CHAPTER II

REVIEW OF LITERATURE

2.1 Proteomic strategies to unveil snake venom composition

Snake envenomation has been one of the major health issues in most of the tropical and sub-tropical Southeast Asian countries [1]. The major intriguing challenge in the treatment of snake envenoming is to understand the complex cocktail of snake venoms and development of effective antivenom to reverse the pathophysiological conditions inflicted by pharmacologically active venom components in victim [2,3]. Previously, the lack of knowledge and technologies for venom de-complexation and identification of non-enzymatic components were the major stringent barriers in the way. However, the introduction of mass spectrometry to characterize snake venoms in the early 21st century brought a revolution in unraveling new paths to address the fundamental questions pertaining to venom complexity [3-10]. A proteomic investigation by Fox and his co-workers in 2002 analyzed the venoms of *Dispholidus typus*, *Crotalus atrox* and *Bothrops jararaca* using 2D SDS-PAGE followed by mass spectrometry identification of the proteins spots in 2D PAGE that provided fascinating insights into the venom composition of these snake species [11]. In the same year, by a combination of RP-HPLC, LC/MS mass fingerprinting, and Edman sequencing, Fry and his co-workers characterized the venom of *Acanthophis* (Death Adder) [4]. Since then, mass spectrometry coupled with robust database search algorithms have become indispensable in the field of snake venom proteomics has that led to the understanding of the complex organization as well as the pathophysiological role of snake venom components [12]. Further, a comprehensive knowledge of venom composition as well as ontogenetic, individual, and geographical venom variability is imperative for selection of venom samples for the development of improved antivenoms.

Essentially, for investigating snake venom proteomes, deconstruction of complex venom mixture has been done based on individual de-complexation or a combination of the following three strategies [7]:

A. 1D or 2D gel electrophoresis (gel based)

B. Liquid chromatography (LC based)

C. Combined liquid chromatography and gel electrophoresis

2.1.1 Gel-based proteomic approach

With the advent of 2D SDS-PAGE during the late 1980s toxinologists began to visually appreciate venom complexity. One of its first applications to visualize snake venom proteomes was reported in 1998 that investigated the venoms of a sea snake, *Laticauda colubrina* and a terrestrial snake, *Vipera russelli* [13]. In this study individual spots containing venom proteins from the 2D gel were excised, digested with trypsin and subjected to tandem mass spectrometric analysis. Notable advantages of the methodology included an elaborate venom de-complexation, and information on isoelectric point and apparent molecular weights of the proteins. However, smaller peptides (<2 to 3 kDa), which can be one of the abundant venom components with relevant bioactivities, were lost from the gel hence remain unidentified [7]. Further, limited dynamic range, difficulty in handling of hydrophobic proteins, and detection of proteins with extreme pI and molecular weights are also some of the major drawbacks of this approach. In addition, the presence of more than one protein in a single spot of the 2D SDS-PAGE gel can render quantification ambiguous [7].

2.1.2 LC-based proteomic approach

LC based proteomic approach, also known as shotgun proteomics is based on chromatographic separation of venom components followed by tandem mass spectrometry (MS/MS) analysis of the resulting fractions. In this method, venom fractions are subjected to trypsin digestion, followed by separation of the tryptic peptides by reversed-phase high-performance liquid chromatography (RP-HPLC) connected in line with a mass spectrometer for MS/MS analysis [7]. MS/MS analysis operates in a data-dependent acquisition (DDA) mode; the first scan is a survey scan (full MS scan) where the precursor ions are isolated and subsequently activated. The obtained fragments are then analyzed in a second stage (data-dependent MS/MS scan) involving collision-induced dissociation (CID) [14,15]. However, in many sample types, including venoms, the complexity and dynamic range of compounds can be very large which poses challenges for the traditional data-dependent workflows, requiring very high-speed MS/MS acquisition to deeply interrogate the sample. To overcome these constraints, data-independent acquisition (DIA) strategies have been used to increase the reproducibility and comprehensiveness of data collection [15]. In DIA mode, an expanded mass isolation window is stepped across a mass range covering the mass-to-charge distribution of peptides, and all ions present at a given time are activated

and dissociated without selection. In this case, instead of the serial CID of peptide ions, parallel CID of ion mixtures occurs. A large mass range can be interrogated in such an LC-MS time frame because of the larger mass steps. To assign subsequent fragments to correct precursors, data are continuously acquired by alternating between high (precursor filtering) and low (dissociation and fragment filtering) voltage potentials [14,15].

