Chapter 1

Peptidomic Analysis of Animal Venoms

Ricardo Bastos Cunha

Additional information is available at the end of the chapter

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1. Introduction

The last two decades have witnessed a growing interest in the discovery of new chemical and pharmacological substances of animal origin. Pharmacological tests of toxins obtained from animal venoms revealed its effects on central nervous system, mainly acting on ion channels in heart, intestine, in vascular permeability, etc. Potential applications of these substances have been proposed ranging from human disease treatment to plague control of agricultural interest. In this scenario, the peptidomic analysis has played an increasingly important role.

Venomous organisms are widespread throughout the animal kingdom, comprising more than 100,000 species distributed in all major phyla. Virtually all ecosystems on Earth have venomous or poisonous organisms. Venoms represent an adaptive trait, and an example of convergent evolution. They are truly mortal cocktails, comprising unique mixtures of peptides and proteins naturally tailored by natural selection to operate in defense or attack systems, for the prey or the victim. Venoms represent an enormous reservoir of bioactive compounds able to cure diseases that do not respond to conventional therapies. Darwinian evolution of animal venoms has accumulated in nature a wide variety of biological fluids which resulted in a true combinatorial libraries of hundreds of thousands of molecules potentially active and pharmacologically useful.

Venom is a general term which refers to a variety of toxins used by certain animals that inoculate its victims through a bite, a sting or other sharp body feature. Venoms of vertebrates and invertebrates contain a molecular diversity of proteins and peptides, and other classes of substances, which together form an arsenal of highly effective agents, paralyzing and lethal, mainly used for predation and defense. We must distinguish venom from poison, which is ingested or inhaled by the victim, being absorbed by its digestive system or respiratory system. Animal venoms, in contrast, are administered directly into the lym-

phatic system, where it acts faster. Only those organisms possessing injection devices (stingers, fangs, spines, hypostomes, spurs or harpoons) which allow the active use of venom for predation can be correctly characterized as venomous. Many other animals secrete lethal substances (insects, centipedes, frogs, fish, etc.), but, as these substances are used primarily for defense purposes, these animals are termed poisonous and cannot be accurately characterized as venomous.

Venomous and poisonous invertebrates include cnidarians [1, 2] (sea anemones, jellyfish and corals), some families of mollusks [3] (mainly Conidae) and arthropods [4] (scorpions, pseudoscorpions, spiders, centipedes, ticks and hymenoptera insects, like bees, ants and wasps). Arthropods inject their venom through fangs (spiders and centipedes) or stingers (scorpions and pungent insects). The sting, in some insects, such as bees and wasps, is a modified egg-laying device, called ovipositor. Some caterpillars have venom defense glands associated to specialized bristles in the body known as urticating hairs, which can be lethal to humans (such as the moth *Lonomia*) [5]. Bees use an acidic poison (apitoxin), which causes pain in those bitten, to defend their hives and food stocks [6]. Wasps, on the other hand, use its venom to only paralyze the prey [7]. In this way, the pray can be stored alive in food chambers for the young. The ant *Polyrhachis dives* produces a poison that is applied topically on the victim for pathogen sterilization [8]. There are many other venomous and poisonous invertebrates, including jellyfish [9], bugs [10] and snails [11-13]. The sea wasp (*Chironex fleckeri*), also called box jellyfish, has about 500,000 cnidocytes in each tentacle, containing nematocysts, a harpoon-shaped mechanism that injects an extremely potent venom into the victim, which causes severe physical and psychological symptoms known as Irukandji syndrome. In many cases, this inoculation leads to death of the victim, that is why sea wasp is popularly known as "the world's most venomous creature" [14].

Loxoscelism is a condition produced by the bite of spiders from the genus *Loxosceles*, and is the only proven cause of necrosis in humans of arachnological origin [15]. *Loxosceles* spiders can be found worldwide. However, their distribution is heavily concentrated at the Western Hemisphere, particularly at the Americas, with more evidence in the tropics. In urban areas of South America, the presence of this type of spider is so evident that loxoscelism is considered a public health problem. Although *Loxosceles* bite is usually mild, it may ulcerate or cause more serious dermonecrotic injurie and even systemic reactions. This injurie is mainly due to the presence of the enzyme sphingomyelinase D in spider venom. Because the great number of diseases which mimic the loxoscelism symptoms, it is frequently misdiagnosed by physicians [16]. Although there is no known fully effective therapy for loxoscelism, research about potential antivenoms and vaccines has been exhaustive, presently also using the peptidomic approach [17], and many palliative therapies are reported in literature [15, 18].

