned. Similarly Exanta, a direct acting and specific thrombin inhibitor, designed from a peptide found in cobra venom, was approved in Europe as an anticoagulant for venous thromboembolism. However, in evaluating a series of studies in the United States and elsewhere, significant hepatic toxicity was observed and the FDA in the United States did not approve the drug for clinical use; the manufacturer then withdrew Exanta from the international market.

There are also stories waiting to be written with venom-derived components. For example, will the disintegrins have a clinical impact as effective anti-invasive/antiangiogenic agents for cancer therapy and will the novel proline-rich antihypertensive decapeptide member of the bradykinin-potentiating peptides from S. American pit viper venom, acting via an ACE-independent mechanism, find clinical application as an activator of argininosuccinate synthetase and advance to the clinic as a unique antihypertensive agent? Also, there is the potential that venom proteins have new and unanticipated activities that could lead to promising therapeutics either directly or indirectly following alterations to the structure or the development of chemical mimetics. For example, the antiviral activity reported for some venom phospholipases is of considerable interest (Muller et al. 2012) and so is the antiviral activity of a venom disintegrin (Hubbard et al. 2012). These are only two examples indicating the great potential that venom proteins/peptides possess that are yet to be fully exploited, but could lead to exciting, novel applications for venom components.

It is expected in the coming generations that there will be many more success stories with peptides and proteins isolated from the venom of different snake species that will find their way to biomedical application, as the structure isolated or a modified form, as probes for possible therapeutic targets or as lead compounds in the development of therapeutic agents for specific type of disease, as a reagent for diagnostic test procedures, or as a specific tool in the study of important physiological or pathological processes.

In closing, the authors apologize to the many venom researchers whose excellent work was not recognized in this chapter due to editorial limitations.

Cross-References

▶ Automated Mass Fingerprinting of Venoms in Nanogram Range: Review of Technology
▶ Biological Activities and Assays of the Snake Venom Metalloproteinases (SVMPs)
▶ Shotgun Approaches for Venom Analysis
▶ Snake Venom Peptidomics
▶ Structure-Function Relationship of Modular Domains of P-III Class Snake Venom Metalloproteinases
▶ Three-Dimensional Structures and Mechanisms of Snake Venom Serine Proteinases, Metalloproteinases, and Phospholipase A_2s

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Industrial Production and Quality Control of Snake Antivenoms 20

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Abstract

The production of snake antivenoms involves stages such as production of venom, immunization of animals to generate hyperimmune plasma, immunoglobulin purification, viral inactivation (or removal), and stabilization of the formulation. In order to manufacture products of satisfactory effectiveness and safety, antivenom design must be validated by preclinical and clinical studies. Moreover, during the industrial production, the quality of the products and of the entire manufacturing process (including management of clean rooms, production of

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water for injection, and sterilization or sanitization of the equipment) must be strictly evaluated. This chapter presents a practical description of the stages involved in the design, production, and quality control of snake antivenoms.

Introduction

Snake antivenoms are formulations of immunoglobulins purified from the plasma of animals immunized with snake venoms. Their parenteral administration is the only therapy whose efficacy to treat snakebite envenomation has been scientifically demonstrated. Being a drug, antivenoms must meet requirements of identity, purity, safety, and efficacy, as requested by the good manufacturing practices for pharmaceutical products. Identity is conferred to antivenoms when the venoms used as immunogens and the immunized animals used as immunoglobulin source are selected. On the other hand, antivenom purity results from a combination of the magnitude of the antibody response generated during animal immunization and the immunochemical separation of immunoglobulins from other plasma proteins (Segura et al. 2012). Safety increases with the antivenom purity; thus, in more purified formulations, therapeutic doses are achieved with a relatively low amount of heterologous protein, hence reducing the probability of adverse reactions. Finally, the efficacy of antivenoms depends on several factors, such as (1) the specificity of the antivenom towards the venom of the snake causing the envenomation, (2) the potency at which the antivenom is formulated, (3) the dose administered, (4) the time elapsed between envenomation and antivenom administration, and (5) the severity of the envenomation. In summary, the quality of antivenoms is constructed step by step during their manufacture, which includes production of venom and hyperimmune plasma, purification of immunoglobulins, virus inactivation (or removal), formulation, preclinical and clinical evaluation, and routine verification of the design compliance of batches coming off a production line. This chapter presents an overview of the different aspects involved in antivenom manufacture and quality control.

Production of Snake Venom

Snake venoms are complex mixtures of proteins and peptides that belong to several families. In the case of viperid venoms, the following protein types have been identified: Zn^{2+}-dependent metalloproteinases (SVMPs), phospholipases A_2 (PLA_2), serine proteinases, L-amino acid oxidases, disintegrins, C-type lectins and lectin-like proteins, natriuretic peptides, cysteine-rich secretory proteins (CRISPs), and some minor components such as vasoactive peptides and proteinase inhibitors (Calvete 2011). Elapid venoms, on the other hand, are particularly rich in low molecular mass neurotoxins and cytotoxins of the three-finger family and in PLA_2s, also containing minor components such as SVMPs and CRISPs (Calvete 2011). Unlike the antigens used for the production of tetanus and diphtheria

antitoxins and rabies immunoglobulin, the venoms of snakes are characterized by an astonishing inter- and intraspecies variability, often presenting significant differences in their composition even within a single species (Chippaux et al. 1991; Gutiérrez et al. 2009).

The presence of a conspicuous pattern of individual, ontogenetic, and regional variation in venom composition requires careful analysis to select the most adequate mixture of venoms to prepare representative reference venom pools for immunization and quality control (WHO 2010). Once the medically relevant important snake species in a particular geographical setting are selected, the establishment of a venom source is required. Nowadays, snake farms or serpentariums are the most common facilities used to produce snake venoms. However, in the future, snake venoms could be produced from culturing secretory cells of venom glands (Carneiro et al. 2006) or by generating recombinant toxins.

For an appropriate management of a serpentarium, it is important to have a well-trained staff for safe handling of snakes. In addition, some major features have to be considered, such as a dedicated independent room for quarantine of new income snakes, where diagnosis of health problems, medical care, and deworming are conducted, together with the implementation of a feeding schedule. The quarantine time usually lasts for 6–8 weeks. On the other hand, the feeding schedule ranges from every week to every 3 weeks, according to the size of the snakes. Additionally, the type of prey provided, such as mice, rats, or chickens, or alternative diets required for particular snake species are of major concern. Likewise, the way in which these prey are provided may affect the welfare of the captive snakes. Thus, whether the prey items are offered alive, dead, or thawed will influence the overall maintenance of the snake colony (WHO 2010).

The establishment of a live collection of captive snakes for venom production allows a strict control of the conditions for venom extraction. Thus, aspects such as individual cages for maintenance, provision of shelters or “hide boxes” for cryptic or burrowing snakes, meticulous cleaning of the cages, the feeding schedule, and the diet provided are important to ensure the overall welfare of the snake collection. When needed, humidity and temperature of the room must be adjusted to simulate the conditions of the natural habitat of the species.

Procedures for venom extraction are performed according to schedules which vary from 2 to 3 weeks to every 3 months. Depending on the snake species, different extraction techniques can be applied to collect the venom. In general terms, massaging the venom glands is one of the most employed and preferred. Briefly, the extraction process might be done by one person (although the presence of two workers is strongly recommended) while the snake is under the effect of a short-acting general anesthesia (e.g., CO_2 inhalation) to facilitate handling. Extraction is performed upon a glass vessel covered by a plastic or parafilm membrane and with siliconized or rubber vessel edges. In addition, the procedure of venom extraction can be associated with other interventions aimed at removing ectoparasites, searching for old patches of skin, and treating the snake with drugs or vitamins. At the end of the process, all the equipment used has to be disinfected before the next snake is submitted to the extraction protocol (WHO 2010).