2.1.3 Combined liquid-chromatographic and gel electrophoresis approach

A workflow combining a first dimension LC separation followed by one-dimensional electrophoresis (1D SDS-PAGE) as the second dimension was introduced by Calvete and his colleagues who referred to it as 'snake venomics' [16,17]. In this approach, initial venom de-complexation is achieved by RP-HPLC separation of venom sample (typically 0.5 to 2 mg) on a C_{18} column of analytical scale. Thereafter, resolved fractions are manually collected, and further separated by 1D SDS-PAGE. The protein bands are excised from the gel, in-gel digested with trypsin, and then submitted to MS/MS analysis for protein identification.

2.2 Quantification of venom toxins

2.2.1 Label-free quantification approach

Although shotgun proteomic approach for deciphering venom proteomes results into an extensive cataloging of snake venom components; however, quantitative estimation of protein abundances can be complicated. With the significant development of non-gel based quantitative proteomics approach and incorporation of isotope-labeled venom toxin peptides as internal standards has made it more easy and accurate. However, the labeling-based shotgun approach for toxin quantification has potential limitations which include increased time and complexity of sample preparation, requirement of higher sample concentration, high cost of reagents, incomplete labeling, requirement of specific quantification software [18]. Therefore, to overcome these shortcomings and achieve faster, cleaner and simpler quantification results, more interest has shifted towards label-free shotgun proteomic quantification strategies [18-20].

Label-free approach for quantification of detected proteins relies on two measurements - peptide peak area or intensity (MS1) and spectral count (MS2) [18]. For relative quantification of proteins by MS1 approach, the peptides are ionized by

electrospray ionization (ESI), and ions with a particular m/z (mass/charge) ratio is detected and recorded with a particular intensity and definite retention time [18]. However, this approach encounters some practical constraints with complex biological samples (snake venoms), such as variable peak intensities of the same sample from run to run, and any experimental and chromatographic shift resulting in inaccurate quantification. Henceforth, several computer algorithms are developed in order to solve these ambiguities and automatically compare the peak intensity data between LC-MS runs comprehensively [18]. In contrast to the chromatographic peak intensity approach, no specific tools or algorithms have been developed for MS2 based spectral counting method due to its ease of implementation. However, normalization of spectral counts by protein length or number of identified peptides and appropriate statistical tests are necessary for accurate and reliable quantification of relative toxin abundances in venom proteomes [5,18,20]. Although the MS-based label-free quantification strategies have become popular due to the relative ease of experimentation and the small amount of sample needed [3,8,21-23], they are limited by database dependency.

2.2.2 Quantification by the area under RP-HPLC curve (AUC)

In this strategy, peak areas under the RP-HPLC chromatogram combined with SDS-PAGE band intensity are used as a surrogate measure for calculating toxin abundances. In this approach, protein elution is monitored at 215 nm to measure the peptide bond absorption [6,7,17]. Comparatively, this approach is slow and requires significant manual work, especially in the collection and subsequent processing of chromatographic fractions [7]. In addition, the absorbance values (at 215 nm) may be influenced by the side chain composition of the toxins, where the aromatic side chains (present in tyrosine, tryptophan, phenylalanine, and histidine) contribute significantly and thus can be biased towards the composition of different proteins eluted in the RP-HPLC peaks [24]. Furthermore, protein components that are present in trace amounts or venom glycoproteins are generally more likely to be overlooked due to traditional staining limitations. In addition, the high molecular weight components are comparatively difficult to elute from the C_{18} column and thereby can go undetected [7].

2.3 Immuno profiling of venom antigens

2.3.1 Enzyme-linked immunosorbent assay (ELISA) and Western blot

An important area within snake venom research deals with the development, preclinical testing, and clinical monitoring of commercial antivenoms used for the treatment of human envenomation [2,7]. Western blot and ELISA are the most popular techniques for assessing the immunoreactivity of antibodies against commercial antivenoms [3,23,25-27]. However, the immunochemical detection of blotted proteins provides a Yes/No response, for example, whether or not a given venom protein is recognized by the antivenom. It is essentially a non-quantitative technique [28]. Further, proteins are denatured to an unknown extent when solubilized by boiling in SDS sample buffer. This treatment may result in loss of conformational epitopes, and artifactual recognition of non-native epitopes. On the other hand, although antibody binding levels are quantified in ELISA-based protocols, this technique does not identify binding and non-binding molecules present in a mixture, thus allowing only the analysis of the overall degree of immunoreactive between venom and antivenom.