Among vertebrates, only few reptiles (snakes and lizards) have developed the machinery for venom production [9], although some fish [9, 19], amphibians [20] and mammals (platypus for example) [21] have venom glands. The best known venomous reptiles are the snakes, which normally inject venom into their prey through hollow fangs. The snake venom is produced by mandibular glands located below the eyes and is inoculated into

the victim through tubular or channeled fangs. Snakes use their venom mainly for hunting, although they can also use it for defense. A snakebite can cause a variety of symptoms including pain, swelling, tissue damage, decreased blood pressure, seizures, bleeding, respiratory paralysis, kidney failure and coma, and may, in severe cases, cause the patient death. These symptoms will vary depending on snake specie. Snakebite is an important medical emergency in many parts of the world, particularly in tropical and subtropical regions. According to World Health Organization (WHO), the incidence of snakebite reaches 5 million per year, causing 2.5 million envenomations and 125,000 deaths [22]. About 80% of envenomation deaths worldwide are caused by snakebite, followed by scorpion bite, which causes 15% [23]. Most affected are healthy people, such as children and agricultural populations, usually in poor resources areas, away from health centers in low-income countries in Africa, Asia and Latin America. As a result, WHO declared snakebite as a health crisis and a neglected tropical disease.

In addition to snakes, there are other venomous reptiles, such as the beaded lizard (*Heloderma horridum*), the Gila monster (*Heloderma suspectum*) and other species of lizards [9]. The composition of the Komodo dragon (*Varanus komodoensis*) venom is as complex as snake venoms [24]. Because of recent studies of venom glands in squamata and analysis of nuclear protein-coding genes, a new hypothetical clade, Toxicofera, is being proposed [25]. This clade would include all poisonous Squamata: suborders Serpentes (snakes) and Iguania (iguanas, agamid lizards, chameleons, etc.) and the infraorder Anguimorpha, represented by the families Varanidae (monitor lizards), Anguidae (alligator lizards, glass lizards, etc.) and Helodermatidae (Gila monster and beaded lizard).

Venoms can also be found in some fish, such as cartilaginous (rays, sharks and chimaeras) and teleostean, including monognathus eel-like fishes, catfishes, rockfishes, waspfishes, scorpionfishes, lionfishes, goatfishes, rabbitfishes, spiderfishes, surgeonfishes, gurnards, scats, stargazers, weever, swarmfish, etc. [9, 19]. Another venomous fish, the doctor fish, also know as "reddish log sucker", is used by some spas to feed the affected and dead areas of the skin of psoriasis patients, leaving the healthy skin to grow. There are venomous mammals, including solenodons, shrews, slow loris and the male platypus [21]. There are few poisonous amphibian species [20]. Some salamanders can expel venom through a rib of a sharp edge. There are even reports of venomous dinosaurs [26]. *Sinornithosaurus*, a genus of Dromaeosauridae dinosaur with feathers, may have had a venomous bite. But this theory is still controversial. The coelophysoid dinosaur *Dilophosaurus* is commonly portrayed in popular culture as being poisonous, but this superstition is not considered likely by the scientific community.

Until recently, the work in toxinology involved prospecting highly toxic or lethal toxins in animal venoms that could explain the symptoms observed clinically. Typically, such an approach involved the isolation and structural characterization of the molecule which causes an specific adverse effect observed when a person is envenomed. However, small molecules with micro-effects that were not easily observed were neglected or poorly studied. This situation changed in recent years with the improvement in sensitivity, resolution and accuracy of mass spectrometry and other techniques used in proteomic toxinology. With the advent

of these new technologies, small peptides from animal venoms with unexplored biological activities started to be studied systematically, emerging, then, this new area of knowledge and scientific research called peptidomics. These molecules are potential candidates for new drugs or compounds with significant therapeutic actions.

2. Chemical composition and strategic importance of venoms

Over 5000 years ago, the Mesopotamians used a cane with a serpent as an emblem of Ningizzida, the god of fertility, marriage and pests. In Christianity, the serpent has always been associated with evil because of the biblical allegory of Adam and Eve. There is also a biblical story in which Moses erected a post with a brazen serpent to release his people from the plague of snakes. Throughout the development of Christianity, this symbol was transformed and the post became a tau.

But not always and not in all cultures, serpents were associated with evil. Many people believed in the cure power of serpents, often associated with its venom. Indeed, the medicinal value of animal venoms has been known since Antiquity. The medicinal use of bee venom, apitoxin, is reported in ancient Egypt and in Europe and Asia history. Charlemagne and Ivan *the Terrible*, for example, would have used apitoxin to treat common diseases. The medical uses of scorpion and snake venoms are well documented in Chinese pharmacopoeia. In an Islamic traditional tale, Muhammed is sick and, in the face of no known cure, it allows the use of snake venom as a last resource.

To Greeks and Romans, the serpent was a symbol of cure because periodically abandons its old skin and seemingly reborn, in the same way that doctors remove the disease of the body and rejuvenate the men, and also because the serpent was a symbol of concentrated attention, which was required to the curers. However, the association of serpents with cure may also be related to its venom, represented symbolically by herbs in the Greek-Roman mythology of Aesculapius, the god of medicine and cure. Called to assist Glaucus, who had been killed by lightning, Aesculapius saw a snake enter the room where he was, and killed it with his staff. Soon, a second serpent entered the room carrying herbs in its mouth, which deposited at the mouth of another dead serpent, making it back to life. Watching this scene, Aesculapius decided to put the herbs into the Glaucus mouth, who also raised from dead. Since then, Aesculapius turned the serpent your pet guardianship. His staff with a coiled serpent became the symbol of modern medicine in a large number of countries and is present even in the banner of the World Health Organization (WHO).