In order to prevent degradation, venoms should be stabilized rapidly after collection. Several procedures have been used to stabilize snake venoms (e.g., sun-drying, vacuum-drying, and freeze-drying). Little differences have been demonstrated between venoms stabilized by different methods. However, depending on the performance of the method, dried venoms can differ in the activity of some toxins, in comparison to the original fresh venom (Meier et al. 1991). Therefore, freeze-drying or desiccation of venoms should be performed under conditions that ensure activity and antigenicity for long-term storage. Venom pools from a number of individuals have to be prepared in order to ensure an adequate representativeness of the geographical variability of venom composition within a species and a geographical range (Gutiérrez et al. 2009, 2010; WHO 2010).

Venom productivity changes throughout life in captivity. For example, gland massage allows the extraction of 500 mg of dry weight of venom from recently collected *Bothrops asper* snakes. However, venom yield decreases to 200 mg in specimens maintained in captivity for several years. Thus, from a well-established colony of ten *Bothrops asper* snakes, which are "milked" every 3 months, it is possible to obtain an annual production close to 8 g of dried venom. On the other hand, 100 specimens of the coral snake *Micrurus nigrocinctus* (each one producing 5 mg of venom per extraction) are required to produce 1.5 g of dried venom in 1 year in which the snakes are submitted to three procedures of venom extraction (our unpublished observations). Therefore, in order to accomplish the venom production goals to provide sufficient material for immunization and quality control, it is necessary to design strategies to maintain an adequate number of snakes.

Conservation of activity and antigenicity of dried venoms has to be evaluated for each venom batch. Activity can be assessed by the pharmacological and biochemical characterization of the venoms, for example, evaluating the lethal, hemorrhagic, necrotizing, proteolytic, phospholipase A_2, coagulant, and fibrinolytic activities (Camey et al. 2002). On the other hand, antigenicity must be evaluated by determining the ability of venoms to induce antibody responses which allow the neutralization of toxic effects induced by venoms. Antigenicity of venoms is particularly important in the venoms used as reference to evaluate the potency of antivenoms.

Production of Hyperimmune Plasma

Animal Immunization

Immunization must be preferably performed by using as immunogen the venoms of snakes constituting a relevant medical problem in the geographical zone where the antivenom is going to be distributed. In cases where the identification of the offending snake is feasible (e.g., by the clinical features of envenomation or by the use of venom detection kits), the immunogen can be constituted by the venom of a single species, thus generating "monospecific" antivenoms. These react towards the toxins of the venom used as immunogen (homologous venom) and neutralize

their toxicity. Moreover, due to antigenic similarities between toxins, monospecific antivenoms can also cross-react with toxins of venoms from phylogenetically related snakes (heterologous venoms). However, cross-reactivity does not necessarily imply cross-neutralization (León et al. 2011).

When the identification of the offending snake is not feasible, due to the similar pathophysiological picture of envenomations by different species, the immunogen must be composed by the mixture of the venoms of several snakes of medical relevance (Gutiérrez et al. 2009; WHO 2010). Antivenoms produced towards the venoms of several species are called "polyspecific." The selection of venoms used as immunogens to produce polyspecific antivenoms requires preliminary studies to determine (1) the clinical relevance of snake species, both in terms of incidence and severity; (2) cross-neutralization between venoms; and (3) effects of venoms on the antibody response towards the other venoms used as co-immunogens. Cross-neutralization studies allow the identification of venoms which could be neutralized by an antivenom despite not being used as immunogen. On the other hand, studies of co-immunogen effects allow the analysis of whether a polyspecific antivenom must be produced by immunizing a single group of animals with a mixture of several venoms or by mixing plasma from different groups of animals, each one immunized with a single venom (WHO 2010; León et al. 2011).

In order to induce high titers of antibodies of high affinity, adjuvants are used during immunization. Traditionally, Freund's adjuvants and mineral salts are the adjuvants of choice in the industrial production of antivenoms. Freund's complete adjuvant is composed of mineral oil, an emulsifier (e.g., Arlacel A), and heat-killed cells of *Mycobacterium tuberculosis* or *M. butyricum*. Freund's incomplete adjuvant is composed only of mineral oil and an emulsifier, without *Mycobacterium* cells. Both Freund's complete and incomplete adjuvants are used to produce water-in-oil emulsions from which the venom contained in the aqueous phase is slowly released. In addition, the presence of bacterial cells in the Freund's complete adjuvant enhances the ability of macrophages to endocyte and present antigens to T_H cells.

Freund's adjuvants have been described to generate the best antibody response towards snake venoms. However, despite their efficacy, Freund's adjuvants have been associated to adverse effects such as arthritis and tissue damage in the site of injection. Therefore, their use is limited to the first stages of the immunization schedule for the production of antivenoms. Subsequent boosters are administered by using other adjuvants such as mineral salts (e.g., aluminum hydroxide, calcium phosphate). Mineral salts are particulate materials which have the ability to stimulate the immune system by the slow release of immunogen adsorbed to their surface (León et al. 2011). There is active research aimed at the application of additional adjuvants for the production of antivenoms.

Some immunization schedules include the administration of boosters without using adjuvant, composed only by the venom dissolved in saline solution. In these cases, antigen deposits are not formed, and each toxin is differently absorbed according to its toxicokinetic properties, accessing different populations of antigen-presenting cells, which may have an effect on the antibody response towards different venom toxins (León et al. 2011).

Most of the antivenom manufacturers around the world use horses as immunoglobulin source (Gutiérrez et al. 2011). Nevertheless, some commercial formulations are prepared using sheep immunoglobulins. Recently, it has been proposed that industrial production of antivenoms could be based in the purification of antibodies from the yolk of hen's eggs. However, hen-derived antivenoms have not been produced at industrial scale (León et al. 2011).

Immunization is performed by administering sequential doses of the immunogen. Generally, the subcutaneous route is employed. The dose administered in each injection must be carefully selected, since high doses of snake venoms produce injury to the animal (León et al. 2011). In order to prevent tissue damage, some researchers have suggested different physicochemical procedures to detoxify the venoms used as immunogens. However, this practice has the risk of affecting venom immunogenicity and, thus, of reducing the efficacy of the antivenom towards the native venom.

Usually, immunization has been performed by employing a single site for the injection of the venom. However, it has been reported that improvement of the antibody response can be obtained by administering the immunogen dose in multiple injection sites (Chotwiwatthanakun et al. 2001). After injection, the immunogen induces edema and a neutrophil-based inflammatory infiltrate which predominate during the first 24 h. Later on, this infiltrate is replaced by one composed predominantly by antigen-presenting cells such as macrophages and dendritic cells (Teixeira et al. 2009).

Antigen-presenting cells internalize venom components by endocytosis and migrate to the regional lymph nodes, where they present the peptides derived from the processed toxins, coupled to the type II MHC molecules, to specific T_H cells. Later, activated T_H cells stimulate B lymphocytes to proliferate and differentiate into memory B cells and plasma cells. Initially plasma cells produce IgM antibodies, and thereafter they shift and mainly produce antibodies of the IgG isotype (León et al. 2011).

Subsequent administration of immunogen boosters allows the maturation of the affinity, which occurs by the selection and expansion of B lymphocyte clones of higher affinity. As a result, an increase in the production of specific IgG is achieved, with the consequent improvement of the capacity of the sera to neutralize the toxic effects induced by the snake venom. As their concentration increases, antivenom antibodies downregulate their production. Consequently, the peak of antivenom antibodies produced by each immunogen booster is followed by a valley, resulting in a wave whose length depends on the design of the immunization schedule (León et al. 2011).

Antibody response occurs simultaneously towards each protein/toxin in the venom. Therefore, different antibody responses might be obtained towards different toxins in the same venom. These differences depend on the physicochemical properties of the toxin, as well on its structure, molecular mass, relative abundance, and their capacity to interfere with the immune response (Sampaio et al. 2005). On the other hand, the antibody response also depends on the host characteristics (e.g., species and individual genetic background and ability to process the toxins)

and the design of the immunization scheme, which includes venom dose, administration route, booster frequency, and use of adjuvants (León et al. 2011).

