CHAPTER I

INTRODUCTION

1.1 Introduction

One of the most enigmatic animals in this world today is snake. The study of snake and snake venom has stimulated the minds and flared imagination of general people as well as scientific community more, than many of the other subjects. Even today, a majority of us are under the tremendous load of misconceptions and have never been tried to reveal the truth and fair sides of these innocent creatures.

India has vast potential and rich diversity of snake fauna, of which only 242 species have been identified including 57 poisonous or harmful species (Sharma, 1998). The evolution of snakes dates back to some 70 million years in the Cretaceous period. It is not possible to decipher the exact ancestry of snakes from available fossil records because such fossil records are virtually non existent. Only the present living species of snakes, mainly the burrowing snakes are available to explain the ancestry of snakes from the lizard-like reptiles (Sharma, 1998).

North-East India has 92 species of snakes under 34 genera, of which 15 species are venomous. Forty six percent of these are pit vipers and the rest belonging to family Elapidae (Mathew, 1998), which comprises cobras, kraits and coral snakes. The variety of cobra snakes, prevalent in Eastern and NorthEast India is Naja Kaouthia and is responsible for a large number of snakebite mortality (Mathew, 1998; Mukherjee and Maity, 2002). Further, treatment of cobra bite patients is a medical emergency.

In recent years, the subject of snake venom has been receiving much more interest from the standpoint of biochemistry, toxicology, pathophysiology, pharmacology, immunology and biomedical research. Besides the production of
antivenom, snake venom has many exciting and wide ranges of medical applications. For example, Contortrostatin, a protein purified from snake venom, may help to stop metastasis of breast tumors (Markland, 2001). Components of pit viper venom have shown a great promise in breaking blood clot, that can help stroke patients, curing osteoporosis and tumor, production of anticoagulants, production of blood pressure medicine, break down of cell membranes that would provide treatment for leukemia and cancer etc (Mara, 1993). Venom protein, 'Atroporin' and 'Laotree', isolated from venom of Crotalus atrox and Naja naja kaouthia respectively showed potential anticancer activity when tested on human and animal cancer cells (Lipps, 1999). It is obvious that these complex enzymes, derived from snake venom could produce potentially huge medical benefits for mankind. Besides protecting these unique creatures as part of a responsible effort to preserve our natural heritage, it seems that protecting venomous snakes is in our own best medical and health interest.

1.2 Medical aspect of snakebite: The snakebite problem

Snakebite is a global problem, especially in the tropical countries. It has been estimated that, 5 million people are bitten by venomous snakes annually around the World, thereby resulting about 1,00,000 fatalities (Chippaux, 1998). Snakebites are not systematically reported in most of the countries. Very few countries possess a reliable epidemiological reporting system capable of providing precise data on snakebites. Instead, scientific reports and publications have to be used to assess the magnitude of the problem posed by snakebites. The datas thus obtained are generally more precise and reliable but often cover limited geographical areas or deal with special aspects (Chippuax, 1998).

1.2.1 Epidemiology of snakebite in Asia

Snakebite reports from Asia are higher than those reported from European countries. In Asia, there is a wide variation in snakebite incidence according to human activities and snake species involved. According to the reports of World Health Organization (WHO) (1981), every year in Asia, 4 million people are bitten by
snake, of which, 50% of them are by poisonous snake. The annual rate of death can be estimated as 1,00,000. The highest mortality rate due to snake envenomation occurs in Myanmar (Formerly Burma) followed by India, Philippines, Sri Lanka and Thailand (Chippaux, 1998). Therefore, it might be inferred that in the Indian subcontinent, snakebite problem is severe and acute one (WHO, 1981). In Nepal, for example, 3189 cases of snakebite including 144 cases of death have been reported between January to December 2000 from 15 districts hospitals of that country (Sharma et al., 2003). Overall death rate among all the cases of snakebite was 4.5% (Sharma et al., 2003).