However, despite the healing power of animal venoms be known for a long time, the systematic investigation of venom components as natural sources for the generation of pharmaceuticals was only performed over the past decades, after a peptide that potencialize bradykinin action was isolated from the venom of the Brazilian snake *Bothrops jararaca* [27]. This led to the development, in the 1950s, of the first commercial drug based on angiotensin I converting enzyme (ACE)-inhibitor (trade name captopril®), for the treatment of arterial hypertension and heart failure [28]. Prialt® (ziconotide) is another example of synthetic drug

successfully isolated from an animal venom [11]. This is a synthetic non-opioid peptide, non-NSAID, non-local anesthetic calcium channel blocker, isolated from the secretions of the cone snail *Conus magus*. Prialt® is used for the alleviation of chronic intractable pain and is administered directly into the spinal cord, due to deep side effects or lack of efficacy when it is administered by the more common routes such as orally or intravenously.

The evolution of the venom secretion apparatus in animals is indeed an impressive biological achievement at the evolutionary point of view. Since venoms components result of biochemical and pharmacological refinement over a long period in evolutionary scale, they have been tuned for optimum activity by the natural evolution. Thus, nature has already prospected huge combinatorial libraries of potential therapeutic drugs. The biochemical evolution of proteins from salivary fluids or venom exocrine glands is remarkable, especially when one considers the highly specialized functions of these proteins and its high specificity with respect to the target molecule.

Several classes of organic molecules have been described in venoms, such as alkaloids and acylpolyamines. However, the main constituents are indeed polypeptides. Venoms of cone snail and arthropods, such as spiders, scorpions and insects, to a lesser extent, seem to be mainly peptidic, while snakes produce protein rich venoms. Snake venoms contain a variety of proteases, which hydrolyze peptide bonds of proteins, nucleases, which hydrolyze phosphodiester bonds of DNA, and neurotoxins, which disable signaling in the nervous system. The brown spider venom contains a variety of toxins, the most important of which is the tissue destruction agent sphingomyelinase D, present in the venom of all species of *Loxosceles* in different concentrations [29]. Only another spider genus (*Sicarius*) and several pathogenic bacteria are known to produce this enzyme.

Some venoms comprise several hundreds of components, which further expands its potential as a source of new medicines. Many components of venoms affect the nervous system and modulate the generation and propagation of action potentials, acting on multiple molecular sites, which include central and peripheral neurons, axons, synapses and neuromuscular junctions [30]. Many of these target receptors play important physiological roles or are associated with specific diseases. Therefore, the components of animal venoms are important biological tools for studying these receptors, and the discovery of molecules in venoms with selective activity for these receptors represents a very attractive approach to the search for new drugs. The venom components may therefore be probed for the development of new therapies for pain management [31], new anti-arrhythmic [32], anticonvulsant [33] or anxiolytic drugs [34], new antimicrobial agents [35-37] or pesticides [38, 39], etc. Even a substance that causes priapism has been isolated from the venom of a Brazilian spider [40], becoming a potential drug candidate to attend erectile dysfunction.

Another reason to study the composition of animal venoms is trying to seek more effective prophylaxis for envenomings. Doctors treat victims of venomous sting with serum, which is produced by injecting into an animal, such as sheep, horse, goat or rabbit, a small amount of specific venom. The animal's immune system responds to the target dose, producing antibodies to active molecules of the venom. These antibodies can then be isolated from the animal's blood and used in envenoming treatment in other animals, including humans.

However, this treatment can be effectively used only a limited number of times for a particular person, since that person will develop antibodies to neutralize the exogenous animal's antibodies used to produce the antiserum (antibodies antiantibodies). Even if that person does not suffer a severe allergic reaction to the antiserum, his own immune system can destroy the antiserum even before the antiserum destroys the venom toxins. Most people will never need an antiserum treatment throughout their lives. However, others, who work or live in risk areas habited by snakes or other venomous animals, such as agricultural areas for example, need that this treatment is available in public health network.

Some treatments are done not with antiserum, but herbal. *Aristolochia rugosa* and *Aristolochia trilobata*, or angelic, are medicinal plants used in Western India and in Central and South America against snake and scorpion bites [41]. Aristolochic acid, produced by those plants, inhibits inflammation induced by immune complexes and non-immunological agents (carrageenan or croton oil). It also inhibits the activity of phospholipases present in snake venoms (PLA2], forming a 1:1 complex with the enzyme. Phospholipases play an important role in the reactions cascade that lead to inflammatory response and pain. Therefore, its inhibition may reduce problems of scorpionism, snakebite and loxoscelism.

