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STUDIES ON SOME BIOCHEMICAL AND PHARMACOLOGICAL PROPERTIES OF TWO PHOSPHOLIPASE A_2 ISOENZYMES ($NK\text{-}PLA_2\text{-}I$ AND $NK\text{-}PLA_2\text{-}II$) FROM INDIAN MONOCLED COBRA (*Naja kaouthia*) VENOM

THESIS SUBMITTED FOR THE DEGREE OF
Doctor of Philosophy

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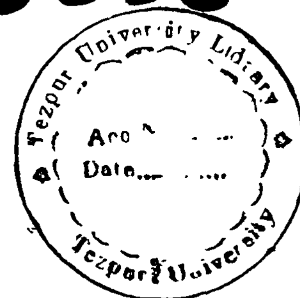
By



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Dedicated to my beloved parents

Late Sri Lalit Kumar Doley
&
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CERTIFICATE BY THE SUPERVISOR

This is to certify that Mr. Robin Doley, M.Sc. has work out the thesis entitled " Studies on some biochemical and pharmacological properties of two phospholipase A_2 isoenzymes (NK-PLA_2-I and NK-PLA_2-II) from Indian monocled cobra (*Naja kaouthia*) venom" under my supervision. He has fulfilled the requirements of the regulations relating to the nature and prescribed period of research at the Tezpur University. The Thesis embodied accounts for his own findings and has not been submitted previously anywhere for any degree whatsoever by either him or anyone else.

AKMukherjee

Date: April 15, 2004

(A. K. Mukherjee)

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Date: 15/04/04 **(Robin Doley)**

Declaration

I hereby declare that due to the lack of proper facility at Tezpur University, following experiments/sample analyses were carried out at other institutes.

1. Circular dichroism (CD) spectrophotometer experiment was kindly performed by Dr. A.K. Mukherjee at the University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT-06032-3305, USA.
2. MALDI-MS analysis was done by Dr. Stephen Eyles, Mass Spectrometry Facility, University of Massachusetts, MA, USA
3. Protein sequencing was done by Prof. Anil K. Lala at National Facility for Protein Sequencing Indian Institute of Technology Mumbai, India.

Date: 15/04/04

(Robin Doley)

List of Abbreviations

AchE = Acetylcholinesterase

ALP = Alkaline phosphatase

ATP = Adenosine triphosphate

ATPase = Adenosine triphosphatase

AMPase = Adenosine monophosphatase

BSA = Bovine serum ablbumin

ρ-BPB =ρ-Bromophenacyl bromide

BCIP/NBT = 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium

β-Btx = β-bungarotoxin

CD = Circular dichroism

CM = Carboxymethyl

CPK = Creatine phosphokinase

DbTx = Daboiatoxin

DEAE = Diethylaminoethyl

DTNB = 5,5'-Dithiobis (2-nitro benzoic acid)

DTT = Dithiothreitol

EDTA = Ethylene diaminetetra acetic acid

ESI-MS = Electrospray ionization mass spectrophotometry

FFA = Free fatty acid

GC-MS = Gas chromatography- Mass spectrophotometry

LDH = lactate dehydrogenase

LD_{50} = Lethal dose for 50% animal

MALDI-MS = Matrix Assisted Laser Desorption/Ionisation Mass Spectrophotometry

MED = Minimum edematous dose

MD = Molecular dynamics

NMR = Nuclear magnetic resonance

NPSC = Nitrophenylsulphenyl chloride

PBS = Phosphate buffer saline

PC = Phophatidyl choline

PE = Phophatidyl ethanolamine

PLA_2 = Phospholipase A_2

PLIs = PLA_2 inhibitor proteins

PLPC= Palmitoyl-2-linoleoyl-sn-glycerol-3-phosphatidycholine

PMSF = Phenyl methyl sufonyl fluoride

PPP = Platelet poor plasma

PS = Phosphatidyl serine

PT = Prothrombin time

PVDF = Polyvinylidene difluoride

RBC = Red blood corpuscles

RP-HPLC = Reverse Phase High Performance Liquid Chromatography

S.D = Standard deviation

SDS-PAGE = Sodium dodecyl sulphate polyacrylamide gel electrophoresis

TBST = Tris buffer saline containing Tween 20

TCA = Trichloroacetic acid

TFA = Trifluoroacetic acid

TLCK = Nα-ρ-Tosyl-L-lysine chloromethyl ketone

TMB/H_2O_2 = Tetramethyl benzidine/Hydrogen Peroxide

TNBS = Trinitrobenzenesulfonate

TPCK = α-Tosyl-L-ρ phenylalanine chloromethyl ketone

UV/Vis = Ultra violet/Visible

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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 form 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 Myanmmar (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 Maharastra, 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 Systemtic classification

Phylum- Chordata
Group- Vertebrata
Subphylum- Gnasthostomata
Class- Reptilia
Subclass- Diapsida
Order- Squamata
Suborder- Ophidia
Infra order- Caenophidia
Family- Elapidae
Genus- *Naja*
Species- *kaouthia*

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 A_2 (PLA_2): A toxic enzyme of snake venom

PLA_2 enzymes are one of the most biologically active proteins present in the snake venom. In addition to the digestion of prey, PLA_2 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 PLA_2 enzymes and till date, 280 PLA_2 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 PLA_2s 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 PLA_2 enzymes, they aggregate and cause problem in purifcation and hence interpretation of their biological activities (Kini, 1997). Therefore, purification and characterization of PLA_2 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 PLA_2 isoenzymes (Shiloah et al., 1973; Kini and Gowda, 1983). ***Although few of the PLA$_2$ 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 PLA$_2$ enzymes from the venom of Naja kaouthia of the Indian origin***.

Therefore, a comparison of PLA_2 isoenzymes from both the venom samples and from the same origin will help us to understand the species specific variation among the PLA_2 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.

CHAPTER II

REVIEW OF LITERATURE

2.1 Snake venom: A general consideration

Snake venom is an evolutionary adaptation to immobilize prey, secondarily use in defense. Venoms are highly toxic secretions produced and stored in specialized salivary glands of snakes. It is a unique mixture with reference to their biochemical and pharmacological properties and mostly consists of non-cellular proteins (Kochva, 1987; Meier, 1990). Snake venoms are either colourless or yellowish in colour. The latter colour is due to presence of L-amino acid oxidase (Thomas and Pough, 1979). The content of the solid matter of venoms of Elapidae, Crotalidae and Viperidae has been found between 18 to 52%, 16 to 51%, and 28 to 31%, respectively (Elliot, 1978). Milked venom also contains tissue debris (Mukherjee and Maity, 1998).

2.1.1 Physical properties of snake venom

The snake venom is slightly acidic in nature and specific gravity ranges from 1.03 to 1.07. The relative viscosity of snake venom varies from 1.5 to 2.5. The solubility of Elapidae venom in H_2O is much higher than that of Viperidae and solubility of all venoms increase in physiological saline (Sarkar and Devi, 1968).

2.1.2 Proteins and polypeptides in snake venom

Over 90% of the solid snake venom components are protein and polypeptides responsible for exerting pharmacological effects in victims (Sarkar and Devi, 1968; Stocker, 1990). Proteins/polypeptides of venom can be further divided into enzymes and non-enzymes.

2.1.2.1 A brief account of enzymes present in snake venom

Activity and number of enzymes present in snake venom varies from venom to venom and about 26 enzymes have been identified. Although no single venom contains all of them, at least 10 of these enzymes are present in every snake venom, while the remaining are found in several combinations in different varieties of snakes (Sarkar and Devi, 1968; Mebs, 1970; Lee, 1979; Bieber, 1979; Iwanaga and Suzuki, 1979; Stocker, 1990). A comparative study on the activities of enzymes in venoms of 42 species comprising Colubridae, Elapidae, Viperidae and Crotalidae snake families led to the conclusion that Elapidae venoms are rich in phospholipases, phosphodiesterase, nucleotidase, ATPase and cholinesterase, whereas Russell's viper and pit viper venoms contain proteases, coagulant, kinin-releasing and argininester hydrolyzing enzymes (Sarkar and Devi, 1968; Mebs, 1970). Enzymes present in the snake venom play an important role in inducing toxicity following bite. Important enzymes present are protease, acetylcholinesterase, ATPase, AMPase, L-amino oxidase, phospholipase A_2, etc. Proteases are responsible for the local effects like haemorrhagic, necrosis and muscular degeneration etc. (Sarkar and Devi, 1968; Soto et al., 1988; Ownby, 1990, Mukherjee and Maity, 1998). The acetylcholinesterase, which is one of the toxic enzymes of cobra venom, acts on acetylthiocholine to liberate the choline and acetate (Guieu et al., 1994). 5′-nucleotidase enzymes are responsible for hydrolysis of terminal phosphate from adenylic acid (AMP). This is a Zn^{2+} and EDTA sensitive enzyme (Sarkar and Devi, 1968; Elliot, 1978; Iwanaga and Suzuki, 1979). Venom ATPase, when injected, is known to give "shock" to the victims due to sudden hydrolysis of ATP (Kini and Gowda, 1982). The L-amino acid oxidase purified from venom of King cobra (*Ophiophagus hannah*), having a molecular mass of 1,35,000 dalton (by gel filtration) causes aggregation of platelets through the formation of H_2O_2, and subsequent thromboxane A_2 synthesis requiring Ca^{2+} but independent of ADP release (Li et al., 1994). Both procoagulant and anti-coagulant enzymes from snake venom have been isolated which affect different steps of blood coagulation cascade (Seegers and Ouyang, 1979; Teng et al., 1984).

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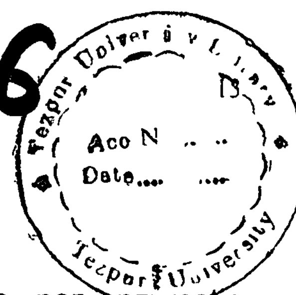

2.1.2.2 Non-enzymatic snake venom proteins

Other than enzymes, snake venom contains numerous non-enzymatic proteins, which play an important role in toxicity of the venom. Various non-enzymatic proteins have been isolated and characterized. Neurotoxins isolated from snake venom have been found to impair the nerve function, mainly neuromuscular transmission (Mebs, 1990). These neurotoxins may be either: - (i) Post synaptically active neurotoxins, which block neuromuscular transmission by binding specifically to the AchE receptor. (ii) Presynaptically acting neurotoxins inhibit transmitter release from nerve terminals or enhance release of neurotransmitter (dendrotoxin) (Mebs, 1990). Cardiotoxins are involved in cardiac arrest, muscle contracture, membrane depolarization, cytolysis, myonecrosis, hemolysis and affect on platelets (reviewed by Condrea, 1974; Stocker, 1990). Cytotoxins are low molecular weight toxic polypeptides, that induce various pharmacological effects like hemolysis, cytolysis, depolarization of muscle membrane and specific cardiotoxicity (reviewed by Stocker, 1990). Myotoxins contribute to the digestion of muscle cells or cause significant skeletal muscle necrosis (Ownby. 1990; Brusses et al., 1993). The mode of action of the myotoxin is the lysis of the plasma membrane of skeletal muscle cells. Nerve growth factor activity has been identified in six Viperdae, nine Crotalidae and five Elapidae species, which induces plasma extravasation and histamine release from whole blood cells (Elliot, 1978; Mebs, 1990; Stocker, 1990). Protease inhibitor are low molecular weight polypeptides of Elapidae and Viperidae venoms consisting of 52 to 65 amino acids and cross-linked by 2 or 3 disulfide bridges. They either act as proteinase inhibitors or represent structural analogous of proteinase inhibitors (Iwanaga et al., 1976; Hokama et al., 1976; Ritonja et al., 1983; Jayanthi and Gowda, 1988).

2.1.3 Non-protein components of snake venom

The non-protein components of snake venom can be divided into two category (a) organic constituents and (b) inorganic constituents (Sarkar and Devi, 1968; Bieber, 1979; Stocker, 1990). The organic constituents are carbohydrates

(glycoproteins), lipids (phospholipids primarily), nucleosides and nucleotides, amino acids, biogenic amines (abundant in Viperidae and Crotalidae venoms) including histamine, seretonin, bufotenine and N-methyl trytophan etc. The inorganic constituents of snake venoms include Ca^{2+}, Fe^{2+}, Mn^{2+}, Na^{+}, Li^{+}, K^{+}, Co^{2+}, and Zn^{2+} beside anions like phosphate, sulphate and chloride. All these substances mentioned above are not found in every type of venom and the amount of each varies from species to species (Stocker, 1990).

2.2 Variation in venom composition: Impact on pathogenesis and antivenom treatment

The variation in venom composition is a common phenomenon and plays an important role in pathophysiological symptoms following bite and deserves medical concern. The venom varies greatly due to variation in individual, geographical origin and age of the snakes (Taborska, 1971; Taborska and Kornalik, 1985; Meier, 1986; Minton and Winstein, 1986; Jayanthi and Gowda, 1988; Daltry et al., 1996; Tsai et al., 1996; Mukherjee and Maity, 1998). Gene mutation, which is the primary cause of evolution plays an important role in variation of venom composition between closely related species or even within the same species of snake (Glenn et al., 1983; Yang et al., 1991; Assukuri et al., 1992; Daltry et al., 1996; Fry et al., 2002).

Due to the variation in venom composition, the pathogenesis developed after a bite is complex in nature. Further, the clinical manifestation depends upon the qualitative composition as well as the quantitative distribution of different components of venom proteins (Stocker, 1990; Warrell, 1989; Mukherjee and Maity, 1998). For example, Russell's viper venom from Southern India differs from that of the Western and Northern India in terms of lethal potencies (Jayanthi and Gowda, 1988). Though *Naja naja* and *Naja kaouthia* are closely related species, but they differ in their venom composition (Mukherjee, 1998; Mukherjee and Maity, 2002). The former venom is more toxic as compared to latter venom and antivenom raised against *Naja naja* is hardly effective in neutralizing the pharmacological effects of *Naja kaouthia* venom (Mukherjee and Maity, 2002). It has been well documented

that this variation in venom composition affects significantly the neutralizing capacity of antivenom as well (Fry et al., 2003). Therefore the variation in the venom composition should be given proper consideration while producing antivenom, because the antivenom raised against the venom of one population of snakes may be less effective against the venom of another population of snakes, which may be of the same species (Fry et al., 2001).

2.3 Snake venom PLA_2 enzymes: Classification, structure and functions

Phospholipase A_2 (EC: 3.1.1.4) are one of the most studied snake venom enzyme due to their pivotal role in inducing various pharmacological effects. They are abundantly found in nature and human pancreas and of course snake venom is the richest source of this enzyme (Kini and Evans, 1989; Dennis, 1994; Hawgood and Bon, 1991). In addition to digestion of prey, snake venom PLA_2s are involved in many pharmacological effects, such as neurotoxicity, cardiotoxicity, myotoxicity, necrotic, anticoagulant, hypotensive, hemolytic, haemorrhage, edema etc. Further they attack various vital organs of human victims (Kini, 1997). However, not all PLA_2 enzymes induce all these pharmacological effects; but an individual PLA_2 enzyme exhibit either one or more specific pharmacological effects. For example OHVA-PLA_2 from *Ophiophagus hannah* venom induces myotoxicity, cardiotoxicity, antiplatelet effects (Haung et al., 1993; Haung and Gopalakrishnakone, 1996; Haung et al., 1997). β-Bungarotoxin (β-Btx) a PLA_2 toxin from *Bangurus multicinctus* venom exhibit presynaptic neurotoxicity (Strong et al., 1976) but failed to show postsynaptic neurotoxicity (Yang, 1978).

2.3.1 Classification of PLA_2 enzyme

PLA_2 enzymes can be classified based on various parameters. Balsinde et al. (1999) classified PLA_2 enzymes utilizing their properties into three main types: secretory PLA_2, cytosolic Ca^{2+} dependent PLA_2 and intracellular Ca^{2+} independent PLA_2. Kini (1997) has classified PLA_2 enzymes depending on their structure and mechanism of catalysis (Fig. 2.1). In general they have a molecular weight in the

range of 1,40,000-4,00,000. Based on the molecular weight they are classified into high molecular weigh and low molecular weight (Kini, 1997).

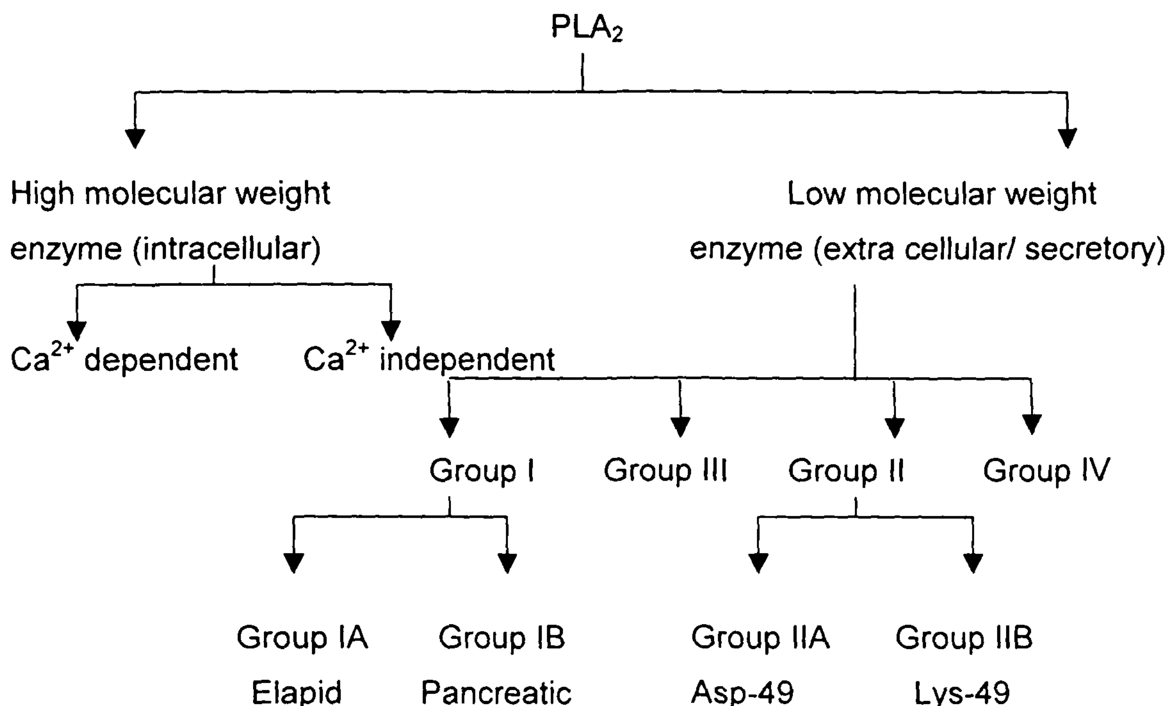

Fig. 2.1. Classification of PLA_2 enzymes (cited from Kini, 1997)

a) **High molecular weight PLA_2**: These groups of PLA_2 enzymes are intracellular in origin and specifically hydrolyze plasmogen at sn-2 position. Basically these groups of enzymes are found in various tissues and snake venom, which is further divided into Ca^{2+} dependent and Ca^{2+} independent.

b) **Low molecular weight PLA_2:** These group of enzymes have a molecular weight in the range of 13,000-15,000 dalton and are extra cellular or secretory enzymes. Snake venom and mammalian pancreas are rich source of these enzymes. They specifically release fatty acid from sn-2 position. Amino acid sequence of more than 150 proteins are known and they can be classified into following four groups based on their primary structures.

i) **Group I PLA_2 enzymes:** PLA_2 enzymes of Elapid and Hydropid snake venom and mammalian pancreas fall under this group. These enzymes typically contain 115-120 amino acid residues and is cystine rich containing 7 disulfide bridges. This group is further subdivided into two, Group I and II, based on the presence or absence of pancreatic loop. PLA_2 of Elapid is under Group I and while that of pancreatic is under Group IB PLA_2.

ii) **Group II PLA_2 enzymes:** This group is isolated form Viperid and Crotalid snake venoms and mammalian cells such as platelets. 120-125 amino acid residues and seven disulfide bridges are present in these enzymes. This enzyme lack pancreatic loop. This group differs from Group I in having an additional C-terminal, which forms an extra disulfide link with a cystine residue in position 49. In some enzyme it is replaced by lysine. Thus this group can be classified into Asp-49 and Lys-49 enzymes.

iii) **Group III PLA_2 enzymes:** This group of PLA_2 enzymes has been isolated from bee venom. They contain 130-135 amino acid residues and are glycoprotein in nature. They does not share any homology with Group I and Group II enzymes, but the three dimensional folding shows significant similarities.

iv) **Group IV PLA_2 enzymes:** This protein was first isolated as the inhibitor of binding of isradipine, a ligand specific for the L-type Ca^{2+} channel of rat neocortical membrane. This enzyme has two long and short chains containing 77 and 42 residues respectively. This group does not share any significant homology with any other group and is Ca^{2+} dependent.

2.3.2 Structure and mechanism of action of PLA_2 enzymes

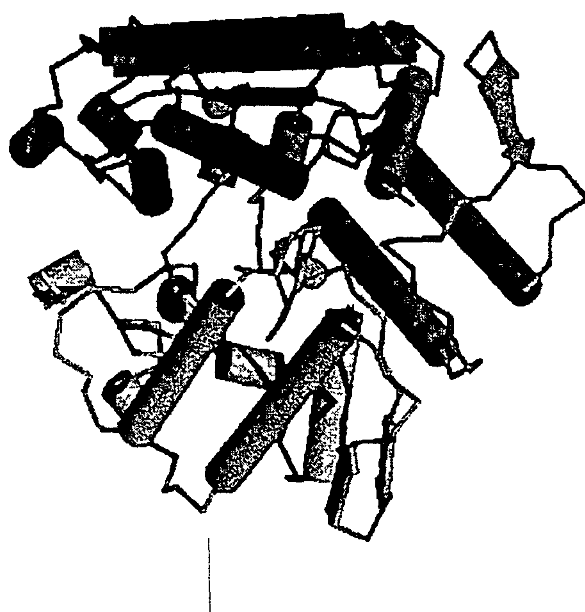

Fig 2.2. Structure of PLA_2 enzyme from *Naja naja naja* (Indian cobra).

Resolution 2.3°, R-factor: 0.174 (Fremont et al., 1993).

