Chapter 1

General introduction and review of literature

1.1 Introduction

Venomous creatures have always remained in our curiosity and fascination from the onset of humanity. They have captured a place from the mythic period to 21st century. Snakes are limbless creatures, which possess one of the most sophisticated integrated defense and predatory system. Venomous reptiles venom glands have evolved more than 60-80 million years ago where extensive accelerated evolutionary tinkering is observed[1]. During this period the myriad of toxins have evolved which allows them to capture, kill and digest their prey. Snake venom is a complex mixture of proteins and peptides. However, the compositions of venom have been found to vary in species to species and within a species. These components exert multiple biological effects on prey or the victims which targets various physiological processes such as neurotransmission, blood coagulation, complement system and homeostasis [2–6].

This biological property of snake venom is of great interest to present world toxinologists which is channelized for a noble cause for humanity. Reptile venoms and toxins have heavy potential for contributing to treatment for various human ailment and diseases. Reports are available for various commercialized venom proteins which are used in modern times as drugs[7, 8]. These toxins can be used as a prototype for therapeutic agents, research tools, diagnosis of diseases and as tools to understand structure function, physiological and pathological processes [9–13].

Apart from snake venom and its usefulness, snakebites remain a potential threat. WHO has termed snakebite as the "neglected tropical disease" and globally, 421,000 envenoming and 20,000 deaths occur each year due to snakebite[14]. In India the "Big Four" *(Naja naja, Bungarus caeruleus, Daboia russelli, Echis carinatus)* are considered as the medically most important snakes which are responsible for most of deaths. *Naja kaouthia*, a medically important snake prevalent throughout South East Asia, is responsible for highest number of fatalities in the region[14–16]. To counter and

eradicate the problem, a thorough study and understanding of venom composition and effect of various venom components on pathophysiology is necessary.

The present chapter outlines a general introduction on snakes, their classification, distribution venom apparatus and most importantly introduction of various venom components and their functions.

1.2 Snakes

The Earth has an abundance of wildlife, which includes a wide variety of reptiles. Amphibians and reptiles appeared on earth millions of years ago and much before human colonizing on this planet. Snakes are under fascination may be due to the way they move, curl-up and coil which is so different from our own methods of locomotion. They have also occupied several places in mythology of various countries and religion well before the Christian era. In Mesoamerica, the feathered serpent *Quetzalcoatl* is a god of wisdom and *Wadjet is* the Cobra Goddess; the Cobra in Ancient Egypt was an important symbol of strength and power[17, 18]. *Nagas*, or snake deities in Hindu mythology, represent death and rebirth, fertility, wisdom, and knowledge [18]. *Nag Panchami* is a snake-worshiping festival still celebrated in parts of India. In Greek Mythology**,** Medusa & her sisters, whose writhing snake locks gave new meaning to the term 'bad hair' day [18]**.** *Ouroboros,* the snake eating its tail, symbolizes eternity, eternal renewal, immortality, and can be found in alchemy, Masonic seals, West African religions, and it has been adapted by modern people around the world [18]. *Jörmungandr* from Norse Mythology is the World Serpent who encircles the earth and can grasp his own tail. When he lets go of his tail, the world will end [18]. The *Hopi* of North America perform a snake dance to influence the weather. After the ceremony, the snakes are released so they can carry the prayers of the priests to the underworld where the rain gods live [17, 18].

Snakes and other distantly related reptiles like the alligators, crocodiles, turtles and lizards play a vital role in maintaining the earth's ecology and occupy a valuable place in the fauna of the ecosystem. Snakes are elongated, legless, carnivorous reptiles of the suborder Serpentes that can be distinguished from most legless lizards by their lack of eyelids and external ears. However, many lizards and most amphibians are also elongate and limbless. In fact, limb reduction,

or total loss of limbs, has occurred many times among lizards including anguimorphs, skinks, dibamids and gekkotans. There are also lizards with forked tongues, some geckos also have immovable eyelids and there are also lizards that lacks external ears, such as the earless monitor lizard *Lanthanotus borneensis* and many skinks [19, 20, 21]. In recent years there have been new and exciting fossil finds of snakes that show a mixture of primitive and advanced characters [22–24]. This has led to a plethora of morphological studies placing these fossils in a phylogenetic context and some controversial hypotheses as to the origin of snakes. Their scales come in all different colors and patterns making them good at camouflage [25, 26]. Molting of the skin in snakes is a regulated process dependent on growth [27]. They have jaws, skin and a flexible rib cage that can all spread wide enough to accommodate prey much larger than their resting diameter [28]. The forked tongues, which they constantly flick is a sensory organ which tests the air for smells [29, 30]. Snake sizes may be as small as worms to giants such as pythons, king cobras, anaconda and boas [31].

1.3 General distribution

Snakes are ectothermic organisms and they requires warm environments for their habitat. Regulation of metabolism in snakes is dependent on the surrounding environment [32, 33]. For this reason they go for hibernation during winter when even food supply becomes scarce [34]. Snakes live in deserts and other warm places butthey can be also found in mild cold areas [35]. However, reports are also available for snakes which can even live in cold climates, eg. *V. berus and V. aspis* [36]. Many snakes live in the trees and are called arboreal snakes [37]. They are well known for their camouflage. Some species of snakes live in the mountain terrain up to 16,000 feet [38], some of them live on the ground or in underground tunnels. Snakes living in water are also known [39, 40]. Many snakes live around swampy and cultivation lands eg. farms where food sources and shelter are easily found. Basically, snakes are present throughout the world except Antarctica (Figure 1.1) [14]. However, islands of the western Mediterranean, Atlantic and Caribbean, Madagascar, New Caledonia and New Zealand, Hawaii and many other Pacific islands, Ireland, Iceland and Chile lack venomous snakes (Figure 1.1) [41].

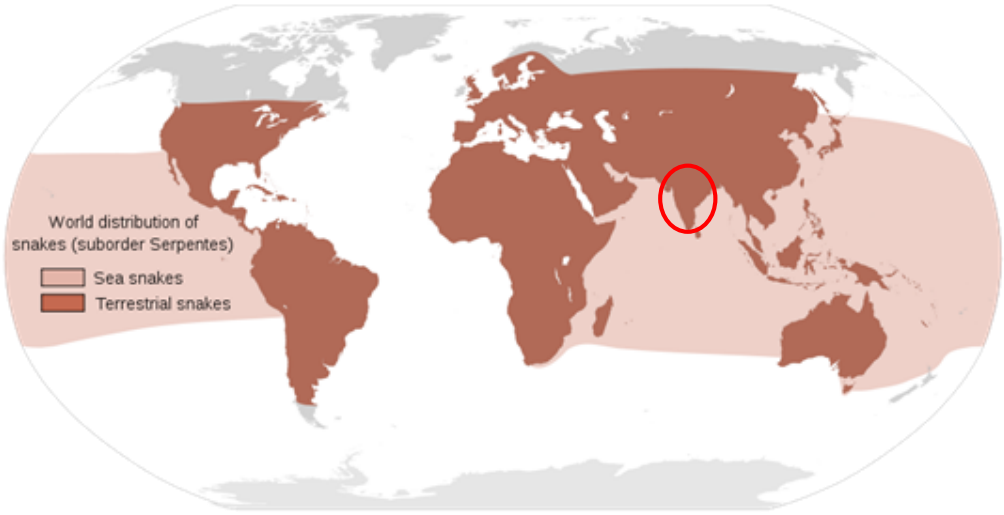


Figure 1.1: World distributions of snakes. The encircled area in red is to emphasize the distribution of terrestrial and sea snakes in India. (http://commons.wikimedia.org/wiki/File:World_Distribution_ofsnakes.svg#/media/File:World_distribution_of_snakes.svg)

In the distribution map of snakes the encircled area in red shows the distribution of snakes throughout India (Figure 1.1).

1.4 Classification and families of snakes

Snakes are in the class Reptilia and order Squamata, , infra-order Serpentes (snakes). At present, 3,496 snake species have been identified worldwide and classified into various families including Colubridae, Elapidae, Homalopsidae, Lamprophiidae, Pareatidae, Viperidae and Xenodermatidae [42]. The present study is based on a venomous species *(Naja kaouthia)* and hence forth we will be describing venomous species. The venomous snake species is comprised of five different families. The systematic classification of venomous snakes in the world has been given in table 1.1[42].

Table 1.1: Classification of venomous snakes in the world [42]

Sl no	Family	Common name	Distribution	Characteristics
1	Elapidae	Kraits, Cobras, Mambas, Coral snakes	Americas, Asia, Africa, Australia	Small head, short and fixed fangs
2	Viperdae	Viper	Europe, Africa, Asia	Flattened triangular head, large grooved fangs on the maxillary bone
3	Crotalinae	Rattlesnakes, Pit vipers	Americas, Parts of Southeast Asia, Southeast Europe	Similar with family Viperdae, but they possess heat-sensitive pits on head
4	Colubridae	Tree snakes	In all parts of the world, except Australia	Short grooved fangs at rear of upper jaws
5	Hydrophiidae	Sea snakes	Asia and Australia	Nostrils dorsally on head, flattened tail (mostly marine)

1.5 Cobras

Cobra is a Portuguese word which stands for snakes and this class of snakes can flatten their neck ribs with flaps of skin and forms a widened hood. They are classified in the elapidae family and are highly venomous and deadly. Cobras under *Naja* genus are termed true cobras and they can raise their body, flattening the neck as a warning signal when threatened. However, the "King Cobra", the longest venomous snake in the world, also can raise its body and merely flatten its hood, but it does not fall in the genus *Naja* and is not called a true cobra. Cobras are distributed all over, Africa and Asia (Table 1.2) [43].

The phenotypic features of cobras are generally long and slender bodies with smooth scales. They vary in size. Typically, a *Naja* species can reach up to 2 m. However, the King Cobra, average 4 m but can reach 5.5 m. Cobras have a shorter hollow fang in the front of the maxilla (proteroglyphous). Some species of cobras can spit venom and their fangs are accordingly modified for spitting. These are termed spitting cobras.

Table 1.2: Genera, distribution and habitat of cobras*.

