Review Article

Structure and Pharmacological Properties of Some Snake Venom Protein Families with Special Reference to Kunitz-Type Serine Protease Inhibitors

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Abstract: Snake venom consists of a large repertoire of pharmacologically active proteins and peptides that play a significant role in capture, immobilization and digestion of prey. Both enzymatic and non-enzymatic toxins exist in snake venom which contributes to the overall toxicity during envenomation. A deep understanding of toxin composition as well as structure and function properties of the families is a pre-requisite to understand the pathophysiological conditions during bite and to combat post-bite consequences in victims. Moreover, this goldmine of proteins can be explored for drug development or its prototype if their functional properties are deciphered at the molecular level. In the recent past, using high throughput technologies the complexities of snake venom has been deciphered to a great extent. This has helped in understanding the variation in venom composition for development of effective strategies to combat envenomation. In this review, the structural and functional aspects of some of the major snake venom toxin families have been summarized. Among the enzymatic proteins, phospholipase A_2, acetylcholinesterase, snake venom thrombin-like enzymes, snake venom metalloproteinase, hyaluronidase are discussed and other non-enzymatic toxins like three finger toxins, snaclecs, cysteine-rich secretory proteins and Kunitz-type serine protease inhibitors are also described. In addition, the mechanism of action and pharmacological effects of some toxins are also discussed in this review. Further research on snake venom in deciphering its complex composition, understanding the underlying mechanism of action would help in developing toxin-specific antibodies.

Key words: Snake venom, enzymatic toxins, non-enzymatic toxins, Kunitz-type serine protease inhibitors.

Introduction

Venomous organisms are widely distributed throughout the animal kingdom, comprising around 1,00,000 species which are found in all major phyla like chordates, echinoderms, annelids, molluscs and arthropods. One of the most fascinating techniques of capturing prey or defending oneself by these animals is the use of their venom. Among the reptiles, venomous snakes dominate their position as predators by using its venom as offensive tool for incapacitating and immobilizing as well as for digesting the prey (primary function) and as defensive tool against their predators (secondary function). These elongated, legless carnivores produce and store their venom in a pair of venom gland, located ventral and posterior to the eye. However, not all snakes are venomous in nature. Out of around 3150 species of snakes found in the world, only 600 are known to be venomous (Vidal *et al.*, 2007). Biochemically, snake venom comprises of a myriad of proteins and peptides, which disturb the activity of critical enzymes, receptors and ion-channels thereby affecting the central as well as peripheral nervous system, cardiovascular system, neuromuscular system, blood coagulation cascade and overall homeostasis of the prey or victim. Thus, envenomation remains as a significant cause of mortality and morbidity worldwide.

Caenophidia, the advanced snakes constitute the most diverse group of living snakes and include medically important venomous snakes that are classified into three families: Atractaspididae, Elapidae and Viperidae. The snakes belonging to Viperidae family are further divided into two subfamilies, Viperinae (True Vipers) and Crotalinae (Pit Vipers) (Tasoulis and Isbister, 2017). There are reports suggesting that the ancestor of all Caenophidia possessed at least some components of the venom delivery system, confirming the evolution of all the front-fanged venomous snakes from non-venomous ancestor, making the venom apparatus a homologous characteristic of all snakes (Jackson *et al.*, 2017; Vonk *et al.*, 2008).

About 90-95% of the dry weight of snake venom is made up of peptides and proteins. Based on their mechanism of action, these proteins can be classified into two families: enzymatic and non-enzymatic. Enzymatic toxins are responsible for lethality, debilitating effects of venom in addition to aiding in digestion of the prey; whereas non-enzymatic toxins contribute towards immobilization of the prey (McCleary and Kini, 2013). They are grouped into several toxin superfamilies on the basis of their structures. The enzymatic family includes: Phospholipases A_2, Acetylcholinesterases, L-amino acid oxidases, Serine proteinases, Metalloproteinases, Nucleotidases, Hyaluronidases, whereas the non-enzymatic family is comprised of three finger toxins, Kunitz-type serine protease inhibitors, Snaclec proteins, natriuretic peptides, nerve growth factors etc. Each snake venom protein family has its respective molecular weight, conserved sequence and multiple biological functions. However, there is a vast diversity within the proteins belonging to the same snake venom protein family. The diverse pharmacological properties of the toxins isoforms may be due to rapid gene duplication followed by accelerated point mutations. Such toxin evolution can be explained by several mechanisms (Kini, 2018) like- increased point mutations in exons due to unstable exon triplets as compared to introns; ii) Accelerated Segment Switch in Exons to alter Targetting (ASSET) wherein the exon segments are replaced by unrelated fragments to alter the molecular surface of the toxin (Doley *et al.*, 2008); iii) Rapid Accumulation of Variations in Exposed Residues (RAVER) in which the molecular surface is altered rapidly by point mutations (Sunagar *et al.*, 2013); iv) alteration at intron-exon boundary (Fujimi *et al.*, 2003); deletion of exon (Pawlak and Kini, 2008); v) domain-swapping by recombination (Casewell *et al.*, 2011; Moura-da-Silva *et al.*, 2011).

Due to their high degree of target specificity, venom toxins have been explored as pharmacological tools as well as prototypes for drug development. Moreover some of the snake venom proteins have been used in diagnostics tools. For instance, Ancrod, a snake venom thrombin-like enzyme from *Agkistrodon rhodostoma* is used for treatment of ischaemic stroke and deep vein thrombosis (Burkhart *et al.*, 1992); Captropril from *Bothrops jaraca* has found its applications in treating hypertension as well as congestive heart failure (Smith and Vane, 2003); Textilinin-1, a plasmin inhibitor from *Pseudonaja textilis* have been used as an alternative anti-bleeding agent to aprotinin in surgeries to prevent blood loss (Flight *et al.*, 2009); Contortrostatin, a disintegrin from *A. contortrix contortrix* has its application as an anti-tumour agent by blocking integrins during tumour progression (Trikha; De Clerck; Markland, 1994); Bothrojararacin, a thrombin inhibitor from *Bothrops jararca* serves as an anticoagulant agent (Zingali *et al.*, 1993). The structure-function relationship of various snake venom toxins has been studied and recent advances in proteomics as well as transcriptomics have made identification of venom toxins possible (Tasoulis and Isbister, 2017). Together, all these have added to the present snake venom protein database as well as our understanding of toxin composition and snake venom evolutionary biology. In the present review, we have highlighted the characteristics of some of the snake venom toxin families including their structure, function and mechanism of action.

Enzymatic venom families

Phospholipase A_2 (PLA_2)

Characteristics: Snake venom PLA_2 consists of a wide range of enzymes that hydrolyze glycerophospholipids specifically at the *sn*-2 position of the glycerol backbone releasing lysophospholipids and fatty acids which are involved in

important downstream processes like cell signaling, phospholipid remodeling and membrane perturbation (Six and Dennis, 2000). They have a molecular weight of around 14 kDa and are notably known to be stable molecules due to the presence of a number of disulphide bonds. The PLA_2 super family is divided into 15 groups which are further sub-divided into many subgroups, each displaying variety of structural and functional properties (Burke and Dennis, 2009; Schaloske and Dennis, 2006). The four main classes are secretory, cytosolic, Ca^{2+} independent and lipoprotein associated PLA_2s.