3. Proteomic and peptidomic analysis: A new approach to study venoms

Proteins are very large molecules formed by amino acids chains linked together as a polymer. Although biological systems uses only 20 amino acids to build their proteins, the different possible combinations among them is virtually infinite, resulting in tens of thousands different proteins, each one with a unique sequence, genetically defined, which determines its specific form and biological function. Furthermore, each protein may undergo a variety of post-translational modifications, which diversifies even more its form and function. Proteins are the main constituents of protoplasm of all cells. As the major components of cells metabolic pathways, proteins have vital functions in organism, such as: catalyze biochemical reactions (eg. enzymes), transmit messages (eg. neurotransmitters), regulate cellular reproduction, influence growth and development of various tissues (eg. trophic factors), carry oxygen in the blood (eg. hemoglobin), defend the body against diseases (eg. antibodies), among countless other achievements. There is no metabolic reaction in which the participation of at least one protein is dispensable.

The term "proteome" is derived from the junction of the word "PROTEin" with the word "genOME" and refers to the set of proteins expressed starting from a genome, i.e., all the proteins produced by an organism. Indeed, the word proteome is often be more related to the set of proteins expressed in a specific organ, or biological fluid, or cell, in a given state (eg. diseased cell). The proteome is therefore the complete complement of a genome, including the "makeup" that proteins receives after being synthesized, i.e., the post-translational modifications, all of them absolutely relevant for that proteins perform their biological function. The proteome of a cell or fluid varies with time and conditions under which the organism is subjected. The human body, for example, can contain more than 2

million different proteins, each one exerting a distinct role. Unlike the genome, which is relatively static, the proteome is constantly changing in response to tens of thousands of intra and extracellular environmental signals. The proteome varies with the nature of each tissue or organ, the cell development stage, the stress conditions to which the organism is subjected, the organism health state, the effects of drug treatment, etc. As such, the proteome is often defined as the proteins present in a sample (tissue, organism, cell culture, biological fluid, etc.) at a given point in time.

The term proteomics consists of comprehensive and systematic study of all proteins present in a given cell state, which was made possible by the huge development of mass spectrometry techniques over the past two decades. Proteomics and genomics run parallel and are interdependent. Genomics without proteomics is only an "alphabet soup", because it can only make inferences about their products (proteins). Moreover, proteomics requires genomics to identify the proteins expressed in a particular cell state. Briefly, genomics provides a static information of the various ways in which a cell may use its proteins, while proteomics gives a dynamic panorama of molecular diversity, showing not only which proteins are more or less expressed (or is not even expressed), but also how these proteins were modified and how these modifications affect its role in the cell theater.

Proteomic technologies can play an important role in new drugs discovery, new diagnostics and molecular medicine, because it is the connection between genes, proteins and diseases. For example, the discovery of defective proteins that cause specific diseases can help develop new drugs that either alter the shape of a defective protein or mimic its action. Most of the most popular drugs today either have proteinaceous nature or have a protein target. Through proteomics, one can create "custom" drugs, i.e., drugs specially designed for specific individuals. Such drugs are supposed to be more effective and cause fewer side effects. Another field to which proteomic studies can contribute is the biomarkers discovery for specific diseases, whose overexpression (or depletion) would indicate, quite early, the disease development. For example, serum levels of prostate specific antigen (PSA) is commonly used in the diagnosis of prostate cancer in men, which makes PSA a biomarker for cancer. Unfortunately, however, the diagnosis based on a single protein biomarker is not very reliable. Proteomics may help scientists to develop diagnostic tests that simultaneously analyze the expression of multiple proteins in order to improve the specificity and sensitivity of these tests.

Over time, new study areas with the suffix "omics" have emerged, such as metabolomics, lipidomics, carbohydratomics, degradomics etc. The term venomics did not slow to appear, and today it is defined as the study of all components (protean or not protean) of a venom. The word peptidomics has also been proposed to set the study of the peptides (instead of proteins) of a cell type or a biological fluid, such as venom. According to Ivanov and Yatskin [42]: "structure and biologic function of the entire multitude of peptides circulating in living organisms, their organs, tissues, cells and fluids comprises the scope of peptidomics". For these authors, "these two multitudes of polypeptides (proteins and peptides) play a dominant role in the functioning of any cellular system, tissue or organ. They are intimately con-

nected with each other and exist in equilibrium as an essential part of homeostasis (i.e., the normal state of any living organism and the basis of life itself)".