After immunization, not all animals reach an antibody response large enough to support the industrial production of antivenoms. Thus, the immunized animals can be classified as good, intermediate, or poor responders. In order to select the animals whose plasma meets the potency specifications, antibody response must be evaluated. This can be done by the lethality neutralization test in a mouse model or by alternative in vitro methods such as ELISA (Rial et al. 2006) or inhibition of enzymatic activities, e.g., inhibition of PLA_2 or proteinase activities (Gutiérrez et al. 1988).

Bleeding of Animals

Before bleeding, the physical condition of immunized animals must be evaluated in order to approve their inclusion in the process. Normally, this evaluation is performed by auscultation of cardiorespiratory and digestive systems, conjunctival and gingival mucosal examination, capillary filling test, overall clinical evaluation, and determination of hemoglobin and hematocrit values. For normal horses, hematocrit and hemoglobin concentrations range between 28–40 % and 9 and 14 g/dL, respectively. However, a drop in hematocrit and hemoglobin can be induced as a consequence of immunization with viperid venoms (Angulo et al. 1997). Only animals having hematocrit values higher than 30 and hemoglobin concentration higher than 10 g/dL should be considered for bleeding for antivenom production.

During transportation, animals may suffer injuries that difficult their management during bleeding procedures. Therefore, facilities for bleeding must be ideally located in the farm where horses are kept. The bleeding must be performed gently, in a quiet environment where the animal is disturbed as little as possible. However, for safety of the staff, animals must be immobilized during the procedure. In order to prevent microbial contamination of the blood, an aseptic technique must be strictly followed during washing, waxing, disinfection, and jugular venipuncture.

Automated plasmapheresis is the method by which plasma with less extent of cellular contamination is obtained (Feige et al. 2003). However, this technology is slow and expensive, thus making it largely inaccessible for most of antivenom producers in developing countries. Normally, the equipment used for blood collection consists of a closed system composed of (1) a #10 needle, (2) an 8 l bag containing an anticoagulant such as ACD, (3) a bag for plasma separation, and (4) a bag for sample collection. Alternatively, bleeding can be performed by using autoclaved glass bottles, but in this case plasma must be separated by aspiration using a mechanical pump to generate suction.

Several protocols can be used for industrial bleeding. In the case of horses, the typical protocol consists of three collections of blood performed in four consecutive days. In the first day, 8 l of blood (when using horses weighing between 450 and 550 kg) are collected and stored at 2–8 °C overnight to allow erythrocytes to sediment. In the second day, plasma is separated from erythrocytes using a press

similar to that used in human blood banks. Plasma is preserved by the addition of thimerosal, phenol, or cresol and stored at 2–8 °C until use. On the other hand, erythrocytes are suspended in saline solution and warmed to 37 °C to be transfused to the corresponding animal (self-transfusion). It has been demonstrated that equine erythrocytes can be stored for at least 35 days before transfusion without apparent damage (Niinistö et al. 2008). Self-transfusion has the objective to prevent anemia and is performed after the horses are subjected to a second extraction of 8 l blood. Procedures in the third day are similar to those described for the second day, but only 4 l of blood are collected. Blood is not collected in the fourth day, and, after the infusion of erythrocyte suspension, 19 l of Ringer's lactate solution are infused in order to hydrate and replenish serum sodium and potassium (Angulo et al. 1997).

Horses bled according to the protocol described in the previous paragraph have a yield of 10–12 l of plasma. Bleeding results in no adverse acute or chronic physiological alterations. However, horses experience a slight drop in hemoglobin, hematocrit, and serum concentrations of protein, sodium, and potassium. Therefore, after finishing the bleeding protocol described, horses must rest during the time required to recover their normal hematological status (generally 2–3 months). Then, they are ready to be submitted to a new cycle of immunization and bleeding. A meticulous veterinary surveillance should be kept during the cycle of bleeding and afterwards.

Purification of Antivenom Immunoglobulins

The current general downstream process for immunoglobulin purification starts with the primary recovery and purification of immunoglobulins by precipitation from hyperimmune plasma. In some protocols, immunoglobulins are further purified by means of chromatography or are subjected to dedicated viral inactivation or removal steps. Enzymatic digestion is included in several protocols in order to obtain immunoglobulin fragments ($F(ab')_2$ or Fab) and is performed either before or after precipitation (WHO 2010; Gutiérrez et al. 2011). These operations encompass a set of solid/liquid separations that normally are accomplished by gravity microfiltration and centrifugation. When working with precipitated immunoglobulins, care should be taken to avoid coprecipitation of impurities, entrapment of impure molecules within the precipitate, and aggregate formation when dissolving the precipitate (WHO 2010).

After purifying the active molecule of the antivenom preparation, the solution is dialyzed or diafiltered for removing low molecular mass components added in the previous stage. This operation comprises dilutions of the antivenom for washing out unwanted products. Also, buffer exchange can be done during this step in preparation for the formulation step. Then, antivenoms are usually concentrated by tangential flow ultrafiltration to reach the neutralizing potency specification. A membrane with a MWCO three to five times smaller than the target protein, usually 30 kDa in the case of IgG or $F(ab')_2$, is used for this purpose to remove water, small molecular mass proteins, and remaining contaminants while retaining immunoglobulins or their fragments. Finally, the solution is formulated before sterilization in order

to add stabilizers and preservatives and adjust the pH, osmolality, and ionic strength, which vary depending on whether the final presentation of the antivenom is freeze-dried or liquid.

Purification of Immunoglobulins from Hyperimmune Egg Yolk

Chicken egg yolk can be used as source of antibodies instead of mammalian plasma. One of the major drawbacks when isolating immunoglobulin Y (IgY) from egg yolk is the high concentration of lipids and lipoproteins, which makes difficult the extraction and purification of the immunoglobulin fraction (Ko and Ahn 2007). For this reason, most of the protocols or extraction methods of IgY share a common strategy that involves the removal or separation of lipids and lipoproteins (delipidation) from a water-soluble fraction (WSF) rich in IgY and other proteins, followed by one or more methods of purification (Kim and Nakai 1998). Among the most common purification strategies are (1) methods that include precipitation with polyethylene glycol (PEG), ammonium sulfate, sodium sulfate, or a combination of caprylic acid and ammonium sulfate and, to a less extent, alternative methods such as (2) ultrafiltration and (3) chromatography.

Purification of IgG, $F(ab')_2$, or Fab Fragments by Precipitation with Sulfate Salts

The general process for IgG antivenom production with ammonium sulfate comprises the addition of a solution of 24 % (w/v) ammonium sulfate, 0.15 M NaCl, and 0.2 % (w/v) phenol to plasma (1:1) at pH 7, followed by 1 h stirring at 22–24 °C. The mixture is left unstirred for 20 h and then microfiltered or centrifuged. The precipitate, which corresponds mainly to fibrinogen, is removed, and the filtrate is adjusted again to 24 % (w/v) ammonium sulfate with half the volume of a 47 % (w/v) ammonium sulfate solution. Again, the mixture is stirred for 1 h and left unstirred for 20 h. The mixture is then microfiltered or centrifuged, and the precipitate, mainly containing the gamma globulin fraction, is dissolved and dialyzed or diafiltered against distilled water for ammonium sulfate removal. The pH of the preparation is adjusted to pH 5.4, stirred for 1 h, and left unstirred for another 20 h. Finally, the precipitate formed is separated by microfiltration or centrifugation, and the remaining solution is formulated by adjusting the pH, NaCl, and phenol to 7.2, 0.15 M and 0.15 % (w/v), respectively (WHO 2010).