The secreted PLA_2 are present in the venom of various snakes, scorpions, bee as well as in the component of pancreatic juice, arthritic synovial fluid and in many mammalian tissues (Six and Dennis, 2000). In general they possess five to eight disulphide bridges and the structure consists of a characteristic Histidine residue at the catalytic site and Ca^{2+} in the active site. Most of the secreted PLA_2 exhibit interfacial activation in presence of a large lipid aggregate as the substrate instead of a monomeric one (Carman; Deems; Dennis, 1995; Gelb *et al.*, 1995). Based on amino acid sequence, three-dimensional structure and disulphide bond pattern, snake venom secretory PLA_2 are categorized into Group I and Group II (Six and Dennis, 2000). Elapid and colubrid snake venom as well as mammalian pancreatic PLA_2 enzymes are classified under group I while that of Viperidae family fall under group II. The characteristic feature unique to group I enzymes is the presence of an elapid loop between 63-67 amino acid residue that links the catalytic~ -helix and the -wing (Six and Dennis, 2000). Group II PLA_2 enzymes have an extended C-terminal loop. Structurally, PLA_2 enzymes consist of three major ~ -helices and two anti-parallel -sheets, which are linked by a disulphide bridge. The conserved structures in the enzyme are the N-terminal helix, calcium binding loop, antiparallel helix, active site and -wing. These enzymes function by a common catalytic mechanism involving a conserved active site dyad of His48 and Asp99 and the Ca^{2+} ion essential for catalysis interacts with third active site residue Asp49 (Fig.1). His48 is crucial for catalysis and is supported by hydrogen bonds from Tyr52 to the side chain of the opposite helix (Asp99). All these together form the active site of PLA_2 enzymes and it follows the proton relay catalytic mechanism wherein deprotonation of an active site water molecule results in attack of hydroxyl ion on the ester bond of the phospholipid (Verheij; Slotboom; de Haas, 1981).

Pharmacological properties: Snake venom PLA_2 enzymes exhibit a wide range of pharmacological effects despite their similar structures. These include neurotoxicity, myotoxicity, anticoagulant effects, cardiotoxicity, edema-inducing properties etc. Such diverse toxic effects are directly related to the multiple functional sites present on the surface of these enzymes and their receptors (Kini, 2003). Most snake venom PLA_2 enzymes exist as monomers, while some of them form complexes (covalent and non-covalent) with other proteins which enhance the overall toxicity of the venom. Among the covalent complexes, the best studied are -bungarotoxins, a major presynaptic neurotoxin component of *Bungarus* venom. These are heterodimer complex between PLA_2 enzyme (A chain) and Kunitz type seine protease inhibitors (B chain), held together by a disulphide bond. The non-covalent complexes include Crotoxin, a heterodimer between a non-toxic, non-enzymatic subunit named Crotapotin and a weakly toxic basic PLA_2. In addition to neurotoxicity, this complex exhibits local and systemic myotoxicity, inhibits inflammatory response, initiates platelet aggregation and analgesic effects. Other complexes include Vipoxin, where two PLA_2 molecules interact and in the acidic subunit, the crucial His48 is replaced by Gln4 and acts as an inhibitor to the basic subunit. Another example is Trimucrotoxin, a homodimeric PLA_2 complex isolated from *Trimeresurus mucrosquamatus* that shows presynaptic neurotoxicity. PLA_2 also exists as a ternary complex named Taipoxin comprising of three subunits:~ , and γ . The~ and subunits are group IA PLA_2 enzymes while the γ subunit belongs to group IB. This complex causes neuromuscular blockade and is one of the most potent animal toxins known so far (Fohlman; Lind; Eaker, 1977).

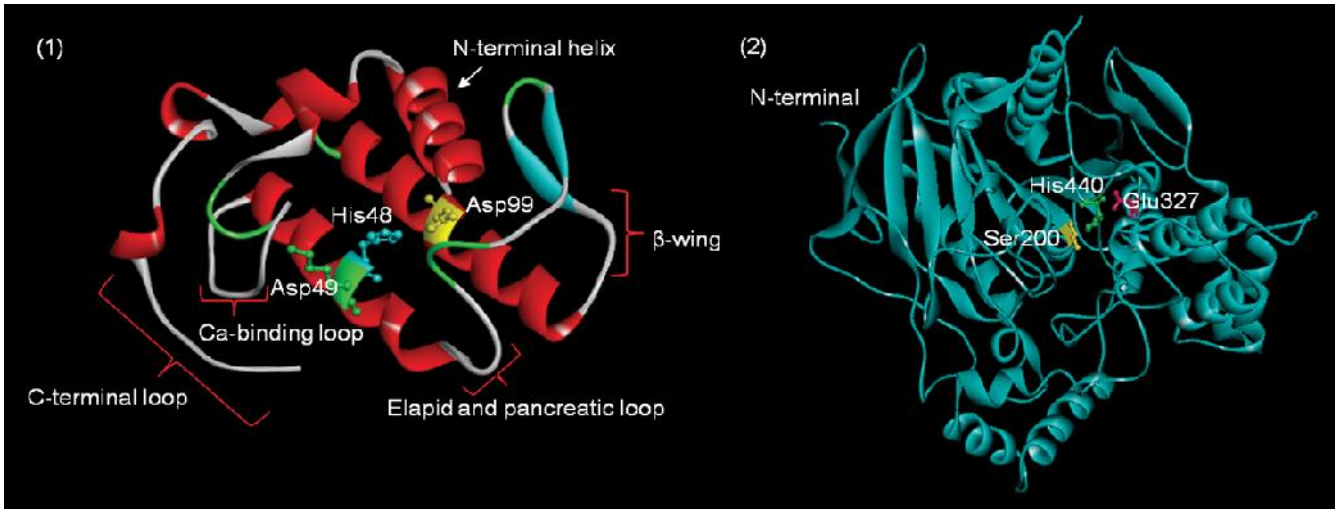

Fig.1. Three-dimensional ribbon structure of a PLA_2 from *Agkistrodon acutus* venom (PDB ID: 1MG6). The conserved structures are highlighted. The active site residues: His48 (blue), Asp49 (green) and Asp99 (yellow) are represented as ball and stick.

Fig.2. Three-dimensional ribbon structure of acetylcholinesterase from *Bungarus fasciatus* venom (PDB ID: 4QWW). The active site residues: Ser200 (yellow), His440 (green) and Glu327 (pink) are represented as ball and stick.

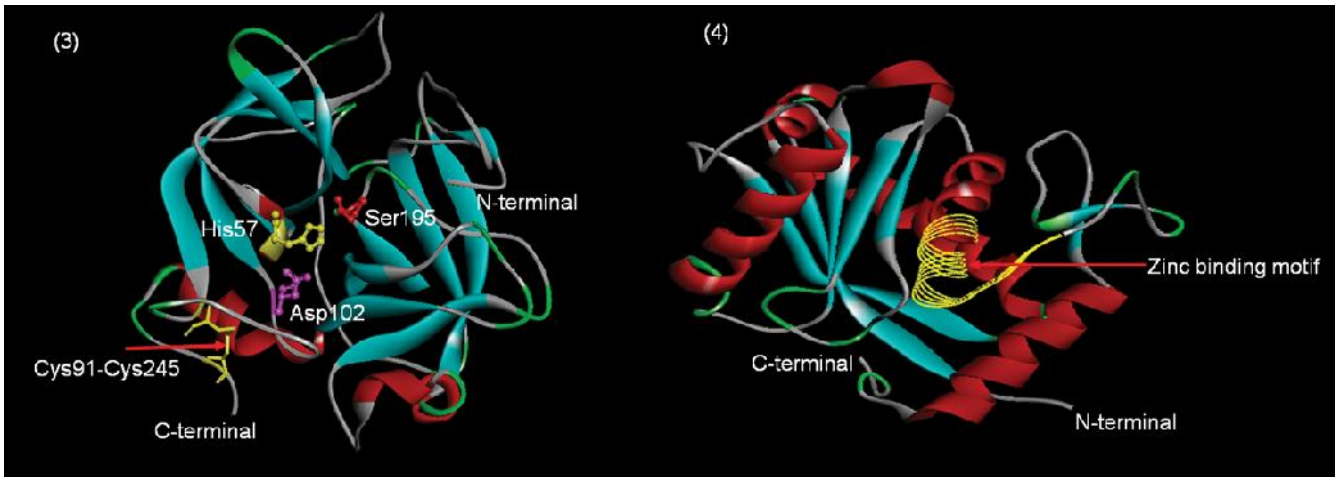

Fig.3. Three-dimensional ribbon structure of thrombin-like enzyme from *Agkistrodonhalys* venom (PDB ID: 4E7N). The active site residues: Ser195 (red), His57 (yellow) and Asp102 (pink) are represented as ball and stick between two â-barrels. The unique disulphide bond between Cys91 and Cys245 is represented as stick (yellow).