Peptidomic analysis has been proposed by several authors [43-55] as a way to access information relevant to clinical diagnosis and/or to monitor the patient biochemical profile during the therapy. The growing interest in peptidomic analysis led some scientists to develop new analytical technologies to improve peptidomic analysis, such as: use of capillary electrophoresis to separate the peptides [46]; use of size exclusion chromatography as a pre-fractionation step [53, 56]; new technologies and methods for sample pretreatment [57], such as methods for isolation rare amino acid-containing peptides, terminal peptides, PTM peptides and endogenous peptides, automated sample pretreatment technologies (automated sample injection and on-line digestion) [58]; development of a new target plate for MALDI-MS for one step electric transfer of analytes from a 1-dimensional electrophoresis gel directly to the target plate [59, 60]; etc. In recent years, in the face of the remarkable development on nanotechnology, many researchers have produced different kind of nanoparticles, such as mesoporous silica nanoparticles [50, 51, 61, 62] and carbon nanotubes [52, 63], for selective peptide extraction (and, hence, its enrichment) from biological fluids for therapeutic purposes (clinical diagnosis and/or novel biomarker discovery).

In the case of animal venoms, however, peptidomics is a highly interesting area for different reasons, since most of the biologically active components of pharmacological interest are of peptidic nature [64]. For example, Biass and co-workers [12] studied the venom peptidomic profile of the cone snail-hunting fish, *Conus consors*, through approaches involving different sample preparation protocols and analysis by mass spectrometry. The cone snail was quoted in the television series *Animal Planet: The Most Extreme*, because it can quickly shoot a harpoon filled with deadly toxins. The conidia (Conidae) constitute a family of several shells divided into subfamilies. It is estimated that this genus produce more than 70,000 different pharmacologically active components, most of peptidic nature, whereas interspecies variations. It is a rich library of neuropharmacology and combinatorial chemistry. Precisely for this reason, the 6th Framework Programme of the European Union funded with € 10.7 million the international project CONCO involving 20 partners and 13 countries [65], whose objective is to explore new molecules therapeutically relevant produced by venomous marine cone snails.

4. The tools to peptidomic analysis

Mass spectrometry is an analytical tool that has evolved dramatically over the past 20 years in terms of sensitivity, resolving power and versatility, and is currently one of the main tools for studying the molecular components of biological systems, including venoms. The development of techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) was essential for allowing polypeptides be analyzed by mass spectrometry. Hyphenation of separation techniques such as high performance liquid chromatography (HPLC) with mass spectrometry was also decisive for this progress. As a conse-

quence, the highly combinatorial nature of venom components and their underlying pharmacologic complexity have been progressively revealed by mass spectrometry. Currently, major challenges remain on samples complexity, lack of biological material and databases absence to peptide and protein identification based on sequence information.

Peptidomic analysis of a sample will consist of essentially four steps: (I) peptides extraction from the sample; (II) separation of these peptides — including their prior separation from other polypetidic components of the sample, i.e., proteins, defined as the protean components with molecular weight above 10 kDa —; (III) peptides detection — which is commonly performed by mass spectrometry —, (IV) and finally identification of the peptides — which usually involves fragmentation of those peptides in a tandem mass spectrometer (MS/MS).

With respect to peptide sequencing for identification purposes, the technique traditionally used is Edman degradation-based sequencing [66, 67]. But nowadays this kind of sequencing is increasingly being replaced by sequencing techniques based on mass spectrometry [68, 69]. This is due to the fact that mass spectrometry is much more rapid and sensitive than Edman sequencing and prenscinde of prior separation of the peptides, which means that peptides can be sucessfully analyzed and sequenced by mass spectrometry from a complex peptide matrix, which is impossible by Edman sequencing. This is only possible because the peptide of interest is selected (i.e., separated from others) in the first mass spectrometer. Then, this parent ion is fragmented in a collision chamber and the daughter ions are analyzed in a second mass spectrometer (MS/MS). Figure 3 gives an example of peptide *de novo* sequencing by tandem mass spectrometry. For more details about this kind of polypeptide sequencing, see reference [69].

In proteomics, the most widely used technique to separate protean components of a sample is the two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). In peptidomics, however, techniques based on liquid chromatography coupled to mass spectrometry (LC-MS) appear to be more popular, since peptides are not well resolved by electrophoresis [70]. Despite this, capillary electrophoresis has also been used successfully in peptidomic analysis, mainly to analyze biological fluids for clinical applications, such as disease diagnosis and response to therapy [46].

As an example, Valente and co-workers [71] ran a two-dimensional gel from the venom of *Bothrops insularis*, an endemic snake specie in Queimada Grande Island, Brazil. The result is shown in Figure 1. This is an example of venomics, i.e., the study of all protean components of a venom. Using the proteomic approach, the authors detected 494 spots in the gel using an image analysis software, from which 69 proteins were identified by current identification techniques, using mass spectrometry and heavy bioinformatics to interpret the mass spectra and also to make a comparative search of protein sequences deposited in databases. The identified proteins include metalloproteinases, serine proteinases, phospholipases A2, lectins, growth factors, L-amino acid oxidases, the developmental protein G10, a disintegrin, a nuclear protein of the BUD31 family, and putative novel bradykinin-potentiating peptides. In the same study, the authors also performed a peptidomic analysis of the venom, by direct analysis of the crude venom by MALDI-TOF-TOF and LC-ESI-Q-TOF. Many new peptides were partially or completely sequenced by both MALDI-MS/MS and LC-ESI-MS/MS. Using

the proteomic approach associated with peptidomic analysis, the authors could speculate about the existence of posttranslational modifications and a proteolytic processing of precursor molecules which could lead to diverse multifunctional proteins.