The general methodology for $F(ab')_2$ antivenom production, which is the most utilized method employed in antivenom manufacturing laboratories, involves immunoglobulin and non-globulin digestion by means of pepsin (usually 1 g pepsin/L) incubation for 30–60 min at 30–37 °C and pH 3.0–3.5. The pH is then raised to 6 to stop further digestion, and then a heat denaturation step of the non-neutralizing degraded proteins is performed (1 h at 55 °C, pH 4.0–4.5, and 12–15 % ammonium sulfate). The mixture is cooled down, and the

thermocoagulated proteins are separated by filtration or centrifugation. The remaining solution is precipitated with 23–24 % ammonium sulfate to precipitate $F(ab')_2$ fragments, which are dissolved in water and formulated (WHO 2010). Alternatively, the 12–13 % ammonium sulfate precipitation is performed before thermocoagulation. Also, ammonium sulfate precipitation can be done before enzymatic digestion in order to digest a pre-purified immunoglobulin fraction. Other modifications to this basic methodology have been described, consisting in variations of the time, temperature, and pH of the enzymatic digestion and the heat treatment (Gutiérrez et al. 2011).

Fab antivenoms are obtained by papain digestion of previously purified gamma globulins from hyperimmune sheep serum or plasma. Previous purification is performed with ammonium sulfate, sodium sulfate, or caprylic acid precipitation (WHO 2010). In a general process, serum proteins are precipitated with 18 % w/v sodium sulfate or 23 % w/v ammonium sulfate at 25 °C under stirring conditions for 15 min. The precipitate is recovered by microfiltration or centrifugation and washed with a solution of 18 % w/v sodium sulfate or 23 % w/v ammonium sulfate. The remaining precipitate is reconstituted in saline (0.15 M NaCl) at pH 7.4, and then IgG is digested at 37 °C for 18–20 h with papain to yield Fab fragments.

Purification of Whole IgG by Caprylic Acid Precipitation

Caprylic acid irreversibly precipitates plasma non-IgG proteins, essentially albumin, while antibodies are recovered in solution. This represents an advantage for antivenom production, as IgG aggregation due to precipitation is avoided. The general methodology consists of the addition of 5 % v/v caprylic acid to hyperimmune plasma (pH to 5.8 adjustment is optional) under stirring conditions (Rojas et al. 1994). The mixture is stirred vigorously for 45–60 min, and then the precipitate is separated and discarded by filtration or centrifugation. The filtrate is then diafiltered, for caprylic acid removal, and formulated. Caprylic acid can be also employed to purify pepsin-digested antivenoms (Gutiérrez et al. 2011).

Purification of Whole IgG by Aqueous Two-Phase Systems (ATPS)

Purification of immunoglobulins by aqueous two-phase systems (ATPS) is based on differential partition of plasma proteins within the two phases. For example, horse hyperimmune plasma can be fractionated in a polyethylene glycol (PEG) 3350/ potassium phosphate ATPS. This process generates two fractions based on protein partition within the two aqueous phases, so that an IgG-enriched upper phase and an albumin-enriched bottom phase are obtained. Albumin fraction can be further purified for obtaining a secondary product. IgG precipitates in the PEG-rich upper phase and, after dissolution, can be further purified with 2–4 % v/v caprylic acid precipitation.

The aqueous two-phase system is generated by the successive addition of NaCl (15 % w/v), potassium phosphate (20 % w/v), and PEG 3350 (9 % w/v).

After addition of each component, the mixture is stirred until complete dissolution and then filtered for separation of the two phases. The IgG is recovered as a precipitate in the upper phase and is dissolved in water for its consequent caprylic acid precipitation. Finally, the filtered solution is dialyzed and concentrated before formulation. When compared with the caprylic acid protocol, this methodology generates a superior antivenom in terms of purity, expressed as potency/total protein (same potency in less total protein), and yield, expressed as vials of antivenom/l (20 % more vials) (our unpublished results).

Polishing of Whole IgG by Chromatographic Techniques

Chromatographic steps are incorporated by some manufacturers to enhance the purity of antivenom preparations (WHO 2010). Traditionally, affinity chromatography and ion exchange chromatography have been the most preferred chromatographic methods for polishing antivenoms out from non-neutralizing proteins or fragments. Ion exchange chromatography is employed to bind either the active substance of the preparation or its impurities. Binding of the antibodies to the adsorbent is generally performed with cationic exchangers (carboxymethyl and sulfonic acid) at low pH (4–5), whereas protein contaminants are eluted in the breakthrough fraction. On the other hand, anionic exchangers, such as DEAE and quaternary ammonium, are used to bind contaminants at pH 7–8 (Gutiérrez et al. 2011).

Affinity chromatography is based on the immobilization of the venom used as immunogen for the antivenom manufacture on an activated Sepharose matrix or a similar affinity gel (Gutiérrez et al. 2011). In this way, antivenom immunoglobulins or their fragments that are directed specifically against venom components bind to the matrix, while other nonspecific constituents pass through. Specific immunoglobulins are then eluted generally by using low pH or changes in ionic strength. Special care should be taken in this step, as these variations may affect protein stability on the final formulation (WHO 2010). Affinity chromatography renders highly pure antivenoms, as this operation removes not only non-IgG contaminants but also IgG towards other antigens different from venom components. Nonetheless, besides considerations on its elevated cost, strict cleaning and storage procedures and monitoring of venom ligand leakages are required (WHO 2010).

Viral Safety of Snake Antivenoms

The risk of pathogen transmission is a common feature of all biotechnology products purified from human plasma, animal plasma, or cell cultures (EMEA 1996). Potential pathogenic contaminants can enter into the production process through raw material (plasma or cell cultures) or through excipients, equipment, water, air, or personnel involved in downstream processes. In the particular case of blood plasma fractionation to obtain therapeutic proteins, strict regulations have been established to prevent that the final purified products present contamination by

bacteria, fungi, protozoa, prions, or viruses. The elimination of bacteria (or their endotoxins), protozoa, and fungi is controlled by an effective system of sanitization of equipment and materials used and a sterilizing filtration process. In the case of viruses and prions, the above processes might not be entirely effective. The producers must address this potential problem through a strategic plan that involves a well-controlled screening stage of raw material, antiviral process validation, and pharmacovigilance (EMEA 1996; WHO 2010).

There are no reports of equine virus transmission to human patients by parenteral administration of antivenoms. However, the introduction of steps aimed exclusively to inactivate or remove viruses, as well as to evaluate if the fractionation steps possess this implicit function, was recommended by the WHO in 2003 (Theakston et al. 2003) and was included in the WHO guidelines for antivenom production and quality control in 2010 (WHO 2010). Among the steps suggested by WHO to reduce the viral load in snake antivenoms are (1) evaluation of the animals involved in plasma production, (2) decrease viral load input into the process, (3) evaluation of the capacity of several downstream processes to inactivate and/or remove residual viruses, (4) introduction and evaluation of strategies exclusively aimed to inactivate and/or to remove virus, and (5) establishment of a program of pharmacovigilance for the clinical management of products that allows a rapid response when a possible viral contamination event occurs.

Design of Validation Tests for Virus Removal and Virus Inactivation

Each snake antivenom producer should design an appropriate methodology to determine the virus removal/inactivation potential of its process. Ideally, the virus selected should be a relevant virus (virus that is present in animal plasma used for production). However, this is not always possible because of biohazard risks and absence of validated techniques to detect these viruses. Therefore, a more suitable option is to work with model viruses, which have similar characteristics to the relevant virus, such as structural features, nucleic acid type, size, resistance to several treatments, and others (Burnouf et al. 2004).

Once the strategies that will be tested for their ability to inactivate/remove viruses have been selected, it is necessary to implement small-scale experiments following the pattern: upstream-strategy-downstream, where upstream is the material that is to be spiked with the virus to evaluate, the strategy refers to the antiviral step that will be evaluated, and downstream is the material resultant after the application of the strategy (Burnouf et al. 2004). Both upstream and downstream materials must be evaluated for the quantity and infectivity of virus by median tissue culture infective dose ($TCID_{50}$) tests, plaque assays, or nucleic acid tests (NATs).