Fig.4. Three-dimensional ribbon structure of snake venom metalloproteinase Adamalysin II from *Crotalusa damanteus* venom (PDB ID: 1IAG). The characteristic Zinc-binding motif in the catalytic domain of the protein is represented by line ribbon (yellow).

Acetylcholinesterase (AChE)

Characteristics: Acetylcholinesterase belongs to the cholinesterase family (Frobert *et al.*, 1997) and is found in both synaptic and non-synaptic locations. It plays a key role in cholinergic transmission in the nervous system by rapidly inactivating the neurotransmitter, acetylcholine. AChE is abundantly found in Elapid venom with exception to Mambas though marking its complete absence from the venom of Vipers and Colubrids (Cousin and Bon, 1997). Presence of high levels of AChE in snake venom may be correlated to selective pressures favourable for the evolution of compounds that disrupt cholinergic transmission in the central nervous system as well as neuromuscular junction of the prey. According to a study, snake venom AChEs are functionally more active than Torpedo and mammalian AChEs in acetylcholine hydrolysis

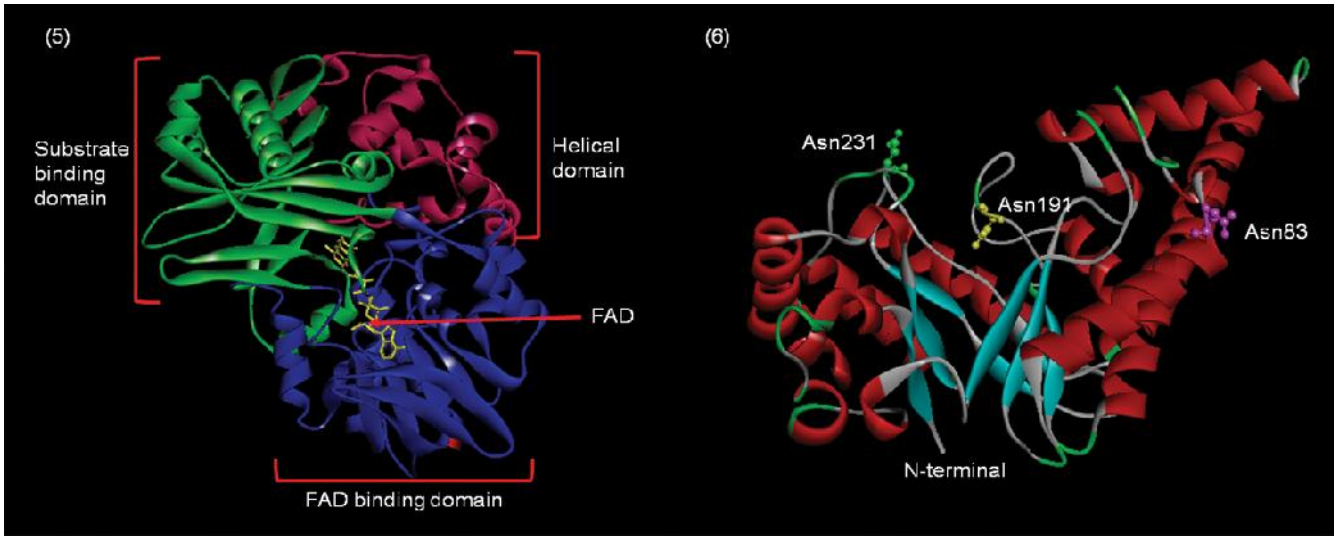

Fig.5. Three-dimensional ribbon structure of snake venom LAAO from *Bothropsatrox* (PDB ID: 5TS5). The structure shows three characteristic domains: FAD binding domain (dark blue), substrate-binding domain (green) and the helical domain (majenta).

Fig.6. Three-dimensional ribbon structure of Hyaluronidase (PDB ID: 1FCQ) from bee venom. The gylcosylation motifs Asn83 (pink), Asn191 (yellow) and Asn231 (green) are highlighted as ball and stick).

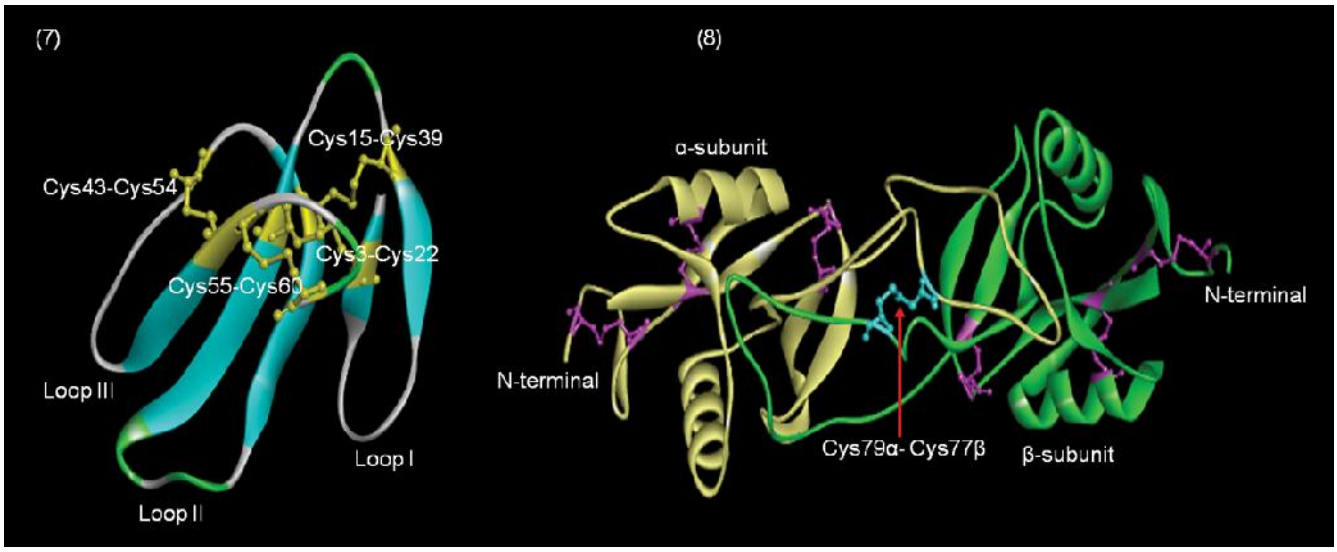

Fig.7. Three-dimensional ribbon structure of Hemachatoxin (PDB ID: 3VTS), a three-finger toxin from the venom of *Haemachatus haemachatus*. The structure is stabilized by four disulphide bridges in the central core region which are represented as ball and stick (yellow). The three loops (I, II, III) extending from central core are also highlighted.

Fig.8. Three-dimensional ribbon structure of Agkisacucetin (PDB ID: 3UBU), a snaclec from *Agkistrodon acutus*. The two subunits á and â are highlighted in yellow and green respectively showing their interchain disulphide bond in blue. The intrachain disulphide bonds in individual subunits are highlighted as ball and stick (majenta).