Sl no	Genus	Taxon author	Species	Common name	Geographic range habitat	Habitat
1	*Aspidelaps*	Fitzinger, 1843	2	Shieldnose cobra	South Africa (Cape Province, Transvaal), Namibia, southern Angola, Botswana, Zimbabwe, Mozambique	Fossorial (subterranean)
2	*Boulengerina*	Dollo, 1886	1	Water cobra	Cameroon, Gabon, Democratic Republic of the Congo, Congo, Central African Republic, Tanzania, Equatorial Guinea, Rwanda, Burundi, Zambia.	Typically aquatic, inhabiting rivers, streams, lakes, swamps and also found in lowland forests
3	*Hemachatus*	Fleming, 1822	1	Spitting cobra (Rinkhals)	South Africa, Zimbabwe, Lesotho, Swaziland	Prefers grassland
4	*Naja*	Laurent, 1768	25	Cobra	Africa, Asia	Various including forests, savannah, semi-desert, cultivated areas, populated areas
5	*Ophiophagus*	Günther, 1864	1	King cobra	Bangladesh, Myanmar, Cambodia, China, India, Andaman, Islands, Indonesia, Laos,	Forest

					Thailand, Vietnam, west, Malaysia, Philippines	
6	*Pseudohaje*	Günther, 1858	2	Forest cobra	Angola, Burundi, Cameroon, Central African Republic, Democratic Republic of the Congo, Congo, Gabon, Ghana, Kenya, Nigeria, Rwanda, Uganda, Sierra Leone, Liberia, Ivory Coast, Togo, Nigeria	Forest (arboreal)
7	*Walterinnesia*	Lataste, 1887	2	Black desert cobra	Egypt, Israel, Lebanon, Syria, Jordan, Iraq, Iran, Kuwait, Saudi Arabia, Turkey	Desert

*Reproduced with due permission from Pest animal risk assessment – Cobra (all species), Department of Employment, Economic Development and Innovation, The State Of Queensland, Australia [43].

1.6 *Naja kaouthia* (Monocled cobra)

Naja kaouthia is commonly known as Monocellate Cobra or Monokel Kobra (true cobra). The scales of this cobra are smooth and arranged in 19-21(usually 21) rows in longitudinal pattern at mid body, with dark color and often a single dark band. The ventro-lateral throat has distinct spots. A pale, oval or circular marking having a dark centre (called as monocled) on the hood in adults is prominent [44]. The species fangs are not modified for spitting, however the venom discharge orifice is large. They are oviparous, ectothermic and amniote vertebrates. The adults can reach a length of 1.35

to 1.5 meters [44]. Incubation period of *N. kaouthia* eggs is 55 to 73 days from January to. It is a highly venomous snake which is responsible for mortality and morbidity in Southeast Asia [44].

N. kaouthia is distributed throughout Southeast Asia (Figure 1.2A). In India, it is prevalent in Uttar Pradesh, Bihar, Kolkata, Orissa and NortheastIndia (Figure 1.2A). The scientific classification of the species is given in figure 1.2B.

A

B

Scientific Classification	
Kingdom	Animalia
Phylum	Chordata
Class	Reptilia
Order	Squamata
Suborder	Serpentes
Family	Elapidae
Genus	*Naja*
Species	*N. kaouthia*

Figure 1.2: A. Distribution of monocled cobra *(N. kaouthia)*. Region colored in orange is resident of the snake species (Reptile database http://reptile-database.reptarium.cz/species?genus=Naja&species=kaouthia). **B.** Scientific classification of *N. kaouthia*.

1.7 *N. kaouthia* of Northeast India, Assam

The Northeastern region in India has diverse flora and fauna. However, the herpetofauna of Assam plains and south hills of the Brahmaputra (Major River in the region) are less well known. *N. kaouthia* is prevalent throughout Northeast India (Figure 1.3) and the common names are "Chakari Feti, Muga Feti (in Assamese), Kharoo (in Manipuri), Nag, Keutae (in Bengali) and Bsein-Iong (in Khasi) [45]. The classification and phenotypic characteristics are the same as explained in section 1.5. The species is a nocturnal predator but is often found basking in the day. They live in primary and secondary forests, agricultural areas and also around human settlements [45]. The common diets of the species are rats, fishes, small snakes and frogs [45]. The species is listed as LC (least concerned) under IUCN status. Cobra bites are common

and hence it is a medically important species in this region. However, deaths due to snakebite are also associated with factors like limited medical facilities, lack of awareness among the local people and poor transportation facilities [46].

Figure 1.3: *N. kaouthia* of Northeast India. Photo credits: Debabrata Phukan, Tezpur University, Tezpur, Assam, India.

1.8 Venom, poison and toxins

Various organisms' secretes different form of chemicals or biological agents (proteins/peptides) either for acquisition/digestion of prey or for defense against potential threats. Venoms are substances of biological origin and secreted by organisms and are injected, which causes various physiological impairment in the prey or victim. However, poisons are the substances which must be ingested (via skin or gastrointestinal tract) which finally affects physiology of the victim. Both venom and poisons are mixtures of toxin molecules and can contain other biologically active and inert substances, but toxins are pure compounds. However, both venom and poison work in a dose-dependant manner [47]. Venom formation and secretion by organisms is an evolutionary process and has ecological significance [48–50]. To consider a substance as venom, there must be an ecological need. B. G. Fry in 2003a reported that oral secretions developed in specialized glands which have biologically active compounds can be considered as venom regardless of any knowledge of its

specific effects [51] for example, snake venom, bee venom, scorpion sting venom etc. Now if we consider the herbivory inhibitors in marine algae which is secreted to keep predators at safe distance [52] does not signifies prey accusation nor digestion and cannot be considered as venom. In contrast, snake venom is a complex mixture of proteins and peptides which exerts multiple pharmacological effects in the target organism either for prey accusation or defense. Snake venom is produced in a specialized gland called venom gland which is connected to an apparatus called fangs for its delivery and injection.

1.9 Snake venom gland and delivery apparatus

Three major cell types such as secretory cells, basal cells and conical mitochondrial-rich cells aids in venom production in snake venom glands [53]. It was reported that the production of venom in venom gland is regulated by own mechanisms [53]. Venom is carried from the main gland and connected through primary duct to the accessory gland (Figure 1.4(1)). The function of the accessory gland was reported to be monitor wasteful flow of venom (Figure 1.4(1)). The snake can control whether to release venom or not is controlled by the accessory gland.. However, it was found that no protein or peptides gets added to venom when analyzed by electrophoresis and RP-HPLC and compared with main venom gland [2].

Snake venom glands are apparatus of high-pressure delivery systems especially in viperids, elapids and attractaspidids (Figure 1.4(2)) [54–56]. Injection of venom into prey or victims is connected with a series of mechanism. The muscle associated with gland as compressor muscle contracts, and then the primary venom gland gets pressurized which leads to expulsion of stored venom bolus. The root of venom gland to exit orifice present at the tubular fang remains closed when activated [57]. The root does not open at ambient pressure. Therefore it develops a high pressure end under striated muscle function which sustains until venom enters into the prey or victim [57]. When fangs does penetrates, the fang sheath gets lifted and opens the root for venom injection causing a fast discharge of venom [56, 58].

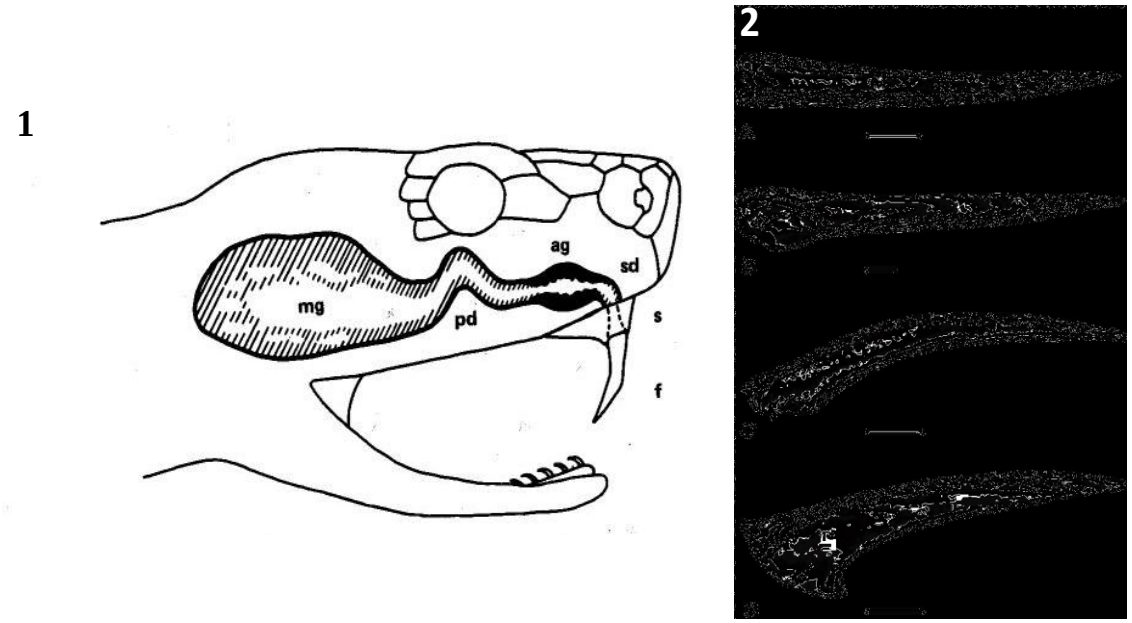


Figure 1.4: 1. Schematic drawing of venom gland connected to specialized fangs for its delivery. Venom synthesized in main venom gland (mg), transported through the primary duct (pd), connected to the accessory gland (ag), which is then connected to the secondary duct (sd), finally joints to the fang sheath pocket (s) and flows when necessary through the fang duct (f). Image adapted from Rattlesnake Venoms, (1982), Marcel Decker Inc. **2. Scanning electron micrographs of fossil and extant venomous snake fangs. A.** fangs of a fossil viper, **B.** fangs of an extant viper, *V. ammodytes* (both the images are from anterior view). **C.** Fossil of a curved elapid fang which is similar to modern elapid fangs, **D.** Fang of a krait, *B. sindanus.* (Both C & D fangs in medial view) have a suture along the anterior surface that extends from the basal end of the venom discharge orifice to the basal end of the tooth. Scale bars correspond to 0.5mm. Reproduced with due permission from Springer publishers [59].

1.10 Snake venom

Snake venom is a complex mixture of protein and peptides and it represents an adaptive trait which comprises both divergent and convergent evolution[61,62]. The cocktail is pharmacologically active proteins and peptides which constitutes over 90-95% of dry weight of the whole venom [62]. However, non-protein content includes amino acids, nucleic acids, metal ions, carbohydrates, biological amines and others [63]. Snake venom proteins acts on diverse molecular targets which involve vital processes of normal physiological systems. They can be termed as a naturally occurring gold mine of biologically active molecules which can be exploited for various therapeutic and other research interest [65, 66]. The composition analysis of

various snake venom reveals that it varies depending on species and also within species, which contributes to varying snakebite effects [66–68]. The pharmacological effects of various snake families are listed in table 1.3 [41]. The local effects of snake venom varies with just not respect to amount of venom injected but also specific biological characteristics of each snake family.