2.3.2.1 Structure of PLA_2 enzyme

Crystal structure of various snake venom PLA_2 enzymes have been determined, including rattlesnake venom (Brunie et al., 1985), Chinese pit viper venom (Wang et al., 1996; Tang et al., 1998), Indian cobra venom (Fremonth et al., 1993; Segelke et al., 1998), Thai cobra *Naja kaouthia* venom (Gu et al., 2002) etc. PLA_2 enzymes of snake venom belong to the Group I $sPLA_2$ with a highly conserved Ca^{2+} binding loop and a catalytic site. Besides these elements, there are six absolutely conserved disulfide bonds and up to two additional unique disulfide bonds, which contributes to the high degree of stability of these enzymes. Substrate hydrolysis proceeds through the activation and orientation of a water molecule by hydrogen bonding to the active site histidine. Adjacent to this histidine, there is a conserved aspartate residue, which, together with the Ca^{2+}-binding loop, acts as a ligand cage for Ca^{2+} (Dennis, 1994). The crystal structures of $sPLA_2$-IB and -IIA have defined conserved active site within a hydrophobic channel lined on one side by the N-terminal helix (Scott et al., 1991). This hydrophobic channel binds a single phospholipid molecule following interfacial binding of the enzyme to the aggregated phospholipid surface (Kudo and Murakami, 2002). However, the relationships between catalytic efficiency of the enzyme and its various pharmacological properties, the identification of pharmacological sites and their characteristic conformations and relationships between conformations and lethalities of these actions are not yet clearly defined. Several attempts have been made to predict the site of toxicity of these enzymes by comparing protein sequences in correlation with their lethalities but it has not yet been understood adequately due to lack of consistency in the correlation (Singh et al., 2001).

2.3.2.2 Mechanism of PLA_2 catalysis

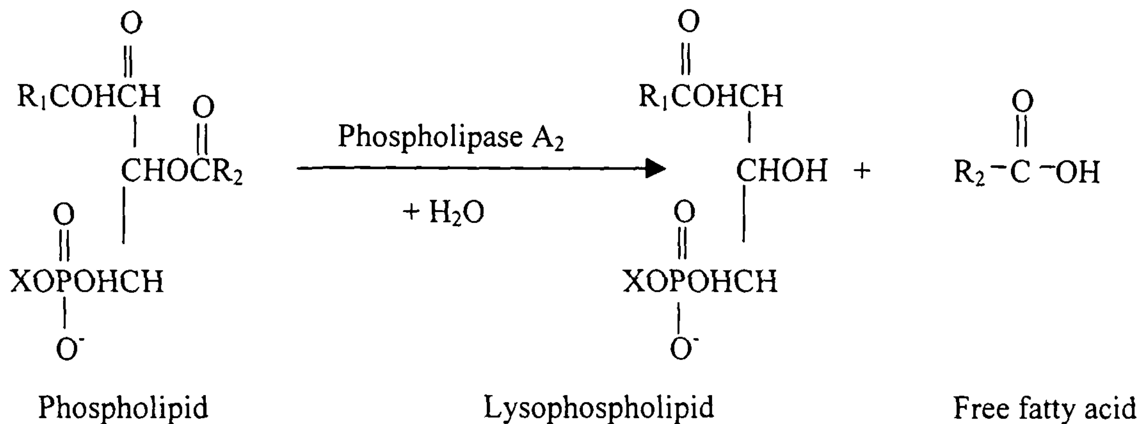

Fig. 2.3. Reaction catalyzed by PLA_2 (cited from Kini, 1997).

Phospholipase A_2 are esterolytic enzymes, which hydrolyze glycerophospholipids at the sn-2 position of the glycerol backbone liberating free fatty acid and lysophospholipid (Dennis, 1983) (Fig 2.3). They can hydrolyze phospholipid in monomeric, miceller or lipid bilayer phases. PLA_2 exhibits distinct head group preference; the catalytic efficiency is determined by the physical properties of aggregation, such as packing density, phase transition, temperature, liquid crystalline and other parameters. When phospholipid is in bilayer, PLA_2 activity depends on several factors such as curvature of the bilayer, the physical state of lipids and the presence of other molecules, such as ionic or non-ionic detergent (Verheij et al., 1981; Veron and Bell, 1992). The active site of the PLA_2 enzyme consists of histidine residue. Histidine residue assisted by Asp, polarize a bound H_2O, which then attacks the carbonyl group. The Ca^{2+} ion, which is bound to the conserved Ca^{2+} loop, is required to stabilize the tetrahedral transition state (Dennis, 1994). Thus Ca^{2+} plays an important role in catalysis by PLA_2.

Catalytic action of PLA_2 in aqueous phase is postulated to occur as follows. The enzyme binds to substrate molecule to form ES complex, followed by formation of product. The enzyme then either goes back to aqueous phase or binds to another

substrate in the same interface (Kini, 1997). It has been found that the enzyme activity is also dependent on the organization of phospholipids in the membrane (Burack and Biltonen, 1994). Several studies have proposed that the presence of defects in the bilayer structure may act as starting point of enzyme activity (Grainger et al., 1989; Vernon and Bell, 1992).

Recently Hyvonen et al. (2001) studied the structural effects induced by PLA_2 hydrolysis on membrane, applying molecular dynamics (MD) system. They documented that the PLA_2 hydrolyze bilayers that had a loosened structure as compared to normal intact 1-palmitoyl-2-linoleoyl-sn-glycerol-3-phosphatidylcholine (PLPC) system and increased penetration of H_2O molecules. The decreased integrity of the bilayer consisting of the hydrolysis products implies structural perturbation in the hydrolyzed bilayer area. This perturbations may also activate PLA_2 by allowing more mobility of the substrate molecules in the membrane-normal direction and accordingly better access to the active site of the enzyme (Apitz-Castro et al., 1982; Jain and de Haas, 1983; Jain and Jahagirdar, 1985; Burack et al., 1993, 1995, 1997; Scheffield et al., 1995; Lehtonen and Kinnunen, 1995; Bell et al., 1996; Honger et al., 1996; Grandbois et al., 1998).

2.3.3. Biochemical properties of PLA_2 enzymes

Table 2.1. *Molecular mass of some purified snake venom PLA_2.*

Snake species	Molecular weight (Dalton)	References
Vipera russelli	11,8000[a] (VRV PL VIIIa)	Kasturi and Gowda, 1989
Daboia russelli	15,000[a] (DbTx)	Maung-Maung-Thwin et al., 1995
Bothriechis schegelii	15,000[a]	Angulo et al., 1997
Hydrophis cyanocinctus	13,588.1[b] (H1) 13,247.2[b] (H2)	Ali et al., 1999
Crotalus atrox	13,779[c]	Tsai et al., 2001
Crotalus .m. molossus	13,723[c]	Tsai et al., 2001
Bothriechis schlegelii	13,671[c]	Tsai et al., 2001
Porthidium godmani	13,836[c]	Tsai et al., 2001
Porthidium nummifer	13,738[c]	Tsai et al., 2001

[a] SDS-PAGE

[b] MALDI-MS

[c] Determined by ESI-MS with SE ± 0.01%

As shown in Table 2.1, the molecular mass of snake venom PLA_2 enzymes generally ranges from 11,000 to 15,000 dalton. They have a very rigid tertiary structure arising from the presence of 5-8 disulfide bonds which helps in stability against proteolysis and resistance to denaturation and allows them to retain their activity in the extracellular fluid where they are found (Balsinde et al., 1999). Further, the presence of large number of disulfide bonds in PLA_2 enzymes may attribute them to be thermostable (Vishawanath et al., 1988; Francis et al., 1995). PLA_2 enzymes require Ca^{2+} *in millimolar concentration for its catalytic activity* (Balsinde et al., 1999). Adjacent to the histidine residue of the active site, there is a conserved aspartate residue, which, together with the Ca^{2+}-binding loop, acts as a ligand cage for Ca^{2+}. Ca^{2+} ion binds to the Ca^{2+} loop, which is conserved and plays an active role in catalysis (Dennis, 1994). Although Ca^{2+} enhance the catalytic activity, other divalent ions such as Ba^{2+}, Sr^{2+} and Mn^{2+} can substitute Ca^{2+} ions (Reynolds et al., 1993). The amino acid sequence of many PLA_2 have been

determined and found to be 40-90% identical, however they differ greatly in their enzymatic and pharmacological effects (Rosenberg, 1979; 1986; 1990; Basavarajappa et al., 1993; Doley et al., 2004). Treatment of ρ–bromophenacyl bromide inactivates cobra venom PLA_2 by alkylating 0.5 histidine residue indicating that histidine 48 is conserved among snake venom phospholipase A_2 enzymes and plays an essential role in the catalysis process (Roberts et al., 1977; Tsai et al., 2001; Doley and Mukherjee, 2003; Doley et al., 2004). Some of the PLA_2 enzyme contains aspartate residue at 49 position which is critically involved in the binding of Ca^{2+}, however, in several enzymes this residue is replaced by lysine, which does not bind to Ca^{2+} efficiently (Maraganore et al., 1984; Maraganore and Hennrikson, 1986; Scott et al., 1990; Francis et al., 1991). Other chemical inhibitors like Potassium cyanate, *o*-Methylisourea, Acetic anhydride and Trinitrobenzenesulphonyl (TNBS) modify the Lys residue (Soares and Giglio, 2003). Modification of the Lys residue distorts the binding ability of the PLA_2 enzyme for the substrate, causing a drastic loss in the enzymatic activity (Chang et al., 1994); Nitrophenylsulphenyl chloride (NPSC) modifies the Tyr. and Trp (Soares and Giglio, 2003). Modification of Tyr residues of *Bothrops myotoxins* have been found to affects lethality, myotoxicity, cytotoxicity and the neuromuscular blocking effect induced by these toxins (Soares et al., 2000a,b; Andriao-Escarso et al., 2000). Chloramine T and Iodoacetic acid modifies the Met. residues. Selective oxidation of Met6 and Met8 of β-bungarotoxin from *Bungarus multicinctus* by chloramine T did not destroy the two Ca^{2+} binding domains, though it modified the toxin to become less effective for binding Ca^{2+}. (Soares and Giglio, 2003).

Most of the reported snake venom PLA_2s are highly stable and resistant to heat, acid and urea but catalytically inactivated at high pH (Bonfim et al., 2001). They have a pH range of 6.9 to 8.0 and optima temperature of 30-55 °C (Rudrammaji and Gowda, 1998; Bonfim et al., 2001). Thermostable PLA_2 enzymes from snake venoms have been reported (Vishawanath et al., 1988; Francis et al., 1995; Ali et al., 1999). CD spectra analysis of thermostable PLA_2 enzymes reveal that the enzymes are of highly compact folded structures, mainly based on the core structure of the disulfide bridges (Dufton et al., 1983; Dufton and Hider, 1983). PLA_2

enzymes of snake venom have a α–helix structure, which is the major secondary structure, this is further confirmed by X-ray crystallography and solution NMR studies (Fremonth et al., 1993; van den Berg et al., 1998; Wang et al., 1996; Han et al., 1997; Perbandt et al., 1997; Segelke et al., 1998; Ali et al., 1999).

2.3.4. Pharmacological properties of PLA_2 enzymes

The fatty acid and lysophospholipids generated during hydrolysis serve as a precursor for lipid-derived mediators with a wide range of biological activities (Gelb et al., 1995, 1999; Tishchfield, 1997; Dennis, 2000), but still the implication of these mode for the pharmacological effects are not clear. The other product of PLA_2, lysophospholipid, may be metabolized to platelet-activating factors which is a potent inflammatory mediator (Kume and Shimize, 1997; Jackson et al., 1998) or to lysophospholipidic acid with mitogenic activities (Fourcade et al., 1998). Snake venom PLA_2 exhibits a wide variety of pharmacological effects despite their similarity in primary, secondary and tertiary structures (Kini, 1997). PLA_2 induce many pharmacological effects which are either, dependent or independent of its catalytic activity and enzymatic activity is partially responsible for, at least, some of these effects (Kini and Evans, 1989). In mechanism where the pharmacological effects are dependent on enzymatic activity, either the hydrolysis of the intact phospholipid or the released product such as lysophospholipid and free fatty acid can cause the pharmacological effect (Kini and Evans, 1989). Whereas in those mechanisms, where they are independent of the enzymatic activity, binding of PLA_2 to the target protein can cause the pharmacological effects by acting as an agonist or an antagonist, or by interfering in the interaction of the target protein with its physiologic ligands (Stefansson et al., 1990; Evans and Kini, 1997; Mounier et al., 2000).

Some snake venom PLA_2 enzyme forms covalent or non-covalent complex with additional venom protein to express their pharmacological effects at full potency, which forms complex. For example, β–Bungarotoxin, crotoxin and Mojave toxin have two subunits (Bon, 1997). Whereas taipoxin and textilotoxin have 3 to 5

subunits (Fohlman, et al., 1976). One of the component is PLA_2 and the other subunit(s) is(are) PLA_2 like molecule with or without catalytic activity (Kini, 1997). Specific effects of PLA_2 enzyme on a particular tissue can be explained by the presence of specific target site on the surface of tissue or cell (Kini and Evans, 1989). These target sites are recognized by specific pharmacological sites of the PLA_2 enzymes which are independent or sometime overlapping with the active site of this enzyme (Rosenberg, 1986). It has been proposed that target sites and pharmacological site are complementary to each other in terms of charges, hydrophobicity and Vander waal's contact surface, hence higher affinity (Kini and Evans, 1989). The proposed target sites would be either membrane lipids or proteins (glycoproteins) (Kini, 2003).

Both lethal and non-lethal PLA_2 from snake venom have been reported. Some of the reported lethal PLA_2 enzymes are CM-II and CM-III (LD_{50} of 10 ± 3 and 4.4 ± 0.8 μg/g body weight) isolated from *Naja naja kaouthia* venom (Joubert and Taljaard, 1980), acidic PLA_2s of *N. naja sputatrix* (LD_{50} of 270 μg/kg) (Tan, 1982), PLA_2-H1 (LD_{50} of 45 μg/kg) isolated from sea snake venom (Ali et al., 1999), Dabiotoxin (LD_{50} of 50 μg/kg) isolated from *Daboia russelli* (Maung-Maung-Thwin et al., 1995). Whereas PLA_2s isolated from Indian cobra (*Naja naja naja*) are reported to be non-lethal up to a dose of 10 mg/kg body weight (Rudrammaji and Gowda, 1998). Other non-lethal snake venom PLA_2s are also reported (Boucheir et al., 1991; Yang et al., 1991; Ali et al., 1999). Basic PLA_2s are reported to be more toxic and enzymatically less potent but the acidic PLA_2 are less toxic and enzymatically active (Rosenberg, 1986), therefore the lethality of snake venom PLA_2 cannot be correlated with the catalytic property (Dhillon et al., 1987).

In presynaptic neurotoxicity, nerve terminal exposed to PLA_2 enzyme shows mitochondrial damage and depletion of synaptic vesicle (Gopalakrishnakone and Hawgood, 1984; Cull-Candy et al., 1976; Landon et al., 1980). It has been postulated that during presynaptic blockade PLA_2 enzyme first binds to presynaptic site followed by perturbation of the presynaptic membrane by PLA_2 near the binding site.

Myotoxin induces myonecrosis that leads to leakage of CPK followed by increase in its level in the plasma of the victim (Mukherjee and Maity, 2002). In addition, myotoxins induce acute muscle cell damage by affecting the integrity of plasma membrane of the target cell, thereby causing hypercontraction and other cellular alteration, leading to the cell death (Gutierrez and Lomonte, 1995). Study has revealed that the catalytic activity of the enzyme has no role to play in the myotoxicity, however, hydrophobic and cationic regions in PLA_2 molecules are responsible for determining the myotoxicity (Kini and Iwanaga, 1986).

Few PLA_2 enzymes exhibit cardiotoxicity (Lee et al., 1977; Fletcher et al., 1981, 1982; Chang et al., 1983) and it has been opined that cardiotoxicity is independent of enzymatic activity. However, the exact mechanism of induction of cardiotoxicity by PLA_2 enzymes has not been well-established (Kini and Evans, 1989).

PLA_2 enzymes are classified into strong, weak and non-anticoagulant enzymes (Verheij et al., 1980; Boffa et al., 1980). Amino acid sequence reveals the presence of anticoagulant region between the 54 and 77 amino acid residue. In strongly anticoagulant PLA_2 enzymes, this region is positively charged. In non-anticoagulant enzyme, lysine is replaced by negatively charged amino acid (Kini and Evans, 1987). According to the model suggested by Kini and Evans (1989), strongly anticoagulant PLA_2 enzyme would bind with high affinity to an unidentified clotting factor, which acts as a target molecule in such a complex, which would interfere the interaction between clotting factors. The enzyme would then hydrolyze phospholipids in the microenvironment. These combine results of binding, hydrolysis and loss of critical phospholipids would slow or stop the normal activation of clotting factor(s), resulting in an anticoagulant effect.

Venom PLA_2 enzymes, which interfere in platelet function, can be classified into two distinct classes- A and B. Class A comprises of platelet affector PLA_2 enzymes which show biphasic effect. These enzymes at a low concentration and short incubation time induce platelet aggregation while at higher concentration and

on prolong incubation inhibit platelet aggregation (Ouyang and Huang, 1984). Class B PLA_2 enzymes cause only the inhibition of platelet aggregation but fail to initiate aggregation (Li et al., 1985; Ouyang et al., 1983). Experimental evidence suggests the presence of distinct target molecule on the platelet surface and separate pharmacological sites exist on the enzyme molecules (Kini and Evans, 1989).

2.3.5. Molecular diversity and evolution of PLA_2 isoenzymes

Occurrence of large number of PLA_2 isoenzymes in snake venom is a common phenomenon (Braganca and Sambray, 1967; Vishwanath et al., 1987, 1988; Takasaki et al., 1990; Ogawa et al., 1992; Subburaju and Kini, 1997; Singh et al., 2000; Shiloah et al., 1973; Sim, 1998). Indian cobra (*Naja naja*) venom has been reported to contain as many as 14 isoenzymes of PLA_2 (Shiloah et al., 1973) whereas Kini and Gowda (1983) reported 9 PLA_2 isoenzymes. These isoenzymes have been reported to share high identity in their amino acid sequence and similar three-dimensional structure (Heinrikson, 1991; Scott and Sigler, 1994). Recently three PLA_2 isoenzymes ($MiPLA_2$-2, $MiPLA_2$-3 and $MiPLA_2$-4) have been purified from *Micropechis ikaheka* venom (Gao et al., 2001) which show similar hydrophobic properties but have different charge states. Snake venom PLA_2 isoenzymes may be either acidic, neutral or basic in nature (Jayanthi and Gowda, 1988). Basic PLA_2 are more toxic as compared to acidic and neutral and contributes significantly to the toxicity of venom (Jayanthi and Gowda, 1988; Mukherjee and Maity, 1998). The higher toxicity of the basic PLA_2 might be due to the presence of positive charge, which have been postulated to be responsible for their penetrability in plasma membrane. This may explain why they are more toxic as compared to acidic or neutral PLA_2 (Verheij et al., 1980).

These isoenzymes exhibit different pharmacological effects and often cause problems in purification and determination of their functional specificity (Kini, 1997). Further, they show similar hydrophobicity but have different charge states. Different isoenzymes present in snake venom are not due to the subspecies polymorphism, but exist in a venom sample collected from single snake (Hazlett and Dennis, 1985).

Although there is dearth of knowledge on the biochemical basis of the diversity of PLA_2, but it is presumed that the diversity in the venom PLA_2 enzymes could result from two types of structural modification: -

(i) Pre-translational modification that leads to difference in amino acid sequence.

(ii) Post-translational modification resulting in the alteration in net charge among PLA_2 enzymes (Dubourdieu et al., 1987).

According to Kini and Chan (1999), multiple PLA_2 isoenzymes found in snake venom are formed by gene duplication and accelerated evolution. Mutation in nature is a random process. Some gene loci mutate more frequently, which are termed as hot spots. However the role of this hot spots in protein evolution is not clearly understood. Examination of three-dimensional structure of PLA_2 enzyme reveals that the residue that are located in the surface mutates more frequently. These surface substitutions play a significant role in the evolution of new PLA_2 isoenzymes by altering the specificity of targeting to various tissues or cells, resulting in the distinct pharmacological effects.

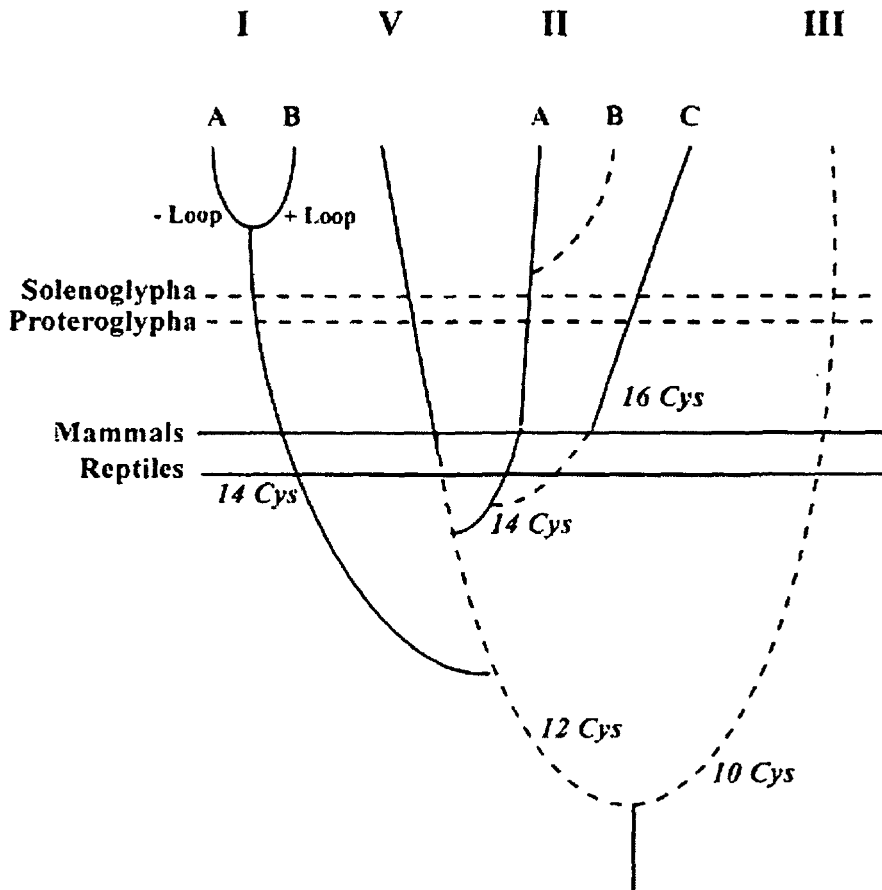

Fig. 2.4. Scheme for evolution of PLA_2 genes leading to the major present day PLA_2. Solid vertical lines indicate ancestral lines associated with individual genes. Branch points indicate duplication of a gene. *Dashed* vertical lines indicate possible radiative events i.e. evidence for a gene duplication awaits evidence that differences are not the result of speciation in the limited number of species examined. Horizontal lines denote that at the time of emergence of the indicated life forms at least as many PLA_2 genes existed as are intersected by the line. Vertical scaling is not correlated with time. The Solenoglypha are movable front-fanged snakes, and the Proteroglypha are fixed front-fanged snakes. (Tischfield, 1997).