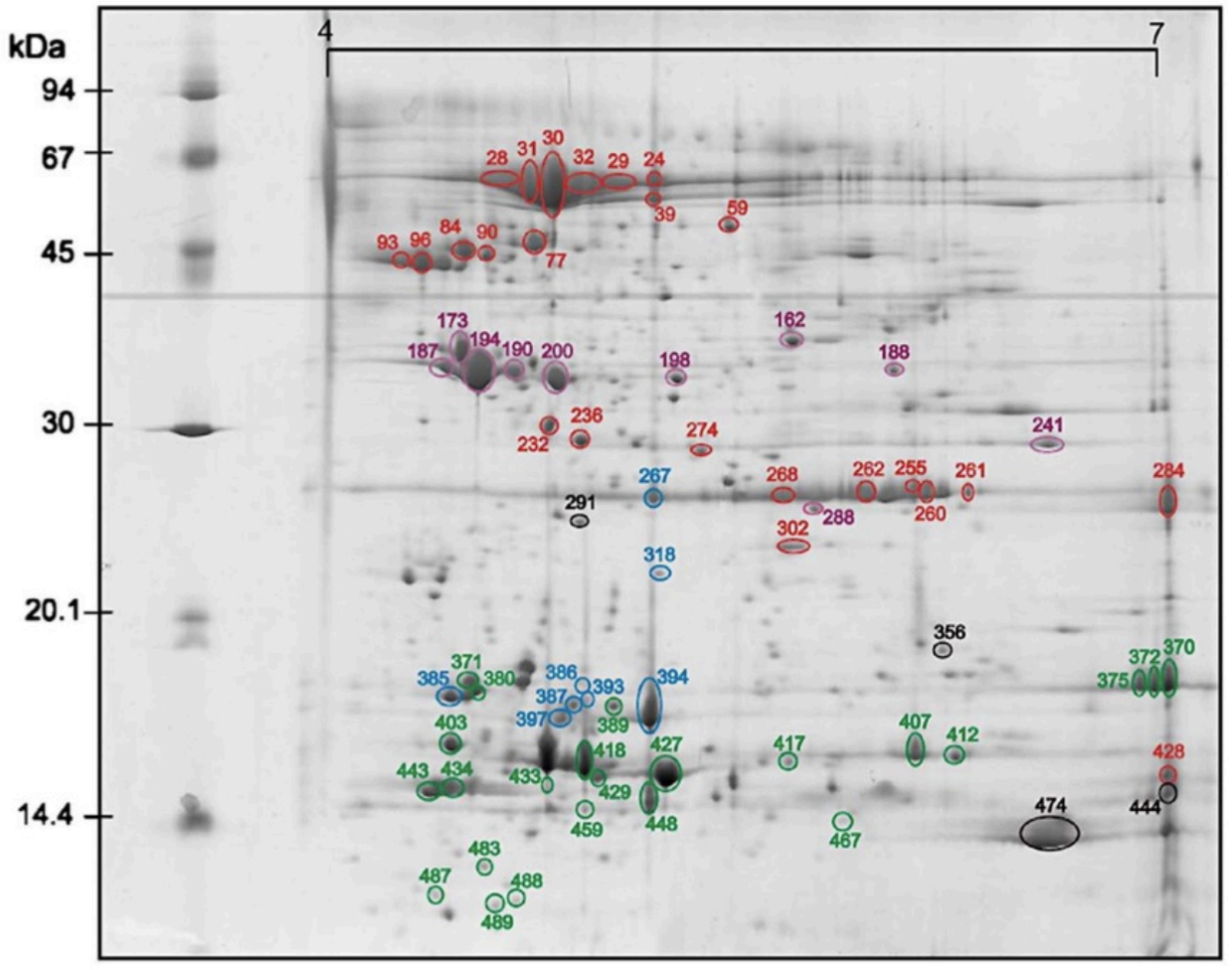

Figure 1. Proteomic profile of *Bothrops insularis* venom: 2D-PAGE reference map (copyed from reference [71]).