(Vigny *et al.*, 1978). The structure of the enzyme basically comprises of several major domains that includes a catalytic active site further composed of two sub sites, the aromatic gorge in which the catalytic active site resides and a peripheral anionic site (PAS) (Rosenberry *et al.*, 2005). Within the active site, there are two binding domains: an anionic site that contains a glutamate residue involved in binding with the catalytic head of acetylcholine (ACh) and an esteratic site that has histidine and serine residues which function as acid/base catalyst domain (Kabachnik *et al.*, 1970; Rosenberry *et al.*,

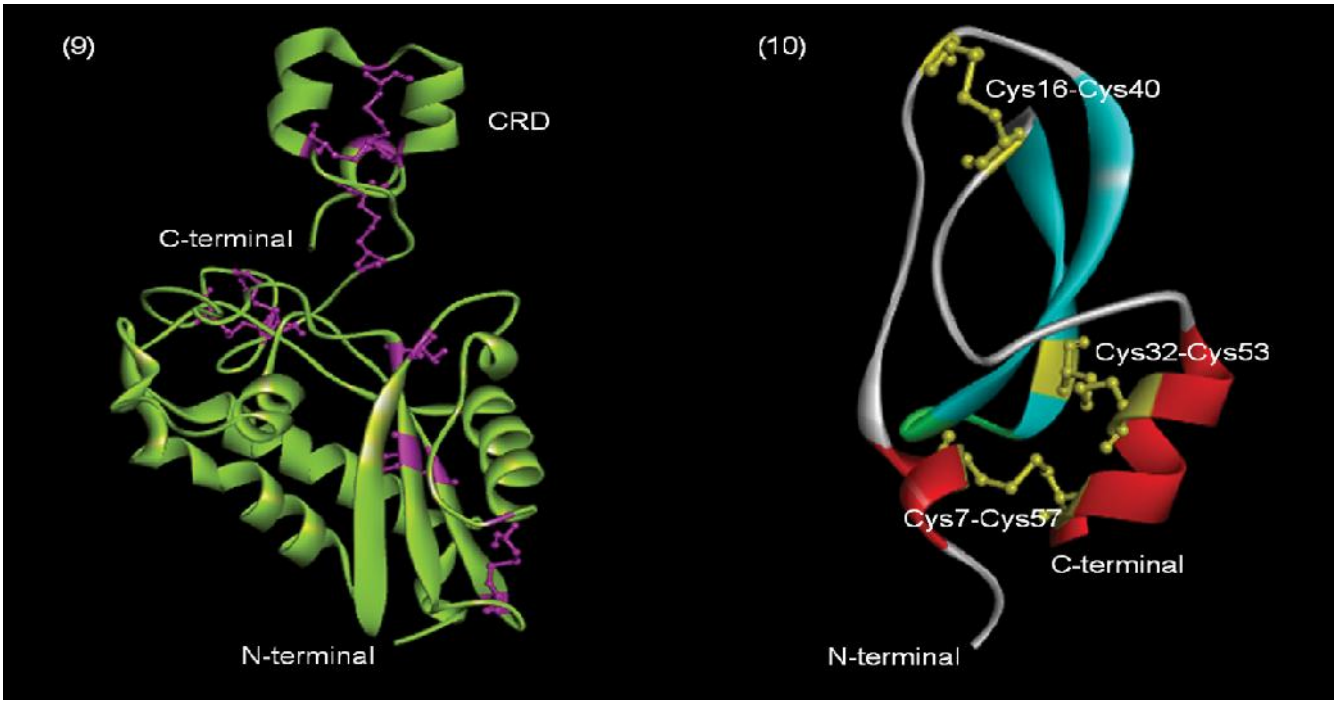

Fig.9. Three-dimensional ribbon structure of Natrin (PDB ID: 1XTA), a CRISP from *Naja atra* venom. The structure consists of 16 cysteine residues that form eight disulphide bridges and are represented as ball and stick (majenta). The cysteine-rich domain (CRD) is at the C-terminal comprising of 10 cysteine residues.

Fig.10. Three-dimensional ribbon structure of Textilinin-1 (PDB ID: 3BYB), a Kunitz-type serine protease inhibitor from *Pseudonaja textilis*. The structure is stabilized by three conserved cysteine residues Cys7-Cys57, Cys16-Cys40, Cys32-Cys53, represented as ball and stick (yellow).

2005; Soreq and Seidman, 2001). Thus the catalytic triad of AChE consists of Ser (200), His (440) and most interestingly a Glu (327) instead of aspartate (Fig.2). The hydrolysis of ACh occurs at the esteratic site and involves serine and histidine. The serine residue acts as a nucleophile while histidine acts as acid/base catalyst during hydrolysis. The overall hydrolysis process of ACh occurs in a very rapid rate within 100 microseconds. Comparative studies of amino acid sequence have demonstrated that the catalytic domain of these enzymes from snake venom shares a high level of homology with other sources of AChE. For instance, *Bungarus fasciatus* AChE shares 60% identity and 80% similarity with that of Torpedo AChE in terms of their catalytic domain (Cousin *et al.*, 1998). *Bungarus* venom AChE has four N-glycosylation sites which correspond to the glycosylated position in Torpedo and mammalian AChEs. Besides, the six cysteine residues in the catalytic triad and tryptophan residue (Trp84) are also conserved in all types of cholinesterases (Weise *et al.*, 1990). The only difference found was in the peripheral site where tyrosine 70 was replaced by methionine and lysine 285 was replaced by aspartic or glutamic acid.

Pharmacological properties: The presence of elevated amounts of AChE in venom might attribute to some functional importance during feeding. According to Mackessy (Mackessy *et al.*, 2006), the evolution of resistance mechanisms by prey is countered by an evolutionary response of the snakes. Hence, the resulting secreted form of AChE in snake venom may have a vital role in catalysing hydrolysis of ACh in muscle cells (Massoulie *et al.*, 1999) and facilitating capture of the prey, making the venom cholinotoxic in nature.

Snake Venom Thrombin-like enzymes (SVTLEs)

Characteristics: Snake venom serine protease (SVSP) family consists of a broad group of enzymes that mainly affect the haemostatic system. A subgroup of SVSPs is structurally and functionally related to thrombin, a key enzyme involved in blood

coagulation cascade. Hence this group is designated as thrombin-like enzymes (Markland, 1998; Wisner; Braud; Bon, 2001). SVTLEs are important components mostly of Viper venom (Mackessy, 2016) with a few exceptions in elapid venom (Jin; Lee; Zhang, 2007). While thrombin acts on fibrinogen to release both fibrinopeptides A and B, TLEs preferentially cleave either fibrinopeptides A or B, but not both. The result is the formation of an easily degradable fibrin clot, a condition called hypofibrinogenemia.

SVSPs act on components of the coagulation cascade, fibrinolytic system, kallikrein-kinin system, complement system, endothelial cells and blood cells causing an imbalance of the overall haemostatic system (Serrano, 2013). They are classified in the clan PA, subclan S, family S1 (Barrett; Woessner; Rawlings, 2012). The catalytic triad consists of serine (Ser195, according to chymotrypsinogen numbering), histidine (His57) and aspartate (Asp102), where serine acts as a nucleophile and histidine both as a proton donor and acceptor. They are considered as trypsin-like enzymes and cleave peptide bonds following Arg or Lys at P1 position. Although they display high degree of sequence identity, SVSPs show exquisite specificity towards different macromolecular substrates (Serrano, 2013). They contain twelve cysteine residues, out of which ten residues form five disulphide bridges (Itoh *et al.*, 1987) and the rest two forms a unique bridge that remains conserved among the SVSPs and includes Cys245e in the C-terminal extension region (Parry *et al.*, 1998). Most SVSPs are glycoprotein in nature with various glycosylation sites throughout the sequence. Depending upon the extent of glycosylation, the molecular size of this protein varies from 26-67 kDa.

The secondary structure of TLEs consists of a / hydrolase fold with a catalytic cleft between the two -barrels (Wisner; Braud; Bon, 2001). The structure is highly stabilized by six disulphide bridges, five of which are conserved across the S1 serine proteinase family while the sixth one (Cys91-Cys245) is unique to SVSPs (Pirkle, 1998). The catalytic mechanism of TLEs involves the highly conserved catalytic triad of Ser195, His57 and Asp102 (Fig.3). Besides, the conserved residues Asp189 and Gly216 found in SVTLEs as well as in trypsin and thrombin (Pirkle, 1998) contribute to S1 and S2 specificity sites, respectively (Lu; Clemetson; Clemetson, 2005; Schmidt *et al.,* 2004). The basic P1 residues (lysine and arginine) of the substrate are the target of the S1 site (Kini, 2005).