2.4 Medicinal plants in the treatment of snakebite patients including PLA_2 inhibitors

In many parts of the world numerous plant species are used as folk medicines to treat snakebite (Moris, 1991; Martz, 1992; Houghton and Osibogun, 1993). In Papua New Guinea, following plants viz: *Alphitonia incanea* (Rhamnaceae), *Cerbera floribunda* (Apocynaceae), *Magnifera minor* (Anacardiaceae), *Maclura sp* (Moraceae), *Melanolepis muttiglandulosa* (Euphorbiaceae), *Osmoxylon micranthum* (Araliaceae) are used for treating bites of two major poisonous snake, the dead adder (*Acanthopis sp*) and the small-eyed snake (*Micropechis ikaheka*) (Mebs, 2000). In NorthWest region of Columbia, traditional healers attend around 60% of the snakebite patients. Based on field interviews, 101 species of plants used against snakebite were identified (Otero et al., 2000). In ancient Indian books, there are various plants recommended for use in snakebite therapy. Around 50 such plants have been indexed (Biswas and Ghosh, 1977) and many others (not include) have also been popularly used against snakebite by villagers, snake charmers and *Ohjas* throughout India including many tribal people of North East India.

Mournfully, clinical and pharmacological tests on alkaloid extracted from well-known and reputed medicinal herbs sometimes show distinctly negative results (Jain, 1996). Such observations should prompt us to reassess these herbs carefully and critically. Literature survey shows that there is scanty of works on exploring the antivenom activity of these medicinal plants. However in recent years, much more attention has been paid to pharmacological screening of the medicinal plants used to treat snakebites (Akunyili and Akubue, 1986; Mors et al., 1989; Gomes et al., 1994; 1998; Alkofahi et al., 1997; Mahanta and Mukherjee, 2001). Houghton and Osibogun (1993) reviewed many such flowering plants useful against snakebites. For example, water extracts of root and leaves of *Eryngium creticum*, a perennial globrous herb of Jordan, have been reported to inactive the haemolytic activities of desert viper (*Cerastes cerates*) and scorpion (*Leiurus quinquesteiartus*) venoms (Alkofahi et al., 1997). Similarly, Mors and his colleagues (1989) reported that lethality and myotoxicity of American rattlesnake venom could be effectively

neutralized by *Eclipta prostrat* plant extracts. Methanol extract of the stem bark of *Parkia biglobosa* (Mimosaceae) has been shown to protect significantly against the neurotoxic, haemorrhagic and cytotoxic effects of two poisonous snakes of Nigeria (*Naja nigricollis*, and *Echis ocellatus*) (Asuzu and Harvey, 2003). Several plant constituents like flavanoids, quinonoids, xanthene, polyphenols and terpenoids possessed protein binding and enzyme inhibiting properties (Havestean, 1983; Selvanayagam et al., 1996), which also inhibits the PLA_2 activities of both Viper and Cobra venom (Alcaraz and Hoult, 1985). Recently, two Indian medicinal plants (*Hemidesmus indicus* and *Pluchea indica*) were identified for their venom inhibitory activity (Gomes et al., 1994). An organic acid isolated from root extract of an Indian medicinal plant sarsaparilla, locally called "Anantmul" (*Hemidesmus indicus* R.Br) possessed Russell's viper venom inhibitory activity. This compound responsible for venom inhibitory activity was isolated from the root extract by solvent extraction, silica gel column chromatography and thin layer chromatography. Spectral analysis confirmed the presence of a benzene ring, methoxy group and hydroxyl group; the molecular weight of the compound was 168. This compound (designated as HI-RVIF) significantly antagonized the Russell's viper venom induced lethality, haemorrhagic, coagulant and anticoagulant activity in experimental rodents. Recently, Mahanta and Mukherjee (2001) reported that the aqueous extract of root of *Mimosa pudica* locally known as "Lajuki late" neutralized the myotoxic and lethality of Cobra *Naja kaouthia* venom. The methanolic root extracts of *Vitex negundo* Linn. and *Emblica officinalis* Gaertn have been found to antagonize significantly the *Vipera russelli* and *Naja kaouthia* venom induced lethal activity, both in *in-vitro* and *in-vivo* studies (Alam and Gomes, 2003).

Since the first report of isolation of a protein from the blood of Habu snake (*Terimerusurus flavoviridis*) by Kihara (1976), research on both venomous and non-venomous snake venom has led to the characterization of series of serum globular proteins which possess the unique ability to neutralize the enzymatic and toxic effects of snake PLA_2 enzymes. Presence of PLA_2 inhibitor proteins (PLIs) has been associated with the resistance of snake to the deleterious effects of their venom PLA_2 effects (Domont et al., 1991; Faure, 2000; Perales and Domont, 2002;

Fortes-Dias, 2002). Some of these inhibitors have also homologous counterparts in mammalians and specific mammalian derived inhibitors have been reported (Rocha et al., 2002). Despite the significance of PLA_2 inhibitors in antivenom therapy, the most significant consideration of these natural inhibitors is their potential therapeutic use, not only as an alternative to antivenom but also as a potential antagonist for PLA_2 activities associated with inflammatory process in human (Lizano et al., 2003). Even if these inhibitors would be an alternative to antivenom this would lead to another limitation: availability of inhibitors. So far inhibitors have been isolated from snake blood but snake themselves would be an insufficient and ecologically unacceptable source of inhibitors for commercial purpose. Further they may elicit immune response which could be deleterious to the patients. Apart form the snake and mammalian origin PLIs, plants are also reported to have these natural inhibitors. A glycoprotein isolated from *Withania somnifera* (Ashwaganda) have been found to be active against scorpion sting envenomation as well as *in-vivo* myotoxic and edematous effect of venom PLA_2 (Mishra et al., 2000; Deepa and Gowda, 2002). Screening of plants for the presence of PLA_2 inhibitory proteins and other compounds would in near future lead to isolation of potent antivenom compound that will have potential therapeutic use as well.

2.5 An overview of Indian medicinal plants used as folk medicine in the treatment of snakebite patients

Agyle mermolos, Linn. (Rutaceae) is a deciduous plant, 6-8 mts in height. Fresh half-ripe fruit is mildly astringent and used to care dysentery, diarrhoea, hepatitis, tuberculosis, and in the treatment of snakebite (Jain, 1991).

Alstonia scholaris, Linn. (Apocyanaceae). It is a common resident of India in deciduous and evergreen forest. The bark is bitter in taste, astringent, acrid, thermogenic, digestive, laxative, antihelmenthic, stomachic, cardiotonic. It is useful in fever, malaria, abdominal disorders, skin diseases, ulcers, asthma etc (Sivarajan and Balachandran, 1994). The dried bark is boiled and the extract is used as an anti-dote to snakebite by the tribal people (N. Taye, personal communication).

Aristolochia indica, Linn. (Aristolochiaceae). It is a twining shrub, slender, woody at base. The roots of this plant are butter, acrid, astringent, thermogenic, purative, digestive, antihelminthes, stomachic, cardiotoxin, anti-inflammatory, diuretic and tonic. They are useful in ulcer, inflammation, skin diseases and all type of poisonous bites and stings (Warrier et al., 1994). The tribal people believe that chewing the whole plant or taking its juice can neutralize the snake poison (N. Taye, personal communication).

Azadirachta indica A. juss (Maliacea) is known for its several medicinal values. The leaves, seeds, roots and bark of the plant possess bitter active principles in different constituents (Oliver-Bever, 1986). The plant has insecticidal properties (Nwude, 1986; Oliver-Bever, 1986). Moreover, the antifertility (Prakash et al., 1988; Bardhan et al., 1991; Upadhyay et al., 1993) and hypotensive with minimal negative chronotropic effect of *Azadirachta indica* have been reported (Thompson and Anderson, 1978). The bitter principles of *Azadirachta indica* are also known to increase the flow of saliva and gastric juice as a result of which the plant is used as stomachics (Oliver-Bever, 1986). *Azadirachta indica* lowered blood glucose level and attenuated gastric ulcerogenesis (Sen et al., 1992). Garg (2000) has indexed this plant as medicinal plant used against snakebite.

Calamus rotang, Linn. (Arecaceae). Climbing palm exhibiting a slender stem ranging from a few millimeters to some centimeter m in diameter, flexible sometimes more or less armed with spines. Roots are astringent, acrid, bitter, expectorant, anti-inflammatory, diuretic and tonic. It is useful in burning, cough, dysentery, and various skin diseases. (Warrier et al., 1994). Fresh root of the plant is crushed and applied on the wound of the snakebite (N. Taye, personal communication).

Carica papaya, Linn. (Caricaceae). The papaya is a short-lived, fast-growing, woody, large herb up to 10 or 12 feet in height. The unripe fruit is used as abortificient. Other uses of the fruit include against bone fracture, indigestion,

ringworm, skin diseases, tooth and gum ache, urine bladder complains and snakebite (Jain, 1991).

Curucuma aromatica, (Zingiberaceae) is a perennial tuberous herb native to India and cultivated in the tropics. Rhizomes are bitter, carminative, appetizer, tonic and are useful in various skin diseases and poisonous bite (Warrier, 1994; Mukherjee, 2001).

Curucuma longa, (Zingiberaceae). This is a perennial tuberous herb native to India and cultivated in large scale. Oral administration of the powdered rhizomes of *C. longa* had been found to be beneficial in case of Asthma and cough (Jain and Dam, 1979). Paste of the fresh rhizome is applied externally to get relief from the inflammation as well as on the wound caused by the snakebite. In other diseases such as indigestion, insect sting, swelling of body, jaundice, etc this has been used extensively by the Tribal people (N. Taye, personal observation).

Leucus lavendulaefolia, Linn. (Laminaceae). It is a common resident of India. The plant is antihistaminic, antipyretic, anticeptic, carminative, febrifuge and wormifuge and is used in anorexia, cough, dyspepsia, fever, helminthic manifestation, jaundice, and other skin diseases. Extract of the plant exhibits strong anti-inflammatory activity on acute and chronic inflammation caused due to snakebite (Sivarajan and Balachandran, 1994; Mukherjee, 2001).

Murraya koenigii, Linn. (Rutaceae). The plant is common in India. The leaves are used for many skin diseases, promote appetite and digestion, destroy pathogenic organism, worm troubles, neurosis and poison (Sivarajan and Balachandran, 1994). The tender leaves are made into paste and used as anti-dote to snakebite by the tribal people (Mukherjee, 2001; N. Taye, personal communication).

Piper longum Linn. (Piperaceae). This plant is commonly found in India in evergreen forest. It is aromatic slender climber. *Piper nigrum*, Linn. (Piperaceae).

This plant is commonly found in India in evergreen forest as well as cultivated by the local people. The fruit are acrid, bitter in taste. The fresh spike of *P. longum* and dried fruits of *P. nigrum* are chewed and externally applied at the site of snakebite. This has been practiced by the Mishing community from long ago and has been passing from generation to generation as folk medicine to treat snakebite patients (N. Taye, personal communication).

Terminalia arjuna, Roxb. (Combretaceae). This is a large evergreen tree. The bark is astringent, sweet, acrid, aphrodisiac, cardiotonic, stypic and urinary astringent. It is useful in fractures, ulcers, cardiopathy, fatigue, asthma, tumors, internal and external haemorrhages (Warrier et al., 1994). The dried bark is boiled and given orally for snakebite by the local healer (Mukherjee, 2001; N. Taye, personal communication).

Zingiber officinale Rosc. (Zingiberaceae) is a perennial herb cultivated throughout India. The rhizome is commonly used as species. Rhizome has a pungent aromatic, lemonly and slightly bitter in taste. The rhizome has been used for various diseases like cough, asthma, cholera, scabies, insect sting, snakebite, throatache etc (Jain, 1991; Mukherjee, 2001).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

Pooled venom samples of *Naja kaouthia* and *Naja naja* from five adult snakes of Eastern India origin were purchased from Calcutta Snake Park, Kolkata. Dried venoms were kept in desicator at room temperature. CM Sephadex C-50, DEAE Sephadex A-50, Sephadex G-50 and Sephadex G-25 were obtained from Pharmacia Fine Chemicals (Sweden). ρ-BPB, TPCK TLCK, PC, PE, PS, leupeptin, PMSF, DTT and Protein Molecular Weight Markers (MW-GF-70, Lot 90K9309) were obtained from Sigma Aldrich, (USA). Freund's complete adjuvant and Freund's incomplete adjuvant were from Difco Laboratories. Horse radish peroxidase, Tetramethly benzidine/H_2O_2, Molecular weight marker, range 14-100 kDa (Cat. No. PMW-M, Lot.-PM 5030) were obtained from Bangalore Genei, Bangalore (India). All other reagents of analytical grade used were purchased from Sigma Aldrich (USA). Human blood samples were donated by healthy volunteers of age group between 21-30 years from the Department of Molecular Biology and Biotechnology, Tezpur University. Goat blood and live chicken were obtained from known local vendors. Polyvalent antivenom was purchased from Bharat Serums & vaccines Ltd. Thane (Lot No. 06/01). In the present study, 13 medicinal plants were selected based on previous report from this laboratory (Mukherjee, 2001) as well as from the information gathered from the Local healer, Mr. N. Taye (Personal communication). Herbarium sheets for each plant were prepared and kept in the department. BALB/C albino mice of both sexes weighing 18-20 g used were obtained from the Department of Biotechnology, Guahati University. Rabbit, used was white albino male rabbit.

3.2 Methods

3.2.1 Comparison of PLA_2 isoenzyme pattern of *N. kaouthia* and *N. naja* venom samples

3.2.1.1 Fractionation of *N. kaouthia* and *N. naja* venoms by cation exchanger

50 mg of either *Naja kaouthia* or *Naja naja* crude venom was dissolved in 4 ml of K-phosphate buffer, pH 7.0 and centrifuged (5000 X 10 min) at 4 °C in a Sorvall RC 5B PLUS centrifuge. The clear supernatant was applied to CM Sephadex C-50 (20 X 60 mm^2) column pre-equilibrated with 20 mM K-Phosphate (K-P) buffer, pH 7.0. Proteins were eluted stepwise, using phosphate buffers of increasing molarities and pH values. The increasing molarities and pH values were (a) 20 mM, pH 7.0, (b) 75 mM, pH 7.5, (c) 90 mM, pH 8.0, (d) 100 mM, pH 8.0, (e) 110 mM, pH 8.0, (f) 130 mM, pH 8.0, (g) 150 mM, pH 8.0 and (h) 180 mM, pH 8.0 at room temperature (~23 °C). The flow rate was adjusted to 24 ml/ hr and 1 ml fraction was collected in each test tube. The protein content and PLA_2 activity were estimated as described in sections 3.2.5.1 and 3.2.5.5.1, respectively.

3.2.1.2 Fractionation of *N. kaouthia* and *N. naja* venoms by anion exchanger

50 mg of either crude venom was dissolved in 4 ml of K-Phosphate buffer, pH 7.0 and centrifuged (5000 X 10 min) at 4 °C in a Sorvall RC 5B PLUS centrifuge. The clear supernatant was applied to an anion exchanger, DEAE Sephadex A-50 (20 X 60 mm^2) column pre-equilibrated with 20 mM K-Phosphate (K-P) buffer, pH 7.0. Protein elution was performed stepwise using phosphate buffers of increasing molarities and decreasing pH values. The increasing molarities and decreasing pH values were (a) 20 mM, pH 7.0, (b) 80 mM, pH 6.5, (c) 120 mM, pH 6.0, (d) 140 mM, pH 6.0, (e) 160 mM, pH 5.5, (f) 180 mM, pH 5.5, (g) 200 mM, pH 5.5, (h) 230 mM, pH 5.0 and (i) 260 mM, pH 5.0 at room temperature (~23 °C). The flow rate

was adjusted to 24 ml/hr and 1 ml fraction was collected in each test tube. The protein content and PLA_2 activity were estimated as described in section 3.2.5.1 and 3.2.5.5.1, respectively.

3.2.2 Purification of NK-PLA_2-I

3.2.2.1 Fractionation of *N. kaouthia* venom on CM Sephadex C-50 cation exchange chromatography

Crude *Naja kaouthia* venom (50 mg) was dissolved in 4 ml of K-Phosphate buffer, pH 7.0 and centrifuged (5000 X 10 min) at 4 °C in a Sorvall (RC 5B PLUS) centrifuge. The clear supernatant was applied to a CM Sephadex C-50 (20 X 60 mm^2) column pre-equilibrated with 20 mM K-Phosphate (K-P) buffer, pH 7.0. The column was washed with three volumes of equilibrium buffer and then bound proteins were eluted stepwise using phosphate buffers of increasing molarities and pH values at room temperature (~23 °C). The increasing molarities and pH values were (a) 20 mM, pH 7.0, (b) 75 mM, pH 7.5, (c) 90 mM, pH 8.0, (d) 100 mM, pH 8.0, (e) 110 mM, pH 8.0, (f) 130 mM, pH 8.0, (g) 150 mM, pH 8.0 and (h) 180 mM, pH 8.0. The flow rate was adjusted to 24 ml/hr and 1 ml fractions were collected in each test tube. The protein content and PLA_2 activity were estimated as described in sections 3.2.5.1 and 3.2.5.5.1, respectively.

3.2.2.2 Fractionation of CM I by Sephadex G-50 gel filtration chromatography

The non-retained acidic fraction, CM I (obtained from fractionation of *Naja kaouthia* venom on CM Sephadex C-50 cation exchanger and showed highest PLA_2 activity) was selected for further purification. CM I was concentrated at –20 °C in a Maxi dry plus (Heto Lab Equipment, Denmark) and applied to Sephadex G-50 gel filtration column (1 X 64 cm^2), pre-equilibrated with 20 mM K-phosphate buffer, pH 7.0. Elution was carried out with the equilibration buffer at room temperature (~23 °C). The flow rate was adjusted to 20 ml/h and 1 ml fractions were collected. The

protein content of each tube was determined according to the method as described in section 3.2.5.1. The fractions were pooled according to protein content. The PLA_2 activity of pooled fraction was assayed by taking 1 μg of protein as described in section 3.2.5.5.1. The gel filtration fraction displaying maximum PLA_2 activity was desalted through Sephadex G-25 (1.0X30 cm^2) column, then lyophilized and was stored at –20 °C until further use. Homogeneity and molecular mass of the purified protein was ascertained as described in section 3.2.4.

3.2.3 Purification of NK-PLA_2-II

3.2.3.1 Fractionation of CM-II by RP-HPLC

Peak CM-II, obtained from fractionation of *Naja kaouthia* venom on CM Sephadex C-50 cation exchanger was desalted by passing through a Sephadex G-50 column and concentrated at –20 °C in a MAXI dry plus (Heto Lab Equipment, Denmark). Peak CM-II was further purified by RP-HPLC using C18-μ-Nova pack column (Waters). About 100 μg of CM-II was pre-incubated with 40 μl of buffer B (0.1 % v/v TFA in acetonitrile) for 30 min at room temperature, passed through Spartan 3 nylon filters before application to C_{18} column previously equilibrated with solution A (0.1% v/v TFA in 5.0% v/v acetonitrile). Proteins were eluted at a flow rate of 1 ml min^{-1} using a linear gradient of 5-60% buffer B over 28 min. Detection was monitored at 220 nm and individual fractions were collected manually. Protein content of individual peak was estimated as described in 3.2.5.1 and each peak was screened for PLA_2 activity.

3.2.4 Criteria of purity and determination of molecular weight

3.2.4.1 SDS-PAGE

SDS-PAGE was carried out with or without reduction of proteins by β-mercaptoethanol as described by Laemeli (1970). 80 μg of crude venom and 30 μg of purified proteins were loaded into 15%. Separating gel containing 5% glycerol.

Electrophoresis was carried out at a constant current of 15 mA until the dye front (bromophenol blue) reached the bottom of the gel. Before staining, proteins were fixed by incubating the gel in 20% TCA for 30 min followed by washing the gel several times in distill water. Protein bands were visualized by staining the gel with 1% (w/v) Coomassie Brilliant Blue R250 in methanol:acetic acid:water (4:1:5 v/v/v) and destained with Methanol:acetic acid:water (4:1:5 v/v/v). Destained gels were scanned in Gel Doc. 1000 (BioRad). Mobility of the purified protein was compared with the following molecular Weight Markers; phosphorylase b (97,400), bovine serum albumin (66,000), ovalbumin (43,000), carbonic anhydrase (29,000), soyabean trypsin inhibitor (20,100) and lysozyme (14,300). Molecular weight of the unknown proteins was calculated using Bio-Rad Multi-Analyst^{TM}/PC version 1.1 software (Bio-Rad).

3.2.4.2 Gel filtration chromatography

Molecular weight of the purified proteins in native state were determined by gel filtration on Sephadex G-50 column (1.0 X 64 cm^2), pre-equilibrated with 20 mM K-Phosphate buffer, pH 8.0. One mg of purified protein dissolved in 0.5 ml of equilibration buffer was loaded into the column and eluted with the same buffer at room temperature (~23 °C). Flow rate was adjusted to 20 ml/h and 1 ml of fraction was collected. Elution of protein was monitored at 280 nm using D®U UV/Vis spectrophotometer (Beckman). The column was calibrated with the following Molecular Weight Markers-aprotinin (6,500), cytochrome C (12,400), carbonic anhydrase (29,000), bovine serum albumin (66,000) and Blue dextran (2,00,000). The molecular weight of the unknown proteins was calculated from the standard curve obtained by plotting Log. Mol. Wt. Vs relative elution volume of the known protein.

3.2.4.3 MALDI-MS

Molecular mass of the purified proteins (NK-PLA_2-I and NK-PLA_2-II) was also determined by matrix-assisted laser desorption/ionization mass spectrophotometry

(MALDI-MS). Analysis was carried out in a sinapinic acid matrix with an average of 50 shots at 30–40% laser power.

3.2.4.4 RP-HPLC

RP-HPLC was performed on a reverse-phase C_{18}-μ-Nova pack column (Waters Corporation, Milford MA, USA). About 40 μg of purified proteins were pre-incubated with 20 μl of 0.1% (v/v) TFA in Millipore water for 30 min at room temperature and passed through Spartan 3 nylon filters before application to RP-HPLC. Protein was eluted with a linear gradient from 0 to 60% acetonitrile containing 0.1% (v/v) TFA over 30 min of time and detection was monitored at 220 nm. Flow rate was adjusted to 0.5 ml/min. Protein peak was collected and tested for PLA_2 activity.

3.2.4.5 Protein sequencing

Partial N-terminal amino acid sequencing of purified proteins by Edman degradation was performed in a gas-phase PPSQ-10 protein sequencer (Shimadzu) connected to an on-line PTH-analyzer and a CR-7A data processor. Protein homology searches were performed using the Swiss-Prot databases (http://us.expasy.org/sprot/).

3.2.5 Biochemical Characterization

3.2.5.1 Quantitation of protein

The protein content was estimated by Folin-Lowry method (Lowry et al., 1951) using BSA as a protein standard. The protein content of the unknown samples were calculated from the standard curve obtained by plotting optical density Vs concentration of BSA (1 mg/ml).

3.2.5.2 Estimation of carbohydrate content

Total carbohydrate was quantitated by Phenol-sulphuric acid method as described by Dubois et al. (1956) using D-glucose as standard. The optical density of the reaction mixture was measured at 490 nm against a reagent blank. The carbohydrate content of the unknown samples were calculated from the standard curve obtained by plotting optical density Vs concentration of D-glucose (0.1 mg/ml).