2.3.2 Antivenomics

Recently, the introduction of proteomic analysis has opened new opportunities to deepen our knowledge on the detailed immuno-recognition of venom components by antivenoms, an area that has been referred to as 'antivenomics' [7]. Antivenomic analyses can reveal which venom proteins are strongly, poorly, or even not immuno-recognized by given antivenom, providing valuable knowledge on the relative immunogenicity of venom components towards commercial antivenom. The original antivenomics protocol [6,29,30] was based on the immuno precipitation of antigen-antibody complexes formed by mixing of venom and antivenom in fluid-phase. Venom antigens are depleted from the supernatant if recognized by antibodies, and the RP-HPLC profile of the supernatant can then be compared to that of a control venom sample in order to assess the degree of immunodepletion of each peak.

A second generation antivenomics protocol was developed switching from a fluid-phase immuno precipitation to a solid-phase interaction provided by immunoaffinity chromatography [7,28]. Antivenom is covalently immobilized onto the beads of an affinity matrix, which is then used to separate bound from unbound venom components. The antivenom-bound or 'immunocaptured' venom fraction is eluted by a

change in pH, and then both fractions, as well as non-venom specific IgG and matrix controls, are analyzed by RP-HPLC to compare their profiles and quantify the degree of immuno-recognition of each venom component [23,28].

2.3.3 Neutralization of enzyme activities and pharmacological properties of venom

Another *in vitro* method for testing the efficacy of PAV is the determination of neutralization potency of commercial antivenom against the wide array of enzymatic activities and pharmacological properties exhibited by venom. In this method, venom and antivenom at different ratios are incubated at 37 ˚C followed by assay of enzymatic activities and pharmacological properties of the venom-antivenom mixture which is compared to the same activities of only venom (control) [3,31-36]. Several proteomic studies from our laboratory tested the neutralization potency of commercial antivenom against enzymatic activities and pharmacological properties of venom and/or its components and established a good correlation between enzyme function and the pathophysiology of snake envenomation [3,23].

2.4 Russell's Viper venom composition - biochemical and proteomic analyses

2.4.1 A comparative analysis of RVV from different geographical regions of India

Variation in venom composition is a ubiquitous phenomenon and it is not surprising that it has been well evident in RVV as well [37,38]. One of the pioneer studies conducted by Jayanthi and Gowda [37] provided the first experimental evidence of variation in RVV composition owing to locale of RV in India. They analyzed RVV samples from V. P. Chest Institute, Delhi (northern India, NI), Haffkine Institute, Bombay (western India, WI), and Irula Snake Catcher's Industrial Cooperative Society Ltd., Chennai (southern India, SI) by SDS-PAGE analysis and cation-exchange chromatography. In addition, some of the biochemical properties such as PLA_2, protease, trypsin, and arginine esterase activities and toxicity of the above RVV samples were also assayed. They reported significant differences in SDS-PAGE banding patterns among the three RVV samples, particularly the SI RVV samples lacked three protein bands corresponding to molecular mass of 66, 39, and 9 kDa. Further, variation in venom composition was also evident from the number of peaks resolved and percent protein recovered from the column chromatography of these venom samples. In addition, these RVV samples exhibited variable toxicities towards experimental mice models [37]. LD_{50} values for the RVV samples from NI (8.0 and 9.0 mg/kg) and WI

(10.0 mg/kg) were twice as high as that of the RVV samples from SI (4.1 and 4.6 mg/kg). Further, acidic phospholipases were found to be absent in SI RVV, while it contained unusual amounts of basic proteins with phospholipase A_2 activity. It exhibited higher lethal potency, albeit lower proteolytic and trypsin inhibitory activities [37].