Table 1.3: Clinical symptoms of envenomation by various snake species. (Adapted from Ismail and Memish, 2003 [41])

Snake family	Clinical manifestations in victims physiology upon envenomation
Colubridae	Vomiting, headache, abdominal pain, renal failure, systemic bleeding, blood clotting failure, hemolysis etc.
Elapidae	Prominent neurotoxic effects characterized by blurred vision, vomiting, paralysis, severe headache, dizziness, hypersalivation and congested conjunctivae. Respiratory failure is common. Bites by spitting cobra victims suffer venom opthalmia and corneal opacification.
Viperidae	Local toxicity is severe. Bleeding and hemorrhage is common. Bruising, blistering and necrosis occur within few days. Immediate swelling of whole limb occurs. Haemostatic abnormalities are characteristic of viper bites. Most of the times renal failure is the major cause of death.
Atractaspididae	Local toxicity characterizing pain, swelling, blistering, necrosis and tender enlargement of local lymph nodes. Nausea, respiratory failure and ischemic ECG changes are observed.
Hydrophiidae	Severe neurotoxic symtoms are observed. Generalized rhabdomyolysis and haemostatic disturbance are seen. The symptoms mostly resemble to elapid envenomation however stiffness and tenderness of muscles and myoglobinuria observed which leads to renal failure. Hyperkalamia causing cardiac arrest is also observed.

1.11 Snake venom protein families

Snake venom proteins families can be broadly classified into enzymatic and non-enzymatic families based on their structural and functional properties. Various enzymatic and non-enzymatic families of snake venom proteins are listed in table 1.4

and 1.5. Some of the important enzymatic and non-enzymatic families which play a major role during envenomation are discussed below.

Table 1.4: Some enzymatic components of snake venom

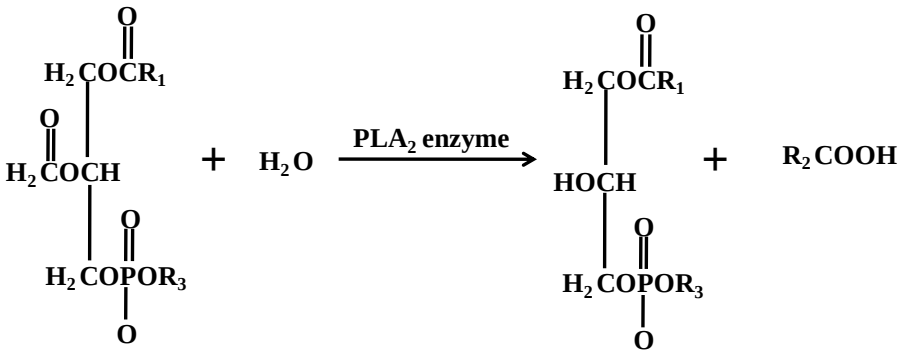
Protein families	~Mol weight (kDa)	Function	Biological activity
Serine proteinases	31-36	Catalysis of fibrinogen hydrolysis	Anticoagulation, disruption of hemostasis [69]
Acetylcholinesterase	55-60	Hydrolysis of acetylcholine	Paralysis [70]
Hyaluronidase	73	Hydrolysis of intestinal hyaluronan	Diffusion of venom components [71]
L-amino acid oxidase (homodimer)	85-150	Oxidative de-amination	Induction of apoptosis, cell damage [72, 73]
PLA_2 enzymes and PLA_2 based pre-synaptic neurotoxins	13- 15	Ca^{2+} hydrolysis of Phosphoglycerides/ fibrinogen	Myotoxicity, Membrane damage, myonecrosis, neurotoxicity [74, 75]
SV metalloproteinases	43 - 60	Hydrolysis of many structural proteins/basal lamina components	Induces DIC, highly toxic [76, 77]
Phosphodiesterase	94-140	Hydrolysis of nucleic acids and nucleotides	Hypotension, Depletion of cyclic, di and trinucleotides [3]
Cystein rich secretory proteins (CRiSPs)/helveprins	21-29	Possibly bocks cNTP-gated channels	Hypothermia, prey immobilization[78]

Table 1.5: Some non-enzymatic components of snake venom

Protein families	**~ Mol weight (kDa)**	**Function**	**Biological activity**
CRISP/Helveprins	21-29	Possibly blocks cNTP gated channels	Induced hypothermia, prey immobilization [79]
Kunitz-type serine proteinase inhibitor	6-7	Ion channel blocker	Inhibits serine proteases and blocks ion channels [80]
Nerve growth factors	14- 32.5	Promotes nerve fibre growth	Unknown apoptosis [81]
C type lectins	27-29	Binds to platelet and collagen receptor	Anticoagulant, platelet modulator [82]
Disintegrins	5.2-15	Inhibits binding of integrins to receptors	Platelet inhibition, promotes hemorrhage [83]
Prothrombin activators (Group C, D and A)	~45 - >250	Activates blood coagulation factor VII or factor X	Induce DIC, highly toxic [3]
3FTx family	6-9	Potent inhibitor of neuromusculer transmission, cardiac function.	Rapid immobilisation , paralysis, death [84–86]

1.11.1 Phospholipase A_2 (PLA_2)

PLA_2 is one of the major protein families present in all families of snake venoms which are hydrolytic in nature. Venom PLA_2 enzymes shares structural and functional similarity with mammalian PLA_2 enzymes. However, unlike the mammalian PLA_2 enzymes venom PLA_2s are toxic and induces wide spectrum of pharmacological effects. This class of protein family has been most extensively studied. They are esterolytic enzymes which hydrolyze glycerophospholipids at *sn*-2 position of the lipid backbone. The hydrolysis releases lysophopholipids and free fatty acids.



PLA_2 enzymes has a molecular weight of ~13-14 kDa with 115- 133 amino acids and 7 conserved disulfide bridges [88, 89, 76] . Structurally it consists of 3 α helices and 2 anti-parallel β sheets held together by the disulfide bridges [90]. The conserved structure in a PLA_2 enzyme are the N-terminal helix, calcium binding loop, anti-parallel helix, active site (His48) and β wing [74]. PLA_2 enzymes superfamily currently consists of 15 Groups and many subgroups. The assignment of PLA_2 enzymes to particular group is based on their catalytic mechanism (His/Asp, Ser/Asp or Ser/His/Asp hydrolase) as well as structural and functional mechanism [91]. These groups includes five distinct types of enzymes, namely the secreted PLA_2s ($sPLA_2$), the cytosolic PLA_2s ($cPLA_2$), the Ca^{2+} independent PLA_2s ($iPLA_2$), the platelet-activating factor acetylhydrolases (PAF-AH), and the lysosomal PLA_2s [91]. The Ca^{2+} ion acts as a cofactor during catalysis for the Ca dependant PLA_2s. The new world snake venom PLA_2 has been assigned to Group I and Group II where both differ by C- terminal end in Group II. Subsequently bee venom PLA_2 was been added to Group III. The sPLA2 and cPLA2 were categorized under Group IIA and Group IVA. [90].

Mostly PLA_2 enzymes exist as monomers but some PLA_2 enzymes interacts among them or with other protein to form complexes [74, 90]. The complexes formed can be covalent or non- covalent and are more potent then monomers [92]. These enzymes are known to target the nervous system, the cardiovascular system, muscular system, and circulatory system. Various pharmacological effects of PLA_2 enzymes are pre- and post synaptic neurotoxicity, Local and systemic myotoxicity, cardiotoxicity, anticoagulant effects, hemolytic activity, internal hemorrhage, convulsant activity, hypotensive activity, edema inducing activity, organ/tissue damage activity, affects cell migration and cell proliferation, bactericidal activity [74, 93–95]. Elapid venom has

been found to contain a major percentage of PLA_2 enzymes in its venom cocktail. *N. kaouthia* from East Indian origin has been well reported with various PLA_2 isoforms. Eg. Doley et al. in 2003, reported a PLA_2 enzymes (NK-PLA_2-I) which showed a preferential hydrolysis of phosphotidylcholine and also exhibited high anticoagulant, indirect hemolysis, liver and tissue damaging activity [88]. Simultaneously, in their further studies they had purified another PLA_2 isoenzyme (NK-PLA_2-II) in 2004 from the same snake species with membrane damaging activity [96]. However both differed in their preference to saturated and unsaturated fatty acids in their initial phase of attack. Further NK-PLA_2-A and NK-PLA_2-B were purified from *N. kaouthia* of the same geographical location which were reported to exhibited anticoagulant and cytotoxic activities [97]. The same group had identified two acidic anticoagulant PLA_2 isoenzymes (Nk-PLA2α and Nk-PLA2β) from *N. kaouthia* venom of Eastern India which was reported to exhibit different potency to inhibit thrombin and factor Xa via phospholipids independent, non-enzymatic mechanism [87]. Recently, venomics study of *N. kaouthia* from Thailand origin have revealed 13.5% PLA_2 enzymes present in the crude venom cocktail [67].

1.11.2 L-amino acid oxidase (LAAO)

It is a flavoenzyme (E.C. 1.4.3.2.) which catalyses the oxidative deamination of L-amino acid forming α-keto acid and ammonia.

$$RCH(NH^{+}_{3})COO^{-} + O_{2} + H_2O \rightarrow RCOCOO^{-} + NH^{4+} + H_2O_2$$

The pale yellow color of the snake venom is because of the presence of riboflavin present as a cofactors of LAAO [98]. LAAO consists of two identical sub units each with a molecular weight of 57-68 kDa. They are glycoproteins with 3-4% of carbohydrate. Structurally snake venom LAAO has three domains: an FAD binding domain, a substrate binding domain and a helical domain [98]. LAAO are known to induce edema, platelet aggregation or apoptosis during envenomation and the toxicity is caused by the H_2O_2 liberated in oxidation reaction [73, 98]. A purified LAAO from the venom of *N. kaouthia* of Thailand was reported to affect hemostasis [99]. Another LAAO from the venom of *N. kaouthia* had

been purified and its properties were studied by Tan and Swaminathan; however, the study of functional properties was not carried out [100].