3.2.5.3 Isolation of total lipid

Total lipid from tissue or tissue fraction was isolated by the method of Folch et al. (1957). 1 gm of tissue or tissue fraction was homogenized with 2:1 (v/v) methanol-chloroform mixture in a final volume of 20 ml. The homogenate was mixed properly and filtered through a Whatman filter paper No. 1. The filtrate was collected in a fresh tube and the residue was re-suspended in 5 ml of methanol-chloroform mixture, vortexed, then re-filtered and the filtrate was collected in a fresh tube. This process was repeated once more and the filtrate was evaporated to dryness. The dried residue was weight and re-suspended in chloroform at a final concentration of 5 mg/ml.

3.2.5.4 Estimation of total lipid

The total lipid was estimated spectrophotometrically as described by Nath and Chatterji (1962). Briefly, 0.2 ml of lipid extract was transferred in 4.8 ml of concentrated H_2SO_4 and kept in a boiling water bath for 10 min. The tubes were allowed to cool at room temperature, then 0.2 ml of the mixture was withdrawn and transferred in a fresh tube containing 3.8 ml of orthophosphoric acid followed by addition of 1 ml of 0.6 % (w/v) vanillin. The reaction was allowed to continue for 10 min at room temperature and the absorbancy of the pink colour was read at 530 nm using a double beam spectrophotometer (Hitachi). The concentrations of the

unknown samples were calculated from the standard curve obtained by using phosphotidylcholine as lipid standard.

3.2.5.5 Assay of PLA_2 activity

Phospholipase A_2 activity was assayed by turbidometric as well titrametric methods.

3.2.5.5.1 Turbidometric method

Phospholipase A_2 activity of crude venom/purified proteins was assayed by the turbidometric method of Joubart and Taljaard (1980) with the following modifications. One egg yolk was suspended in 250 ml of 0.9% (w/v) NaCl containing 0.02% (w/v) Sodium azide. The egg yolk suspension was stored in 4 °C until further use. Before experiment, 1 ml of suspension was mixed with 10 ml of 0.1 M Tris-HCl pH 8.0 and the absorbance of the resulting mixture was adjusted to 1.0 at 740 nm with the same buffer. For the enzyme assay, 1 μg of crude venom/fraction/purified protein was mixed with 2 ml of reaction mixture and the decrease in turbidity after 10 min was monitored at 740 nm against the reagent blank. One unit of PLA_2 activity is defined as decrease in 0.01 absorbance in 10 min at 740 nm.

3.2.5.5.2 Titrimetric method

For titrimetric assay of PLA_2 activity of crude venom/purified protein samples, procedure of Deems and Dennis (1981) was followed. Briefly, 1 μg of protein was incubated with 1 mM of phospholipid suspension (PC/PS/PE) for 10 min at 37 °C in a final volume of 3 ml, adjusted with 100 mM Tris-HCl buffer, pH 8.0. Following incubation, the reaction mixture was titrated with 0.1 N NaOH with one drop of phenopthalin as indicator. One unit of enzymatic activity (U) is defined as one microlitre of O.1 N NaOH required to neutralize the reaction mixture. Reaction mixture without venom protein is treated as the control.

3.2.5.6 Assay of acetylcholinesterase activity

Acetylcholinesterase activity of crude/purified proteins of *Naja kaouthia* venom was determined by the method of Ellman et al. (1968). 10 μg of protein was added to the reaction mixture containing 300 μl of 10 mM acetylthiocholine iodide in a final volume of 2.990 ml (adjusted with 70 mM Tris-HCl buffer, pH 8.0). 10 μl of DTNB (freshly prepared in 100 mM Tris-HCl buffer, pH 7.4 containing 15 mg Na_2CO_3). Was added immediately and change in absorbance at 412 nm after 1 min was monitored against a reagent blank. One unit (U) of AchE activity is defined as micromoles of thiocholine formed per min.

3.2.5.7 Assay of protease activity

Casienolytic activity was evaluated calorimetrically by the method of Ouyang and Teng (1976) as modified by Mukherjee and Maity (1998). 1% (w/v) of casein in 0.1 M potassium-phosphate buffer, pH 8.0 was incubated with specific amount of crude venom/purified proteins for 90 min at 37 °C followed by addition of 0.5 ml of 10% (w/v) ice-cold TCA to stop the reaction. After centrifugation of the mixture, supernatant was transferred to a fresh tube and 2 ml of 2% (w/v) Na_2CO_3 in 0.1 N NaOH was added and the reaction was allowed to continue for 10 min at room temperature followed by addition of 0.5 ml of Folin-Ciocalteu's reagent (1:2 dilution). After 30 min, absorbance was measured at 660 nm. Casienolytic activity of the crude venom/purified proteins was calculated from the standard tyrosine curve. One unit (U) of casienolytic activity is defined as n mole equivalent of tyrosine formed per minute.

3.2.5.8 Assay of adenosine monophosphatase activity

AMPase or 5′-nucleotidase activity was assayed by the method of Campbell (1962) as modified by Mukherjee and Maity (1998). The typical reaction mixture contain 100 mM Tris-HCl buffer, pH 8.0, 100 mM $MgCl_2$, 3 mM 5′-adenosine monophosphate and 100 μg crude venom/purified proteins. After incubation for 30

min at 37 °C, 0.5 ml of 10% (w/v) TCA was added to stop the reaction and the tubes were centrifuged at 300 rpm for 15 min. For the estimation of liberated Pi, 0.2 ml of supernatant was transferred to another tube containing 0.25 ml of 1% (w/v) ammonium molybdate in 5 N H_2SO_4 and 0.25 ml 1% (w/v) ρ–methyl amino phenol sulphate containing 1.5% (w/v) sodium bisulphate. Final volume was adjusted to 5.0 ml with Millipore water. Absorbance was read at 660 nm after 30 min against a reagent blank. One Unit (U) is defined as μg of Pi liberated per 30 min at 37 °C.

3.2.5.9 Assay of adenosine triphosphatase activity

ATPase activity of crude venom/purified proteins was determined by the method of Williams and Esnouf (1962). 5 μg of venom/purified protein in 800 μl of reaction mixture was incubated for 30 min at 37 °C. The reaction mixture (1 ml) consists of 2 mM $MgSO_4$, 50 mM KCl, 50 mM Tris-HCl buffer pH 8.0 and 1 mM ATP. The reaction was stopped by adding 0.2 ml of 10% (w/v) ice-cold TCA and mixture was centrifuged at 3000 rpm for 10 min. The filtrate (0.2 ml) was transferred to a fresh tube containing 0.25 ml of 1% (w/v) ammonium molybdate and 0.25 ml of 1% (w/v) metol and final volume was adjusted to 5.0 ml with distilled H_2O. The reaction was allowed to stand for 30 min at room temperature and then the absorbance was measured at 735 nm against a reagent blank. The ATPase activity of the crude venom/purified proteins was calculated from the standard curve of inorganic phosphate. One unit (U) of the ATPase activity is defined as the amount of enzyme required to liberate 1 μ mole of inorganic phosphate in 30 min at 37 °C.

3.2.5.10 Enzyme kinetics

3.2.5.10.1 Substrate specificity of PLA_2 enzymes

To determine the substrate specificity and phospholipid head-group preference of PLA_2 enzymes, different phospholipid substrates viz. PC, PS and PE were used at a final concentration of 1 mM and incubated with 20 nM of enzyme at

37 °C for desired time periods. The PLA_2 activity was assayed as described in the section 3.2.5.5.2.

3.2.5.10.2 Effect of substrate concentration on PLA_2 activity

Effect of substrate concentration on PLA_2 activity was determined by titrimetric method using phosphatidylcholine as substrate (Reynolds et al., 1991). Graded amount of substrates (PC/PS/PE) ranging from 0.5-2.5 mM were incubated *with 1 μg of purified protein for 5 min at 37 °C and then PLA_2 activity was assayed* by the titrimetric method as described in section 3.2.5.5.2.

3.2.5.10.3 Effect of enzyme concentration on PLA_2 activity

To determine the effect of enzyme concentration on catalytic activity, graded amount of enzyme (0.5 to 10 μg) was added to the reaction mixture and the PLA_2 activity was assayed by titrimetric method as described in section 3.2.5.5.2.

3.2.5.10.4 Determination of K_m and V_{max} for the enzyme catalyzed reactions

The K_m and V_{max} values of purified enzymes were calculated using Lineweaver-Burk representation (Zubay et al., 1995). Phospholipase activity was estimated by using PC as a source of substrate as described in section 3.2.5.5.2. By plotting the values of 1/v as a function of 1/s, a straight line was obtained that intersect the vertical line at a point which is the $1/V_{max}$ (since 1/[S] =0 therefore $1/v=1/V_{max}$). Extension of the straight line results in intersecting the horizontal axis (1/[S]) at the point, which is $-1/K_m$.

3.2.5.10.5 Temperature optimum for PLA_2 catalyzed reactions

Effect of temperature on the PLA_2 activity of purified proteins was assayed by incubating the reaction mixture (egg yolk phospholipid and 100 mM Tris-HCl, pH

8.0) containing purified enzyme at different temperature for 10 min followed by assay of PLA_2 activity by turbidimetric method as described in section 3.2.5.5.1.

3.2.5.10.6 pH optimum for PLA_2 catalyzed reactions

Effect of pH on the PLA_2 activity of purified proteins was assayed by turbidimetric method as described in section 3.2.5.5.1. The different pH values were obtained as follows 0.1M sodium acetate buffer, pH 5.5-6.5; 0.1M phosphate buffer, pH 7.0-7.5 and 0.1 M Tris-HCl buffer, 8.0-9.5.

3.2.5.11 Heat-inactivation of PLA_2 enzyme

Purified proteins (2 mg/ml) were dissolved in 100 mM Tris-HCl buffer, pH 8.0 and incubated for 20 min at different temperatures ranging from 37-100 °C. The protein solutions were cooled to room temperature immediately post heating and PLA_2 activity and pharmacological properties of the heated and native PLA_2 enzymes were analyzed.

3.2.5.12 Circular dichroism

Circular dichroism (CD) measurement was performed using a Jasco J-720 spectropolarimeter (Tokyo, Japan). Protein samples (native, heated at 100 °C for up to 45 min and chemically modified) were dissolved at room temperature (25 °C) in Millipore water at a final concentration of ~1 mg /ml. CD spectra were recorded using a quartz cuvette with an optical path length of 0.5 mm. Each CD spectrum represents an average of 5 scans in the range of 195–250 nm, collected at 0.2-nm intervals, with a spectral band width of 0 5 nm and 4 s integration time. The CD spectra were corrected by subtraction of water blank and expressed in molar ellipticity [θ] (degrees cm^2 $dmol^{-1}$), using 113 as mean residue molecular weight (Ali et al., 1999).

3.2.5.13 Chemical modification of PLA_2 enzyme by ρBPB

Chemical modification of PLA_2 enzymes were done as described by Roberts et al. (1977). Purified proteins in a concentration range of 0.025-0.05 mg/ml were incubated with 3.3 mM ρBPB (final concentration) in 25 mM Tris-HCl buffer, pH 8.0 at 4 °C for 24 h. The reaction mixture was desalted in Sephadex G-25 column (1X30 cm^2) to remove the excess reagent. The modified proteins were assayed for PLA_2 activity as well as for other pharmacological properties.

3.2.5.14 Chemical modification of PLA_2 enzyme by other inhibitors

To investigate the effect of other inhibitors on PLA_2 enzyme activity, 1 μg of purified enzyme in 25 mM Tris-HCl buffer, pH 8.0 was incubated with different inhibitors viz; PMSF (serine-protease inhibitor), TCPK, TLCK (serine and cysteine inhibitor), Leupeptin (protease inhibitor), EDTA (metal chelator), DTT (reducing agent for disulphide bridge) at different molar concentrations at 37 °C for 30 min. After incubation, PLA_2 activity and other pharmacological properties of modified proteins were assayed.

3.2.6 Pharmacological characterization

3.2.6.1 LD_{50} determination

LD_{50} of crude venom/purified proteins was determined by intravenous (i.v.) injection of different concentrations of venom/purified protein in 0.2 ml of physiological saline into the tail vein of BALB/C albino mice (20 ±2 g). Four mice were used at each venom dose. Venom/purified proteins dose of each group was increased by 1 μg of protein until 50% mortality was observed within 24 h of venom injection (Larsen and Wolf, 1968)

3.2.6.2 Assay of plasma clotting activity

3.2.6.2.1 Ca-clotting time

The plasma clotting activity was assayed by the method of Angulo et al. (1997). Platelet poor plasma (PPP) from goat/healthy human donor was prepared by centrifuging (2,500 X g) the citrated blood (1:9) twice for 15 min at 4 °C and used within 4 h of collection. To assay the recalcification time, specific amount of crude/purified proteins (in a final volume of 30 μl) were added to 300 μl of PPP pre-incubated at 37 °C. The mixture was incubated for 2 min at 37 °C and 40 μl of 250 mM $CaCl_2$ was added to this reaction mixture. The clotting time of plasma was recorded with the help of stopwatch based on the first appearance of a fibrin clot. As a control, plasma aliquot were incubated with 30 μl of phosphate buffer saline (PBS) and coagulation time was determined identically.

3.2.6.2.2 Prothrombin time test

Prothrombin time (PT) of goat PPP was determined according to the method of Quick (1966) using commercial kit (Batch No. V-076) obtained from Tulip Diagnostics (P) Ltd. For the assay, 0.1 ml of PPP was taken in a clean and dry test tube and pre-incubated for 5 min at 37 °C in a water bath. Various amount of crude venom/purified proteins were added and incubated for 5 min in a water bath at 37 °C followed by addition of 0.2 ml of Liquiplastin reagent and appearance of the first clot was noted using a stop watch. 0.9% (w/v) NaCl served as control.

3.2.6.3 Assay of direct and indirect hemolytic activity

Hemolysis was assayed by the semiquantitative method of PLA_2 assay (Sharp et al., 1989). Direct hemolytic activity of crude/purified proteins were tested by incubating 10 μg of crude venom protein and 100 nM of either purified enzymes with 5 % (v/v) of human erythrocyte suspension in 100 mM phosphate buffer, pH 7.4 in a final volume of 3 ml. After incubating for 60 min at 37 °C, the reaction

mixture was centrifuged and released hemoglobin was measured spectrophotometrically at 540 nm. For indirect hemolytic assay, 10 μg of egg yolk suspension was added to the reaction mixture (as prepared for direct hemolytic assay) as a source of phospholipids. Total hemolysis (100%) was achieved by adding 2.7 ml of deionized water (Millipore) instead of venom protein to the erythrocyte suspension in phosphate buffer. Hemolysis was expressed as percent total hemolysis. To check the effect of purified PLA_2 enzymes on PC, PS and PE enriched human erythrocyte; the method of Diaz et al. (2001) was followed. Briefly 300 μl of 5% (v/v) human RBC suspension was pre-treated with 5 mM of either PC, PS or PE for 1 h at 4 °C. The pretreated cells were then incubated with 10 μg of PLA_2 enzyme for a period of 3 h at 37 °C either in presence or in absence of 1.5 mM $CaCl_2$ and percentage of hemolysis was determined as described above.

3.2.6.4 Edema-inducing activity

Edema-inducing activity of crude venom/purified PLA_2 was measured either by the method of Yamakawa et al. (1976) or Lomonte et al. (1999). For the method of Yamakawa et al. (1976), group of 4 mice (20 ± 2 g) was injected into the right footpad of the hind limb with different dose of purified venom protein in a total volume of 50 μl. The left footpad received only 50 μl of 0.9% saline and served as control. The minimum edema dose (MED) is the amount of protein required to cause edema ratio (weight of edematous leg X 100/weight of normal leg) of 120%. For the method of Lomonte et al., (1999) a group of four mice (20-21 g) received subcutaneous injection of 25 μg of PLA_2 enzymes in 100 μl of PBS, or 100 μl PBS alone, in their right food pad. After a regular time interval, increase in the thickness of the footpad was measured using a spring capillary.

3.2.6.5 Assay of *in-vitro* tissue damaging activity

For the assay of *in-vitro* tissue damaging activity, procedure of Datta and Bhattacharyya (1999) was followed with the following modifications. Fresh chicken liver/heart/lungs was washed with 0.9% NaCl, cut into small uniform sized pieces,

patted dry with tissue paper and 300 ±10 mg of tissue were taken. The tissues were pre-incubated with 1 ml of 0.2 M K-Phosphate buffer, pH 7.4 for 45 min at 37 °C. The tissue were washed twice with the same buffer and incubated with 25 μg of either crude venom or purified protein in a final volume of 2.5 ml of 0.2 M K-Phosphate buffer, pH 7.4 for 5 h at 37 °C. After incubation, the reaction mixture was centrifuged for 5 min at 3000 rpm and the absorbance of the supernatant was read at 540 nm by an UV/Vis spectrophotometer (Hitachi). The percentage of hemoglobin released i.e. the *in-vitro* tissue damaging activity of crude venom/purified protein was calculated with respect to tissue incubated with 0.1% (v/v) Triton X-100 (100% activity).

3.2.6.6 Assay of myotoxicity

3.2.6.6.1 Histological observation

Myotoxicity induced by crude venom/purified proteins was evaluated by histological observation. Mice were anaesthetized with ether, and a single i.m injection of twice the amount of LD_{50} of crude venom/purified protein in a volume of 100 μl of normal saline was given into the calf muscle of mice. Control mice received only 100 μl of normal saline. Animals were killed post 4 h of injection by cervical dislocation, muscles were removed from the site of injection and fixed in Bouin's solution followed by dehydration in different grades of alcohol (Babu and Gowda, 1991). The processed tissues were embedded in paraffin wax and 5 μm thick sections were cut using microtome. The sections were then subjected to redehydration and dehydration in different grades of alcohol and finally stained with hematoxylin-eosin stain. Areas exhibiting pathological changes were photographed with Kodak film.

3.2.6.6.2 Assay of plasma LDH activity

To determine the amount of LDH released into the blood, a group of 4 mice were injected with 25 μg of crude venom/purified protein (i.v) in 50 μl of PBS and

control group received 50 μl of PBS only. After 3 h of injection, blood samples were collected from the tail vein of mice. The amount of LDH released was determined calorimetrically (King, 1965). Briefly, 0.3 ml of serum was transferred in a tube containing 0.1ml of 3.5 mM NADH and the final volume was adjusted to 3 ml with 0.1 M K-Phosphate buffer pH 7.4. The reaction mixture was incubated for 10 min at 37 °C and the decrease in O.D. at 340 nm was recorded immediately post adding 0.1 ml of 21 mM pyruvic acid. One Unit (U) of LDH activity is defined as one μmol of LDH release/min/ml.

3.2.6.7 Assessment of neurotoxicity

For neurotoxicity assessment the method of Mukherjee and Maity (2002) was followed. Different concentration of crude/purified proteins (in a final volume of 50 μl) was injected (i.v) into the tail vein of BALB/C albino mice (20 ±2 g). All animals were constantly observed for the appearance of neurotoxic symptoms e.g. convulsion, hind limb paralysis, lacrination, urination and respiratory distress post injection of crude/purified protein.

3.2.6.8 Assay of antibacterial activity

Antibacterial (bactericidal) activity of crude venom and purified PLA_2s against *Escherichia coli* (DH5α) (Gram negative) and *Bacillus subtilis* (Gram positive) bacteria was assessed by plate count technique (i.e. time course experiment) as described by Haeberli et al. (2000) as well as by zone of inhibition. For plate count technique, 1 ml of mid-log phase culture of either bacteria ($OD_{630} \cong 0.27$) was inoculated with graded amount of crude venom/purified protein samples, dissolved in growth media and incubated at 30 °C for 5 h. The change in the optical density at 630 nm was recorded at different time intervals. For control experiment, equal volume of medium was used instead of protein sample. For observing the colony forming units, serial dilution of bacterial culture treated with either crude venom or purified PLA_2 was plated on nutrient agar plate. The plates were incubated at 30 °C for overnight and the next day, number of colony formed were counted. To check

the zone of bacterial growth inhibition, bacterial culture were grown on LB medium at 37 °C for 12-24 h till saturation and were maintained on nutrient agar slants. Serial dilutions were prepared in sterile distilled water and the agar surface was spreaded with 0.1 ml of bacteria (ca. $3X10^8$ cells/ml). Various amount of samples (1-100 μg in a volume of 2 μl) were applied at different points on the plate and incubated for 24 h at 37 °C. The diameter of the clear zone at around the point of application of crude venom/purified enzyme, an indication of inhibition of bacterial growth, was measured. The experiment was carried out in triplicate.

3.2.6.9 Isolation of chicken liver mitochondria

Mitochondria were isolated from fresh chicken liver as described by Valente et al. (1998). All the operations were carried out at 4°C, unless otherwise stated. Liver tissues were homogenize in 0.25 M sucrose containing 20 mM Tris-HCl pH 7.4 and 1 mM EDTA (isolating buffer), centrifuged for 10 min at 460 X g to sediment the nuclei. The supernatant was transferred to another tube and centrifuged at 12,500 X g for 7 min. In appearance, the centrifuge tube has three distinct layers. The middle layer containing the mitochondria was dislodged very gently, re-suspended in 4 ml of isolating buffer and centrifuged at 12,500 X g for 7 min at 4°C. Finally, the pallet was re-suspended in isotonic buffer (20 mM Tris-HCl pH 7.4 containing 0.3 M sucrose) to give a final protein concentration of 80-100 mg/ml.

3.2.6.9.1 Assay of mitochondrial swelling

To check the effect of native/chemically modified PLA_2 enzymes on mitochondrial swelling the method of Valente et al. (1998) was followed. Isolated mitochondria were suspended (0.5 mg/ml) in 0.25 M sucrose containing 20 mM Tris-HCl, pH 7.4 and incubated with increasing doses of PLA_2 enzymes either in presence or absence of Ca^{2+} at room temperature (~25°C) for 30 min. Mitochondrial swelling was monitored spectrophotometrically by the decrease in absorbance at 520 nm using a double beam spectrophotometer (Hitachi, Tokyo, Japan). The change in the optical density at 520 nm was recorded at different time interval. One

unit of mitochondrial swelling is defined as decrease in 0.01 OD at 520 nm after 30 min per mg of mitochondria. Basic test system without PLA_2 enzyme served as control.

3.2.6.9.2 Analysis of free fatty acids by GC-MS

For quantitative analysis of free fatty acids liberated form the mitochondrial membrane, 100 mg equivalent of mitochondria (Mitochondria isolated from 100 mg wet weight of tissue) from chicken liver was incubated with 100 nM of either enzymes. The liberated free fatty acids were isolated as described in section 3.2.5.3 and were scraped into glass tubes and methylated as described by Shukla and Hanahan, (1982). Briefly, 1 ml of 0.5% (v/v) H_2SO_4 in methanol was added. The tubes were sealed and incubated at 70 °C for 1 h with intermittent mixing. At the end of the incubation, 1 ml of distilled water was added and fatty acid methyl esters were extracted four time with 2 ml of *n*-hexane and the fatty acid methyl esters were analyzed on a Varian GC-MS 3800, Saturn 2000 system. Samples were injected with splitless mode into a chrompack capillary GC column CP-Sil 8 CB low bleed (30 m x 0.25 mm x 0.25 μm) coupled with a CP-Sil 5 CB low bleed /MS (30 m x 0.25 mm x 0.25 μm) column with helium as a carrier gas. The column temperature was 80-240 °C for 30 min with 5 °C/min increament and hold at 240 °C for 30 min, the injector temperature was 240 °C and the transferline temperature was 300 °C. The data were acquired in chemical ionization mode (70 eV). Mass spectra of unknown methylated fatty acids were identified by comparing with the retention time of authenticated fatty acids, using the Saturn 2000 MS library search where 99% matching was observed.