Liao and co-workers [72] also applied proteomic and peptidomic approaches together to analyze the venom of *Chilobrachys jingzhao* (a type of tarantula; one of the most venomous spiders in southern China). They developed a protocol which consists in run a gel filtration of the crude venom and then divide the fractions in two parts. The fraction containing protean components with molecular mass above 10 kDa they underwent proteomic analysis, which consisted of 2-DE, in gel trypsin digestion, MALDI-TOF-TOF and ESI-Q-TOF analysis of the spots, protein identification by PMF, *de novo* sequencing of the peptides, and protein identification by MS BLAST sequence similarity search. The fraction containing protein components with molecular weight below 10 kDa was used for peptidomic analysis, consisting in separation of the peptides by ion-exchange HPLC followed by reverse phase HPLC, MALDI-TOF analysis of the chromatographic fractions, Edman peptide sequencing, and peptide identification by MS BLAST. The authors reported that peptides were the predominant com-

ponents [69%) of the dry crude venom, while proteins accounted only for 6%. Nonprotean components (low MW inorganic and organic molecules, such as polyamines, salts, free acids, glucose, etc.) complete the remaining 25% of the crude venom.

Another good example of peptidomic analysis was presented by Rates and co-workers [73], who studied the *Tityus serrulatus* (a specie of scorpion whose venom has been most extensively studied) venom peptide diversity. In this work, the authors fracionated the venom by gel filtration followed by reverse phase chromatography of each fraction obtained in the first separation. The results are shown in Figure 2. Then, the chromatographic fractions were analyzed by MALDI-TOF-TOF. The peptides were sequenced using *de novo* methodology (Figure 3) and the sequences obtained were compared with protein databases in sequence similarity searches. The authors also reported the finding of novel peptides without sequence similarities to other known molecules.

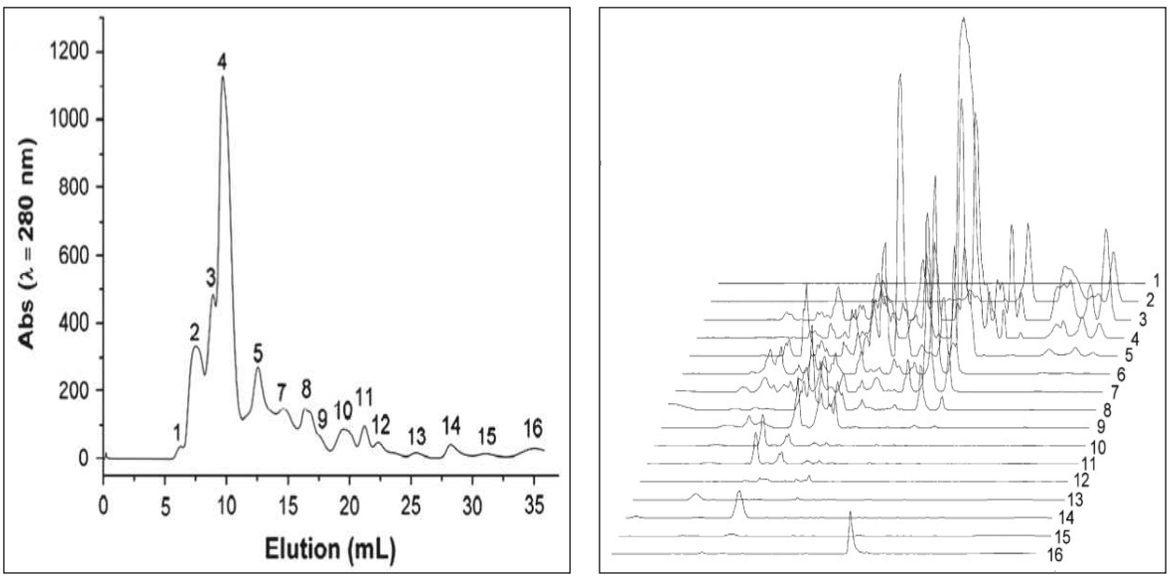

Figure 2. *Tityus serrulatus* venom fractionation through gel filtration (A) and re-chromatography of each fraction by reverse phase chromatography (B) (copyed from reference [73]).

One of the biggest difficulties currently encountered by researchers working with peptidomic analysis of animal venoms is that organisms with unsequenced genomes, including venomous animals, still represent the overwhelming majority of species in the biosphere. Fortunately, Andrej Shevchenko, from Max Planck Institute of Molecular Cell Biology and Genetics, at Dresden, Germany, paved the way for homology-driven proteomic approaches to explore proteomes of organisms with unsequenced genomes [74-76]. Through this new methodology, the search against sequences databases is made not by the exact sequence, but by sequence similarity to other protein sequences deposited in the database. This new approach does not fully solve the problem, but allows peptides to be positively identified in peptidomic experiments through cross-species identification. Wang and colleagues [77] developed an alternative strategy to circumvent the problem of absence of

systematic online database information, and used this technique to analyze the peptidome of amphibian skin secretions. Although amphibian skin secretion is not exactly a venom, it is still a biological model also very promising for the search of new pharmacologically active substances. First, the authors deduced all of putative bioactive peptide sequences by shotgun cloning the cDNAs encoding peptide precursors. Then, they separated the entire peptidome by UPLC/MS/MS, and confirmed those sequences deduced before by *de novo* MS/MS sequencing.