1.11.3 Proteases Snake venom Metalloproteinase (SVMP)

SVMP are zinc-dependant enzymes of varying molecular weight (mol wt. 22-100 kDa) with multi-domain organization [101], and they are present in families Viperidae, Elapidae, Colubridae and Atractaspidae. SVMP comprises a protease domain and some contain a disintegrin-like domain, cysteine-rich domain and lectin domain. They have evolvedfrom ADAMs (A disintegrin and metalloproteinase) and SVMPs are classified into class P-I, P-II, P III and P IV based on their domain structure [76]. The first group, P-I, consists of only metalloproteinase domain; P-II has metalloproteinase domain followed by a disintegrin like domain; P-III are comprised of metalloproteinase, disintegrin like and cysteine rich domains and the P-IIIb group consists of metalloproteinase, disintegrin-like, cysteine and an additional lectin-like polypeptide linked by a disulfide bridge to the metalloproteinase containing polypeptide chain [101].

Because of such differences in their domain composition, their molecular weight range from 22 kDa to over 100 kDa [101]. Elapid venoms have been reported to contain metalloproteinases in the transcriptome of their venom gland [102]. They exhibit proteolytic activities both directly and indirectly. Haemorrhage, platelet aggregation, myonecrosis, blistering, impair muscle regeneration and ADP-induced platelet aggregation [76, 103].

1.11.4 Hyaluronidase

Hyaluronidases are enzymes which breaks hyaluronan or hyaluronic acid present in interstitial space. They are endo β-glycosidases (mol. Wt.- 73 kDa) with 5 conserved N-linked glycosylation sites [104]. Three of the sites have high potential for glycosylation. They are classified into 3 categories based on their mechanism of action and end product analysis: 1) hyaluronate 4-glycanohydrolase/ endo β-N-acetyl hexosaminidase (E. C. 3.2.1.35), which hydrolyses β-1,4 glycosidic bonds yielding tetrahexasaccharides as major product. 2) Hyaluronate 3-

glycanohydrolase/endo β-gluconoridase (E.C. 3.2.1.36.) which hydrolyses β-1, 3 glycosidic bonds yielding tertrasaccharides and hexasaccharides. 3) Hyaluronate lyases/ β-N-acetylehexosaminidase (E. C. 4.2.99.1.) which hydrolyses β-1, 4 glycosidic bonds yielding disaccharides [104, 105]. They primarily degrade hyaluron and increase the potency of venom components effects [106]. Venomic analysis of elapid venom reveals its presence in two Asiatic kraits (*Bungarus candidus* and *Bungarus fasciatus*) [107]. Also, venom gland transcriptomic analysis of Malaysia king cobra reveals presence of hyaluronidases with low mRNA expression [108].

1.11.5 Acetylcholinesterases (AChE)

Acetylcholinesterases are the enzymes which catalyses the hydrolysis of acetylcholine to choline and acetate (E.C. 3. 1. 1. 7.). The enzyme plays a role in function of peripheral neuromuscular junction. Venom acetylcholinesterases are stable and stereospecific in terms of hydrolysis of actyle- β- methylcholine [109]. They consist of non amphiphilic monomers. Snake venom contains the soluble globular form of AChEs. The catalytic site is composed of two subsites, the aromatic gorge where catalytic site is present and one peripheral anionic site [110]. Studies has showed that there might be two binding domain in active site having a glutamate residue which binds with cationic head of ACh and esteric site containing serine and histidine residues functioning as acid or base catalytic domain [109]. They are involved in depletion or accumulation of ACh (acetylcholine), resulting in flaccid or tetanic paralysis [109, 110]. Elapid venom is reported with acetylcholinesterases. Recently, venom from elapids (*Bungarus candidus* and *Bungarus fasciatus*) has been reported with presence of small amounts of high molecular weight enzymes such as LAAO, hyaluronidases, and acetylcholinesterases through venomics study [107].

1.11.6 Snake venom nerve growth factors

Nerve growth factors (NGF) are the proteins which are reported to stimulate the differentiation and maintenance of sympathetic and embryonic sensory neurons [81]. They are the member of neutrophin family which helps in maintenance and survival of neuronal cells. Neutrophins are a diverse class of proteins which are structurally similar. They are characterized by major families such as the nerve growth

factors (NGF), brain-derived neurotrophic factors (BDNF) and neurotrophin-3 (NT-3) [111]. NGF is reported to have extremely important conserved role in vertebrate homeostasis [111]. They are secreted as chemical weapon by the venomous advanced snake family elapidae (and to a lesser extent viperidae) which have characteristics consistent with the typical accelerated molecular evolution of venom components [111]. This is reported to include a rapid rate of diversification under the significant influence of positive selection. The majority of the positively-selected sites are found in the secreted β-polypeptide chain (74%) and on the molecular surface of the protein (92%); however, the core structural and functional residues remain highly constrained [111] whichgenerates active residues on the toxin molecular surface and are capable of inducing a myriad of pharmacological effects [111]. They have a moleculer weight of 14-32.5 kDa. NGFs are protomers containing 3 antiparallel β strands forming a flat surface to interact the subunits (mol weight ~ 13 kDa). The variability of NGFs are defined and confined to four loops which influence receptor specificity. They help in promoting nerve growth. Snake venom NGFs are seen to induce unknown apoptosis. In 2010, Wijeyewickrema coworkers isolated a NGF from the venom of *N. kaouthia*, which was found to act as metalloprotease inhibitor [112].

1.11.7 Cysteine-rich secretory proteins (CRISP)

Cysteine-rich secretory proteins (CRISP) are a class of glycoproteins in which primary structure is rich in cysteine residues. The cysteine residues are involved in disulfide bond formation and stabilize the tertiary structure of the protein for the proteins that are secreted in extracellular medium [79, 113]. They have 16 conserved cysteine residues which form 8 disulphide bridges. They are mainly associated with the ion channel activity of cysteine-rich domain (CRD). CRISPs bind to Zn^{2+} at their N-terminal are pathogenesis related (PR-1) domain; however the function is still not clear. Similarly, Zn^{2+} binding site were found to exist in all CRISP family [114]. These proteins have a moleculer weight of 21-29 kDa. Some CRISPs are involved in blockage of cNTP gated channels, leading to induced hyperthermia and prey immobilization [113, 78, 115]. Cobra venom has been reported to contain CRISP. In 2005, Osipov et al. identified four variants of CRISP in the venom of *N. kaouthia* and three

from *Naja haje* venom using combinations of liquid chromatography. However no functional characterization was carried out [113].

1.11.8 Snaclecs

Snake C-type lectins are a class of non enzymatic proteins which are found in many animals and binds to mono and oligosaccharides. The binding to carbohydrates is Ca^{2+} dependant [82, 116]. There are two major divisions in C type lectin family of proteins based on their structural and functional characterization namely, snake C-type lectin like family (CLPs) and sugar binding snake lectin [116]. CLPs are composed of homologus heterodimers which exists in monomeric or oligomeric forms $(\alpha\beta)_x$ whereas snake lectins are sugar binding proteins made of homodimers and are called classic sugar binding proteins. The molecular mass of C-type lectin is ~26-28 kDa homodimer while CLPs are heterdimers of subunits of α and β homologs, each of the subunit has a moleculer weight ~13-18 kDa [117, 118]. The globuler subunits are stabilized by 2 α-helices, 5 β-strands and a long loop to interact with other subunits. CLPs are found in αβ, $(\alpha\beta)_2$ or $(\alpha\beta)_4$ oligomeric forms [82, 116]. The snake lectins or classic sugar binding proteins are carbohydrate recognising proteins which causes agglutinations of the erythrocytes [117, 118].

1.11.9 Kunitz-type serine protease inhibitor

Kunitztype serine protease Inhibitors (KTSPI) belong to the functionally diverse bovine pancreatic trypsin inhibitor (BPTI) family and are characterized by conserved fold of approximately 60 amino acids, stabilized by 3 disulphide bonds [119]. Based on their function they can be divided into 2 groups, 1) Non-neurotoxic KTSPI: Trypsin and Chymotrypsin inhibitors. 2) Neurotoxic KTSPI: K^+ and Ca^{2+} channel inhibitors [80]. Non-neurotoxic Kunitz inhibitors interact with serine proteases by binding to their active site via exposed binding loop [119]. The neurotoxic domain of kunitz/BPTI serine protease inhibitors are present in snake neurotoxins, such as dendrotoxins, calcicludine and the B chain of b-bungarotoxin (b-Bgt).

1.11.10 Disintegrins

Disintegrins are non-enzymatic group of molecules with a molecular mass ranges from 4-14kDa. They are highly selective in nature and binds to integrin molecules. Mostly they exists as monomers, however homodimers and heterodimers are also reported [120]. Disintegrin domains are found to form part of metalloproteases PII with exception as observed in the chain of acostatin [121]. The dimmers are found to be with 60-67 amino acid residues with 10 cysteine residues. 8 cysteine residues form the intra- chain disulfide bridge and rest 2 forms the inter-chain disulfide bridge [122]. Most disintegrins contains a RGD motif at the tip of a loop which acts as adhesion factor to bind integrin molecules [123]. They are known to induce various biological and pharmacological activities such as platelet aggregation, angiogenesis, metastasis,and tumor growth through their interaction with various integrins [124–127]. Kaouthiagin, a metalloproteinase from *N. kaouthia* venom which cleaves von Willebrand factor reported to be comprised of the metalloprotease, cysteine rich and disintegrin like domain in its primary sequence. This toxin had an HDCD sequence in the disintegrin-like domain and uniquely had an RGD sequence in the Cys-rich domain [128].