A robust inactivation strategy must reduce the viral infectivity by at least 4 $\log_{10}$ in the $TCID_{50}$ test. However, in addition to the reduction of the viral infectivity, there are other conditions that must be met to conclude that a strategy fulfills the condition of providing viral security (EMEA 1996). It is also important to measure

sample volumes, viral loads upstream and downstream, sample cytotoxicity, and possible interferences, in order to determine the reduction factor of each strategy.

Caprylic Acid Precipitation as Antiviral Strategy

The non-ionized form of caprylic acid (CA) is lipophilic and has the ability to penetrate and disrupt the lipid bilayer of enveloped viruses and the proteins associated with it. The result is the inactivation of the virus. This non-ionized CA form can be maintained at a minimum concentration in which it is still inactivating through varying the proportion of total CA over a range of pH between 4.5 and 8.0 (Dichtelmüler et al. 2002). CA represents an effective and low-cost strategy to inactivate enveloped viruses. However, it has the limitation of its low ability to inactivate non-enveloped viruses (Gutiérrez et al. 2011).

In several equine immunoglobulin preparations, CA has been validated as an effective antiviral strategy. Two snake antivenom producers have shown separately in their production process that CA achieved significant reduction factors in virus infectivity. At pH 5.8, CA induced more than 6 logarithmic reduction factor (LFR) for all the three model viruses assessed (Burnouf et al. 2007). In another study, the incubation of immunoglobulins with 0.02 M CA was performed at pH 4.5–4.6 for 30 min. The LRF obtained for several model enveloped viruses was in the range of 4.3–6 (Caricati et al. 2013). In both cases, non-enveloped viruses tested showed insufficient LFR to meet required specifications.

Thermal Treatments as Antiviral Strategy

Pasteurization is the most widely thermal treatment used and is defined as the application of heat at 60 °C during 10 h on purified proteins. Its performance depends on a combination of several factors: temperature, exposure time, homogeneous exposure to heat, and the use of excipients that provide thermostability to proteins (Burnouf et al. 2004). As antiviral treatment, pasteurization has the challenge of inactivate viruses without inducing damage to proteins. This could be achieved by the use of protein stabilizers. However, this could also stabilize the virus. Pasteurization is very effective on enveloped viruses. It has been suggested that the mechanism of inactivation is the lipid bilayer transformation by the heat, from solid to a liquid state, and therefore the virion becomes unstable (Kempf et al. 2007). In the case of non-enveloped viruses, some of them are very sensitive to pasteurization, whereas others are not inactivated even after many hours of heat exposure.

Low pH Treatment as Antiviral Strategy

Incubations of intermediate and final formulations at pH between 3.8 and 4.5 have been used since the 1980s in human blood products to inactivate viruses.

This incubation was originally used in IgG solutions to reduce anticomplementary activity and dimer formation. A combination of incubation at low pH (4–4.5) at temperatures of 30–37 °C and traces of pepsin seems to be the ideal conditions to inactivate several enveloped viruses (Burnouf et al. 2004). However, there are also reports of non-enveloped virus inactivation during treatment with low pH. This suggests that one possible mechanism of viral inactivation by low pH treatment is through denaturation of glycoproteins associated with either the lipid bilayer of enveloped viruses or the capsid of non-enveloped virus (Kempf et al. 2007).

Based on the experience of human plasma fractionation, some antitoxin producers have evaluated the potential of low pH treatment for viral inactivation. It was demonstrated that the incubation of horse plasma spiked with West Nile virus, at pH 3.2 for 20 min, is sufficient to inactivate it, decreasing the virus titer below the limit of detection. However, it has also been reported that the Sindbis virus is not inactivated by low pH treatment alone, thus requiring the combination of pepsin and low pH to achieve a complete inactivation (Lazar et al. 2002).

Caricati et al. (2013) also evaluated the combination of pepsin and low pH treatment in intermediate products of snake antivenom purification. They assessed the incubation of the spiked products for 60 min with pepsin (1 mg pepsin/80 mg total protein) at pH 3.1–3.2 and demonstrated the inactivation of five enveloped virus and one non-enveloped virus in 4 LRF or more. However, the inactivation of two non-enveloped viruses did not achieve 4 LRF (Caricati et al. 2013).

The antiviral potential of low pH treatment alone for viral inactivation in snake antivenoms was assessed by the incubation of whole IgG formulation at pH (4–5) during 24 h at 37 °C. This treatment inactivated herpesvirus (enveloped virus) in more than 5.5 $\log_{10}$ after 6 h of treatment. A non-enveloped virus, poliovirus, resulted less sensible to this treatment, achieving less than 4 $\log_{10}$ after 24 h (Solano et al. 2012).

Solvent/Detergent Treatment as Antiviral Strategy

Combinations of organic solvents and detergents have the capacity of disrupting the lipid bilayers of enveloped viruses. The reagents frequently used are combinations of tri-(n)-butyl phosphate (TnBP) and an anionic detergent like Triton X-100 or Tween 80. Commonly, the treatments involve incubations of plasma or downstream protein fractions with low percent of TnBP/Triton (1 %/1 %). A moderate stirring and constant temperature (30 °C or 37 °C) are also necessary to achieve best results in virus inactivation. Subsequently, both reagents should be removed because of their high toxicity. This step is performed using extractions with mineral oil or chromatography in C18 sorbents.

The only report of S/D treatment evaluation in snake antivenoms is the incubation of equine plasma with 1 % TnBP and 1 % Triton X-45 (Segura et al. 2009a). This work evaluated the impact of S/D treatment on the quality of whole IgG snake antivenom. The S/D treatment on plasma, upstream of fractionation, has a positive impact on the final product quality. In addition, the extraction with oil mixings was

successful. Although this strategy has been reported as successful for inactivating enveloped viruses, this investigation did not evaluate this issue.

Phenol Treatment as Antiviral Strategy

Phenol and cresol are preservatives routinely used in snake antivenoms as bacteriostatic reagents (WHO 2010). Recently, the antiviral effect of phenol in snake antivenoms production process was evaluated. It was demonstrated that phenol (2.5 g/L) has a high capacity of virus inactivation for enveloped and non-enveloped viruses (Caricati et al. 2013). This is a very important finding for snake antivenom producers, because phenol is the most widely used preservative in liquid preparations of snake antivenoms. Other manufacturers use cresol; since its chemical structure is similar to phenol, it is likely that it possesses a similar antiviral effect.

Stabilization of Antivenoms

Antimicrobial Preservatives

In antivenoms, the addition of preservatives is widely used to protect the formulation from microbial growth or from microorganisms, particularly bacteria and fungi, which are introduced inadvertently during or following the manufacturing process. However, antimicrobial preservatives should not be used in substitution of good manufacture practices (WHO 2010).

The most currently used antimicrobial preservatives are m-cresol and phenol. In general, phenol concentration is adjusted to 2.5 g/L, and concentration of cresols should be less than 3.5 g/L (WHO 2010). At these concentrations, these preservatives do not affect the potency of antivenoms. However, they could result in the appearance of turbidity in some formulations, caused by the formation of protein aggregates of high molecular mass (Segura et al. 2009b). Moreover, at this concentration, phenol impairs leukocyte-endothelial interactions and induces transient symptoms in mice, such as tremor and dyspnea (Zychar et al. 2008). In rats, a pro-inflammatory activity on the mesenteric microcirculation induced by phenolic compounds administered chronically has been described (Macedo et al. 2006), and phenol has been described to cause transitory hypotension in mice.

The amount of preservative added to the formulation should be the lowest concentration at which bacteriostatic effectiveness has been demonstrated. Antimicrobial effectiveness, whether inherent to the product or obtained by the addition of an antimicrobial preservative, must be demonstrated with an antimicrobial efficacy tests. Guidance for performing these tests is provided in the United States Pharmacopeia and the European Pharmacopoeia.

The elimination of preservatives from antivenom formulations is not currently feasible for some manufacturers since this may hamper the safety of the product.

However, in the future, it is expected that all antivenoms will be produced in aseptic manufacturing plants in conditions that will allow the elimination of the need of preservatives.