1.2.2 Epidemiology of snakebite in India

Every year over 3,00,000 incidences of snakebite occur in India of which about 10,000 people die (Jena and Sarangi, 1993). Despite many reports on the snake envenomation in India, however, most of the datas were not collected from actual field survey but were from hospital records and therefore did not represent the true picture (Hati, 1984). Moreover the account of death due to snakebite, as reported by previous investigation, was probably copied from books after books, some were being exaggerated (Hati, 1984, 1992). Swroop and Grab (1954) initiated a systematic study of the snakebite problem in India. They statistically analyzed the data available on snakebite from different parts of the country, covering a period from 1940 to 1949 and came to the conclusion that, in India, West Bengal (eastern zone province) has the highest snakebite mortality cases. In Maharatra, the annual incidence of severe envenomation is about 70 per 1,00,000 inhabitants and the mortality is about 2.4 per 1,00,00 people per year (Gaitonde and Bhattacharya, 1980). According to Chippuax (1998), incidence of snakebite in India ranges from 66-163 victims per 1,00,00 people, out of which 1.4-68 deaths occur every year.

1.2.3 Epidemiology of snakebite in other countries

In Great Britain approximately 200 people have been hospitalized every year from snakebites but no death has been reported since 1975. Moreover, in France,
the number of such cases is higher (Warrell, 1986). Chippaux (1995) reported an annual incidence of approximately 5 cases per 1,00,000 residents in the Department of Yonne (150 Km South of Paris) and similar incidence has been reported elsewhere in the country. In Switzerland, the morbidity is very low corresponding to approximately 0.1 case per 1,00,000 residents per year (Stahel et al., 1985). In rural areas of Southern Europe morbidity rates are higher. In Spain and Italy the annual incidence of snakebites can reach 5 per 1,00,000 people. Mortality of 0.2 per 1,00,000 population was recorded in Costa Rica during the year 1990-1993 (Rojas et al., 1997).

In Canada and USA the annual incidence of snakebites is similar to that observed in Europe. In North America each year approximately 45,000 snakebites occur out of which 15 individuals thus bitten die. In Central and South America, the prevalence of snakebites is significantly higher. In Brazil, during 1990 to 1993 about 20,000 snakebite cases have been reported out of which 90 cases were fatal (da Silva et al., 2003). However in Australia, the snakebite cases are very few; every year 300 cases are reported those requiring treatment for envenomation with between 1-4 fatalities (Steward, 2003).
Fig. 1.1. Indian monocled cobra (*Naja kaouthia*)
1.3 Indian monocled cobra (*Naja kaouthia*): Systematic classification and distinctive features

1.3.1 Systemic classification

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1.3.2 Distinctive features

Medium-sized; smooth, shiny scales; wide head and neck; distinctive hood marking different from that of the spectacled cobra.

1.3.3 Description

The skin of the Monocled cobra is shinier; the hood is rounder and smaller than that of the spectacled cobra. The colour varies widely from yellowish to greenish brown to black with ragged bands. The commonest nuchal pattern is an annular marking with a black central rim: the single ‘eye’ or ‘monocale’. The underside is yellowish white. Monocled cobra superficially resembles with Spectacled cobra, but there are many small differences. The former cobra inhabits wetter areas like in pond, lakes etc and feeds mostly on aquatic animals like small fish, frog, small snakes etc. Average length of *Naja kaouthia* is 1.5 m (Whitaker, 1978).
1.3.4 Distribution

Indian monocled cobra (*Naja kaouthia*) is distributed only in some parts of West Bengal, Assam, Andaman Islands, Sikkim, Nepal, Thailand etc (Whitaker, 1978).

1.4 Evolution of snake venom: A general consideration

In venoms of Elapidae, mostly non-enzymatic toxins in addition to enzymes are present, whereas in Viperidae and Colubridae venoms, different enzymes are predominant. Numerous evidences indicate that non-enzymatic toxins found in Elapidae snake venoms have evolved from digestive enzymes (Strydam, 1979; Kochva et al., 1983; Kochva, 1987).