1.11.11 Three finger toxins (3FTxs)

Three finger toxins (3FTxs) are well characterized snake venom protein family. They are abundantly found in elapids and hydrophiids venom and recently, they have been also reported in the transcriptome of Viperidae and Colubridae family [51, 129–131]. They are non-enzymatic polypeptides of 60-74 amino acid residues and four or five disulfide bridges. They are called "three finger toxins" (3FTx) as the three β stranded loops extends from the central hydrophobic core containing 4-5 disulfide bridges resembling three stretched fingers of a hand [132]. Additional disulfide linkage is also found in the first (non conventional toxins) and second (long chain α-neurotoxins and κ-neurotoxins) loop [86, 102] . The first report on snake venom 3FTx was isolation of α- bungarotoxin (α-bgt) was half a century ago by Chang and Lee [133]. Mostly 3FTxs exist as monomers eg. fulgimotoxin from Green Vine Snake *Oxybelis fulgidus,* a taxon-specific neurotoxin which targets nAChRs of skeletal muscle [134], Candoxin from Malayan Krait *Bungarus candidus* produces a postjuctional neuromuscular blockade and blocks nAChRs in oocyte expressed rat

muscle [135], Denmotoxin, from *Boiga dendrophila* (mangrove catsnake), a bird specific postsynaptic neurotoxin irreversibly inhibits indirectly stimulated twitches in chick biventer cervicis nerve-muscle preparations [136], β-cardiotoxin from *Ophiophagus hannah*, which targets β andregenic receptors and is a natural exogenous β blocker [137], Hemachatoxin (P-type cardiotoxin) from *Hemachatus hemachatus* venom, with highest similarity to particularly P-type cardiotoxins that are known to associate and perturb the membrane surface with their lipid binding sites [132]. However, dimeric 3FTxs are also reported eg. κ-neurotoxins from *Bungarus* species which shows highest homology with curaremimetic postsynaptic long neurotoxin. However, some striking differences between κ-bungarotoxin and other members of this particular group exists. This may explain its unusual ability to block neuronal acetylcholine receptors. Hemextin AB from *hemachatus hemachatus* venom which prolong clotting by inhibiting extrinsic tenase activity [138], haditoxin, from *Ophiophagus hannah* which target neuronal $\alpha_3\beta_2$ and $\alpha_4\beta_2$ nAChRs [139], irditoxin from *Boiga irregularis* (Brown Treesnake)[140] exhibits taxon specific neurotoxicity by producing a potent neuromuscular blockade at the avian neuromuscular junction etc. Functionally they have multiple pharmacological effects on the prey or the victim's physiology. They are reported to be neurotoxic, cardiotoxic, cytotoxic, anticoagulant, myotoxic, platelet aggregation inhibition etc [136–139, 141]. Further various activities of 3FTxs appeared as conserved and suggested to may have resulted from accelerated segment switch in exon to alter targeting (ASSET) from the recent analysis of 3FTx genes present in viperid *Sistrurus catenatus edwardsii* (Desert Massasauga) [131]. 3FTx family constitutes the best example of a unique structural scaffold to support multipotent biological functions. However despite of their common scaffold, they exhibit a wide array of potent toxic effects. Various biological properties of 3FTxs are given in table 1.6.

Table 1.6: Diversity in biological properties of 3FTxs and their molecular targets.

Sl no	Toxins	Targets/function
1	α- neurotoxins	Binds to muscle nicotinic acetylcholine receptors (nAChRs) [142]
2	κ- bungarotoxins	Binds to neuronal nAChRs [143]
3	Muscarinic toxins	Binds to different types of muscarinic receptors [144]
4	Fasciculins	Inhibits acetylcholinesterase [145]
5	Calciceptine and FS2	Blocks L-type calcium channels [146]
6	Cardiotoxins	Interact with phospholipids and forms pores on cell membrane, some cardiotoxins also binds to heparin, potassium channel-interacting proteins or $\alpha_v\beta_3$ integrin [147, 148]
7	Dendroaspins	Interacts with $\alpha_{IIb}\beta_3$ and inhibits platelet aggregation [149]
8	Hemextin	Inhibits factor VIIa [150]
9	β- cardiotoxins	Binds to β1- and β2- andrenergic receptors [137]
10	AdTx1, MTα and others	Antagonists of α-adrenoreceptors [151, 152]
11	Orphan group 3FTxs	Functional property is not known yet [51]

1.11.12 Accelerated evolution of 3FTxs

Venom proteins from snakes are found to be evolving more rapidly than other proteins by accelerated changes in the coding regions. 3FTxs, which are abundant in elapids and colubrid snake venoms undergo changes due to ASSET [153]. Through transcriptomic studies, 3FTxs were reported from viperid that have undergone extensive ASSET [131]. 3FTxs are evolved through ASSET in far greater ratio than other protein families [153]. Due to such exon exchange changes, functionally important residues might change which will impact on the overall property of the particular toxins. Such changes may also lead to novel functions. There are replacements in critical amino acid residues as well as changes in loop conformation which impacts the biological function and interaction of loops to specific target (Figure 1.5). Some of the toxins which undergone accelerated evolution has been found with demonstrated novel functions [146, 154]. As mentioned in section 1.11.12, 3FTxs have four conserved disulfide bridges. However, presence of fifth disulfide bond in their first or second loop categorizes them as non-conventional and long chain neurotoxins [86, 155]. This insertion of fifth disulfide bond in case of long chain neurotoxins was found to be due to intron-exon boundary [153] which was reported to

be due to insertion of a single nucleotide "A" in intron 2 causing a shift in splicing site [156], which leads to the insertion of a short segment (S5) containing cysteine residue [153]. Further frame deletion of nucleotide mutation at segment S4 leaded to a different sequence containing a new cysteine residue. These two cysteine residues formed the fifth disulfide bridge and the cyclic structure at loop II of long chain neurotoxin are important for α7 receptor binding with high affinity [86]. However, in case of non-conventional 3FTxs, the fifth disulfide bond at loop I was found to be due to ASSET which was found to have functional implications [86, 153]. Also, the changes in the amino acid residues within the identical segments are due to accelerated point mutations [153]. Therefore, as observed, it can be concluded that both ASSET and accelerated point mutations have been responsible for the diverse functional differences among the 3FTxs in elapids [153].

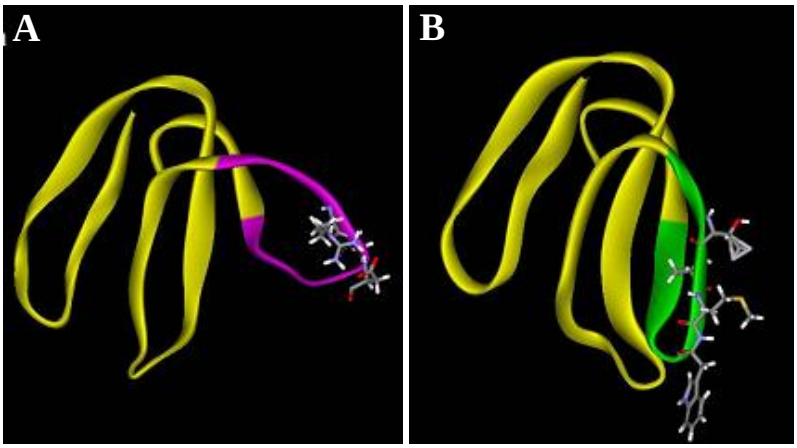


Figure 1.5: Ribbon model of A. dendroaspin (PDB ID: 1DRS) and B. FS2 (PDB ID: 1TFS). In dendroaspin, the segment CFTPRGDMPGPY is shown in magenta, whereas in FS2, the segment CPTAMWPYQTA is shown in green. Side chains of RGD and TAMW, the important residues in their functional motifs, are shown. This segment exchange significantly affected its activity: dendroaspin is a potent antiplatelet protein interacting with αIIbβ3, whereas FS2 is a potent blocker of L-type Ca^{2+} channels. Reproduced and adapted with due permission from R. Doley, 2009 [153].

1.11.13 Structural variations in 3FTxs

All 3FTxs have conserved residues for the proper folding and stabilization of their structure. There are four conserved disulfide bonds found in the hydrophobic core region (Figure 1.6). Some conserved aromatic amino acid acid residues (Tyr25 or Phe27) and charged residues (eg. Arg39 in erabutoxin-a and Asp60 in α-cobratoxin) also have significance in stabilizing the native conformation [85, 90, 157]. The stabilization of conformation is done by forming a salt link with C or N-terminus of the toxin molecule. It was proposed that the hydrophobic core from which the three loops emerge are responsible for binding to different functional groups where the functionally important amino acid residues are present [85, 157].

Mostly, 3FTxs exists as monomers (Figure 1.6A) with minor differences in their loop length and conformation including turns and twists. A minor structural variation in 3FTxs can lead to significant impact in their functions. In case of long chain 3FTxs and non-conventional group of 3FTxs, a fifth disulfide bond is present either in loop I or loops II (Figure 1.6B) [86, 155, 158]. The fifth disulfide bond at loop II of long chain neurotoxin and κ- neurotoxins gives a turn which leads to the formation of a short helical segment at the tip of the loop (Figure 1.6B) and the disulfide bridge at loop I of non-conventional 3FTxs twist and pushes the tip of the loop in an orthogonal position (Figure 1.6C).

3FTxs also have extensions at their C or N-terminal (Figure 1.6D). The colubrid 3FTxs are reported to have an extended N-terminal with additional seven amino acid residues capped by pyroglutamic acid. Long chain neurotoxins (LNTX) have 2-9 residues at C-terminal end additionally [136]. Many short chain neurotoxins from sea snakes and Australian elapids have additional free Cys residue in loop I (4^{th} position) (Figure 1.8), SNTX-1, Cys residue underlined). The structure and function of this cysteine residue which exists as free thiol is not known [159].

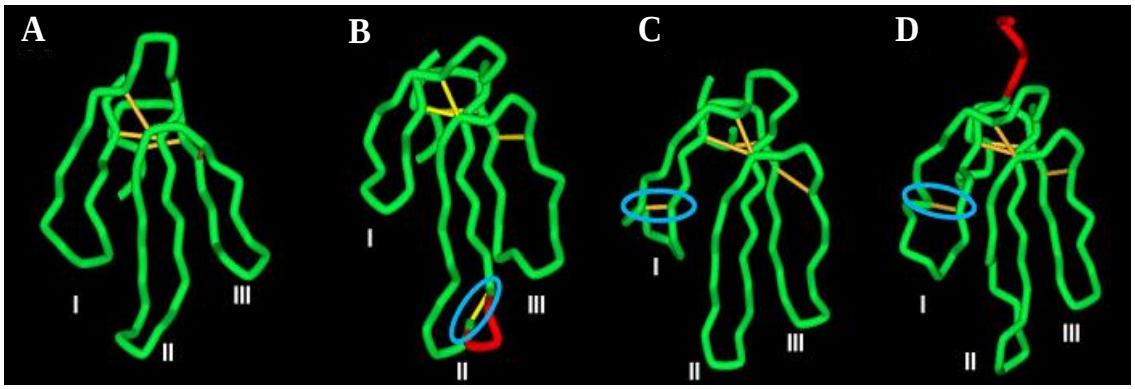


Figure 1.6: Three-dimensional structures of 3FTxs showing loops and disulphide bridges. A. Short-chain (Erabutoxin (1QKD)), **B.** Long-chain (k-bungarotoxin (1KB A)), **C.** Non-conventional toxin (Candoxin (1JGK)) and **D.** Non-conventional toxin with N-terminal extension (Denmotoxin (2H5F)). The extension of second loop in long-chain 3FTx due to fifth disulphide Bridge and extension of N-terminal of denmotoxin is shown in red color. Reproduced and adapted from Kini and Doley, 2010.