2.4.2 Biochemical and pharmacological characterization of eastern India (EI) RVV

Subsequently, some biochemical properties and pathological effects of RVV and its gel filtration fractions from Burdwan district of West Bengal, eastern India (EI) was presented in a study by Mukherjee and his colleagues [38]. They reported strong trypsin inhibitory, phospholipase A_2 and pro-coagulant activities in addition to moderate AMPase and ATPase activities of EI RVV. The study also conducted clinical and epidemiological survey on RV bite victims admitted to Burdwan Medical College and Hospital, West Bengal, India over 10 years and individual patients from 1992 to 1996 for closely examining the clinical manifestation of RV envenomation. High incidence of intravascular hemolysis in addition to hemostasis, haemoptysis, and hematuria were reported as the most prominent features of RV envenomation from EI [38].

Although these studies were instrumental in understanding the RVV complexity and compositional variation, however, information on the non-enzymatic sub-proteome of this venom was yet to be encompassed.

2.4.3 Proteomic characterization of RVV from different geographical locations of the Indian sub-continent and Myanmar

A schematic diagram of the proteomic workflows employed for characterization of the RVV proteomes from different geographical locales of the Indian sub-continent and Myanmar is shown in Fig. 2.1.

2.4.3.1 Myanmar RVV

The first RVV proteome to be deciphered was that of *D. r. siamensis*, the Siamese RV which is distributed throughout Myanmar, Thailand, Cambodia, China, Taiwan, and Indonesia [39]. The venom proteome was analysed by 2D SDS-PAGE followed by MS/MS analysis of the protein spots against all entries of NCBInr database that identified six protein families *viz.* serine proteinases (SVSP), metalloproteinases (SVMP), PLA_2, L-amino acid oxidases (LAAO), vascular endothelial growth factors (VEGF), and C-type lectin-like proteins (snaclec). The venom toxin composition was

correlated with the clinical manifestation and pathological effects of RV bite such as coagulopathy, edema, hypotensive, necrotic and tissue-damaging effects and neurotoxic effects. The predominant venom components in Myanmar RVV are proteinases that are capable of activating blood coagulation factors and promoting rapid clotting of the blood, and neurotoxic PLA_2s. Further, the study reported a lower level of toxin diversity in the proteome of *D. r. siamensis* than the proteomes of other viperid snakes. In comparison to the venoms of *Vipera ammodytes ammodytes* and *V. a. meridionalis*, the venom from *D. r. siamensis* showed quantitative differences in the proteolytic, PLA_2, LAAO and alkaline phosphatase activities [39].