Presumably, the evolving venom glands started to produce enzymes those were already secreted by the pancreas of the respective snake ancestors and against which inhibitors are present in the blood (Kochva et al., 1983). Interestingly, remarkable sequence homology between mammalian pancreatic phospholipase A$_2$ and certain Elapidae venoms have been detected. It may thus be speculated that, phospholipase A$_2$ toxin having a presynaptic mode of action found in at least some same species of all the families of venomous snake as well as post synaptic neurotoxins found in Elapidae, which has evolved from an ancestral dimeric protein, possessing both phospholipase and ribonuclease activity (Strydam, 1979). In another example, the enzyme crotalase from eastern diamond rattle snake (*Crotalus adamanteus*) is homologous to β-chain of thrombin, a highly advanced serine protease. These enzymes share homologies with many other serine proteinases (Meier, 1986). These evidences reinforce the hypothesis that enzymes of pancreatic origin were subsequently produced by reptilian oral gland (Kochva et al., 1983). This may explain the well-known resistance of snakes against their own venoms. Presumably, the ancestors of snakes had a pancreas secreting phospholipase and other enzymes with corresponding inhibitors in their blood to prevent noxious effects. In consequence, a molecular co-evolution of enzymes and their inhibitors seems plausible (Kochva et al., 1983).
Evidences have recently been presented to show that variation in the venom composition of pit viper *Calloselasma rhodostoma* (Serpents: Viperidae) is closely associated with its diet reinforcing that diet plays an important role in snake venom evolution (Daltry et al., 1996). The mutational changes in the gene which is the primary basis of evolution also contributes significantly to the venom variation that occurs between closely related species or even within a species (Glenn et al., 1983; Yang et al., 1991; Assakura et al., 1992; Daltry et al., 1996; Fry et al., 2002). During the venom evolution, toxin encoding genes undergo frequent gene duplication, followed by diversification into different structures and functions (Kordis and Gubensek, 2000). In contrast to the mitochondrial protein encoding genes, toxin-encoding genes do not favour one codon for an amino acid over another (Fry et al., 2003). Further, mutation in the codon are likely to occur in position 1 rather than 2 or 3. These small changes in the amino acid bring profound effect upon the specificity and multiplicity of the venom component. Thus, the fundamental molecular basis of venom evolution favours a multiplicity of actions and consequently a multiplicity of toxins (Fry et al., 2003).

1.5 Phospholipase A₂ (PLA₂): A toxic enzyme of snake venom

PLA₂ enzymes are one of the most biologically active proteins present in the snake venom. In addition to the digestion of prey, PLA₂ enzyme is involved in many pharmacological effects, for example, neurotoxicity, myotoxicity, cardiotoxicity, anticoagulant, hemolytic, internal hamorrhage, edema etc which disturb the normal physiological processes of victim (describe in detail in section 2.3.4 Chapter II).

Snake venom contains large number of PLA₂ enzymes and till date, 280 PLA₂ enzymes have been purified and characterized from various snake venoms (Danse et al., 1997; Tan et al., 2003). These isoenzymes are found to share common homology in their catalytic site and three dimensional structure, but differ in their spectrum of toxin effects. The distinctive functional difference among PLA₂s cannot be correlated with their structural differences and the structural similarities make the structure-function relationship subtle, complicated and challenging (Kini, 1997).
Further, due to protein-protein interaction between PLA₂ enzymes, they aggregate and cause problem in purification and hence interpretation of their biological activities (Kini, 1997). Therefore, purification and characterization of PLA₂ isoenzymes from the same venom will contribute in better understanding of their structure-function relationship as well as the mechanism by which they induce various pharmacological effects in victims. It has been reported that depending upon the geographical origin, venom of Indian spectacled cobra (*Naja naja*) contains as many as 9 to 14 PLA₂ isoenzymes (Shiloah et al., 1973; Kini and Gowda, 1983). Although few of the PLA₂ enzymes were purified and characterized from the venom of *Naja kaouthia* (Joubert and Taljaard, 1980, Wang et al., 2001), but mournfully there is a dearth of knowledge on the biochemical properties and biological activities of PLA₂ enzymes from the venom of *Naja kaouthia* of the Indian origin.

Therefore, a comparison of PLA₂ isoenzymes from both the venom samples and from the same origin will help us to understand the species specific variation among the PLA₂ isoenzymes between these two venomous snakes and the impact of this variation in the pathogenesis following bite.
1.6 Aims and objectives of present study

a. A comparison of PLA$_2$ isoenzyme pattern of _Naja naja_ and _Naja kaouthia_ venom samples.

b. Isolation and purification of two major PLA$_2$ isoenzymes from venom of _Naja kaouthia_ of Indian origin.

c. Characterization of some biochemical properties and pharmacological activities of purified PLA$_2$ enzymes.

d. Pharmacological screening of medicinal plants of North-east India to ascertain their inhibitory activity against purified phospholipase A$_2$ enzymes of _Naja kaouthia_ venom.