Osmolytes

The most common method for stabilizing liquid protein formulations is the use of soluble and nontoxic excipients. Sorbitol is the main stabilizer used to protect proteins from aggregation. However, other excipients, such as glycerol, mannitol, and glycine, have been used successfully in the stabilization of liquid protein formulations.

The thermal stability of liquid snake antivenoms has been assessed in some studies. The storage temperature and the concentration of preservative are the main factors that affect the stability of immunoglobulins, since they determine the generation of turbidity and the formation of high molecular mass protein aggregates. The use of 1 M sorbitol decreased the formation of protein precipitates in antivenoms stored at 70 ºC for 90 min, without perturbation of the antibody structure, as demonstrated by circular dichroism. Similarly, the use of 2 M sorbitol prevented the appearance of turbidity in a caprylic acid-purified equine IgG antivenom, after 1 year of storage at 37 ºC (Segura et al. 2009b). Nevertheless, the use of sorbitol (20 g/L) or mannitol (50 g/L) had no effect in stabilizing a Fab antivenom at room temperature (Al-Abdulla et al. 2003).

The mechanism involved in the stabilization of proteins by osmolytes has been described as a “preferential interaction mechanism,” which means that osmolytes are preferentially excluded from the surface of native proteins, and these are preferentially hydrated. In this mechanism, protein stabilization arises from destabilization of the denatured state by the excipients, leading to a larger free energy change (Xie and Timasheff 1997).

The stabilizing effect of osmolytes is usually concentration and protein dependent. It has been demonstrated that sugars and polyols can protect proteins from chemical reactions like oxidative degradation. The use of reducing sugars in protein stabilization should be avoided because these types of excipients have the potential to react with amino groups in proteins to form carbohydrate adducts, especially at high temperatures (Wang 1999). This “browning” pathway could affect the stability of liquid and solid antivenom formulations.

Freeze-Drying Stabilization

Currently, there are around 45 public and private laboratories that manufacture antivenoms in the world. Most of these antivenoms are commercialized as liquid formulations, which represent a drawback in remote areas of developing countries, where an adequate cold chain cannot be guaranteed. Despite the need to produce more stable and easy to distribute antivenoms, this issue has received little attention by

antivenom producers, and although some commercial formulations are freeze-dried, there is a very limited body of published literature on freeze-drying of snake antivenoms.

Freeze-drying or lyophilization is the most commonly used method for preparing solid proteins, which are physically or chemically unstable in aqueous solution. This process involves two general steps: (1) freezing of a protein solution and (2) drying of the frozen solid under vacuum.

During the freezing step, the solutes are concentrated in the regions between ice crystals, until the system increases in viscosity and is transformed into a solid amorphous system (Pikal 2004). The temperature at which this reversible transition occurs has been referred to as the glass transition temperature of the maximally concentrated freeze concentrate (Tg′). This temperature can be determined by differential scanning calorimetry (DSC), and it is a critical parameter for the development of freeze-drying formulations, including snake antivenoms.

Tg′ is characteristic of each formulation and largely determines the conditions of the drying stage. For an equine IgG formulation, without any kind of excipients, the glass transition temperature is observed as a weak transition around −13 °C (our unpublished observations). This temperature suggests that during the drying phase, the product temperature must not exceed this parameter (Schersch et al. 2010).

The drying step of a freeze-drying process is divided in two phases: primary drying, which removes the frozen water, and secondary drying, which removes the bound water. As mentioned above, the primary drying or sublimation must be conducted to a shelf temperature that brings the product to the optimum target temperature. Usually, the primary drying is the longest step of the freeze-drying process, and the optimization of this stage has a large impact on process economy and product quality. On the other hand, the objective of the secondary drying is to reduce the content of residual humidity to an optimal level for stability. For snake antivenoms, a residual humidity content of less than 3 % is usually recommended, but optimal moisture should be established for each formulation and for each manufacturer through its own stability studies (WHO 2010).

International guides provide a shelf life of 3 years for liquid antivenoms and 5 years for freeze-dried formulations (WHO 2010). However, the stability of freeze-dried antivenoms is characteristic for each formulation and depends on the stability during processing and the storage conditions. During freeze-drying, antibodies can experiment physical and chemical modifications which may cause irreversible changes that have an impact on neutralizing potency, aggregation, and reconstitution (Sarciaux et al. 1999).

The reconstitution time of freeze-dried antivenoms is relevant at the clinical setting because the time lapse between the snakebite and the beginning of therapy has an impact on the evolution of the envenomation. Reconstitution times of 30 and 90 min must be considered as inadequately prolonged, since properly lyophilized antivenoms should be reconstituted in less than 5 min.

It has been demonstrated that retention of native structure of proteins in solid state is critical for long-term storage stability. Antivenom stabilization can be achieved through the use of additives to protect immunoglobulins from freezing

and dehydration. Sorbitol, sucrose, and trehalose have been used successfully in the stabilization of several types of monoclonal antibodies (Andya et al. 2003).

In the solid state, several factors can affect the stability of the antivenoms. These include glass transition temperature, storage temperature, residual moisture, and content and type of excipients. The glass transition temperature of the solid proteins determines the storage temperature. For example, it was found that freeze-dried antibodies formulated with sucrose (Tg~59 °C) suffered significant aggregation when they were stored at 60 °C, while a formulation with trehalose (Tg~80 °C) was stable at the same conditions. These observations highlight the relevance of choosing the most adequate type of stabilizer for a particular product (Duddu and Dal Monte 1997). The effect of moisture on the stability of freeze-dried antivenoms has not been reported. Generally, increasing the moisture content of a freeze-dried antibody preparation will gradually increase the rate of degradation during storage (Wang et al. 2007), but this effect is highly complex and demands further investigation.

Quality Control of Antivenom Design

Quality control involves the set of operations and analyses performed to verify that an antivenom batch in the production line meets the product specifications. However, this term also encompasses the evaluation of the design of the manufacturing line, before the start of the routine production of antivenoms. Stability, safety, and effectiveness of antivenoms must be demonstrated at both preclinical and clinical levels, especially in the cases (1) of the development of new antivenoms, (2) when changes are performed in the immunization scheme or in the formulation, and (3) when existing antivenoms are introduced to a new geographical setting for the neutralization of either homologous or heterologous venoms (WHO 2010).

Stability Studies

Stability studies should be performed in order to establish the shelf life of the antivenom, when either a new product is developed, a change is introduced in the manufacturing process, or a new formulation for an existing is designed. These studies are intended to prove that the product remains stable and efficacious until the expiry date (WHO 2010). Antivenoms are more stable in refrigerated conditions (2–8 °C) than at room temperature, leading to the recommendation that liquid antivenom preparations have to be stored at 2–8 °C (Theakston et al. 2003).

It is recommended that manufacturers perform stability studies of their own antivenoms, in order to assess the actual stability at room temperature, and the real shelf life at 2–8 °C (WHO 2010). Regardless of whether antivenoms are prepared as liquid or freeze-dried products, real-time stability tests should be performed under the expected storage conditions of the antivenom. These tests could also be performed under worst-case storage conditions (WHO 2010) in order to obtain

data useful for actual conditions of product storage and transportation at high temperatures (EMEA 2002). Accelerated stability studies may be useful for determining whether accidental exposures to storage conditions other than those recommended (e.g., during transportation) are harmful to the product, but they are not a substitute of real-time stability data (ICH 1996; Wang et al. 2007).

Evidence should be provided on the consistency of production on at least three consecutive full-scale production batches, preferably not derived from the same bulk material. This should include information on the intermediates, final bulk, finished product, and in-process controls (EMEA 2002; WHO 2010). Samples that enter the stability study may be part of a matrix system for selection and/or by bracketing. Data from pilot-plant scale batches of drug product may be provided for regulatory purposes with a commitment to place the first three manufacturing scale batches into the long-term stability program, as long as the pilot batches are representative of the material used in preclinical or clinical studies (ICH 1996).