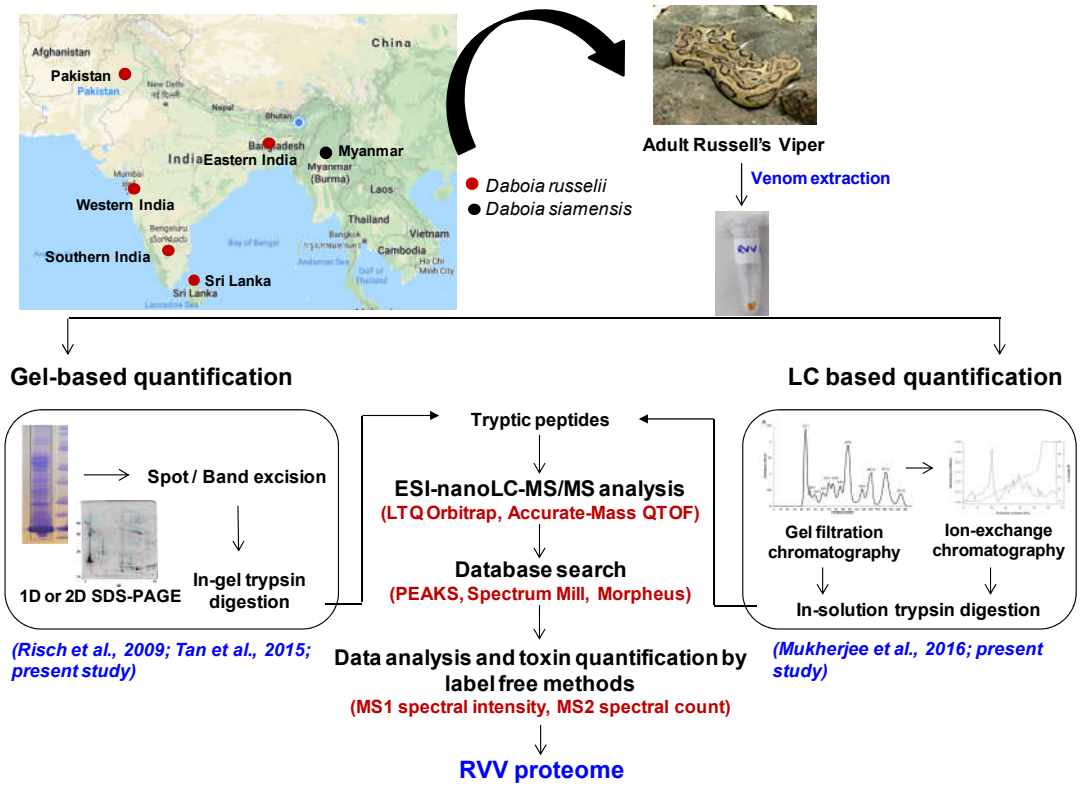

Fig. 2.1. A schematic representation of the proteomic workflow to analyze RVV samples from different regions of the Indian sub-continent. Map of India is adapted from Map data© 2018 Google. Retrieved from https://www.google.com/maps/place /India/@28.1445493,73.5173445.

2.4.3.2 Southern Indian RVV

The proteomic investigation on Indian RV (*D. russelii*) venom started with the works of Sharma et al. [40] in which SI RVV was de-complexed by gel filtration

chromatography and the proteins present in the fractions were identified by in-solution trypsin digestion and tandem mass spectrometry analysis. By this approach, Sharma et al. [40] identified 63 different proteins belonging to 12 families in SI RVV. Apart from the protein families reported in *D. r. siamensis*, the study additionally identified cysteine-rich secretory proteins (CRISP), Kunitz-type serine protease inhibitor (KSPI), disintegrin (Dis), nucleotidase (NT), phosphodiesterase (PDE), and nerve growth factor (NGF) in this RVV sample [40].

Although the above mentioned studies explored the venom proteomes of Siamese and Indian Russell's Vipers, they lacked the necessary information on the quantitative distribution of different proteins/toxins in these venoms. While the proteome composition of SI RVV was merely expressed in terms of number of proteins identified (qualitative analysis) by LC-MS/MS analysis [40], the relative abundance of different protein families in Siamese RVV was not represented [39]. The quantitative information on venom proteome is very crucial because it dictates the differences in severity of pathogenesis and clinical symptoms following snake envenomation. Further, these quantitative data on RVV proteomes across the country can even be critical in the development of effective antivenoms.

2.4.3.3 Sri Lankan RVV

The first proteomic investigation of *D. russelii* venom that included a quantitative analysis of the identified protein families was led by Tan and his co-workers [8]. They studied the proteome composition of Sri Lankan RVV by 1D SDS-PAGE followed by LC-MS/MS analysis of the gel sections against Serpentes entries of NCBI and a private in-house protein database on the venom-gland transcriptome of an adult RV captured in Sri Lanka. Relative abundances of the RVV toxins were calculated by normalized MS1 based spectral intensity coupled to SDS-PAGE band intensities. The study identified 41 different venom proteins distributed in 11 protein families among which PLA_2 (35.0%), snaclec (22.4%), SVSP (16.0%), and SVMP (6.9%) were the major classes (Fig. 2.2). The venom proteome was reported to be consistent with the tested enzymatic and toxic activities of the venom, and with the clinical manifestations observed in RV-envenomed patients of Sri Lanka. In addition, the unique neurotoxic symptom exhibited by RV-envenomed patients from Sri Lanka was well correlated with the abundance of neurotoxic and basic PLA_2 isoenzymes (>30%) in its venom [8].