Though SVTLEs are referred to as thrombin-like, they share less than 40% homology to thrombin (Kini, 2005). In contrast they resemble trypsin more closely in terms of the features of catalytic cleft. While thrombin possesses an additional three-residue S1 loop in its amino acid sequence (Castro *et al.*, 2004), neither SVTLE nor trypsin possesses this domain which might affect the substrate specificity. Further, both TLEs and trypsin possess a serine or threonine at the 190 position which acts as a proton donor and acceptor; on the other hand, alanine is present at the same position in case of thrombin and it is also not involved in any proton exchange (Castro *et al.*, 2004; Di Cera and Cantwell, 2001; Di Cera; Dang; Ayala, 1997). Also Trp215 is conserved in almost all TLEs whereas the S2 site of thrombin is occupied by conserved 215 and 217 residues (Castro *et al.*, 2004). Unlike in TLEs, the active site of thrombin is surrounded by surface loops that form flexible appendages extending from the fixed core (Fuglestad *et al.*, 2012). The residues in the loop surrounding active site of TLEs are however less conserved in comparison to the remaining sequence and are unique in each TLE (Huang *et al.*, 2011; Ullah *et al.*, 2013). All these differences might be responsible for different substrate affinities of TLEs as they affect the charges and shape of the catalytic cleft (Huang *et al.*, 2011). Therefore, their conserved structures and functional diversities may aid in protein engineering. Also comparison between TLEs and thrombin might provide useful insights into the mechanism behind the release of fibrinopeptides from fibrinogen.

Pharmacological properties: SVTLEs act on blood plasma and results in the formation of a soft, translucent clot due to the lack of factor XIIIa activity on fibrin which is required for stabilizing the clot. Since the fibrin clot is not cross-linked, it is readily removed by the fibrinolytic system. Unlike thrombin, TLEs do not act on other coagulation cascade factors (Aronson, 1976). Although TLEs share some homology with thrombin,

both the enzymes are structurally as well as functionally dissimilar (Bell, Jr., 1997; Hutton and Warrell, 1993; M+¬nez, 2002). Because of their unique physiological properties, many SVTLEs are explored for use as therapeutic agents: ancrod (Arvin®) from *Calloselasma rhodostoma;* batroxobin (Defibrase®) from *Bothropsa troxmoojeni* (Stocker and Barlow, 1976), bothrombin from *Bothrops jararaca*, Calobin from *Crotalus atrox* etc.

Snake Venom Metalloproteinases (SVMPs)

Characteristics: Proteomic analyses have revealed the abundance of metalloproteinases in the venom of most of the Crotalids and Vipers. They constitute almost 30% of the total venom, suggesting their potential in envenomation-related pathogenesis such as bleeding, necrosis, intravascular clotting, edema, inflammation (Bjarnason and Fox, 1994; Fox and Serrano, 2005; Fox and Serrano, 2009; Iwanaga and Suzuki, 1979). Majority of SVMPs are considered to be haemorrhagic in nature (Takeya and Iwanaga, 1998). They function by hydrolysing the key components of basement membranes leading to disruption of vessel walls and allows the blood to escape to the stroma (Gutierrez *et al.*, 2005). Phylogenetically, SVMPs are closely related to mammalian ADAMs (A Disintegrin-like And Metalloproteinase) and together they constitute the M12 subfamily of metalloproteinases.

SVMPs are monozinc endopeptidases with a molecular size ranging from 20 to 100 kDa. Structurally, they contain a Zn-binding motif in their catalytic domain characterized by the consensus sequence HEXXHXXGXXH, followed by a methionine turn (Fig.4) (Bode; Gomis-Ruth; Stockler, 1993). Based on the presence of various domain structures, SVMPs are classified into four groups: P-I, P-II, P-III and P-IV (Hite *et al.*, 1994). The P-I group is comprised of SVMPs that have only metalloproteinase domain; the P-II group is characterized by the presence of a disintegrin domain with RGD motif in most of the cases; the P-III group contains metalloproteinase, disintegrin-like and cysteine-rich domains whereas the P-IV group possesses an additional snaclec domain and is included in P-III group as subclass P-IIId and is considered as post-transcriptionally modified form of P-III group of SVMPs (Fox and Serrano, 2008). The gene structure of all these SVMPs contains a pre and a pro domain before the metalloproteinase domain. The pro domain helps in maintaining the latent form of the proteinase during maturation by cysteine switch mechanism (Grams *et al.*, 1993).

The active site cleft of the enzyme contains the catalytic zinc, co-ordinated by three histidine residues (His 334, His338 and His 444) and a water molecule (Ramos and Selistre-de-Araujo, 2006). During catalysis, the water molecule, clamped between the carboxylic groups of the catalytic Glu335 and the zinc ion, goes through a polarization shift. After the transfer of hydrogen atom to Glu, water serves as the nucleophile and attacks the carbonyl group of the substrate scissile bond, bringing the metal to a pentacoordinate transition state. In the consequent step, the carboxyl hydrogen from glutamate is transferred to the nitrogen of the scissile bond resulting in the cleavage of the peptide bond. Finally the enzyme is regenerated with the incorporation of a new water molecule (Stocker *et al.*, 1995).

Pharmacological properties: Majority of the SVMPs are associated with haemorrhage or disruption of the haemostatic system, which are mainly mediated by the proteolytic activity of the metalloproteinase domain. These proteins interrupt the interaction between endothelial cells and the basement membrane by degrading basement membrane and extracellular matrix components like collagen type IV, laminin and fibronectin thereby causing bleeding, hemorrhage in victims upon envenomation (Bjarnason and Fox, 1994). Recent studies have indicated some additional functions of this group of enzyme some of which are as follows: fibrin(ogen)olytic activity (Retzios and Markland, Jr., 1988; Willis and Tu, 1988); activation of prothrombin (Kini, 2005; Kornalik and Blomback, 1975; Yamada; Sekiya; Morita, 1996); factor X activation (Siigur *et al.*, 2004; Tans and Rosing, 2001); apoptotic (Brenes *et al.*, 2010; Han *et al.*, 2007; Trummal *et al.*, 2005); inhibition of platelet aggregation (Kamiguti; Hay; Zuzel, 1996; Laing and Moura-da-Silva, 2005; Moura-da-Silva; Butera; Tanjoni, 2007; Wang; Shih; Huang, 2005; Zhou; Dangelmaier; Smith, 1996) and inactivation of blood serine protease inhibitors (Kress, 1986; Kress and Catanese, 1980; Kress and Hufnagel, 1984).

L-amino acid oxidases (LAAO)

Characteristics: This group of enzymes are widely distributed in different species like insects, bacteria, fungi as well as in snakes. Viperidae, Crotalidae and Elapidae snakes are the richest source of LAAOs. They exhibit catalytic specificity for long chain hydrophobic and aromatic amino acids and are known to be active in wide range of pHs and temperatures (Izidoro *et al.*, 2014). The yellow colour of snake venom is because of the presence of flavins in LAAOs and contributes to the toxicity of the venom through oxidative stress by the production of hydrogen peroxide (Ciscotto *et al.*, 2009; Doley and Kini, 2009; Lomonte *et al.*, 2008; Lu *et al.*, 2002; Sant'Ana *et al.*, 2008; Stabeli *et al.*, 2004; Vieira Santos *et al.*, 2008; Wei *et al.*, 2003).