The analyses include validated biological, chemical, and immunological methods for quality control performed at regular preestablished time intervals, as well as methods to detect and identify impurities (ICH 1996; EMEA 2002). Essential parameters to be followed include venom neutralization potency, turbidity, and content of aggregates as a sign of purity, since these are especially prone to be altered upon storage (WHO 2010). In addition, visual appearance, detection of particulate matter, determination of pH, moisture level of dry products, sterility testing or alternatives, presence of pyrogens, and degradation of excipients (e.g., stabilizers, preservatives) have to be controlled, and the container/closure system should be carefully evaluated (ICH 1996). Each product should retain its specifications within established limits for each quality control test throughout its proposed shelf life. These limits should be derived from all available statistical information and should be supported by adequate data to demonstrate that the clinical profile of the product is not affected (ICH 1996).

Data on these parameters should be generated every 3 months during the first year of storage, every 6 months during the second year, and annually thereafter (WHO 2010). Where there is previous data indicating that product stability is not compromised, the producer is encouraged to submit a protocol which supports elimination of specific test intervals for long-term studies (ICH 1996). Any change in the protocol has to be consulted with the appropriate regulatory department of each country to confirm the proper application of stability studies prior to their clinical use approval.

Preclinical Studies of Antivenom Efficacy

The effectiveness of antivenoms is limited to the group of venoms that are antigenically related to those used in the immunization process (León et al. 2011). Therefore, evaluation of immunoreactivity towards the target venom is the first stage in the preclinical evaluation of antivenoms. Several techniques can be used to study immunoreactive profile of antivenoms (e.g., immunodiffusion, ELISA, or Western blot).

In the last years, a novel analytical platform, "antivenomics," has been introduced; it consists of the use of proteomic tools to the study of venom proteins recognized, or not recognized, by antivenoms (Calvete 2011; Gutiérrez et al. 2013).

Determination of the immunoreactivity profile allows the prediction of which venom activities could be neutralized by the antivenom. These predictions can be verified by in vitro tests such as neutralization of PLA_2, proteinase, or coagulant activities. In the cases in which the antivenom recognizes relevant components in a venom and shows neutralizing capacity in these in vitro tests, this justifies the use of animal models to test the neutralization of relevant toxicological effects. The most important effect to be analyzed in vivo is the neutralization of lethality. In addition, assays have been adapted for the evaluation of hemorrhagic, defibrinogenating, myotoxic, necrotizing, edema-forming, and neuromuscular blocking activities, depending on the toxicological profile of the venom being investigated (Gutiérrez et al. 2013). In the case of elapid venoms whose main effect is neurotoxic paralysis, neutralization of lethality is sufficient, whereas testing antivenoms against viperid venoms which induce hemorrhagic, myotoxic, defibrinogenating, and edema-forming effects demands a more elaborated set of preclinical tests (WHO 2010; Gutiérrez et al. 2013). There is an urgent need to find surrogate tests for these in vivo assays in order to reduce the number of animals used in antivenom evaluation.

In general, preclinical neutralization studies are performed by mixing and incubating a constant amount of venom with several dilutions of antivenom at particular conditions during a period of time (e.g., 30 min at 37 °C). Then, aliquots of the mixtures containing a "challenge dose" of venom are tested in groups of mice or in the corresponding in vitro tests, depending on the assay. Hemorrhagic activity is assessed by measuring the hemorrhagic halo in the skin after intradermal injection. Myotoxic activity is evaluated by quantification of the plasma activity of creatine kinase (CK), whereas defibrinogenating activity is assessed by determining whether blood clots or remains unclotted. Likewise, coagulant activity is determined in vitro by quantifying the coagulation of citrated plasma. The experimental protocols for these preclinical tests are described in WHO (2010). For the neutralization of lethality, the usual challenge dose is four or five median lethal doses (LD_{50}, i.e., the amount of venom that induces lethality in 50 % of the injected animals during the observation time). Lethality experiments are performed using either the intravenous or the intraperitoneal routes of injection (WHO 2010; Gutiérrez et al. 2013). All tests must be controlled with animal groups in which antivenom is substituted by saline solution or normal equine IgG. Neutralization capacity of antivenoms for lethal, hemorrhagic, and myotoxic activities is expressed as median effective dose (ED_{50}), which corresponds to the antivenom/venom ratio in which the activity of the venom was reduced by 50 %. In the case of defibrinogenating activity, neutralization is expressed as effective dose (ED), which corresponds to the minimum antivenom/venom ratio in which the effect was neutralized in all animals tested.

Only when an antivenom has been shown to be effective in such preclinical testing, in addition to the demonstration of its compliance with accepted parameters in chemical and physical tests, can the product be used in clinical trials for assessment of its efficacy and safety in human patients.

Clinical Studies

Well-designed and well-performed clinical studies are the only way to determine whether an antivenom is safe and effective for administration in humans and, consequently, the introduction of an antivenom in a country should be supported by such clinical evidence (WHO 2010). Performing clinical trials that fulfill international requirements is a difficult and challenging task since (1) they require large groups of patients, (2) there are variations in the clinical presentation and severity of envenomations, (3) it is difficult to identify the offending snake and thus to correlate the clinical picture and the therapeutic success with a particular snake species, (4) the costs and complexity of multicenter studies are high, and (5) the inaccessibility of areas where snakebite cases occur further complicates the performance of these studies (WHO 2010).

Clinical studies of antivenoms should be registered in the appropriate national regulatory bodies and approved by scientific and ethical committees of the corresponding health centers where the study is to be performed. They should address, at least, three specific issues: (1) the optimal initial dose of antivenom, (2) the efficacy of the antivenom for halting the main clinical manifestations of envenomation, and (3) the safety of the antivenom (i.e., incidence and severity of early and late adverse reactions). Good clinical practices have been described in detail as standard guides for clinical studies (ICH 1996; WHO 2005). In the case of antivenoms, standard phase I studies are not appropriate since they are primarily designed to detect unanticipated adverse events in healthy volunteers, and there is extensive experience on high risk of adverse events with antivenom treatment.

Dose-finding studies (phase II studies) are aimed to find the optimal initial dose of antivenom required, to establish its safety, and to give an indication of efficacy. Preclinical testing can be used to estimate the starting doses, and dose regimens can be determined using observational studies. The selection of clinical end points to assess antivenom efficacy is highly relevant. In the case of viperid snakebite envenomations, halting of bleeding and restoration of blood coagulability have been used as markers of effectiveness in clinical studies (Abubakar et al. 2010). In the case of envenomations by elapid species inducing neurotoxicity, prevention or improvement of neurotoxic manifestations constitutes a useful parameter of therapeutic success (WHO 2010). Safety is evaluated by the study of both early and late adverse reactions. Early reactions occur within 24 h of antivenom administration, and their pathogenesis involves the participation of bacterial endotoxins (pyrogenic reactions) or immunologic mechanisms (e.g., IgE-mediated reactions and non IgE-mediated reactions). These are characterized by urticaria,