Dimeric 3FTxs such as κ-bungarotoxins and haditoxins exists as non-covalent homodimers [139, 160]. The subunits are placed in anti-parallel arrangement where the dimeric interface is formed at outer strands of third loop. However, covalently linked heterodimers and homodimers of 3FTxs are well reported, such as irditoxin, a covalently linked heterodimer, where each subunit contain an additional cysteine residue involved in forming the inter-chain disulfide bridge [140]. Also, covalently linked homodimers of α-cobrotoxin (α-CT-αCT) and covalently linked heterodimers of α-cobrotoxin with cytotoxins from *N. kaouthia* are also reported [161].

3FTxs mostly do not undergo any post-translational modifications. However, glycosylation of cytotoxin 3 isolated from *N. kaouthia* of Thailand was reported [162]. The N-linked carbohydrates were found to attach at Asn29. Comparative analysis of these cytotoxins with unglycosylated cytotoxins was found to be lower at two orders of magnitude which infers to the functional impact of glycosylation [162]. Also, transcriptomic analysis of *Sistrurus catenatus edwardsii* venom glands cDNA library reveals presence of 1-3 potential N-glycosylation sites [163]. But no protein from the crude venom has been isolated which corresponds to these transcripts. Figure 1.7, as adapted from Kini, 2002, explains the functional diversity of various 3FTxs [157]. The

important point to mention is that this superfamily of proteins is structurally similar but functionally different.

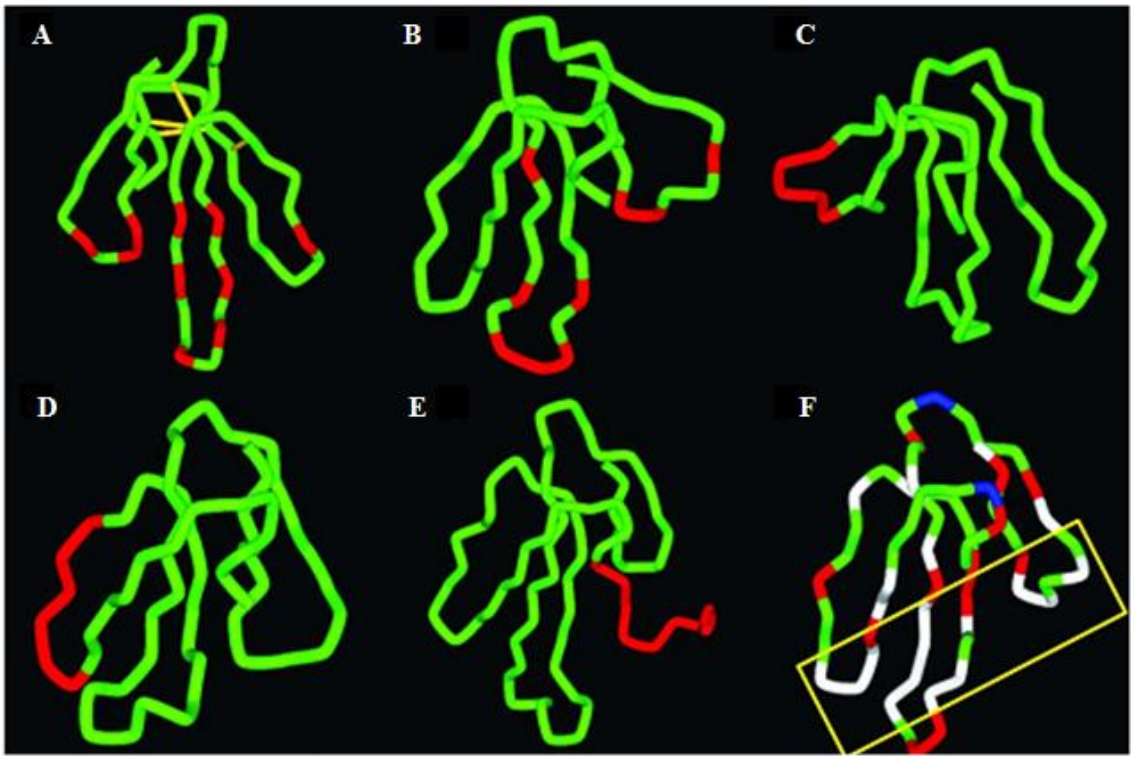


Figure 1.7: Functional sites in 3FTxs. **A.** The residues involved in the binding of erabutoxin a (1QKD) to muscle nAChR, **B.** fasciculin (1DRS) to AChE, **C.** mambin (or dendroaspin, 1DRS) to platelet receptor αIIbβ, **D.** and FS2 to L-type calcium channel are shown in red. Only in erabutoxin a conserved disulfide bridges are shown. **E.** Analgesic site of hannalgesin is also shown in red. **F.** Cytolytic site of CTx has a hydrophobic region (white residues highlighted in yellow box) and cationic residues (shown in red). Acidic residues in CTx are shown in blue. All structures are in the same orientation as shown in Figure 1.15 and Figure 1.16. Adapted from Kini, 2002 [157] and reproduced with due permission.

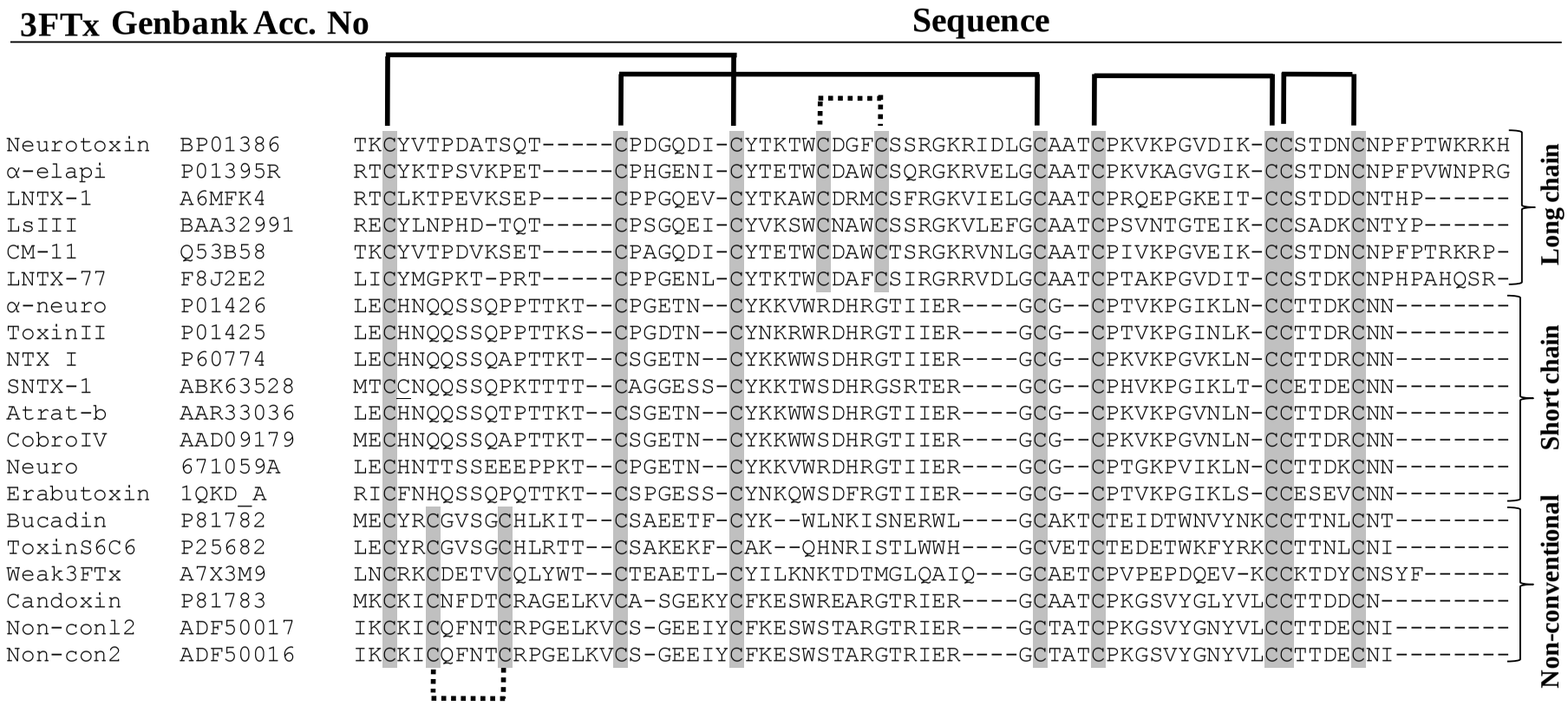


Figure 1.8: Multiple sequence alignment of amino acid sequences of 3FTxs. Alignment of snake venom short-chain, long-chain and non-covalent 3FTxs. The conserved cysteine residues are highlighted and the disulphide linkages are shown in solid lines whereas the fifth disulphide linkage in long chain and non-covalent 3FTx are shown in dotted lines. The N-terminal extension of colubrid 3FTx is highlighted in grey color and the gaps are filled with dashes.

1.11.14 Classification of 3FTxs and their functional properties

3FTxs have conserved three dimensional structure with three β-structured loops extending from a hydrophobic core. However, the 3FTxs differs in their functional properties. Based on their functional properties these toxins are classified into:

1.11.14.1 Neurotoxins

Most of the snake venom neurotoxins has a three finger fold and are members of 3FTx family. Neurotoxic 3FTx interferes with chemical transmission or cholinergic transmission at postsynaptic cleft of PNS (Peripheral nervous system) and CNS (Central nervous system). These neurotoxins have been further classified based on their receptor selectivity into: a) α-neurotoxins which targets nAChRs (nicotinic acetylcholine receptors), b) κ-neurotoxins which targets neuronal nAChRs and c) muscarinic toxins targets various subtypes of muscarinic receptors respectively, d) These toxins have been extensively utilized in identification, characterization and understanding of several subtypes AChRs in nervous systems.