LAAOs (EC 1.4.3.2) are flavoenzymes that belong to the oxidoreductase class of enzymes. These enzymes function by catalysing the stereospecific oxidative deamination of their substrate L-amino acids. The product of the reaction is an ~ -keto acid, ammonia and hydrogen peroxide. Structurally, LAAOs are homodimeric FAD (Flavin adenine dinucleotide) or FMN (flavin mononucleotide) binding glycoproteins and consists of two identical subunits, each with a molecular weight of 57-68 kDa and pI in the range of 4.4-8.5 (Du and Clemetson, 2002; Huang and Xu, 2009; Samel *et al.*, 2006; Zhang and Wu, 2008). X-ray structural analysis demonstrated the presence of a dynamic active site and three domains: FAD-binding domain, a substrate-binding domain and a helical domain (Fig.5) (Pawelek *et al.*, 2000). The FAD-binding domain consists of residues Asp 2 to Val 72, Gly 238 to Arg 322 and Tyr 425 to Ala 483 forming nine ~ -helices and eleven -strands. The substrate binding domain consists of residues Lys 73 to Arg 129, Arg 232 to Val 237 and Ser 323 to Lys 424 and contains six ~ -helices and twelve -strands; whereas the residues Val 130 to Lys 231 form the helical region consisting of ten ~ -helices (Feliciano *et al.*, 2017).

They are highly specific for the L-enantiomers of amino acids. The enzymatic reaction requires the presence of a free primary ~ -amino group. Generally the best substrates are L-Leu, L-Met, L-Phe, L-Tyr and L-Trp, while L-Lys, L-Ser, L-Thr, L-Asp and L-Glu either undergo slow or no hydrolysis at all (Izidoro *et al.*, 2006; Ponnudurai; Chung; Tan, 1994; Samel *et al.*, 2006; Souza *et al.*, 1999; Stabeli *et al.*, 2004; Tonismagi *et al.*, 2006). The catalysis of L-amino acid by LAAO takes place in two consecutive steps. During the reductive half-reaction, the ~ -hydrogen atom of the amino acid is abstracted by FAD, forming ~ -imino acid as the intermediate product, which then reacts with water to form the keto-acid.

Pharmacological properties: LAAOs are considered to be multifunctional enzymes showing cytotoxic, apoptotic, platelet aggregation effects, edema and bactericidal as well as anti-parasitic activities (Zuliani *et al.*, 2009). These activities are because of the production of hydrogen peroxide with free oxygen radical formation thereby causing oxidative stress. Because of all these biological activities, this group of enzymes have gained attention as new therapeutic agents for the treatment of cardiovascular disease, bacterial infections, tumours, parasite and virus-induced diseases and could come up as a promising biotechnological agent (Guo *et al.*, 2012).

Hyaluronidase

Characteristics: Upon envenomation the process of efficient diffusion of the venom from the bite site to different tissues is accomplished by Hyaluronidase, commonly known as the "spreading factor". It not only enhances the venom toxicity but also causes damage to the bite site causing severe morbidity. However, in contrast to other snake venom proteins, limited research has been done on this group of enzymes. Hyaluronidases are basically endoglycosidases and are known to degrade hyaluronan, a glycosaminoglycan (GAG) present in the extracellular matrix (ECM) (Kemparaju and Girish, 2006). This results in the distortion of the ECM of local tissue and paves the way for the spreading of the venom.

These enzymes exist as multiple isoforms (Girish *et al.*, 2002; Girish *et al.*, 2004b) and are widely distributed in almost all venoms, including snake venom. Originally hyaluronidase activity was common in Viperid venom because of the presence of high molecular weight toxins and thus facilitates the spread of these toxins. It shows less or no detectable activity in case of elapid venom with

some exceptions where the activity of the enzyme is higher than that in viperid venom. The molecular size of hyaluronidase ranges from 33 to 110 kDa with a pI greater than 9.0. They show optimal activity between pH 5.0-6.0 and fall under neutral class of enzymes. Most snake venom gland transcriptomes possess hyaluronidase transcripts and the translated proteins have 440 to 450 amino acid residues (Harrison *et al.*, 2007). Furthermore, the presence of a conserved cystinyl scaffold in snake venom hyaluronidases indicates similarity to other hyaluronidases (Kreil, 1995). The structure of hyaluronidase from bee venom comprises of ten ~ -helices, eleven -strands and six 3_{10} helices. The overall fold of the globular protein resembles that of a classical (/~)$_8$ TIM barrel, a feature common to most of the hyaluronidase (Markovic-Housley *et al.*, 2000) (Fig. 6).

Pharmacological properties: Although hyaluronidase degrades the hyaluronan of the ECM at the bite site, it is considered to be non-toxic. Nevertheless, the presence of this enzyme in almost all snake venom strongly supports it role in envenomation. In addition to its spreading effect, there are several indirect effects of hyaluronidase. The hyaluronan fragments generated by this enzyme most likely participate in acute pharmacological effects such as inflammation which is common in case of envenomation. Also production of antibodies and search for synthetic and natural inhibitors of hyaluronidase activity are the topics of current research for improvement of anti-venom therapy (Girish *et al.*, 2004a; Girish and Kemparaju, 2006; Yingprasertchai; Bunyasrisawat; Ratanabanangkoon, 2003).

Non-enzymatic toxin families

Three finger toxins (3FTxs)

Characteristics: The three finger toxins are non-enzymatic proteins found mostly in the venom of elapids (cobras, kraits and mambas), colubrids and hydrophids (Pawlak *et al.*, 2006; Pawlak *et al.*, 2009). They consist of 60 to 74 amino acid residues with four or five disulphide bridges, out of which four are conserved in all 3FTxs (Endo, 1991). They share a common structure of three stranded loops extending from a central hydrophobic core cross-linked by four disulphide bridges that stabilizes the overall structure (Menez, 1998; Tsetlin, 1999). These three loops resemble three outstretched fingers of the hand and hence the name three finger toxin (Fig.7). The structure of this group of toxins is highly stabilized due to the presence of conserved domains. Apart from eight conserved cysteine residues found in the central core area, the aromatic amino acids Try25 or Phe27 is also conserved in most toxins and is crucial for proper folding of the protein (Antil; Servent; Menez, 1999; Dufton and Hider, 1983) and stability of the anti-parallel -sheet structure (Torres *et al.*, 2001). Other amino acids like Arg39 in erabutoxin-a and Asp60 in ~ -cobratoxin are also conserved and forms a salt link with the C- or N-terminus of the protein for stabilizing the native conformation (Endo, 1991). Despite their conserved structure, there are some minor variations that can have significant impact on their function. For instance there is an extra fifth disulphide bridge either in loop I or II. Also some 3FTxs have extended N- and C-terminal regions. All colubrid 3FTxs have an extended N-terminal region with additional seven residues capped by a pyroglutamic acid (Pawlak *et al.*, 2006).

Pharmacological properties: 3FTxs exhibit a wide range of biological properties in spite of their conserved structural features. Members of 3FTx family includes both short chain and long chain ~ -neurotoxins which antagonize muscle nicotinic acetylcholine receptors (nAChR) (Chang, 1979; Changeux, 1990; Tsetlin, 1999); © -bungarotoxin that targets neuronal nicotinic receptors (Grant and Chiappinelli, 1985); muscarinic toxin that act as antagonists of muscarinic acetylcholine receptors (Jerusalinsky and Harvey, 1994); fasciculin which inhibits Acetylcholinesterase (Cervenansky, 1991); calciseptin which functions by blocking L-type Ca^{2+} channels (Albrand *et al.*, 1995; de Weille *et al.*, 1991); cardiotoxin/cytotoxin which helps in formation of pores in cell membranes (Bilwes *et al.*, 1994) and dendroaspin which are antagonists of cell adhesion processes (McDowell *et al.*, 1992). Such varied biological functions might have occurred during evolution when 3FTx gene had undergone duplication several times giving rise to different types of toxins through accelerated evolution (Doley *et al.*, 2008). Although the central hydrophobic core is conserved, the functional sites of 3FTxs

lies either on the surface of the molecule on different loops and/or C-terminal tail. All these functional variations along with changes in the position of functional sites offer opportunities to decipher and understand the structure-function relation of 3FTxs.