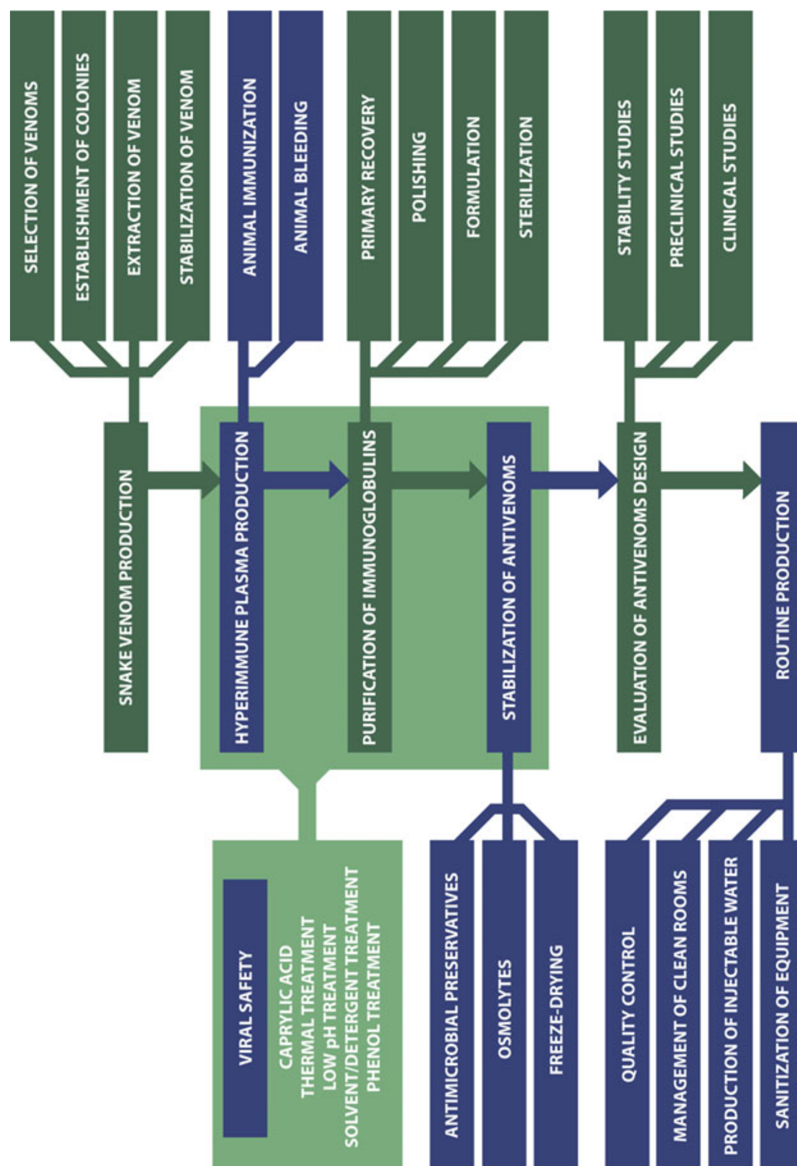

Fig. 1 Key aspects of the design, manufacture, and quality control of snake antivenoms

itching, fever, hypotension, and bronchospasm. On the other hand, late reactions, i.e., serum sickness, may occur between 5 and 24 days of antivenom administration and manifest clinically by fever, urticaria, arthralgia, lymphadenopathy, proteinuria, or neuropathy (Warrell 2010; WHO 2010).

Once an appropriate antivenom dose has been established and the safety of the antivenom has been demonstrated in these trials, large-scale phase III trials are performed. These trials usually compare the new antivenom with the existing standard antivenom treatment (see, e.g., Abubakar et al. 2010). The use of placebo controls in assessing antivenom efficacy and safety is ethically unacceptable. Phase III trials should be controlled, double-blind, and randomized studies. Predefined limits of the acceptable performance should be established based on previous experience, and the number of patients required in each trial arm must give sufficient statistical power to the study (WHO 2010). These trials might compare (1) different formulations of an antivenom, (2) antivenoms from different manufacturers with the same specificity, or (3) antivenoms obtained with different purification processes.

Post-marketing surveillance, phase IV, studies are very important in the case of antivenoms. They apply mostly after the introduction of either a new antivenom or an existing antivenom to be used in a new geographical setting. Since there are antivenoms already positioned in the market with clinical studies, post-marketing surveillance and preclinical testing against medically relevant venoms are minimal requirements for the introduction of these antivenoms to new regions (WHO 2010). Post-marketing surveillance might include aspects such as establishing national or regional systems of surveillance, creating sentinel sites to optimize scarce resources, or performing observational and historical studies. Phase IV studies are critical for detecting antivenoms of poor efficacy or safety and cases of incorrect use of these products. Clinicians, public health authorities, and manufacturers should work closely in surveillance activities. Moreover, regional and international collaborations between national health authorities and expert groups should be promoted in order to develop clinical studies with antivenoms in various regions of the world.

Conclusions

The present chapter is an overview of some of the key aspects of antivenom design, manufacture, and quality control. The diversity of activities involved in antivenom production (i.e., production of venom and hyperimmune plasma, immunoglobulin purification, virus inactivation or removal, formulation and stabilization, preclinical and clinical evaluation, and routine verification of the design compliance), summarized in Fig. 1, implies that the quality assurance of antivenom design, manufacture, and performance demands the concerted participation of multidisciplinary teams ranging from people in charge of keeping the snakes and collecting the venom to veterinary and farm personnel dedicated to the attention and bleeding of horses, to laboratory staff in charge of plasma fractionation and quality control, to clinicians

and public health authorities responsible for the clinical testing and the application of regulatory issues necessary to ensure that only antivenoms of proven efficacy and safety are used in a country.

Cross-References

► Applications of Snake Toxins in Biomedicine
► Snake Venom Proteopeptidomics: What Lies Behind the Curtain

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Erratum to: Phylogenetics of Scorpions of Medical Importance

Adolfo Borges and Matthew R. Graham

Unfortunately Fig. 1b was blurred in the first published version of this book. The labeling has been corrected and the quality has been improved, see page 84.

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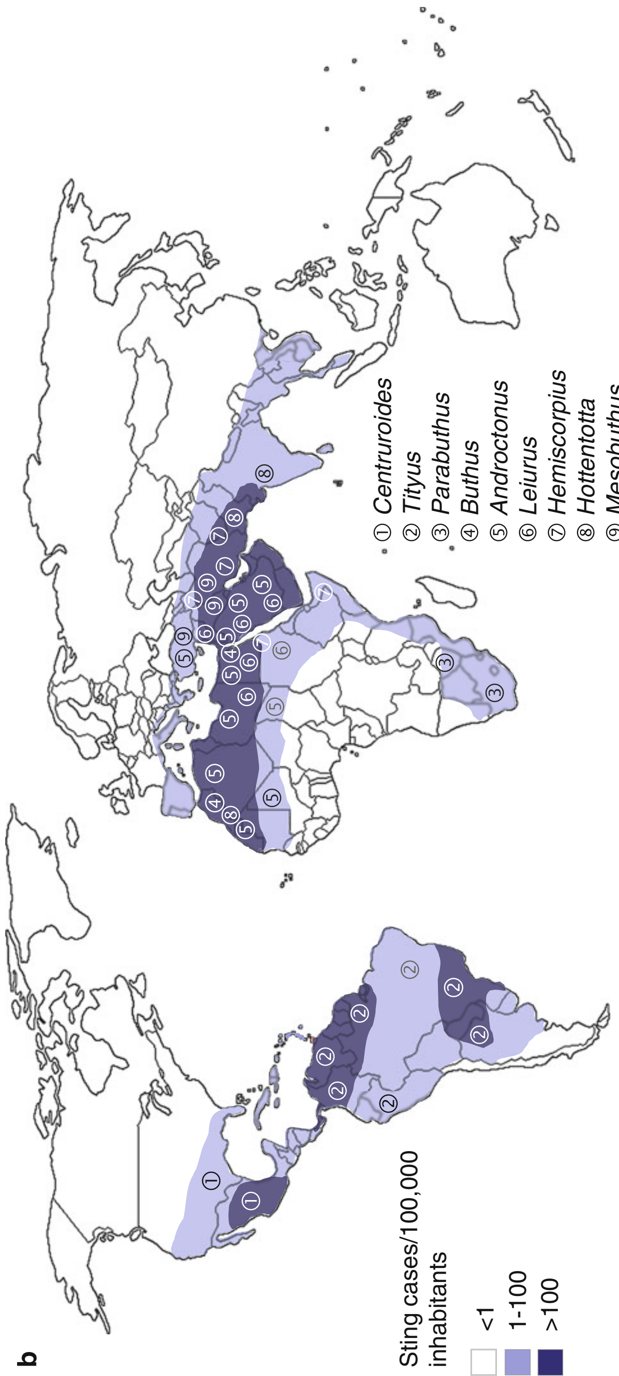

Fig. 1b

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