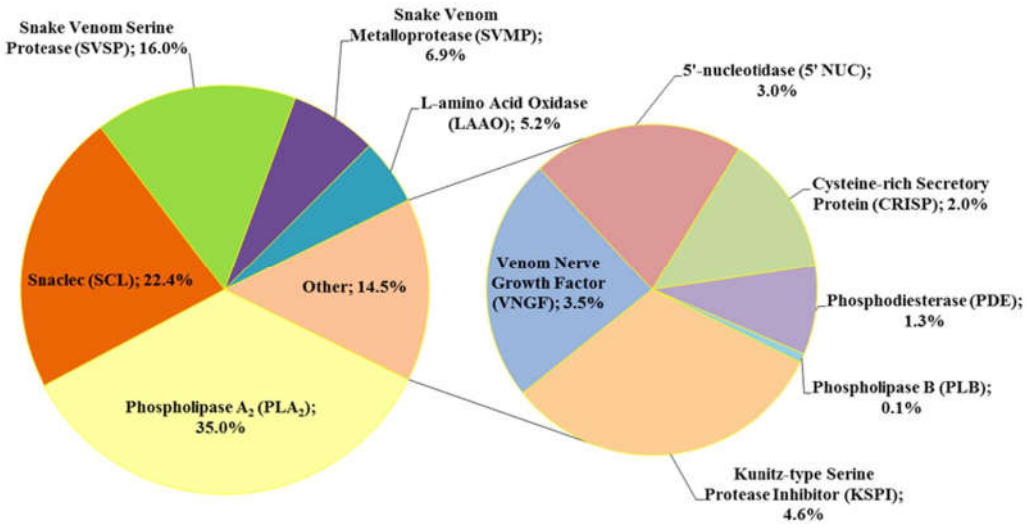

Fig. 2.2. The venom proteome of Sri Lankan *Daboia russelii* (adapted from Tan et al. [8]).

2.4.3.4 Pakistan RVV (captive specimens)

Subsequently, the venom proteome of captive specimens of Pakistani RV was analyzed by gel filtration chromatography followed by ESI-LS/MS/MS analysis of the venom fractions against Viperidae entries of NCBI database that led to the identification of 75 proteins distributed among 14 distinct snake venom protein families (Fig. 2.3) [3]. The protein families were quantified using MS2 based spectral count method and the relative abundances were shown to be consistent with the percent intensities of the SDS-PAGE bands. The major protein families identified in this study were PLA_2 (32.8%), KSPI (28.4%), and SVMP (21.8%) while the rest of the families accounted for minor percentages (6.5–0.2%). The study reported the occurrence of aminotransferase (AMT), and hyaluronidase (Hya) in RVV for the first time. The data presented in this study also provided strong evidence of protein-protein interactions among RVV proteins. Further, the proteome composition of Pakistan RVV was well correlated with the tested biochemical activities and pharmacological properties of the venom fractions. In addition, Pakistan RVV was found to cross-react with commercial Indian PAV (from Bharat Serums and Vaccines Ltd.) as well as MAV (from VINS BioProducts Ltd.); nonetheless, immunological cross-reactivity determined by ELISA and neutralization of pro-coagulant/anticoagulant activity of RVV and its fractions by MAV was found to be superior to that of PAV. However, due to the lack of published clinical data, a

correlation between the venom composition and pathophysiology of RV envenomation in Pakistan could not be drawn [3].