C-type lectin-like proteins (Snaclecs)

Characteristics: C-type lectin-like proteins form an important group among the haemorrhagic components of snake venom. They are named so because of their homology (15-40%) with the carbohydrate recognition domains of C-type lectins (Morita, 2005). These are non-enzymatic Ca^{2+} dependent proteins that typically bind sugar residues (Clemetson; Lu; Clemetson, 2005). The 'C' is from Ca^{2+} dependent and 'lectin' from sugar-binding properties. However, majority of the snake venom C-type lectin-like proteins lack the classical calcium or sugar binding loop. Recently this group of proteins were renamed as Snaclecs (Snake venom C-type lectins) in a nomenclature proposal by the Exogenous Factor Committee of the International Thrombosis and Haemostasis Society (Clemetson; Morita; Manjunatha, 2009). Snaclecs have a heterodimeric structure with two subunits ~ and that is covalently connected via an inter-chain disulphide bridge between Cys79 from ~ -subunit and Cys77 from -subunit (Fig. 8). They can often form larger and complex multimeric structures either by covalent or non-covalent interactions (Clemetson, 2010).

Pharmacological properties: Snaclecs exert their functions by binding to a wide range of coagulation factors, proteins involved in haemostasis and membrane receptors on platelets and other cells. Thus their role is obvious in adhesion, endocytosis as well as pathogen neutralization (Ogawa *et al.*, 2005). Adhesion receptors of platelets such as the von Willebrand factor (vWF)-binding GPIb-complex, the collagen-binding GPVI and integrin ~ 2 1, and the fibrinogen receptor integrin ~ IIb 3 are involved in processes like platelet activation and aggregation. Thus some C-type lectins have anti-coagulant property; some are anti-thrombotic whereas others are known to activate blood coagulation factors (Lu *et al.*, 2005). Owing to its multiple functions, several new Snaclecs have been isolated with promising biomedical potential. Nowadays they are also utilized as tools for studying haemostasis, thrombosis and platelet function. Additionally, they could also serve as targets for developing new anti-thrombotic and anti-coagulant drugs.

Cysteine-rich secretory proteins (CRISPS)

Characteristics: Cysteine rich secretory proteins are widely distributed in numerous animal tissues (Yamazaki and Morita, 2004) and their origin was from epididymis of mammals (Kierszenbaum *et al.*, 1981). Although many CRISPs are found in snake venom, little is known about their biological targets and role in envenomation (Utkin and Osipov, 2007). Snake venom CRISPs are single chain polypeptide with a molecular weight of around 20 to 30 kDa. Like other snake venom toxins, these protein also exhibit highly conserved molecular scaffold and consists of sixteen cysteine residues that form eight disulphide bridges and ten out of these cysteine residues form the cysteine-rich domain (CRD) at the C-terminus (Fig.9) (Yamazaki and Morita, 2004). This protein family can be divided into four classes on the basis of their amino acid sequence homology: CRISP1, CRISP2, CRISP3 and CRISP4. Of these, the first three are found in most mammals; CRISP1 is an acidic epididymal glycoprotein, CRISP2 is testis specific protein 1, and CRISP3 is a specific granule protein, while the fourth type CRISP4 is found only in case of mice (Sunagar *et al.*, 2012). CRISPs are believed to be comprised of two domain-like structures based on amino acid sequence similarity with other proteins. There are around 160 amino acid residues at the N-terminus, showing high degree of homology with group 1 plant pathogenesis-related proteins (PR-1) and the insect venom antigen 5 proteins (Ag 5), which are involved in plant stress-resistance and venom allergens, respectively. This region is followed by the cysteine rich domain of the protein (Shikamoto *et al.*, 2005).

Pharmacological properties: In spite of the limited knowledge regarding the role of CRISPs, there are some reports that have designated them as ion-channel blockers (Brown *et al.*, 1999; Nobile *et al.*, 1994; Nobile *et al.*, 1996;

Wang *et al.*, 2010; Yamazaki and Morita, 2004). Some other CRISPs are capable of blocking L-type Ca^{2+} and/or K^+ channels as well as cyclic nucleotide gated ion channels which ultimately prevent the contraction of smooth muscle cells (Brown *et al.*, 1999; Wang *et al.*, 2010; Yamazaki *et al.*, 2002; Yamazaki *et al.,* 2003). Such an activity in blocking different types of ion channels suggests the neurotoxic nature of these toxins. Apart from ion channel blocking, CRISPs have several other biological roles. For instance Patagonin from *Philodryas patagonienses* venom induced myotoxicity when injected into gastrocnemius muscle (Peichoto *et al.*, 2009); Crovirin, a CRISP from *Crotalus viridis viridis* venom demonstrated significant activity against protozoa like *Trypanosoma* and *Leishmania* (Adade *et al.*, 2014); another CRISP, Natrin from *Naja atra* venom was involved in the regulation of expression of adhesion molecules in endothelial cells thereby influencing inflammatory process (Wang *et al.*, 2010). Hence, further studies on these non-enzymatic minor toxins could help in better understanding of their biological targets and also in development of effective strategies in fast recovery of snake bite victims.

Kunitz-type serine protease inhibitors (KSPI)

Characteristics: Serine proteases inhibitors have been the most extensively studied among all the proteinase inhibitors and are classified into several families based on their structure, mechanism of action, location of active site and sequence homology. Among them, the Kunitz family of protease inhibitors is the one which contains potent serine protease inhibitors and are ubiquitously distributed from invertebrates to mammals (Delfin *et al.*, 1996; Kunitz and Northrop, 1936). Sequentially, the Kunitz-type toxin usually has a peptide chain of around 60 amino acid residues with six cysteine residues (Chang *et al.*, 2008), arranged in a conserved sequence motif of C-8X-C-15X-C-4X-YGGC-12X-C-3XC (Gojobori and Ikeo, 1994) and the structure is stabilized by three conserved disulphide bridges (Fig.10) with the bonding pattern of C1–C6, C2–C4, and C3–C5 (Kwong *et al.,* 1995). The C1-C6 and C3-C5 disulphide bonds are responsible for maintaining the native conformation of the protein (Creighton, 1975) while the third one, C2-C4 stabilizes the two binding domains (Laskowski, Jr. and Kato, 1980). Structurally, it consists of a classical Kunitz-type fold with ~ / /~ structural motif and a conserved active site residue (P1) which is primarily involved in its interaction with respective serine proteases. Bovine pancreatic trypsin inhibitor (BPTI) is the classic member of this family of proteins and was the first among the Kunitz-type protease inhibitors to be described (Kunitz and Northrop, 1936). Hence, Kunitz-type inhibitors are considered to be BPTI-like and belong to the I2 family of peptidase inhibitors (Rawlings *et al.,* 2004).

On the basis of their three-dimensional structure, KSPIs can be generally of two types: canonical Kunitz-type inhibitors (e.g. BPTI) and anti-coagulant proteins (Chen *et al.*, 2001). The difference between the two groups lies in the folds of some loops in the latter, specifically at the N-terminus (Antuch *et al.*, 1994). Based on their function, snake venom Kunitz inhibitors are classified into non-neurotoxic and neurotoxic inhibitors. Basically trypsin and chymotrypsin inhibitors are the non-neurotoxic Kunitz inhibitors while the other group includes the inhibitors that have retained their neurotoxic property (Cardle and Dufton, 1997). During evolution the neurotoxic homologs have lost their inhibitory role and act as K^+ and Ca^{2+} channel blockers (Harris and Harvey, 1991; Harvey, 2001; Schweitz *et al.*, 1994)

Most of the KSPIs carry out their function via the highly conserved antiproteinase site. The P1 position (Schechter and Berger, 1967) is the major determinant of energetics and specificity towards any serine protease (Laskowski, Jr. and Kato, 1980). The enzyme-inhibitor complex interacts mainly at the P1 site which penetrates into the S1 specificity binding pocket of the serine protease. This docking of P1 site into the S1 site forms the basis of the energetics of protease recognition (Czapinska and Otlewski, 1999). Presence of a Lys or Arg residue at P1 site of KSPI is responsible for trypsin inhibition whereas Leu, Met, Phe, Tyr, Trp and Asn at P1 site has a tendency to inhibit chymotrypsin (Laskowski, Jr. and Kato, 1980). Some of the snake venom KSPIs with different P1 site residues are listed in Table 1 below.