1.11.14.1.1 α-neurotoxins

Three finger α-neurotoxins are responsible for blockade of neuromuscular transmission at skeletal neuromuscular junction by binding to postsynaptic nAChRs [142, 164]. Till date they have been extensively reported from elapid venom [155, 164–167]. Considering the amino acid sequence and presence of fifth disulfide bond, they are classified into short chain neurotoxins (60-62 amino acids, four conserved disulfide bond) and long chain neurotoxins (66-74 amino acids, four conserved disulfide bond and addition fifth disulfide bond at second loop) [157]. They are also known as curaremimetic neurotoxins for mimicking neuromuscular blocking effect of plant alkaloid d-turbocurarine (also known as "arrow poison") [168]. However, the blocking effects of α-neurotoxins are approximately 15-20 fold higher than d-turbocurarine and have poor reversibility [168]. Chemical modification and genetic engineering techniques have revealed the structure function relationship of α-neurotoxins. Previously, the functional site of erabutoxin-a (short neurotoxin) was identified by mutagenesis approach by Tremeau and co-workers in 1995 from the venom of sea krait *Laticauda semifasciata* [169]. The crucial amino acid residues were found to be Ser8, Glu10, Lys27 and Arg33. A set of peripheral residues His6, Ser9, Tyr25, Phe32

and Gly34 were also found to surround the functional site which plays a minor role in determining toxins affinity towards receptor. Another study of α-neurotoxin (α-cobratoxin) from the venom of *N. kaouthia* was performed by Antil et al., in 1999 (Figure 1.20). It is a long chain neurotoxin and they have revealed a functional site residing at central loop of the 3FTx. The crucial amino acid residues were found to be Lys23, Asp27, Arg33 and Lys49. The two toxins differ by:

i) The first loop of the toxin is not important for functional attributes in α-cobratoxin whereas in case of erabutoxin the tip of the first loop is crucial for binding with its receptor.

ii) The C-terminal end is critical for α-cobratoxin; however it has no function in case of erabutoxin [170].

iii) The fifth disulfide bond in α-cobratoxin forms a helix-like conformation which is reported for binding to α7 nAChRs in long chain neurotoxins.

1.11.14.1.2 κ-neurotoxin

It is a protein with ~66 amino acid residues and 10 cysteine residues (five disulfide bonds) [171]. The arrangement of the cysteine residues in its primary structure is similar with the long chain α-neurotoxins however they have short C-terminal tail [172]. κ-neurotoxins are reported to exist as homodimeric complex [160]. The dimers are interlinked by hydrogen bonds and at the dimeric interface the two monomers involves six main chin-main chain hydrogen bonding [172]. However, the two chains of the dimer are not identical in its folded conformation. The interacting monomers are not identical in its folded conformation. Subtle difference was observed at the tip of the central loop (Cys27-Pro36) [160]. Arg34 at the tip of the central loop is crucial for activity of α and κ-neurotoxins activity. Functionally, κ-neurotoxins recognize α3β2 and α4β1 (not α1[172]) which binds to nAChRs and specifically interacts with neuronal α3β4 nAChRs [171, 172]. However, the role of dimer in binding specific receptors is still not understood.

1.11.14.2 Cardiotoxins

In nature, polycationic membrane-active peptides are devoid of a disulfide bond which fuses and penetrates cell membranes. They are reported to remain unordered in solution but adopt helical structure when bound to phospholipid membranes [173]. However, snake venom cardiotoxins (CTXs) are structurally different, characterized by a three finger fold. They are mostly reported from elapid venom and constitute about 50% of the dry weight in some cobras. They possess ~60 amino acid residues with eight conserved cysteine residues (four conserved disulfide bonds). Unlike α and κ-neurotoxins, CTXs are amphiphilic and cytotoxic against a variety of cells, including cancerous cells. CTXs are involved in inducing a wide arrays biological effects in the prey or victims. Some of the biological and pharmacological activities of CTXs are listed below:

i) Contraction of smooth muscle and skeletal muscle in humans and rats.

ii) Induction and inhibition of platelet aggregation.

iii) Hemolysis.

iv) As channel blockers deactivating ATPase (Na^+/K^+ activated) pump responsible for regulating ion concentration inside the nerve axon.

v) Inhibit ACTH stimulated lipolysis in isolated fat cells and steroidogenesis in isolated adrenal cells.

vi) Act as an anticoagulation agent.

vii) Are also involved in binding to $\alpha_v\beta_3$ integrins and inhibit bone resorption

The structure-function relationship and mechanism of action of CTXs are poorly understood. However, CTXs possess potent cytotoxic and hemotoxic activity and are thought to be interacting with membranes and penetrating cells [174]. The functional site of CTXs is a continuous hydrophobic patch of almost 40% of the molecular surface which starts from loop I and covers large parts of loop II and loop III [175]. The conserved basic residues helps in recognizing negatively charged head groups of phospholipids present in cell membrane. Internalization of CTXs through cell membrane is by a hydrophobic interaction between loop I of CTXs and membrane [175].

1.11.14.3 Anticoagulant 3FTxs

3FTxs with antiplatelet and anticoagulant effect were initially reported from cardiotoxins isolated fron *Naja nigricollis crawshawii* venom (spitting cobra) [176]. The structure-function relationship and mechanism of action of these proteins are well elucidated [177–179].

A novel anticoagulant protein complex was isolated and characterized from *Hemachatus haemachatus* (African Ringhals cobra) venom [138, 150]. This complex has two 3FTxs, hemextin A and hemextin B, which have identical molecular diameter in gas and solution phase. Both monomers has β-sheet structures and it is the only known heterotetrameric complex of two 3FTxs [138]. The complex assembly of hemextin AB tetramer is enthalpically driven with some conformational changes which also accompany the complex formation. The complex is stabilized by electrostatic and hydrophobic interaction [138]. Functionally, the protein complex affects blood coagulation in a synergistic manner by prolonging the prothrombin time. However, the dissection approach (In this approach blood coagulation cascade is initiated 'upstream' from the inhibited step which will result in elevated clotting times, while initiating the cascade 'downstream' from the inhibited step will not affect the clotting time)[180, 181] reveals that hemextin A prolonged the coagulation time but hemextin B did not had any effect on blood coagulation. The complex was not found to affect Stypven time (thrombin time) and hence it was proposed that the complex targets the extrinsic tenase complex (TF-FVIIa). Hemextin A was found to inhibit reconstituted TF-FVIIa but hemextin B did not. However, hemextin B was found to increase the inhibitory effect of hemextin A by complex formation. The inhibition was non-competitive with a K_i value of 50nM [150]. When tested with various serine proteinases, hemextin A and hemextin AB complex were found to specifically inhibit FVIIa and its complexes. Additionally, it was also found to inhibit plasma kallikrein activity [150]. This is the first three finger anticoagulant complex isolated from snake venom.

1.11.14.4 Fasciculins

These are the class of basic 3FTx family with a net charge of +4. Only three fasciculins from *Dendroaspis* of Elapid family have been characterized. Fasciculins

are composed of 61 amino acid residues in a single polypeptide held together by 4 disulfide bridges [182]. The 3 fasciculins differs from each other on the basis of varying position of the amino acids Tyr, Asn, Ile, Lys, Thr, Asp and Met in a single polypeptide. Fasciculins inhibits acetylcholinesterase with high selectivity and potency by binding to the peripheral anionic site of the enzyme. Presently they are being used as pharmacological tools to inhibit acetylcholinesterase selectivity [183].

1.11.14.5 Muscarinic toxins

Acetylcholine receptors play a major role in nervous system and are the prime targets of nicotinic and muscarinic neurotoxins. Nicotinic acetylcholine receptors (nAChRs) are blocked by curare and stimulated by nicotine, whereas muscarinic acetylcholine receptors (mAChRs) are blocked by atropine and stimulated by muscarine. Five types of mAChRs (M1-M5) control various physiological processes, including function of heart, smooth muscles, neurotransmitter release, gene expression, glandular secretions, arousal, eye movements while sleeping, and cognitive functions such as learning and memory. Binding and interacting with these receptors by snake venom toxins brings blockade of normal physiological processes. MT1 and MT2 are the first reported muscarinic toxin from the venom of *Dendroaspis angusticeps* (Eastern green mamba) [184]. The muscarinic toxins are divided into two structural groups A (65 amino acids) and B (66 amino acids) [185]. The sequences of these toxins are reported to have 30 identical amino acid residues [185]. A toxin (M_2-toxin) from group B was sequenced and was found to contain 63 amino acid residues with four conserved disulfide bonds [186].

mAChRs act through GTP binding proteins (G proteins) to stimulate or inhibit intracellular physiology by interacting with G protein coupled receptors (GPCR) [185, 187]. MT1 and MT2 inhibited binding of non selective ligand [^{3}H]-QNB to rat synaptosomal membranes by about 50%. This study indicated both toxins could not recognize all the subtypes of muscarinic receptors. However, several toxins from the same venom and three toxins (MTα, β and γ) from *Dendroaspis polylepsis* have been isolated. Although there are hundreds of conventional small molecular muscarinic antagonists available, where, none of them is specific to one subtype of mAChR. Most of them have equal affinity towards two or three of muscarinic subtypes [188].

Muscarinic toxins are highly selective and this has made them invaluable research and diagnostic tools for various biomedical application [185, 188, 189].

1.11.14.6 Non-conventional 3FTxs

The non-conventional 3FTxs are isolated from Elapidae and rear fanged Colubridae venom [190]. They are monomers and consist of 62-68 amino acid residues with 8 conserved cysteine residues (four disulfide bonds) in its hydrophobic core. Additionally one disulfide bond is present in its first loop unlike in α and κ-neurotoxin. This class of toxin is also referred as melanoleuca type toxin [191]. Many non-conventional toxinspreviously isolated from various *Naja* species, such as CM9a from *N. kaouthia* [191], WTX from *N. kaouthia* [192], neurotoxin-homologue NNA2 *(Naja n. atra*) [193] and synthetic weak toxin Wntx-5 *(Naja sputatrix),* were studied for various pharmacological characterization [194]. This class of toxin was found to show low and variable lethality (LD_{50} ~5-80mg/kg) compared to highly lethal neurotoxins (LD_{50} ~0.04-0.3mg/kg). They are referred to as weak toxins due to low lethality and produce a weak inhibition of both muscle $\alpha_2\beta\gamma\delta$ and homopentameric α7 receptors in micromolar inhibitory concentrations [164]. However, there is evidence which suggests the above convention might not be universal to all types. γ-bungarotoxin, a postsynaptic toxin isolated from the venom of *Bungarus multicinctus* showed a lethal potency (LD_{50}) of 0.15mg/kg which is compared to the curaremimetic toxins [195]. The lethal potency of bucandin, another non-conventional toxin isolated from *Bungarus candidus* venom was not lethal in mice even at 50mg/kg [196].