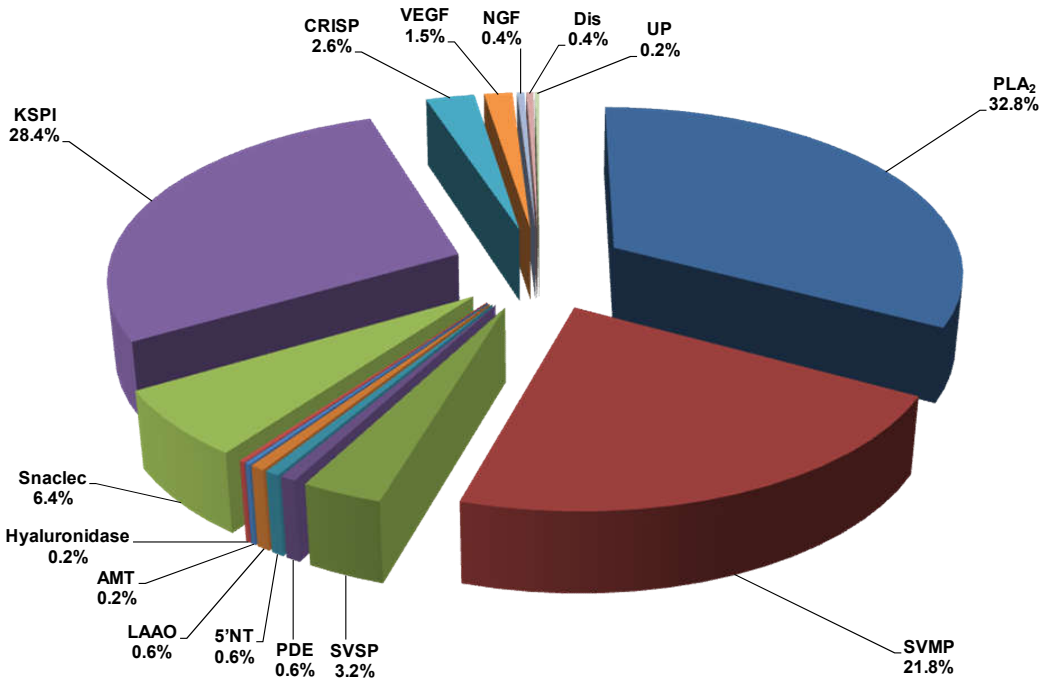

Fig. 2.3. The venom proteome of captive specimens of Pakistani *Daboia russelii* (adapted from Mukherjee et al. [3]).

2.4.3.5 Pakistan RVV (wild specimens)

Recently, Faisal et al. [25] investigated the venom proteome of wild Pakistani RVV samples and observed striking variation in venom composition between samples from wild and captive RV. Proteomic analysis of the RP-HPLC fractions of wild Pakistani RVV identified a total of 54 venom proteins that were clustered into 11 protein families (Fig. 2.4) [25]. Notably, the relative abundance of PLA_2 (63.8%) was exceptionally high in this RVV proteome and that of SVMP (2.5%) was quite low compared to the captive specimens. This implies that venom variability is not only confined to geographical and phylogenetic factors but may also be noted within the same species of snake due to differences in conditions (wild or captive), feeding behavior and/or prey items that may alter the venom production [25]. Further, ELISA experiments suggested that the Indian commercial PAV (from VINS BioProducts Ltd.) could cross-react with the Pakistani RVV sample; however, KSPI, VEGF, and PLA_2-dominating low molecular weight (<20 kDa) RP-HPLC fractions exhibited poor

immuno recognition [25]. In addition, the PAV moderately neutralized the pro-coagulant activity and lethality of wild Pakistani RVV samples.

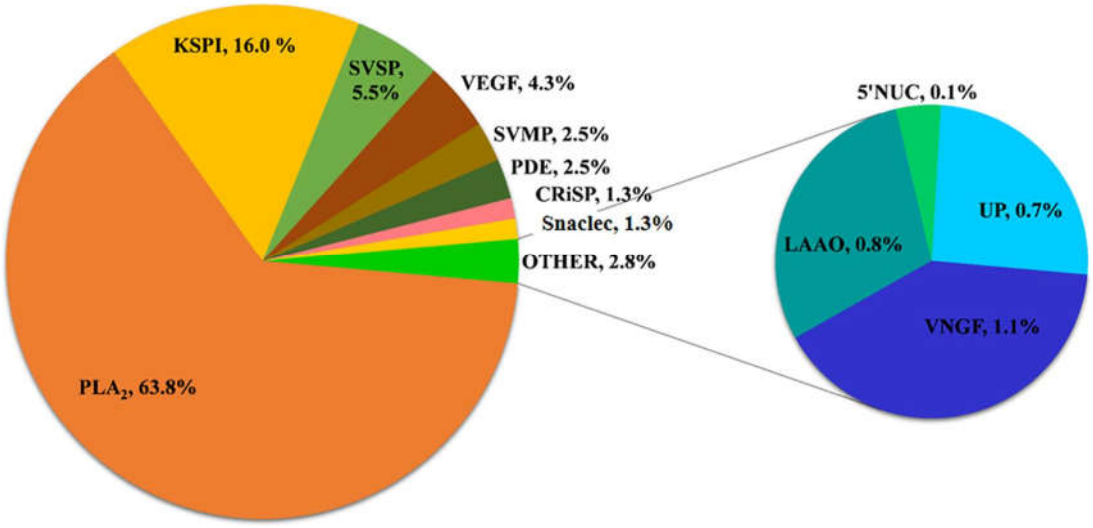

Fig. 2.4. The venom proteome of wild specimens of Pakistani *Daboia russelii* (adapted from Faisal et al. [25]).

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