Table 1. P1 site residues of different snake venom Kunitz-type serine protease inhibitors.

KSPI	Snake	P1 site residue	Function	Reference
BF9	*Bungarus fasciatus*	N	Inhibits chymotrypsin	(Liu; Wu; Lo, 1983)
Bungaruskunin	*Bungarus fasciatus*	H	Inhibits chymotrypsin, trypsin, elastase	(Lu *et al.*, 2008)
Textilinin-1	*Pseudonaja textilis textilis*	R	Inhibits plasmin, trypsin	(Masci *et al.*, 2000)
TSPI	*Oyuranus scutellatus*	R	Inhibits plasma kallikrein	(Earl *et al.*, 2012)
Trypsin inhibitor	*Naja naja naja*	K	Inhibits trypsin	(Shafqat *et al.*, 1990)
OH-TCI	*Ophiophagus hannah*	K	Inhibits chymotrypsin, trypsin	(He *et al.*, 2008)
PILP-1	*Bungarus multicinctus*	K	Inhibits trypsin	(Chang *et al.*, 2008)
BBPTI-1	*Daboia siamensis*	L	Inhibits chymotrypsin	(Guo *et al.*, 2013a)

However, residues surrounding the P1 site and within the secondary binding loop can also effect the association energy (Czapinska *et al.*, 2000; Laskowski *et al.,* 2000; Scheidig *et al.*, 1997).

The mechanism of protease inhibition is similar to that of enzyme-substrate Michaelis complex. It involves a tight, non-covalent interaction where the Kunitz inhibitor is capable of directly blocking the active site of the serine protease without any conformational changes and form an anti-parallel -sheet between the enzyme and the inhibitor (Ranasinghe and McManus, 2013). The hydrophobic core of the inhibitor is the platform that supports the convex, extended and solvent-exposed canonical binding loop. This loop is highly complementary to the concave active site of the enzyme and spans from position P3 to P3′ (Krowarsch *et al.*, 2003). This loop is thus, responsible for the extreme stability of the interaction with the target enzyme in a substrate-like manner (Laskowski; Qasim; Lu, 2000). In addition to P1 residue, the P1′ residue influences protein-protein association energy. Some inhibitors like BPTI have a high tendency of having an alanine (Ala) residue at the P1′ position and seldom replaced by glycine (Gly), while in case of non-inhibitory snake venom Kunitz toxins and ion channel blockers, there is a high variability in this P1′ residue. This might be the primary reason that prevents protease inhibitory function of the inhibitor (Grzesiak *et al.*, 2000). Furthermore, the presence of a conserved phenylalanine (Phe) residue at the P′18 position in most Kunitz inhibitors also determines the stability of the reactive site through internal hydrophobic interactions (Chand *et al.*, 2004).

Pharmacological properties: Despite structural similarities, snake venom Kunitz-type inhibitors exhibit a wide variety of biological functions involving inhibition of one or more serine proteases, blocking of ion channels, interference with the blood coagulation cascade, inflammation as well as fibrinolysis (Guo *et al.*, 2013b; Morjen *et al.*, 2013; Mukherjee and Mackessy, 2014; Mukherjee *et al.*, 2014; Shafqat *et al.*, 1990; Zhou *et al.*, 2004). On the basis of their function, non-neurotoxic Kunitz-type inhibitors can inhibit classical serine protease trypsin (Guo *et al.*, 2013b; Morjen *et al.*, 2013; Mukherjee and Mackessy, 2014; Mukherjee *et al.*, 2014) or chymotrypsin (Shafqat *et al.*, 1990; Zhou *et al.*, 2004) or both (Lu *et al.*, 2008; Zhou *et al.*, 2004). Snake venom Dendrotoxins like DTX I, DTX K, ~ -DTX, and δ -DTX, Kunitz-type homologs found in mambas, are potent blockers of potassium channels but without any inhibitory activity against the serine proteases (Harvey, 2001). Several other snake venom Kunitz toxins show diverse type of protease inhibition but without any ion channel blocking activity. For instance, OH-TCI from *Ophiophagus hannah* venom showed dual inhibition of both trypsin and chymotrypsin (He *et al.*, 2008); Bungaruskunin from *Bungarus fasciatus* exhibited inhibitory activity against trypsin, chymotrypsin and elastase (Lu *et al.*, 2008); Pr-mulgins isolated from the venom of *Pseudechis australis* were found to be potent inhibitors of trypsin, chymotrypsin as well as plasmin (Inagaki *et al.*, 2012); another KSPI named Nikobin identified in the venom gland cDNA library of *Vipera nikolskii* had

some protease inhibitory activity (Ramazanova *et al.*, 2011); PIVL, another serine protease inhibitor isolated from *Macrovipera lebetina* venom showed anti-tumor effect (Morjen *et al.*, 2013); Textilinin-1 from *Pseudonaja textilis* was capable of inhibiting trypsin as well as plasmin (Millers *et al.*, 2009). Due to its significant inhibition of plasmin it could emerge as an alternative to aprotinin, an anti-bleeding drug used in surgery; another Kuntiz type toxin BF9 from the venom of *Bunagrus fasciatus* retained the dual function of inhibiting serine proteases as well as blocking of potassium ion channels, giving a clue to the convergent evolution of this group of toxins (Yang *et al.*, 2014).

Phylogeny: Phylogenetic analysis of snake venom Kunitz/ BPTI inhibitors constitute three clusters based on their function (Zupunski *et al.*, 2003). Non-neurotoxic inhibitors from Elapidae and Viperidae family belong to the first cluster. The Kunitz inhibitors of Elapidae family showed high level of diversity when compared to that of Viperidae, suggesting that they belong to multigene protein families. The second cluster consists of Dendrotoxins (DTxs) and some Elapidae non-neurotoxic Kunitz inhbitors. DTxB and DTxE from *D. polylepis polylepis* and their homologs from *Dendroaspis angusticeps* (ε -DTxs) show strong protease inhibitory property but weak potassium channel inhibition (Sigle; Hackett; Aird, 2002; Strydom and Joubert, 1981; Tytgat *et al.*, 2001). Further, it has been proposed that ε -DTxs have intermediary characteristics between protease inhibitors and their neurotoxic homologs (Sigle; Hackett; Aird, 2002).The third cluster is made up of B-chain of -bungarotoxin, a heterodimer of phospholipase A_2 and KSPI. The B-chain do not have their own inhibitory or neurotoxic activity, however in synergy with PLA_2, it takes the form of a potent neurotoxin, a major component of *Bungarus* venom (Harris and Harvey, 1991).

Till date a large number of protease inhibitors from snake venom have been isolated and characterized. These non-lethal Kunitz toxins are highly specific towards their physiological targets like trypsin, chymotrypsin, elastase and plasmin. Besides, many other Kunitz toxins contribute to snake venom pathophysiology and lethality in combination with other snake venom components. Therefore, further study on Kunitz-type toxins may lead to development of more potent target-specific inhibitors for therapeutic applications and also to investigate the underlying structure-function relationship.

Conclusion

Though snake venom represents a toxic cocktail of proteins and peptides, it contains components that are of great pharmacological significance. With the advent of transcriptomics, proteomics and venomics, characterization and identification of various important venom components have become possible. The structure-function relationship of these diverse snake venom toxins have led to proper understanding of the overall toxin composition. Additionally snake venomics have provided useful insights into its evolutionary relationship, role in envenomation as well as biological functions. Despite their conserved structural feature, snake venom toxins have evolved to portray a wide variety of pharmacological effects upon envenomation. Such diversity of toxins within each snake venom protein family suggests their emergence by gene duplication prior to divergence thereby maintaining high level of functional variations in venom proteins. Furthermore, these toxins exhibit high specificity towards their targets which makes them an excellent natural source of novel drug leads or prototypes for drug development.

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