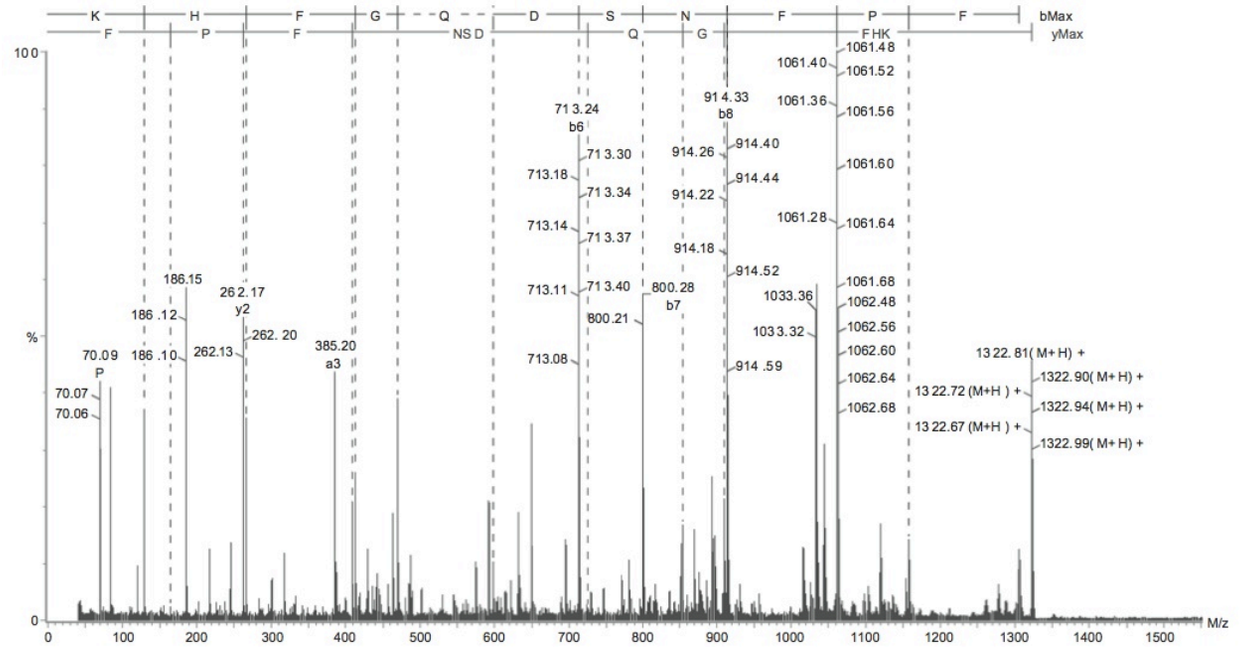

Figure 3. MS/MS spectra interpretation (*de novo* sequencing) for peptide NH_2-FPFNSD(K/Q)GFH(K/Q)-CO_2H (copyed from reference [73]). The K/Q denotes a doubt about the possibility of being lysine or glutamine, as these two amino acids are isobaric. However, as trypsin cleaves on C-terminal sides of arginines and lysines, it is likely that the middle amino acid is glutamine and the last one is lysine.

5. Concluding remarks

Animal venoms are true "cocktails" of substances normally harmful, but that can be explored with intelligence for medical use. Many authors even use the word "cornucopia" to define a venom. The cornucopia — junction of the Latin "*cornu*" (horn) with "*copiae*" (strength) -, also called "horn of plenty", is a symbol of nourishment and abundance in classical mythology, usually represented by a large horn-shaped container overflowing with products such as flowers, dried fruit, other foodstuffs, and other types of wealthiness. Nowadays it is particularly associated with the Thanksgiving holiday. In toxinology, cornucopia represents the chemical wealth of animal venoms, where one can find thousands of such substances with interesting biological effects that can be explored as candidates for future pharmaceutical products.

Much (perhaps the largest) of this pool of substances is of peptidic nature, i.e., polypeptides with molecular weight below 10 kDa. These are biologically active peptides with diverse functions, ranging from heart hypotensors to erectile dysfunction controllers. Thus, the peptidomic analysis of animal venoms is an emerging and promising area of science, and can be considered a frontier area as it includes researchers from toxinology, proteomics, pharmacology, therapeutics, drug discovery, peptide chemistry, analytical chemistry, etc.

In this chapter we tried to show the importance of animal venoms for molecular toxinology and its potential use for biomedical applications. We also sought to demonstrate the recent advent and rapid growth of peptidomic analysis as the main tool to explore the molecular features of these venoms, not only to produce more efficient antisera against venomous bites but also and mainly to characterize the components of peptide nature in search for new products of pharmacological interest. Although this new science is still in its early stages of development, it is already very mature. This is a science field that has enough potential to grow and provide creative solutions to problems that affect human health. Hopefully more and more researchers become interested on this topic. Medicine has much to gain from it.

Author details

Ricardo Bastos Cunha

Bioanalytical Chemistry Laboratory, Division of Analytical Chemistry, Institute of Chemistry, University of Brasília, Brazil

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