3.2.7 Immunological Characterization

3.2.7.1 Raising of polyclonal antibody against NK-PLA_2-I in rabbit

0.2 mg of lyophilized protein in 0.2 ml of phosphate buffered saline, pH 7.4, was mixed with equal volume of Freund's complete adjuvant and injected intradermally into an inbred male albino rabbit (2.0 kg) at several sites on the dorsal surface of the neck. Four booster doses were administered at weekly intervals at the same concentration, but with equal volume of Freund's incomplete adjuvant. After resting the animal for 10 days, blood was drawn from the marginal ear vein. Antisera was separated by allowing the blood to stand overnight at 4 °C. Gamma-globulin (anti-NK-PLA_2-II IgG) was isolated from the antiserum using ammonium sulfate precipitation and precipitated antibodies were detected by Ouchterlony agar gel double diffusion technique.

3.2.7.2 Isolation of IgG

To two volumes of IgG containing serum was mixed with 1 volume of saturate ammonium sulfate solution (33 ml of 33% (v/v) ammonium sulfate solution and 67 ml of PBS, pH 7.0) at 4°C mixing constantly. It was allowed to stand at 4°C for 4 h with constant agitation to form precipitation. The precipitate was centrifuged at 12,000 X g for 20 min, then pellet was washed with ice-cold 33% saturated ammonium sulfate solution equivalent to volume of original IgG containing mixture and centrifuged to obtain the pellet. The pellets were resuspended in 1 volume of PBS and dialyze for 48 h at 4°C with three changes of PBS using dialysis tube (MWCO, 12,000 t0 14,000, Sigma). The antibody solution was removed and centrifuged to remove the remaining debris. The concentration of the antibodies was determined by Lowry's et al. (1951) and stored in presence of 0.02% (w/v) sodium azide.

3.2.7.3 Immunological cross-reactivity

3.2.7.3.1 Gel-immunodiffusion

Antigenic cross reactivity between the purified protein (NK-PLA_2-I and NK-PLA_2-II) and antiserum raised against *Naja naja* or NK-PLA_2-I was analyzed by agarose gel immunodiffusion. 1% (w/v) agarose was prepared in PBS containing 0.02% (w/v) sodium azide. Wells of suitable diameter were prepared on the plate using a puncher, one in the centre and four surrounding the central well. The central well was filled with antiserum while the surrounding wells were filled with antigens of different dilutions. Gel plates were incubated in humid chamber at 37 °C for 1-2 days till the appearance of precipitin line. After the formation of precipitin line, the gels were extensively washed for 24 h with several changes of normal saline, dried and stained with 1% (w/v) Coomassie brilliant blue R-250.

3.2.7.3.2 Immunoelectrophrosis

Briefly, 1% (w/v) agarose was prepared containing 0.1% (w/v) sodium azide in 0.05 M citrate phosphate buffer, pH 6.2. The molten agarose solution was poured on a clean dry microscopic slide approximately to a thickness of 2 mm. The gel was cooled in a humid chamber at 4 °C for 1 h. Two holes were punched and appropriate amount of antigen (purified protein) was loaded. Electrophoresis was carried out in horizontal electrophoresis chamber in 0.05 M citrate phosphate buffer, pH 6.2. It was run for 3 h at 20 mA current. After electrophoresis a trough was cut along the slide between the two holes and 20 μl of antibody (1mg/ml) against NK-PLA_2-I raised in rabbit was added and incubated in a humid chamber at 37 °C for 48 h. After the formation of precipitin line, the gels were extensively washed for 24 h with several changes of saline, dried and stained with 1% (w/v) Coomassie brilliant blue R-250.

3.2.7.3.3 Western blotting

Purified proteins under reduced condition were separated on 15% SDS-PAGE according to Laemmli, (1970). Proteins were electroblotted into 0.45 μm Immobilon-P (Millipore) membrane at 150 mA for 3 h in a Mini transblot Cell (Pharmacia) using the buffer system described by Towbin et al. (1979). Membrane bound proteins were reversible stained with Ponsceau S to check the transfer efficiency. Strips were cut, blocked with fat free milk and incubated with either antiserum against *Naja naja* or antiserum against NK-PLA_2-I (1:4000 dilution in TBST) at 37 °C for 1 h. Bound antibodies were detected by incubating with anti-horse IgG conjugate or Goat anti-rabbit IgG ALP conjugate for 1 h at room temperature using Tetramethyl benzidine/hydrogen peroxide (TMB/H_2O_2) or BCIP/NBT as substrate. Washing in plenty of H_2O stopped further development of the colour.

3.2.8 Preparation of plant extract

Plant extracts were prepared according to Mahanta and Mukherjee (2001). Fresh leaves/roots/bark were shade dried and made to coarse powder. Two g of powder of leaves/roots/bark was taken in a beaker and soaked with 100 ml of H_2O with continuos stirring for 2 h at room temperature. The extract was filtered through a muslin cloth and filtrate was concentrated at 40 °C under vaccum. The dried extract was dissolved in normal saline (0.9% NaCl) at a concentration of 1 mg/ml and kept at 4 °C until further use. However for alcohol/chloroform extraction, same amount of powder was soaked in 100 ml of alcohol/chloroform with continuos stirring for 2 h. After filtration, concentration and drying the residue was suspended in normal saline at a concentration of 1 mg/ml and stored at 4 °C until further use.

3.2.9 Neutralization of catalytic activity and pharmacological properties of PLA_2 enzyme

3.2.9.1 By polyvalent antivenom against crude *N. naja* venom/anti-NK-PLA_2-I IgG

For neutralization of PLA_2 activity, graded amount (μg) of polyvalent antivenom/anti-NK-PLA_2-I IgG was incubated with a fixed amount of crude *venom/purified proteins at 37 °C for 30 min. followed by assay of* PLA_2 *activity and* pharmacological properties. Activity without polyvalent antivenom/anti-NK-PLA_2-I IgG was served as control (100% activity) and compared with other values.

3.2.9.2 By plant extract

Neutralization of PLA_2 activity of *N. kaouthia* crude venom/purified proteins by plant extracts was studied by pre-incubating with graded amount (μg) of plant extracts (1mg/ml) with fixed amount of venom protein/purified enzymes for 30 min at 37 °C. PLA_2 activity of venom-plant extract mixture was assayed as described in section 3.2.5.5.1. PLA_2 activity without plant extract was treated as control (100% activity) and compared with other values.

CHAPTER IV

A COMPARATIVE STUDY OF PLA_2 ISOENZYME PATTERNS FROM THE VENOM SAMPLES OF *Naja kaouthia* AND *Naja naja* OF THE EASTERN INDIA ORIGIN

4.1 Results

4.1.1 Fractionation of *Naja kaouthia* and *Naja naja* venom by cation exchanger

25 mg of *N. kaouthia* and *N. naja* venom samples were fractionated on a CM Sephadex C-50 cation exchanger, pre-equilibrated with 20 mM K-Phosphate buffer, pH 8.0. Elution was done stepwise with basic K-Phosphate buffer of increasing molarity and pH values. 1 ml of fraction was collected in each tube and fractions were pooled according to the PLA_2 content. Fractionation reveals the presence of seven basic PLA_2 isoenzymes in both *N. kaouthia* and *N. naja* venom samples. The *N. kaouthia* venom PLA_2 isoenzymes were designated as NK CMa – NK CMh whereas the *N. naja* PLA_2 isoenzymes were named as NN CMa – NN CMh. The non-retained, acidic PLA_2 isoenzymes of both the venom samples were eluted as a single peak (NK CMa / NN CMa) (Fig. 4.1). A Comparison of the enzymatic activity of the isoenzymes of both the venom samples revealed that *N. naja* PLA_2 isoenzymes were catalytically more active in hydrolyzing egg yolk phospholipids as compared to *N. kaouthia* PLA_2 isoenzymes under identical conditions (Table 4.1). Further, the protein content of the basic PLA_2 isoenzymes of *N. naja* was also higher than that of the *N. kaouthia* (Table 4.1). Among the basic PLA_2 isoenzymes of the either venom, peak NK CMe and NN CMe, eluted by K-Phosphate buffer of 110 mM and pH 8.0 have the highest protein content, whereas peak NK CMb and NN CMb displayed highest enzymatic activity. The basic PLA_2 isoenzymes of both *N. kaouthia* and *N. naja* constitute 30.19% and 31.46% of the total venom protein respectively. The summary of the fractionation of *N. kaouthia* and *N. naja* venom samples is shown in table 4.1.

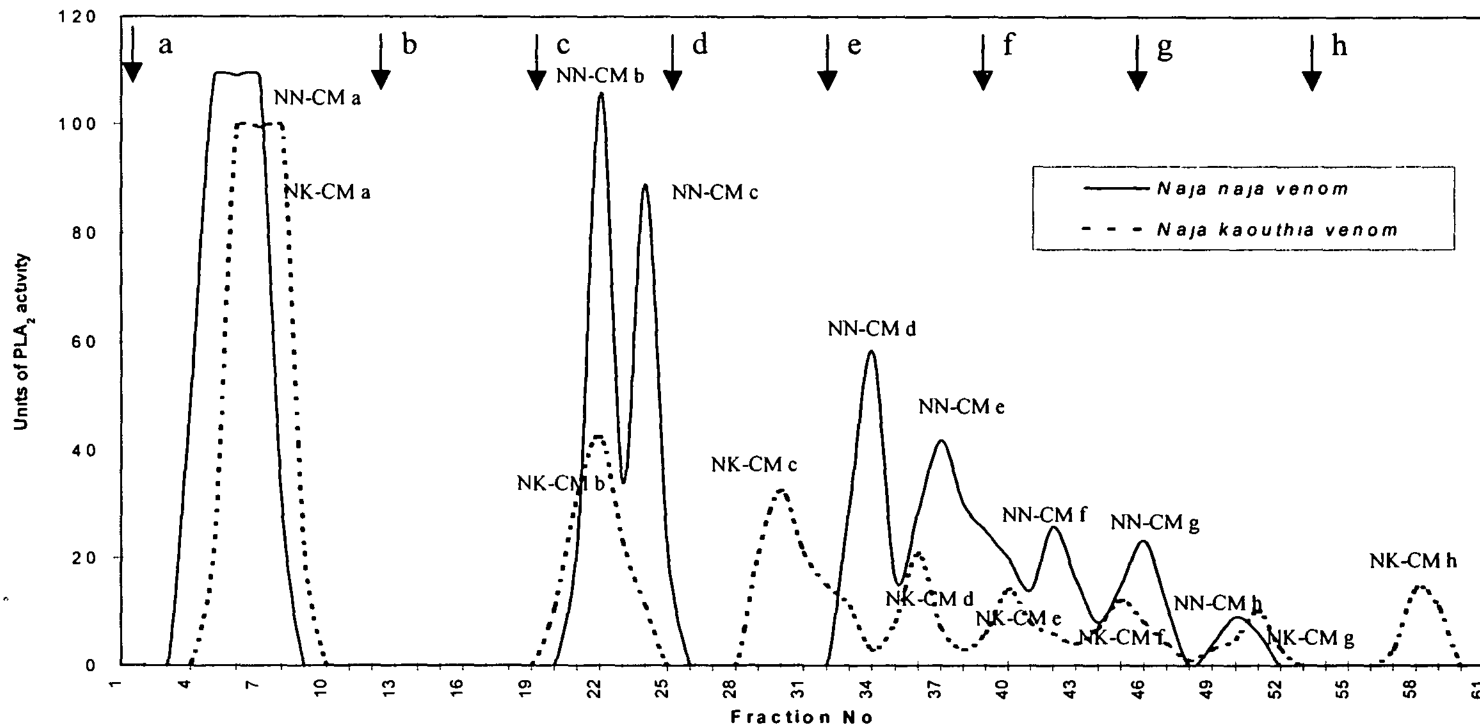

Fig. 4.1. CM-Sephadex C-50 chromatography of *N. kaouthia* and *N. naja* venom. The column (2.5 X 2 cm^2) was equilibrated with 20 mM K-phosphate buffer (pH 7.0) and loaded with 25.0 mg of either venom dissolved in 2.0 ml of the same buffer. The non-retain fraction was eluted during the washing step and the bound fractions were eluted stepwise with phosphate buffers of various molarities and pH values at 23 °C. The increasing molarities and pH values were (a) 20 mM, pH 7.0, (b) 75 mM, pH 7.5, (c) 90 mM, pH 8.0, (d) 100 mM, pH 8.0, (e) 110 mM, pH 8.0, (f) 130 mM, pH 8.0, (g) 150 mM, pH 8.0 and (h) 180 mM, pH 8.0. 1 ml fraction was collected at a flow rate of 24 ml/hr. The elution of protein was monitored at 280 nm using D®U UV/Vis spectrophotometer (Beckman). Individual fractions were screened for PLA_2 activity as described in section 3.2.5.5.1.

Table 4.1. Summary of fractionation of *N. kaouthia* and *N. naja* venom samples on CM Sephadex C-50 column.

Values are from a typical experiment.

Peaks	*Naja kaouthia*		*Naja naja*	
	% recovery of protein[a]	**PLA_2 specific activity[b]**	**% recovery of protein[a]**	**PLA_2 specific activity[b]**
Crude venom	100	87.95×10^3	100	93.4×10^3
NK CMa / NN CMa	18.8	99.5×10^3	17.74	108.9×10^3
NK CMb / NN CMb	5	42.6×10^3	1.106	105.9×10^3
NK CMc / NN CMc	3.2	32.6×10^3	1.33	89.3×10^3
NK CMd / NN CMd	3.78	20.9×10^3	1.08	58.36×10^3
NK CMe / NN CMe	10.12	14.19×10^3	14.07	41.916×10^3
NK CMf / NN CMf	4.52	12.93×10^3	7.20	25.9×10^3
NK CMg / NN CMg	1.39	10.06×10^3	3.58	23.3×10^3
NK CMh / NN CMh	2.18	12.45×10^3	3.09	9.06×10^3

[a] Protein amount was estimated by the method of Lowry et al. (1951).

[b] One unit of PLA_2 activity is defined as decrease in 0.01 absorbance at 740 nm as compared to control.

A: absent

4.1.2 Fractionation of *Naja kaouhtia* and *Naja naja* venom samples on DEAE Sephadex A-50

Fractionation of 15 mg of *N. kaouthia* and *N. naja* venom samples on DEAE Sephadex A-50 anion exchanger results separation of acidic PLA_2 isoenzymes. Fractionation reveals presence of two acidic PLA_2 isoenzymes in *N. kaouthia* venom sample and three acidic PLA_2 isoenzymes in *N. naja* venom sample (Fig. 4.2). The first unbound proteins, NK-I and NN-I are those of the basic isoenzymes as the anion exchanger did not retain them. Acidic PLA_2 isoenzymes of *N. kaouthia* venom were designated as NK-II and NK-III while that for *N. naja* venom are NN-II, NN-III and NN-IV. Comparison of catalytic activity of the acidic PLA_2 isoenzymes revealed that, *N. kaouthia* acidic isoenzymes were less active than those of the *N. naja* counterparts. The protein content as well as the specific activity of the *N. naja* acidic PLA_2 isoenzymes were also higher than that of the *N. kaouthia*. The summary of the fractionation of both the venom samples is shown in table 4.2.

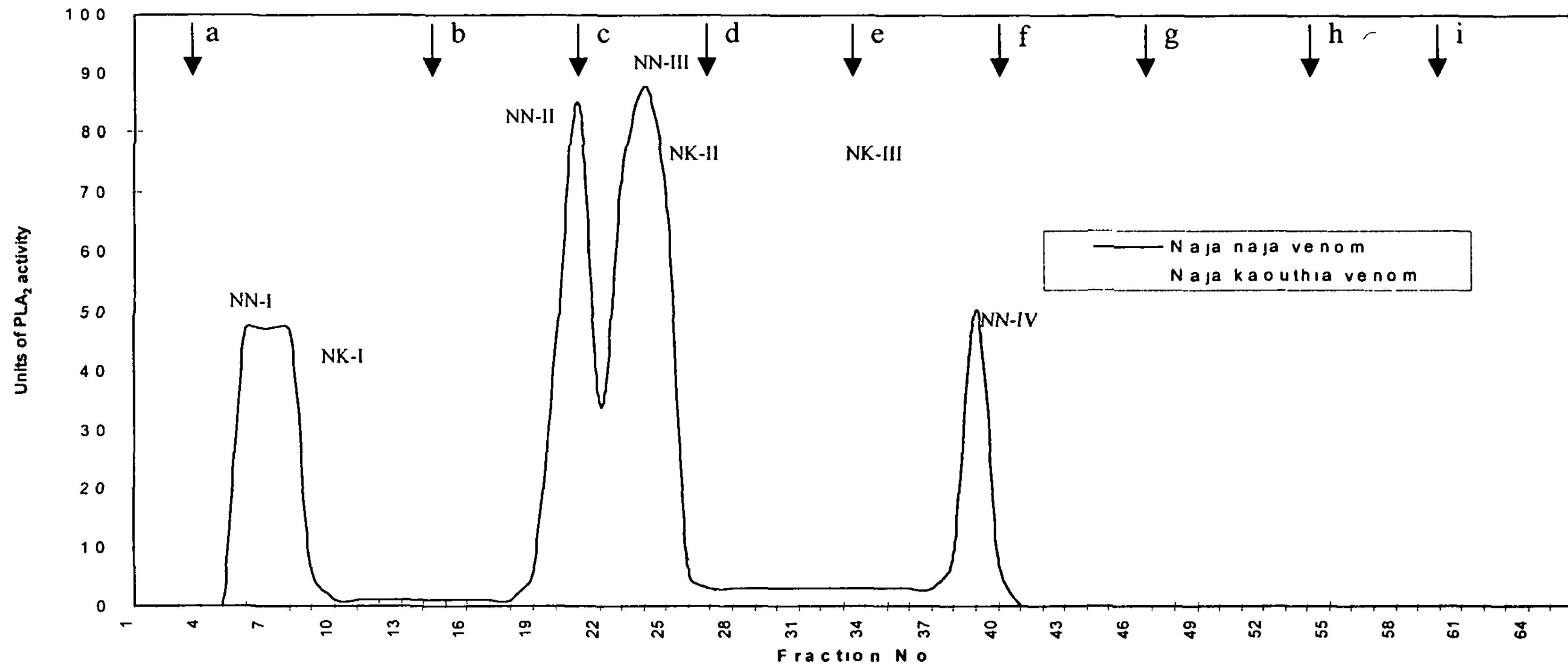

Fig. 4.2. DEAE Sephadex A-50 chromatography of *Naja kaouthia* and *Naja naja* venom samples. The column (2.5 X 2 cm^2) was equilibrated with 20 mM K-phosphate buffer (pH 7.0) and loaded with 25.0 mg of either venom dissolved in 2.0 ml of the same buffer. The non-retain fraction was eluted during the washing step and the bound fractions were eluted stepwise with phosphate buffers of various molarities and pH values at 23 °C. (a) 20 mM, pH 7.0, (b) 80 mM, pH 6.5, (c) 120 mM, pH 6.0, (d) 140 mM, pH 6.0, (e) 160 mM, pH 5.5, (f) 180 mM, pH 5.5, (g) 200 mM, pH 5.5, (h) 230 mM, pH 5.0 and (i) 260 mM, pH 5.0. 1 ml fraction was collected at a flow rate of 24 ml/hr. The elution of protein was monitored at 280 nm using D®U UV/Vis spectrophotometer (Beckman). Individual fractions were screened for PLA_2 activity as described in section 3.2.5.5.1.

Table 4.2. Summary of fractionation of *N. kaouthia* and *N. naja* venom samples on a DEAE Sephadex A-50 ion-exchange column.

Values are from a typical experiment.

Peaks	*Naja kaouthia*		*Naja naja*	
	% recovery of protein[a]	**PLA_2 specific activity[b]**	**% recovery of protein[a]**	**PLA_2 specific activity[b]**
Crude venom	100	87.95×10^3	100	93.4×10^3
NK I / NN I	23.11	45×10^3	30.35	47×10^3
NK II / NN II	5.36	81.2×10^3	7.81	85.07×10^3
NK III / NN III	1.68	76.2×10^3	3.43	87.85×10^3
NK IV / NN IV	A	A	1.35	15.5×10^3

[a] Protein amount was estimated by the method of Lowry et al. (1951)

[b] One unit of PLA_2 activity is defined as decrease in 0.01 absorbance at 740 nm as compared to control.

A: absent

CHAPTER V

ISOLATION, PURIFICATION AND CHARACTERIZATION OF NK-PLA$_2$-I

5.1 Results

5.1.1 Fractionation of *Naja kaouthia* venom on CM Sephadex C-50

50 mg of *N. kaouthia* crude venom was fractionated in CM-Sephadex C-50 cation exchanger (20 x 60 mm^2) at ~23 °C, using basic buffers of increasing molarity and pH values. As depicted in fig. 5.1, fractionation resulted separation of venom components into eight fractions, a major unbound protein peak of acidic components (CM I), followed by one major and six minor peaks of basic proteins (CM II–CM VIII). Screening of these peaks for phospholipase A$_2$ activity revealed that maximum enzymatic activity was associated with CM-I followed by CM II. Peak CM I that displayed the highest PLA$_2$ activity, constitutes about 18.8% of the total venom protein. CM I and CM II were concentrated in Maxi Dry Plus (Heto Lab Equipment) at –20 °C and further purified. The summary of the fractionation of crude *N. kaouthia* venom on CM-Sephadex C-50 ion exchange chromatography is shown in Table 5.1.