Functionally, Arg33 is reported to be the crucial amino acid residue for α/κ-neurotoxins to interact with α7 receptors [197] and also for κ-bungarotoxin for binding to neuronal $\alpha_3\beta_2$ receptors [198]. Candoxin, a non-conventional toxin isolated from the venom of *Bungarus candidus,* is also found to carry the crucial Arg33 residue. This toxin is reported to produce an irreversible blockade in nerve-muscle preparations. The toxin irreversibly blocks rat neuronal α7 receptors at nanomolar concentrations. In long chain neurotoxins, a cyclized helix conformation at tip of the second loop reported to be crucial for interacting with α7 receptors. However, candoxin possesses a fifth disulfide bridge on the first loop. This shows candoxin, despite sharing a common scaffold with long chain neurotoxins, binds with α7 receptors, which might be due to additional determinants present in the toxin. Neuronal nAChRs are known

to mediate subtle brain functions, such as memory, attention, cognition and nociception [199]. They are also implicated in pathology of Parkinson's disease, Alzheimer's disease, schizophrenia, epilepsy and Tourette's syndrome, and there is less understanding of distribution and their physiological roles [198–201]. These venom peptides with subtype specificity to several neuronal receptors can be of significant value in research and studying various neuronal receptors.

1.11.14.7 L-type calcium channel blockers

This group of 3FTxs includes calciseptines and FS2 isolated from the venom of *Dendroaspis polylepsis polylepsis* (Black mamba) [202, 203]. Both toxins are reported to contain sixty amino acid residues with four conserved disulfide bridges [202, 203]. Calciseptine and FS2 significantly block L-type calcium (Ca^{2+}) channels in heart and skeletal muscle [202, 203]. Kini and Evans in 1996 predicted the functional site of polypeptides between residues 42 and 47 by searching for proline residues which mark the flanks of protein-protein interaction sites [204]. Further, experiments were performed to determine the functional site present on FS2 and calciseptine (Figure 1.9).

Previously, calciseptine has been reported to inhibit spontaneous contraction induced by 40mM K^+ and Bay K8644 on rat portal vein and uterus contraction of rat thoracic aorta and cardiac preparation respectively. The mechanism of action of calciseptine was confirmed by observing inhibitory effects on voltage-clamped A7r5 cells and ventricular myocytes where the toxin blocked Ca^{2+} channels [202]. The toxin binds to the 1,4-dihydropyridine recognition site on L-type calcium channel of rat synaptosomal membranes [202].

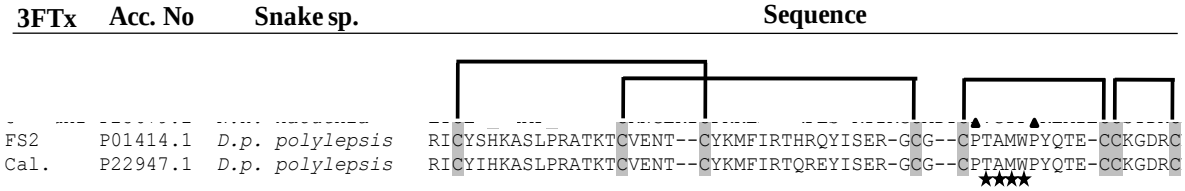


Figure 1.9: Amino acid sequence of FS2 and calciseptine. The proline residues forming the bracket are marked with triangles. The crucial amino acid residues for binding to L-type Ca^{2+} channels are marked with stars. The cysteine residues are highlighted in grey and the solid lines depict the four conserved disulfide bonds.

FS2 is a highly homologues toxin with calciseptine which also inhibits L-type Ca^{2+} channels [205]. The amino acid sequence of calciseptine, consisting the four amino acid residues (Thr43, Ala44, Met45 and Trp46) bracketed by two proline residues is the protein-protein interaction site. The prediction of functional site in calciseptine was tested using synthesized peptide L-calchin (L-type calcium channel inhibitor) [146]. This synthetic peptide inhibited L-type Ca^{2+} channel current in patch-clamped, isolated, ventricular myocytes in a voltage-independent manner. This experiment suggested an L-type Ca^{2+} channel blocking effect of FS2 and calciseptine [146]. Furthermore, the functional site of toxin binding to L-type Ca^{2+} channel has been identified. Unlike other reported toxins, the functional site in this toxin is located in the third loop.

11.14.8 β-cardiotoxin

This is a family of 3FTxs, isolated from the venom of *Ophiophagus hannah* with β-adrenergic receptor antagonist property [137]. β-cardiotoxin shows 55% identity to conventional cytotoxins/cardiotoxins (CTXs); however, it was found to be to distinct in its functional and structural properties. The secondary structure of β-cardiotoxin was found to be unique when compared with group 1 CTX nanixain and group 2 CT, CM18 [137]. Conventional CTXs have conserved amino acid residues apart from the conserved cysteines, among which Tyr22 and Tyr51 in CTXs play a vital role in maintaining structural and functional properties of CTXs [206]. However, in the case of β-cardiotoxin, Val23 and Val53 are present which homologues to Tyr22 are and Tyr51 in CTXs is. These changes may explain the changes in unique secondary structure of β-cardiotoxin. Functionally, β-cardiotoxin was found to be non-lethal up to 10mg/kg, whereas CTXs are highly lethal proteins [137]. β-cardiotoxin do not show hemolytic activity on washed RBCs, whereas CTXs are found to have potent hemolytic activity. CTXs are reported to produce increased heart rate in anesthetized rats, whereas β-cardiotoxin decreased heart rate in a dose-dependant manner [137]. Decrease in heart rate was showed by competitive binding assay due to binding of β-cardiotoxin to β-adrenergic receptors (ARs) responsible for rapid cardiac contraction. The adrenergic receptors (ARs) are abundantly expressed in cardiomyocytes. β-blockers are the drugs of treatment for many cardiovascular diseases (CVDs) [137]. At

present all of the β-blockers are a small molecule which belongs to aryloxy propanolamines [207]. However, these drugs are reported with adverse side effects and hence newer specific β-blockers are pursued actively [207]. Hence, understanding more details of structure-function relationships of β-cardiotoxin will help in designing novel therapeutics and can be an effective promise in future in treating CVDs.

1.11.14.9 Platelet aggregation inhibitors

3FTxs with antiplatelet activity have been reported with dendroaspin [208] and mambin [154], both isolated from the venom of *Dendroaspis jamesoni kaimosae* (eastern Jameson's mamba) and *Dendroaspis viridis* (western green mamba) [209]. Structurally the proteins contain fifty nine amino acid residues and four conserved disulfide bond forming the conserved three finger fold. Dendroaspin has been reported with Arg-Gly-Asp (RGD) tripeptide sequence in its molecular structure which is known to be involved in adhesive function of several proteins. Dendroaspin interferes with fibrinogen and its receptor glycoprotein GP IIB-IIIa complex ($\alpha_{II}\beta_3$) and results in potent inhibition of platelet aggregation (IC_{50} ~170nM) [154, 157]. Integrins are the heterodimeric class I family transmembrane receptor proteins which regulates cell matrix and cell-cell adhesion processes. The β_3 integrin subgroup protein $\alpha_{II}\beta_3$, is essential for platelet function and platelet aggregation in thrombosis and hemostasis [210]. The structure function relationship of dendroaspin by mutational studies have revealed that replacement of Arg-Gly-Asp sequence by Arg-Tyr-Asp and Arg-Cys-Asp tripeptide sequences promoting selective inhibition of β1 and β_3 integrins [211]. Another study by Schleifer in 1997, reveals that substitution of Arg with residues such as Lys, His, Gln and Ala alters its intigrin specificity [212]. The functional site of dendroaspin was found to be located at the tip of the third loop. Another 3FTxs, Gamma-bungarotoxin, isolated from *Bungarus multicinctus* venom containing 68 amino acids, including 10 cysteine residues and a TAVRGDGP sequence at 30-37 position was isolated [213]. The protein was found to carry the tripeptide Arg-Gly-Asp (RGD) sequence at the second loop and is similar to that of other RGD-containing proteins. However, inhibition of platelet aggregation by gamma-bungarotoxin was found to be only with an IC50 value of 34 μM[213]. Both dendroaspin and disintegrins are found to compete for binding to these receptor proteins. There is no structural or sequence similarity between the two except for presence of RGD tripeptide motif [214].

1.11.14.10 Hannalgesin

Hannalgesin was isolated from the venom of *Ophiophagus hannah* which have similarities with neurotoxins and its molecular structure is made up of 72 amino acid residues. The protein exhibits neurotoxicity and potent analgesic effect in experimental mice [215]. The toxin was injected intraperitoneally at a dose of 24ng/g to mice and analgesic activity was determined. Analgesic activity of hannalgesin was also determined by using other injection routes such as intravenous, periocular and intra cerebroventricular which suggested effect of the toxin in central nervous system [215]. The functional site of this toxin was reported to present in C-terminal end of the toxin and was predicted using proline bracket hypothesis [146, 215].

1.11.14.11 Orphan toxins

3FTxs isolated from snake venom whose function is not known are referred as "orphan toxins". It was observed that a long term evolutionary process has given rise to the diversity of 3FTxs which are consistent with birth and death model of multigene family [216]. They are grouped in 20 different clades (I-XX) [217]. Gene duplication and accelerated evolution can be accounted for such a diverse and large number of toxins.

1.12 Aim and scope of the thesis

Snake venom have been a natural source of bioactive proteins. Over the years, these proteins and peptides have been contributing in venom research, antivenom development and understanding various molecular pathways. Present day snake venom research using proteomics and transcriptomics approaches has certainly attributed in obtaining new leads in venom research. However, most of the toxins have not beenthoroughly explored. Northeast Indian monocled cobra *(N. kaouthia)* is one of the medically important snakes of this region having an "O" shaped hood mark [44] similar to the Asiatic cobras. *N. kaouthia* from South East Asia have been extensively studied and are reported to have abundant 3FTxs and other major proteins such as PLA_2 [67]. However, venom composition of these species has been found to vary among the snakes from different locations [68]. Evolutionarily, the prey has been reported to play an important role for such variations in venom composition [218]. Although venom of *N. kaouthia* from other parts of India has been studied, so far

only few toxins have been isolated and characterized. For example, 3FTxs with antiplatelet activity, fibrinolytic activity and cytotoxicity, PLA_2s with strong anticoagulant activity have been reported from *N. kaouthia* of Eastern India origin [87, 141, 219–221]. Also, a detailed proteomics study of *N. kaouthia* of Indian origin has not been carried out.

Hence, the present work has been carried out with the following objectives to understand the biochemical and biological properties of unexplored *N. kaouthia* venom of Northeast Indian origin as well as isolate and characterize one of the isoform of 3FTx family:

Aims and objectives:

1. **Biochemical and biological characterization of crude *N. kaouthia* venom**
2. **Partial compositional analysis of crude *N. kaouthia* venom**
3. **Purification of Nk-3FTx, a 3FTx from *N. kaouthia* venom**
4. **Functional characterization of purified Nk-3FTx**