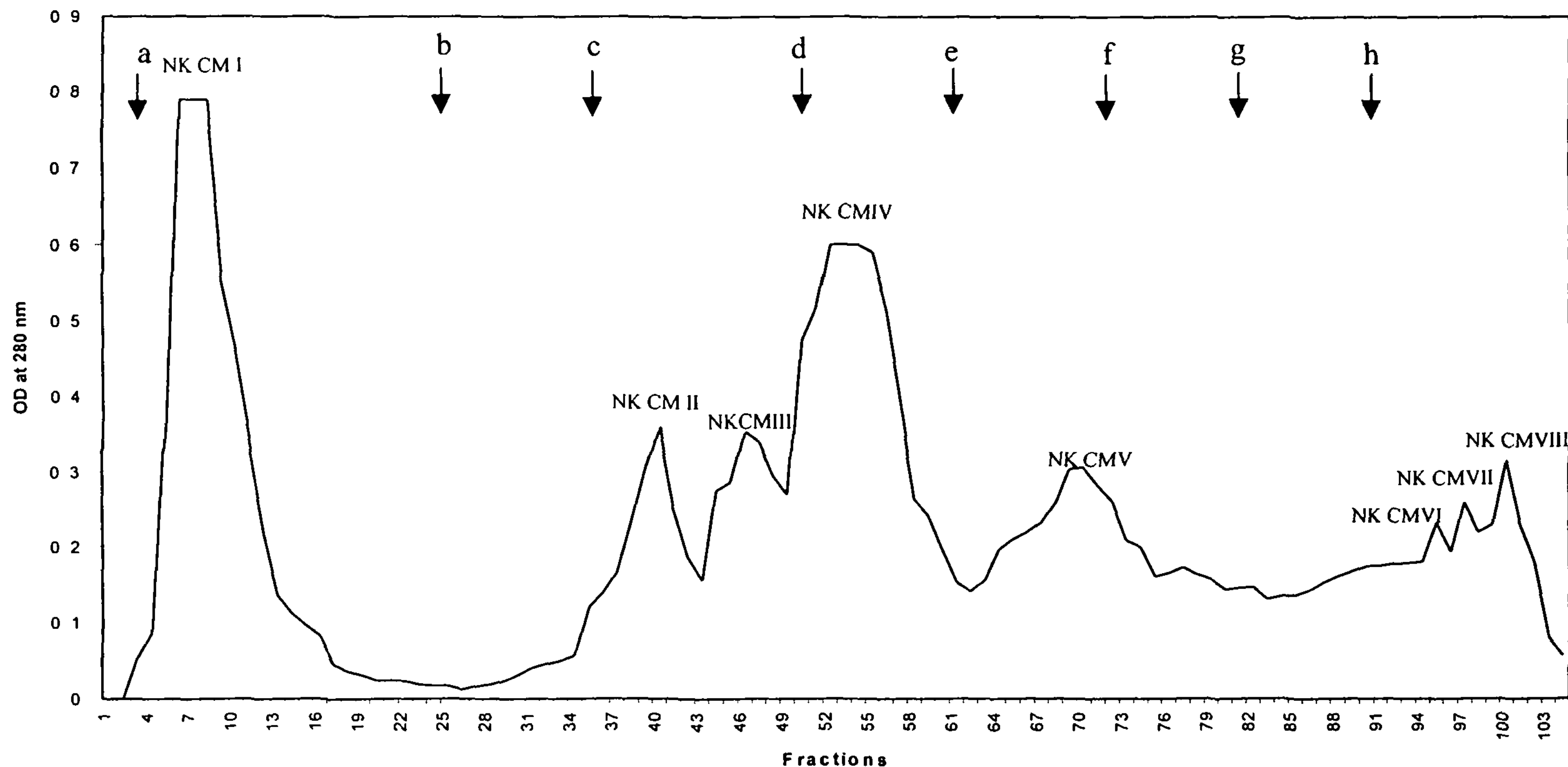

Fig. 5.1. CM-Sephadex C-50 chromatography of *Naja kaouthia* venom. The column (20 X 60 mm^2) was equilibrated with 20 mM K-phosphate buffer (pH 7.0) and loaded with 50.0 mg venom protein dissolved in 4.0 ml of the equilibration buffer. The non-retained fraction was eluted with the equilibration buffer during the washing step and the bound fractions were eluted stepwise with phosphate buffers of various molarities and pH values at ~23 °C. (a) 20 mM, pH 7.0, (b) 75 mM, pH 7.5, (c) 90 mM, pH 8.0, (d) 100 mM, pH 8.0, (e) 110 mM, pH 8.0, (f) 130 mM, pH 8.0, (g) 150 mM, pH 8.0 and (h) 180 mM, pH 8.0. 1 ml fraction was collected at a flow rate of 24 ml/hr. The elution of protein was monitored at 280 nm using D®U UV/Vis spectrophotometer (Beckman).

Table 5.1. Summary of fractionation of *N. kaouthia* venom on CM Sephadex C-50 cation exchanger.

Values are from a typical experiment.

Peaks	Total protein (mg)	% Yield of protein	PLA_2 activity	
			Total activity (Units)[a]	Specific activity (Units/mg)
Whole venom	50.00	100	418.5 X 10^4	83.70 X 10^3
NK-CM I	9.4	18.8	876.08 X 10^3	93.20 X 10^3
NK-CM II	2.5	5.0	106.5 X 10^3	42.60 X 10^3
NK-CM III	3.02	6.04	63.11 X 10^3	20.90 X 10^3
NK-CM IV	8.08	16.16	114.65 X 10^3	14.19 X 10^3
NK-CM V	0.26	0.52	3.35 X 10^3	12.90 X 10^3
NK-CM VI	1.116	2.232	11.22 X 10^3	10.06 X 10^3
NK-CM VII	1.106	2.212	13.769 X 10^3	12.45 X 10^3
NK-CM VIII	1.23	2.46	11.143 X 10^3	9.06 X 10^3

[a] One unit of PLA_2 activity is defined as decrease of 0.01 absorbency at 740 nm per 10 minute

5.1.2 Fractionation of CM I by Sephadex G-50 gel filtration column

Fractionation of 9.4 mg of CM-I in Sephadex G-50 column resulted in separation of two fractions, NK-GF-I and NK-GF-II (Fig. 5.2), which constitutes 5.2% and 6% of the total venom protein, respectively. NK-GF-II, displaying the considerable PLA_2 and anticoagulant activities, was termed as NK-PLA_2-I. This purified protein was lyophilized and stored at –20 °C for further characterization. Summary of purification of NK-PLA_2-I is shown in Table 5.2.

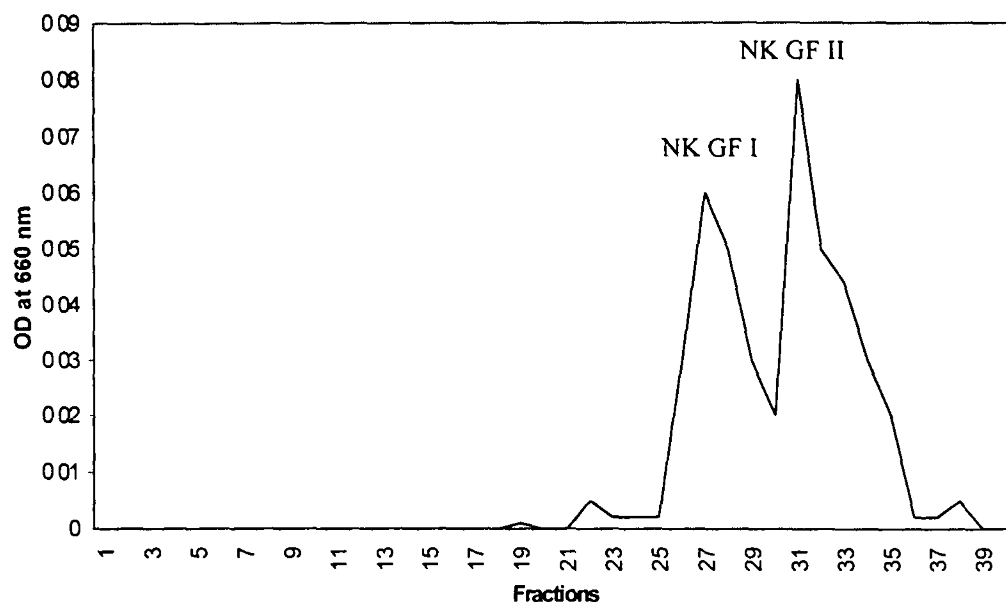

Fig. 5.2. Gel filtration of NK-CM I on Sephadex G-50 (1 X 64 cm^2) column. Elution of proteins was carried out with the equilibration buffer at a flow rate of 20 ml/h. Protein elution was monitored as described in section 3.2.5. and PLA_2 activity of each fraction was assayed as described in the section 3.2.5.5.1.

Table 5.2. Summary of purification of NK-PLA_2-I from *Naja kaouthia* venom. Datas are from a typical experiment.

Fraction	Total protein (mg)	Yield of protein (%)	PLA_2 activity	
			Total activity (Units)	Specific activity (Units/mg)
Whole venom	50.0	100.0	418.5×10^4	83.7×10^3
NK-CMI	9.4	18.8	876.1×10^3	93.2×10^3
NK-GF I	2.6	5.2	$393 64 \times 10^3$	75.7×10^3
NK-GF II	3.0	6.0	$568 2 \times 10^3$	94.7×10^3

[a]One unit of PLA_2 activity is defined as decrease of 0.01 absorbency at 740 nm per 10 minute

5.1.3 Homogeneity and molecular weight of NK-PLA_2-I

5.1.3.1 SDS-PAGE

Under both the reduced and non-reduced conditions, NK-PLA_2-I displayed sharp bands, whereas the whole venom depicted several sharp and diffused bands under reduced condition (Fig 5.3a). NK-PLA_2-I displayed a major band corresponding to a subunit with apparent molecular weight of 13.6 kDa. While in non-reduced condition it migrated as a single band of molecular weight 19.3 kDa. The calibration curve is shown in figure 5.3b.

5.1.3.2 Gel filtration

The native molecular weight and purity of the PLA_2 preparation were also judged by running the NK-PLA_2-I on a Sephadex G-50 column (1 X 64 cm^2) calibrated with molecular weight marker proteins. NK-PLA_2-I was eluted as a sharp, symmetrical peak, indicating purity of the preparation (Fig. 5.4a). From the calibration curve, molecular mass of NK-PLA_2-I, was determined as 12.9 kDa (Fig. 5.4b).

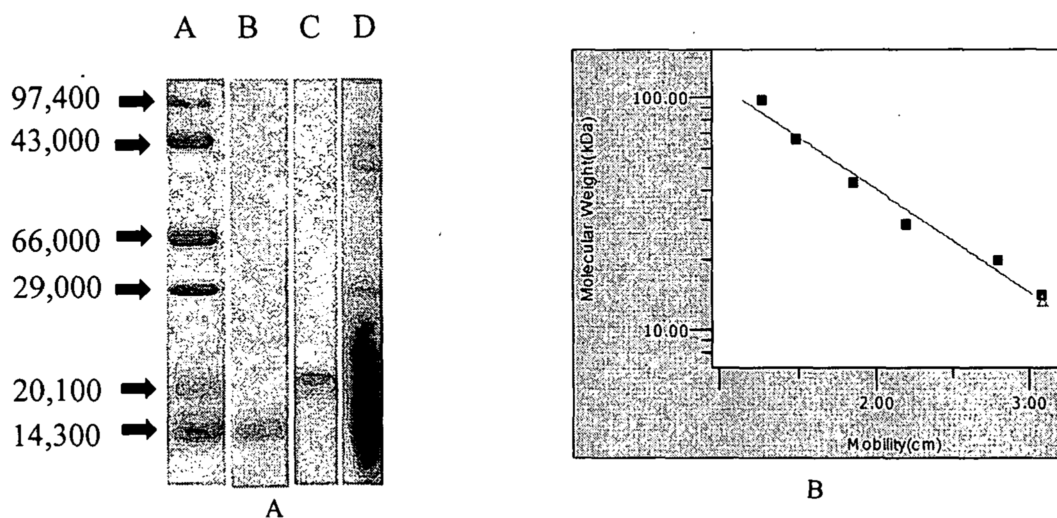

Fig. 5.3 a. 10% SDS-polyacrylamide gel electrophoresis. Lane A: molecular weight marker, Phosphorylase b (97,400), BSA (66,000), Ovalbumin (43,000), Carbonic anhydrase (29,000), Soyabean trypsin inhibitor (20,100) and Lysozyme (14,300); Lane B: Reduced NK-PLA$_2$-I (30 μg); Lane C: Non-reduced NK-PLA$_2$-I (30 μg) and Lane D: Crude *N. kaouthia* venom (30 μg).

b. Calibration curve of 10% SDS-PAGE with molecular weight marker protein. Graph was obtained using Bio-Rad Multi-Analyst™/PC version 1.1 software (Bio-Rad). ■ Indicates the molecular weight marker while ▲ indicates the molecular weight of NK-PLA$_2$–I (13.6 kDa).

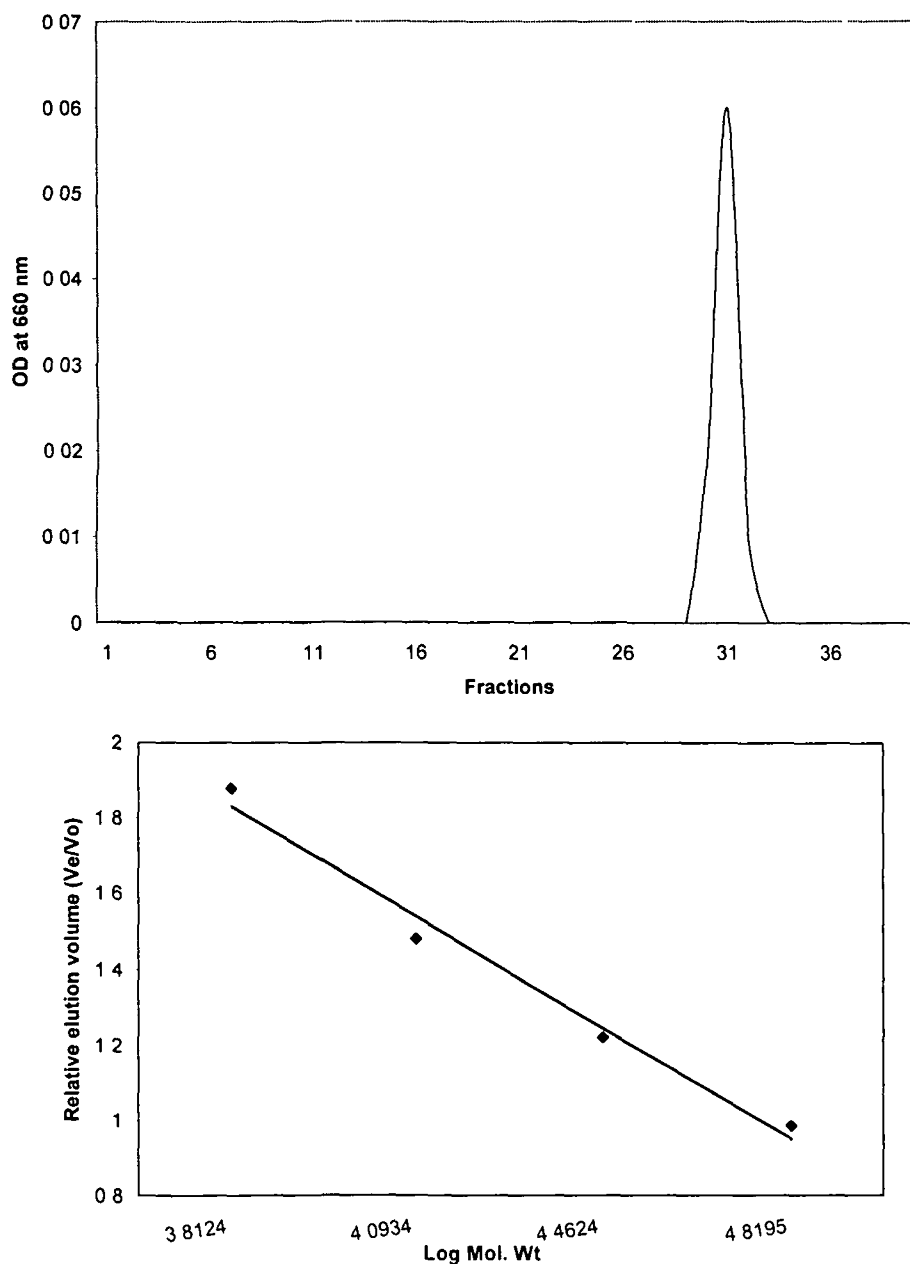

Fig. 5.4 a Elution of NK-PLA$_2$-I in Sephadex G-50 column. Elution of protein was carried out with equilibration buffer and monitored at 220 nm as described in section 3.2.4.2.

b. Calibration curve of Sephadex G-50 gel filtration column chromatography. Column was calibrated with following Molecular Weight Marker; Aprotinin (6,500), Cytochrome C (12,400), Carbonic anhydrase (29,000), BSA (66,000) and Blue dextran (2,00,000).

5.1.3.3 RP-HPLC

30 μg of NK-PLA$_2$-I was subjected to RP-HPLC. The elution of the protein with gradient of 5-60% buffer B over 28 min resulted into two peaks with a retention time of 1.152 min and 14 min respectively (Fig. 5.5). The former peak was that of solvent, which was confirmed by a blank run of only solvent, while the later peak showing PLA$_2$ activity was that of NK-PLA$_2$-I.

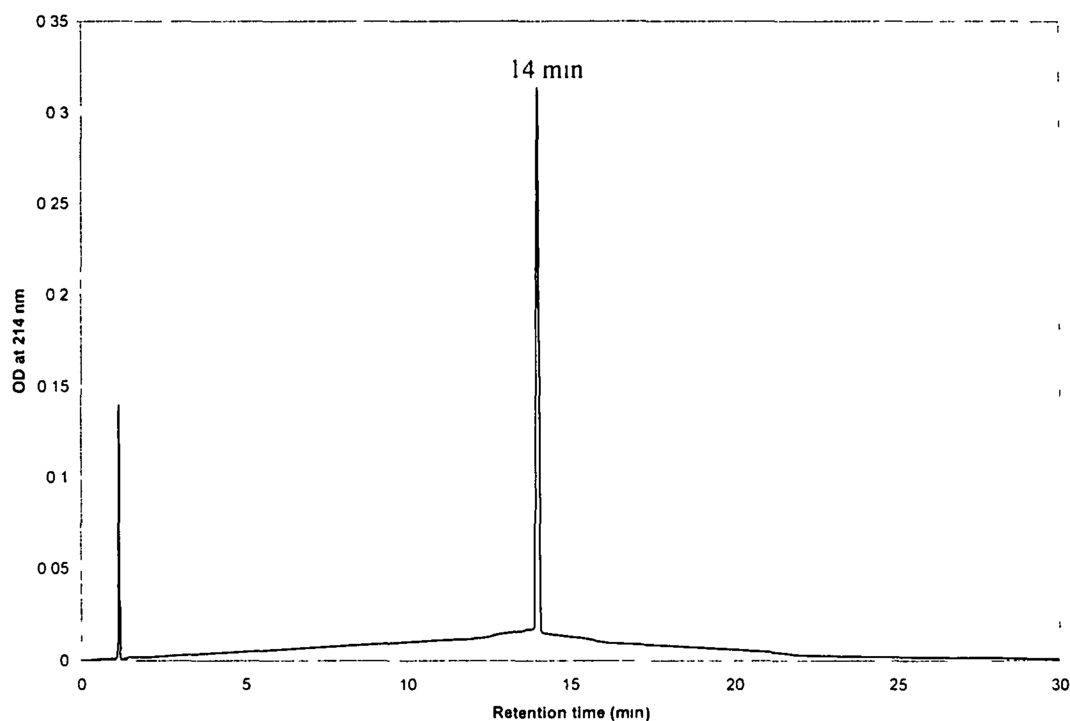

Fig. 5.5. RP-HPLC of NK-PLA$_2$-I on Waters reverse phase C_{18}-μ-Nova pack column. Buffer A was 0.1% (v/v) TFA and buffer B was 0.1% (v/v) TFA in 100% CH_3CN. Elution was performed at a flow rate of 1ml/min (0-60% B) and protein elution was monitored at 214 nm.

5.1.3.4. MALDI-MS

The purity and molecular mass of NK-PLA_2-I were further confirmed by MALDI-MS, which revealed protonated molecular ion [MH^+] at m/z 13,786.205 Da, indicating molecular mass of this protein was 13.78 kDa which was quite close to the mass determined by SDS-PAGE. MALDI-MS normally yields single charged states, but a lower intensity, doubly charged [MH^{2+}] peak at m/z 7193.1025 Da was also noticed (Fig 5.6).

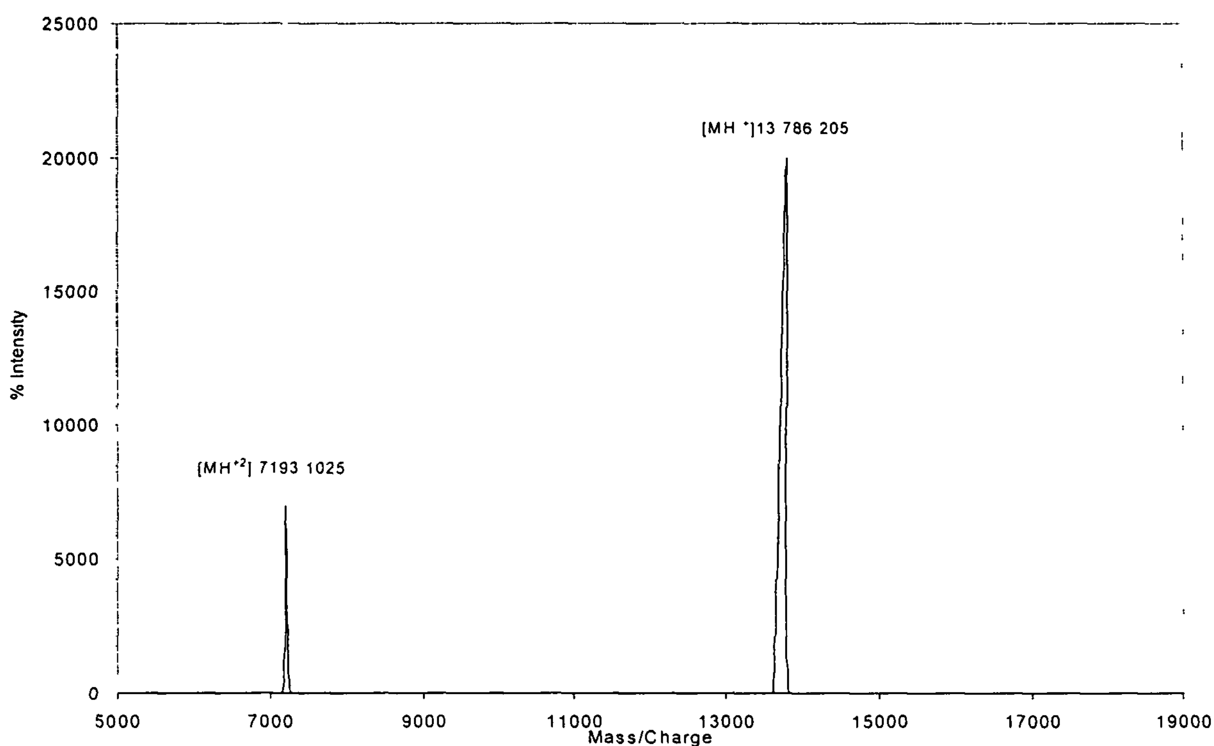

Fig. 5.6. MALDI-MS spectra of NK-PLA_2-I. Matrix: Sinapinic acid; number of shots 50; Laser power 30-40%. The spectra show both single [MH^+ = 13,786.205] and doubly charged [MH^{+2} = 7193.1025] molecules, giving a molecular weight of 13.786 kDa.

5.1.4 Biochemical characterization

5.1.4.1. Partial N-terminal amino acid sequencing

The NH_2 –terminal amino acid sequence of NK-PLA_2-I was determined up to 10 amino acid residues. The amino acid sequence, NIYQFKNNIQ was remarkably similar to another *N. kaouthia* venom PLA_2 isoenzyme i.e. NK-PLA_2-II. A comparison of sequence listed in the Gene bank protein database indicated that NK-PLA_2-I sequences share substantial homology with sequences of some of the already described snake venom phospholipase A_2 enzymes (Fig. 5.7).

PLA_2 enzyme	Amino acid sequence	% identity
NK-PLA_2-I	**NI YQFKNMIQ**	
PA2_NAJSP	**NLYQFKNMIQ**CTVPNR	90
PA21_MICNI	**NLYQFKNMIQ**CTTKRSVLEFMEYGCYC	90
PA22_NAJKA	**NLYQFKNMIQ**CTVPNRSWWHFADYGCFCGYGGSGT	90
PA2X_BUNFA	**NLYQFKNMIQ**CAGTRLWVAYVNYGCYCGKGGSGT	90
PA2T_AUSSU	**NLYQFKNMIQ**CANHGRRATWHYLDYGCYCGPGGLGT	90
PA2A_PSEPO	**NLYQFKNMIQ**CANKGSRSWLDYVNYGCYCGWGGSGT	90
PA22_NAJME	**NLYQFKNMIQ**CTVPNRSWWHFANYGCYCGRGGSGT	90

Fig. 5.7. N-terminal amino acid sequence of NK-PLA_2-I in comparison to other PLA_2s from other snake venom. PA2_NAJSP, *Naja sputatrix*, (Miyoshi et al., 1996); PA21_MICNI, *Micrurus nigrocinctus*, (Mochca-Morales et al., (1990); PA22_NAJKA, *Naja siamensis*, Chuman et al., 2000); PA2X_BUNFA, *Bungarus fassciatus*, (Liu et al., 1988); PA2T_AUSSU, *Austrelaps superbus*, (Singh et al., 2000); PA2A_PSEPO, *Pseudechis porphyriacus*, (Schmidt and Middlebrook, 1989) and PA22_NAJME, *Naja melanoleuca*, (Joubert, 1975) amino acid residues in bold indicates the total homology among the PLA_2s and an asterisk (*) indicates the difference in amino acid residue.

5.1.4.2 Other enzymatic activities

Even at a concentration of 25 µg/ml, no detectable protease, acetylcholinesterase and adenosine triphosphatase or adenosine monophosphatase activity was associated with NK-PLA_2-I. (Table 5.3).

Table 5.3. Other enzymatic activities of NK-PLA_2-I in comparison to crude venom.

Results are mean ± S.D of six determinations.

Enzymatic activities	Crude venom (25 µg/ml)	NK-PLA_2-I (25 µg/ml)
Caseinolytic activity[b]	5.2 ± 0.04	Absent
Acetylcholinesterase activity[c]	26 ± 1.5	Absent
Adenosine monophosphate activity[d]	118 ± 9.0	Absent
Adenosine triphosphate activity[d]	100 ± 5.5	Absent

[a]= Units/mg protein

[b]= nmole equivalent to tyrosine released.

[c]= µg Pi liberated per minute per ml at 37 °C.

[d]= µmole of thiocholine formed per minute per ml.

5.1.4.3 Carbohydrate content

Carbohydrate content of NK-PLA_2-I was found to be 0.52 mole of glucose per mole of enzyme, when determined by the method of Dubois et al. (1956).

5.1.4.4 Substrate specificity

To check the substrate specificity of NK-PLA$_2$-I, phospholipids with different head groups viz. PC, PS and PE were used as source of substrate. The assay reveals that NK-PLA$_2$-I has a distinct preference for phosphotidylcholine over phosphotidylserine or phosphatidylethanolamine (Table 5.4).

Table 5.4. Substrate specificity of NK-PLA$_2$-I. Substrate specificity of NK-PLA$_2$-I using purified phospholipids as source of substrate was determined as described in section 3.2.5.10.1 Results are mean ± S.D. of three determinations.

Phospholipid Substrate (Final concentration 1 mM)	PLA$_2$ specific activity (Units/min/mg)
Phosphatidylcholine	220.5 ± 24
Phosphatidylserine	101.2 ± 10
Phosphatidylethanolamine	61 ± 7.0

One unit of PLA$_2$ activity is defined as one μl of 0.1 N NaOH required to neutralize the liberated fatty acids from the phospholipid substrates.

5.1.4.5 Effect of substrate concentration

Effect of substrate concentration on catalytic activity by NK-PLA_2-I is shown in the figure 5.8. using PC as a source of substrate. With an increase in substrate concentration, hydrolysis increased until saturation was reached at 2 mM substrate concentration.

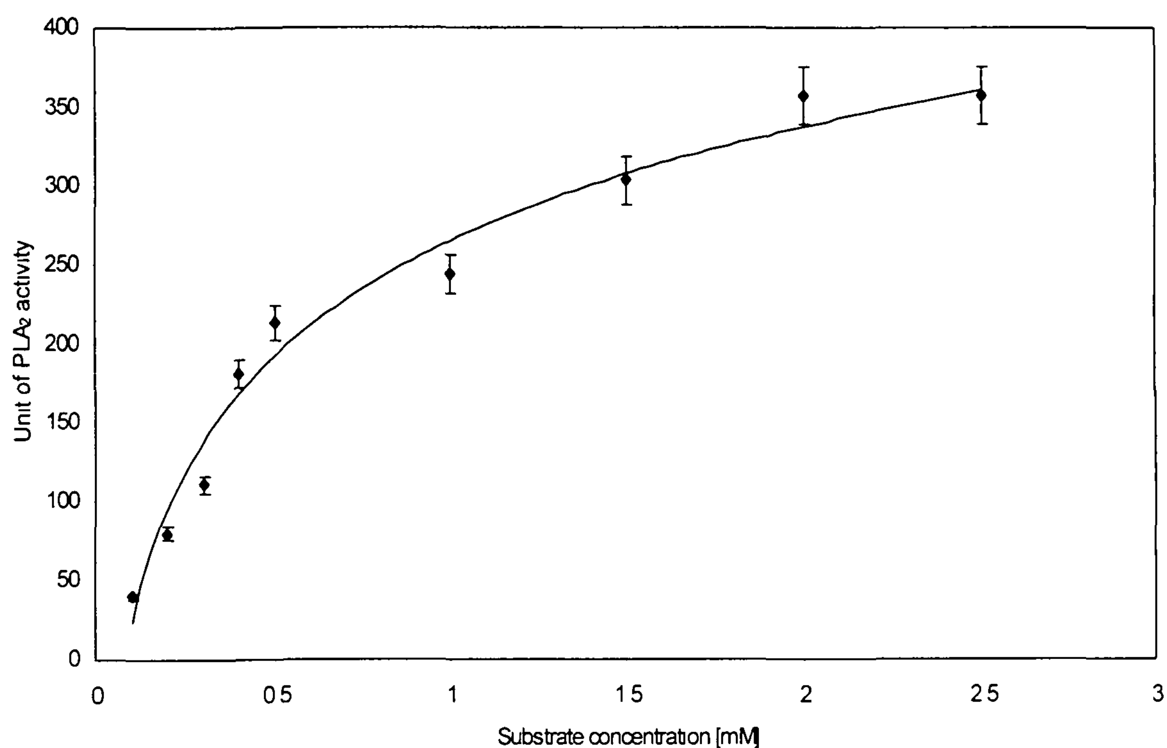

Fig. 5.8. Effect of substrate concentration on PLA_2 activity of NK-PLA_2-I. Effect of substrate concentration on PLA_2 activity of NK-PLA_2-I was determined as described in section 3.2.5.10.2. Results are expressed as mean ± S.D of four determinations.

5.1.4.6 Effect of enzyme concentration

With the increase in enzyme concentration, the hydrolyzing capacity of NK-PLA_2-I on purified phospholipid (PC) increases. The enzymatic activity shows maximum hydrolysis on purified phospholipid with 8 μg/ml enzyme concentration. With further increase in enzyme concentration, a saturation was reached (Fig. 5.9).

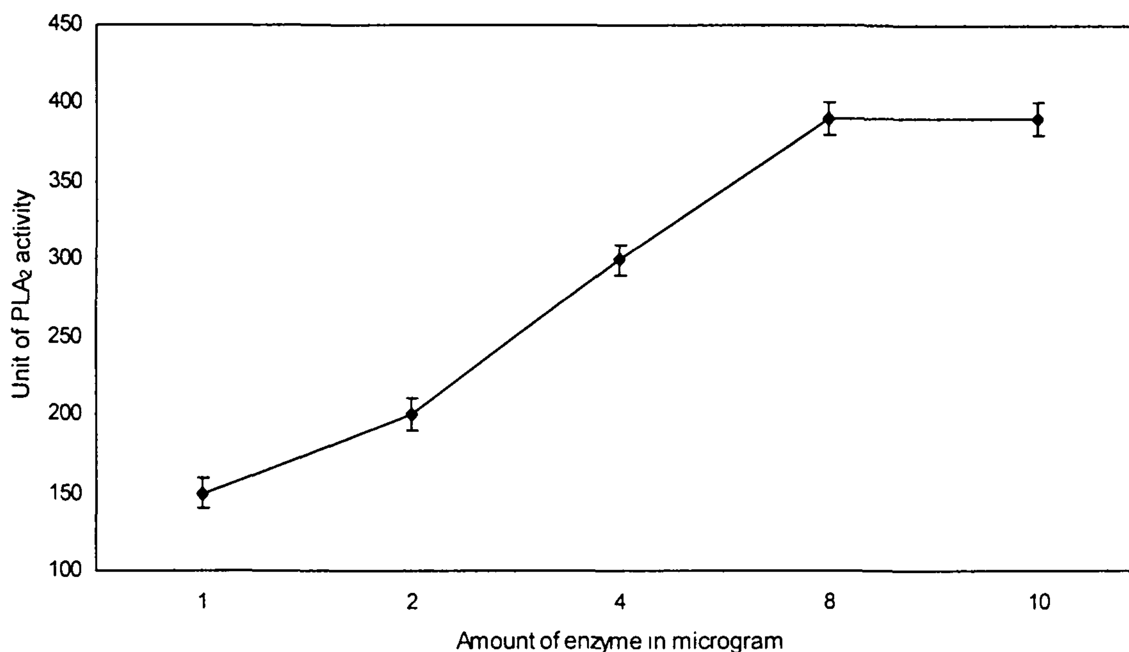

Fig. 5.9. Effect of enzyme concentration on PLA_2 activity of NK-PLA_2-I. Effect of enzyme concentration on PLA_2 activity of NK-PLA_2-I was determined as described in section 3.2.5.10.3. Results are expressed as mean ± of four determinations.

5.1.4.7 Determination of K_m and V_{max}

Using Lineweaver-Burk representation, the Michaelis constant (K_m) and maximum velocity (V_{max}) of the PLA_2 catalyzed reaction were determined. By plotting the values of 1/v as a function of 1/[S] (Fig. 5.10), a straight line was obtain, that intersect the vertical line at a point which is the $1/V_{max}$ (since 1/[S] =0, we have $1/v=1/V_{max}$). If we extent the straight line then the horizontal axis (1/[S]) intersect at *the point which is* $-1/K_m$.

From the graph we have the equation y= 1.4212x +14.047

When y=0, Then x = -14.047/1.4212 or -9.883 i.e $-1/K_m$ = -9.883,

i.e. K_m = 0.10118 mM or 1.0118 X 10^{-4} M i.e K_m = 0.988 X 10^{-4} M

$1/V_{max}$ = 14.047 i.e V_{max} = 0.07118 X 10^{-3} mM μg^{-1} or 71.18 X 10^{-3} µmol μg^{-1} or 71.18 µmol mg^{-1}

i.e V_{max} = 1.40 X 10^{-2} µmol mg^{-1}.

The apparent K_m and V_{max} value of $NK\text{-}PLA_2\text{-}I$ are 0.988 X 10^{-4} M and 1.40 X 10^{-2} µmol mg^{-1} respectively.

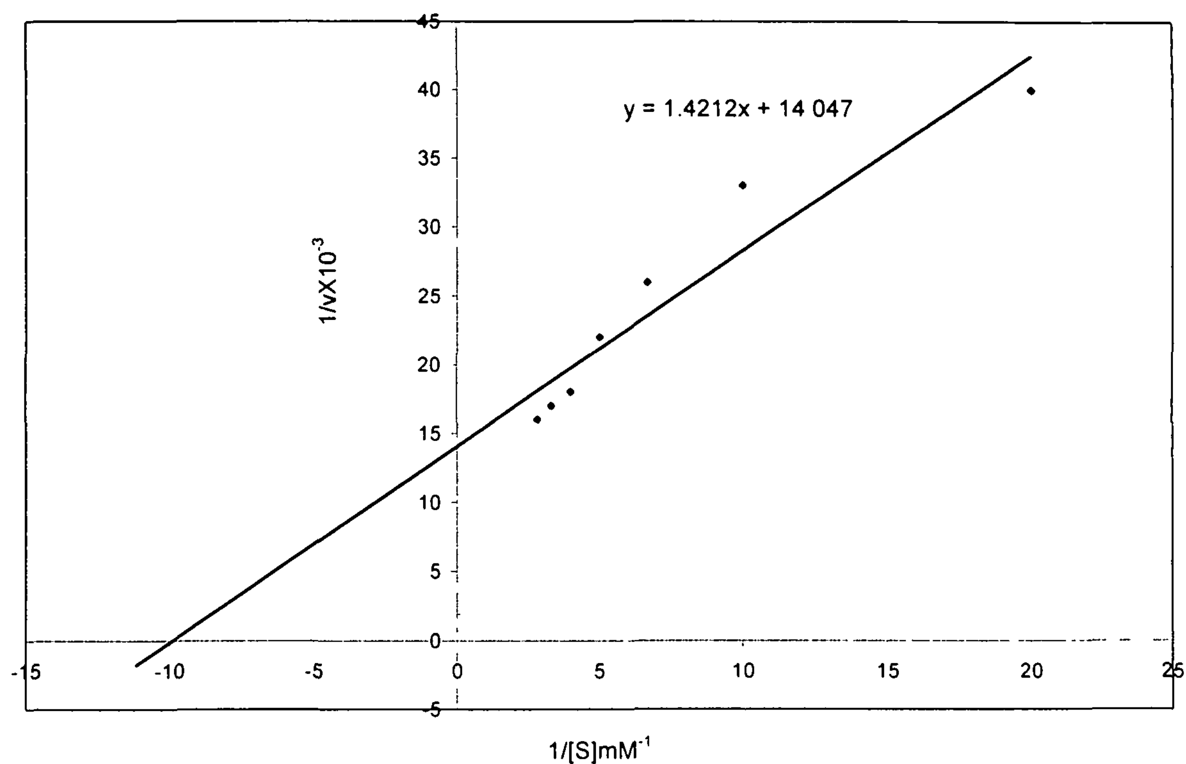

Fig. 5.10. Determination of K_m and V_{max} of NK-PLA_2-I using Lineweaver-Burk plot. Substrate used was purified PC. Values are mean of four determinations.

One unit of PLA_2 activity is defined as one μl of 0.1 N NaOH required to neutralize the reaction mixture.

5.1.4.8 Optimum temperature

The effect of temperature on the catalytic activity of NK-PLA_2-I is shown in figure 5.11. The enzyme displays maximum catalytic activity in the temperature range of 37-40 °C.

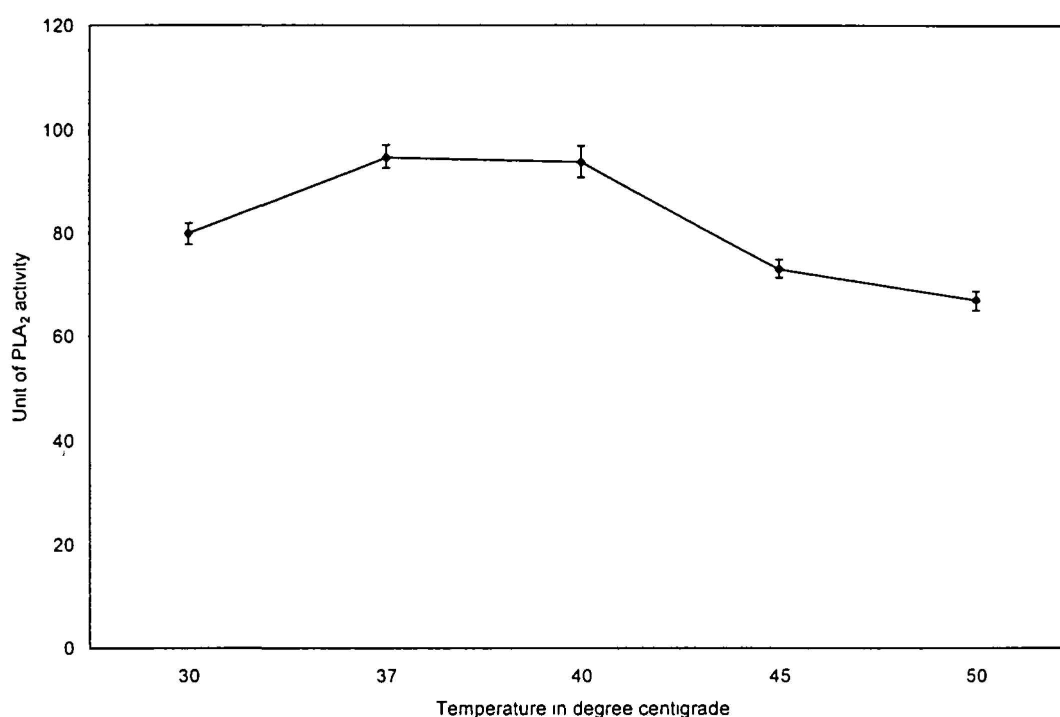

Fig. 5.11. Effect of temperature on PLA_2 activity of NK-PLA_2-I. PLA_2 activity of NK-PLA_2-I at different temperature was determined as described in section 3.2.5.10.5. Results are expressed as mean ± S.D. of three determinations. One unit of PLA_2 activity is defined as decrease in 0.01 absorbance at 740 nm per 10 min.

5.1.4.9 Optimum pH

The optimum pH for enzymatic activity of NK-PLA_2-I was assayed at a pH range of 6.0-9.0 using egg yolk phospholipids a source of substrate (Fig. 5.12). Maximum catalytic activity was displayed at pH 8.0 (131.85 units) and at pH lower than 7.0 the enzyme exhibited very low catalytic activity. The PLA_2 activity at pH 6.5 and 6.0 was determined as 15.5 units and 9.0 units respectively.

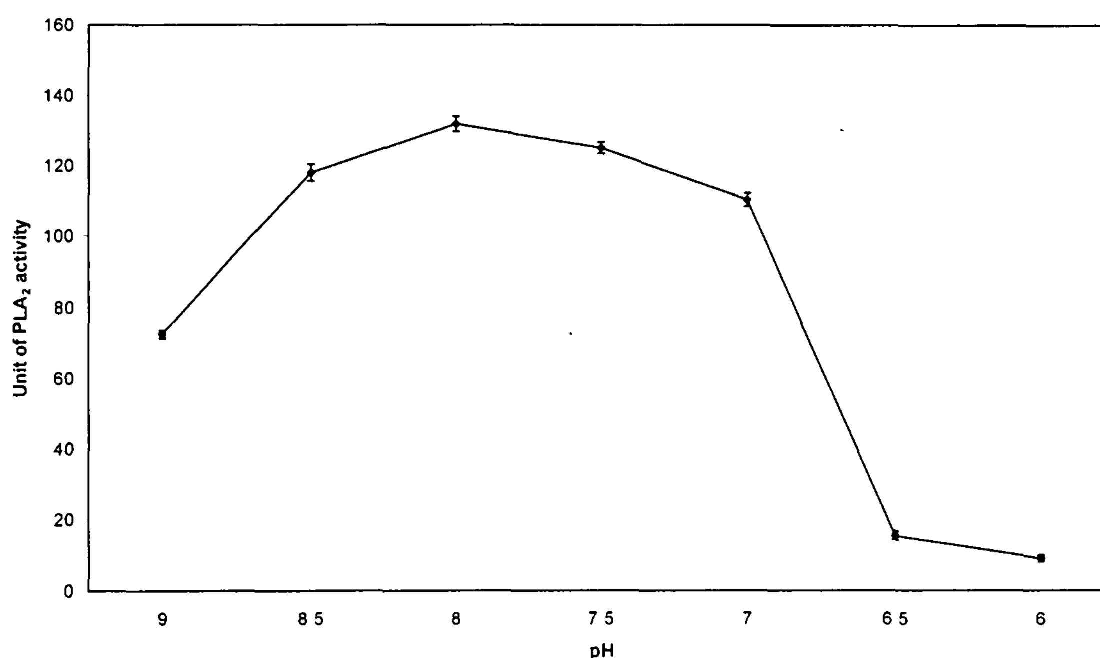

Fig. 5.12. Effect of pH on PLA_2 activity of NK-PLA_2-I. PLA_2 activity of NK-PLA_2-I at different pH was determined as described in section 3.2.5.10.6. The various pH values were obtained as follows; 0.1M Sodium acetate, pH 6.5, 0.1M K-Phosphate pH 7.0-7 and 0.1 M Tris-HCl pH 8.0-9.5. Results are expressed as mean ± of three determinations.

5.1.4.10 Heat-inactivation of catalytic activity

When NK-PLA_2-I, (dissolved in Tris-HCl buffer, pH 8.0) was heated at 100 °C for 10 min, there was no effect on catalytic activity, however, heating for 45 min in the same temperature resulted in loss of 47 % of original activity (Fig.5.13)

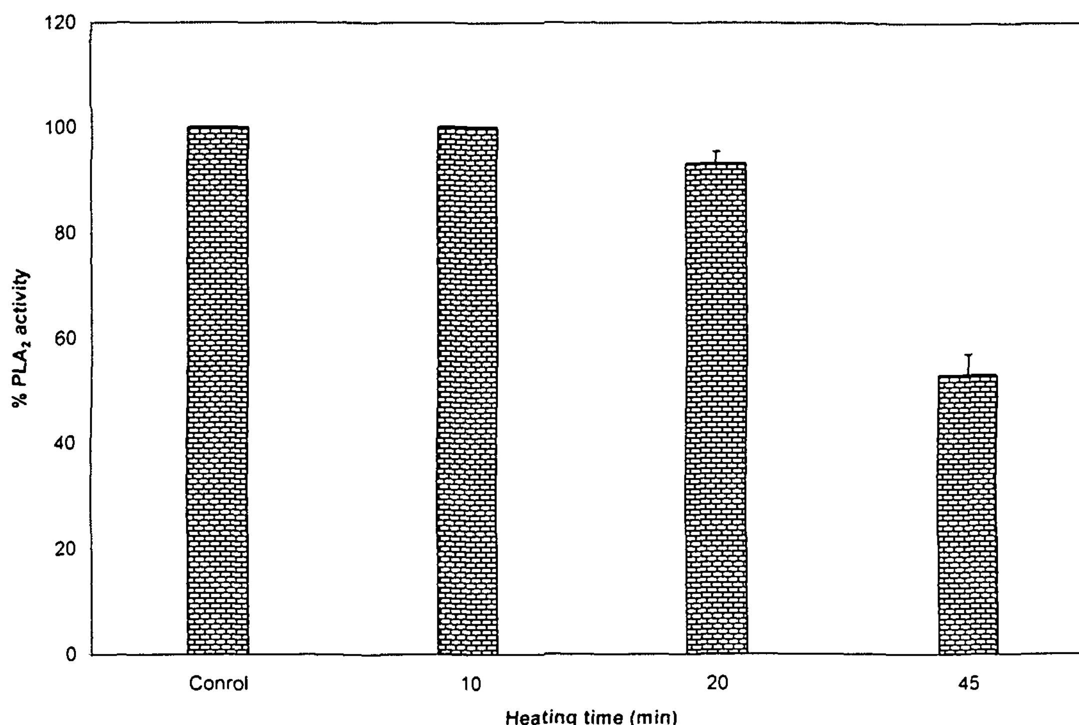

Fig. 5.13. Heat inactivation of NK-PLA_2-I. PLA_2 activity of heat-inactivated NK-PLA_2-II was determined as described in section 3.2.5.11. PLA_2 activity of NK-PLA_2-I without heating was considered as control. One unit of PLA_2 activity is defined as one μl of 0.1 N NaOH required to neutralize the liberated fatty acids.

Results are expressed as mean ± S.D of four determinations.

5.1.4.11 Circular dichroism

CD spectrum of native NK-PLA_2-I demonstrated defined minima at 210 and 222 nm, indicating a strong α–helical contribution to the CD signal (Fig.5.14).

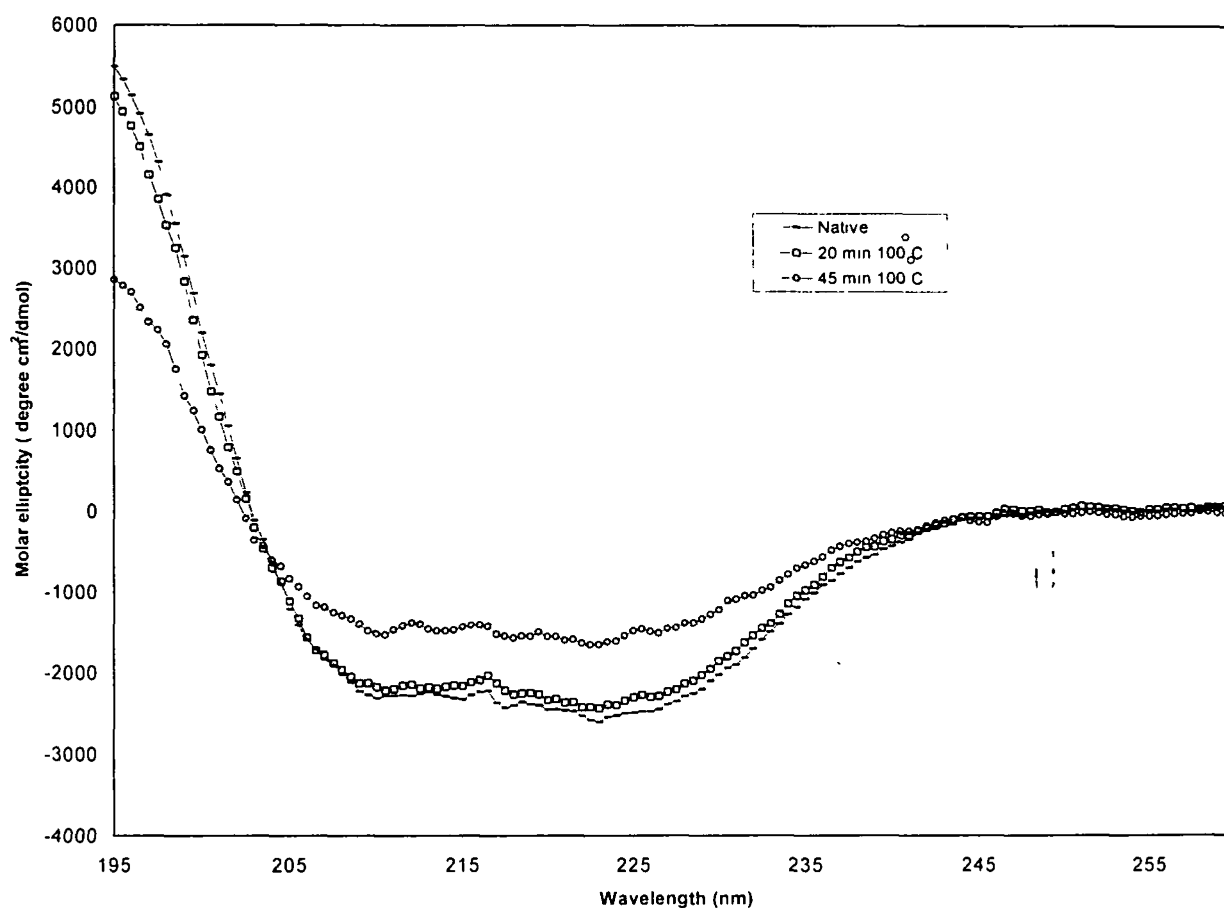

Fig. 5.14. CD spectra of NK-PLA_2-I. (▬) Native PLA_2, (□) PLA_2 after heating for 20 min at 100 °C and (O) PLA_2 after heating for 45 min at 100 °C. CD measurements were performed as described in section 3.2.5.12. The CD signals are expressed as mean residue ellipticity [θ] (degrees cm^2 $dmol^{-1}$), using 113 as the mean residue molecular weight.

5.1.5 Pharmacological Characterization

5.1.5.1 Lethality and toxicity

In contrast to the crude venom, NK-PLA$_2$-I was not lethal to albino mice even at a concentration of 10 mg/kg body weight and did not show any sign of neurotoxicity such as respiratory distress, paralysis of limb, lacrination and profuse urination etc (Table 5.5).

5.1.5.2 Anticoagulant activity

In-vitro condition, NK-PLA$_2$-I increases the ca-clotting time of platelet poor goat in a dose dependent manner (Fig. 5.15). The anticoagulant activity of NK-PLA$_2$-I was higher as compared to that of the crude venom (Table 5.5). Further, with an increase in the pre-incubation time of plasma with NK-PLA$_2$-I, clotting time was concomitantly increased (Fig. 5.16).

5.1.5.3 Prothrombin time test

To check the effect of NK-PLA$_2$-I on the inhibition of extrinsic pathway of blood coagulation, anticoagulant activity of NK-PLA$_2$-I on PPP of goat was assayed using commercial prothrombin time test kit. NK-PLA$_2$-I was hardly effective in delaying the coagulation time of PPP of goat (21±0.16 sec) as compared to coagulation time of control plasma (19.2±1.4sec) when assayed by this method (Table 5.5).

Table 5.5. Pharmacological properties of NK-PLA$_2$-I compared to Crude venom.

Results are expressed as mean ± S.D of four experiments.

Properties	Whole venom	NK-PLA$_2$-I
LD_{50} (i.p mg / kg)	0.7 ± 0.09	No
Toxicity	Present	Absent
Direct hemolytic activity (% Hb released by 10 μg of protein)	16.85 ± 1.45	0.23 ± 0.04
Indirect hemolytic activity in presence of egg yolk (% Hb released by 10μg of protein)	8.0 ± 0.95	25.2 ± 2.46
Anticoagulant activity (Ca clotting time in sec by 1 μg protein)*	89 ±1	100 ± 3
Prothrombin time test (Clotting time in sec by 1 μg protein) **	20 ± 0.34	21 ± 0.16
In-vitro tissue damaging activity (% of Hb released by 25 μg protein)		
a. Liver	3.5 ± 0.5	16.7 ± 1.09
b. Heart	13.86 ± 0.98	24.43 ± 1.67
c. Lungs	14.87 ± 1.3	10.0 ± 1.1

* Ca-clotting time of normal plasma is 87± 1 sec.

** PT of normal plasma is 19.2 ± 1.4 sec

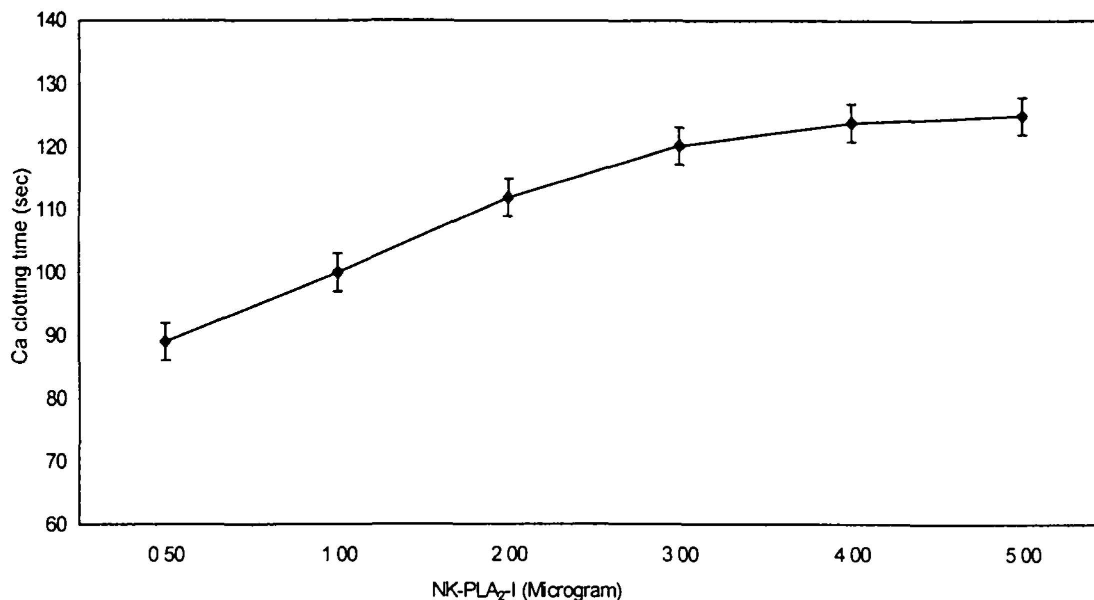

Fig. 5.15. Dose dependent anticoagulant activity of NK-PLA$_2$-I. Values represent mean ± S.D of four experiments. Ca-clotting time of control human plasma is 87±1 sec.

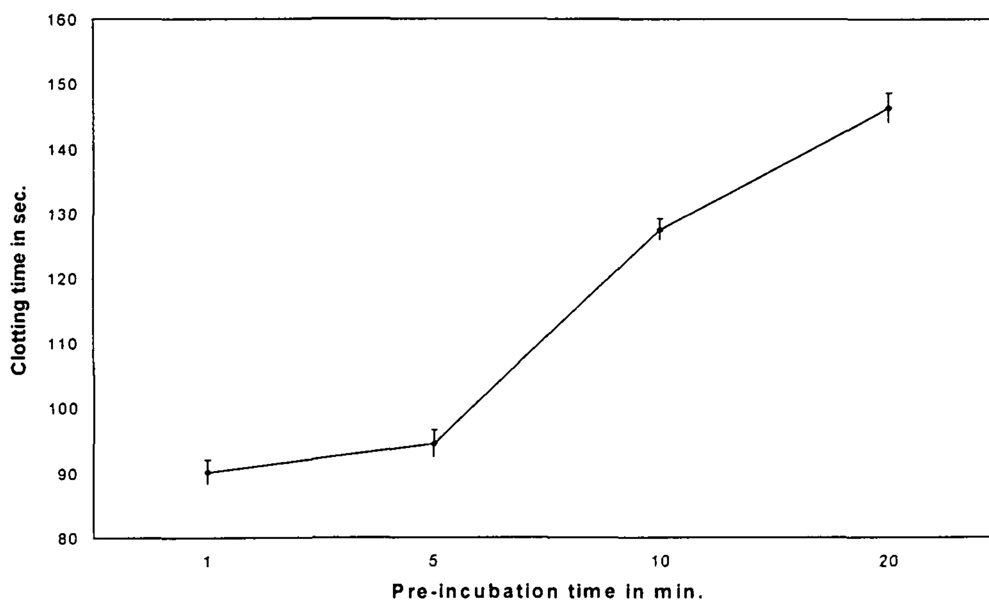

Fig. 5.16. Effect of pre-incubation time on anticoagulant activity of NK-PLA$_2$-I. Values represent mean ± S.D of four experiments. Ca-clotting time of control human plasma is 87±1 sec.

5.1.5.4 Edema-inducing activity

Injection of 25 μg of NK-PLA$_2$-I into the footpad of mice resulted in immediate onset of swelling and inflammation of injected area (edema), which reached a saturation post 45 min of injection and then gradually diminished (Fig. 5.17). However, potency of edema-induction by this PLA$_2$ was significantly less as compared to the whole venom (Table 5.5).

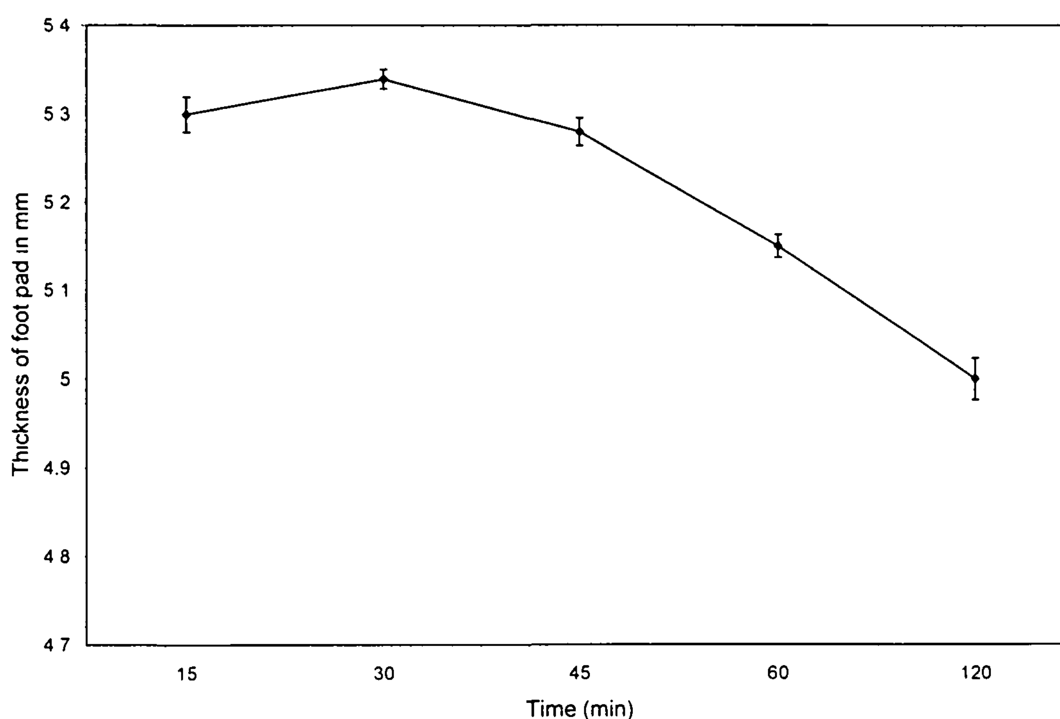

Fig. 5.17. Time course of the foot pad edema of albino mice induced by 25 μg of NK-PLA$_2$-I. Experiment was done as described in section 3.2.6.4. 0.9% normal saline served as control. Values are mean ± S.D of three determinations.

5.1.5.5 *In-vitro* tissue damaging activity

In-vitro heart and liver tissue damaging activity of NK-PLA$_2$-I was higher as compared to that of the whole venom, but the extent of lung tissue damaging effect of NK-PLA$_2$-I was less than that of the whole venom (Table 5.5).

5.1.5.6 Mitochondrial swelling and free fatty acid release

The effect of NK-PLA$_2$-I on change in the mitochondrial matrix volume (an indicator of mitochondrial swelling) was measured at 520 nm (Fig. 5.18). NK-PLA$_2$-I hydrolyzes the mitochondrial phospholipid membrane in a very characteristic manner without any lag phase of initiation of reaction. Although the presence of millimolar of Ca^{2+} could enhance the mitochondrial damage by NK-PLA$_2$-I, however, its presence was not an absolute requirement for the PLA$_2$-induced mitochondrial swelling to occur (Table 5.6). In order to gain further insight into the mode of attack on the mitochondrial membrane by the NK-PLA$_2$-I, NK-PLA$_2$-I-induced fatty acids release pattern was analyzed by GC-MS (Fig. 5.19). Within the initial 15 min of attack, fatty acids of carbon chain length C16 and C18 were the major FFA released by NK-PLA$_2$-I (Table 5.6).

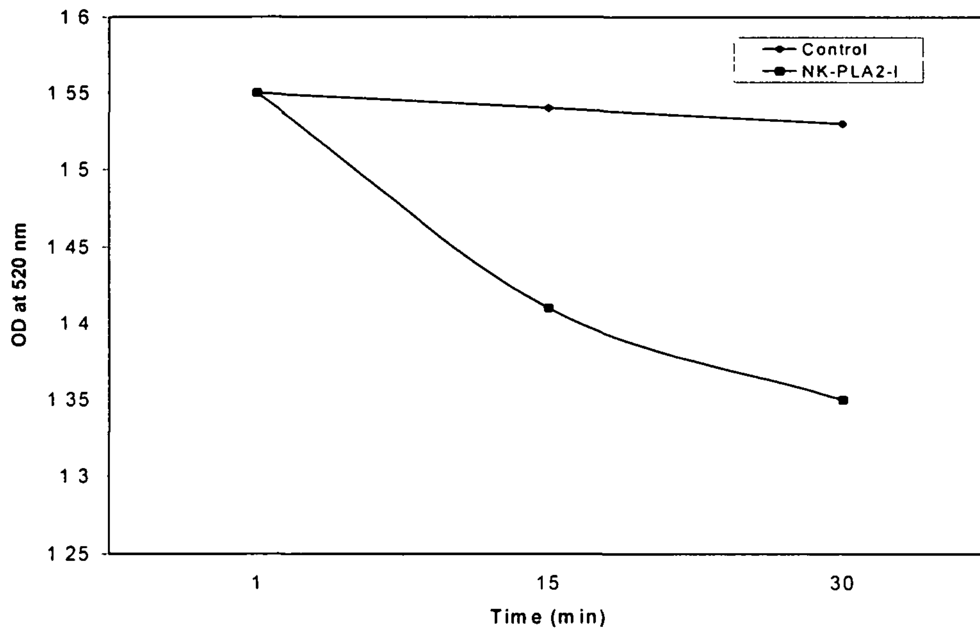

Fig. 5.18. Effect of NK-PLA2-I on mitochondrial swelling. About 100 mg equivalent of mitochondria (mitochondria obtained from 100 mg wet weight of tissue) from chicken liver was previously incubated with 100nM NK-PLA2-I and decrease in absorbance at 520 nm was recorded from 0 to 30 minute. Results represent mean of at least three determinations.

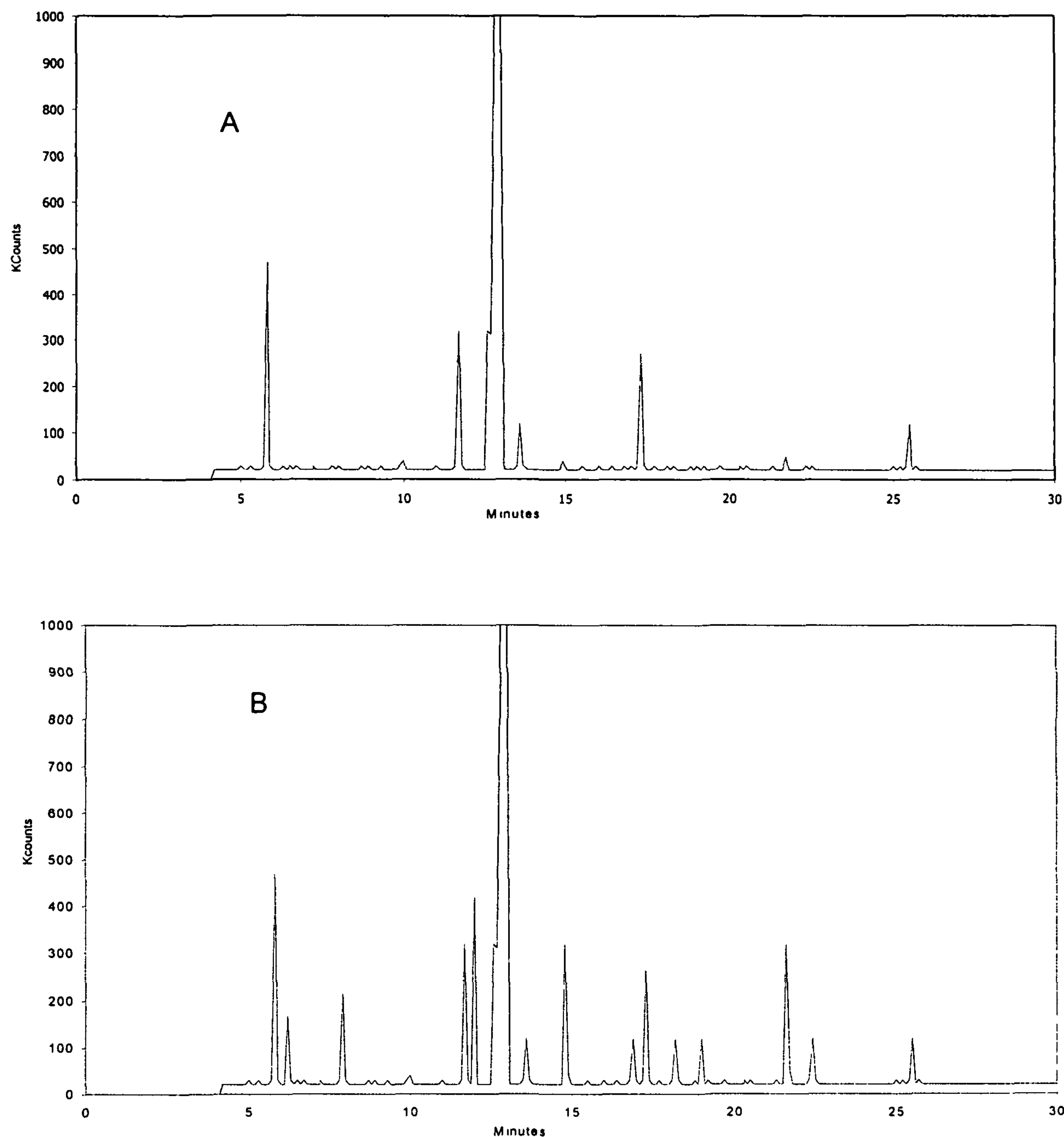

Fig. 5.19. Gas chromatography of methylated free fatty acid. Liberated free fatty acids were extracted from 100 mg equivalent mitochondria (mitochondria obtained from 100 mg wet weight liver tissue), methylated and analyzed by GC-MS as described in section 3.2.6.9.2. A: without enzyme treatment (Control); B: incubated with 100nM NK-PLA_2-I. Results are from a typical experiment.

Table 5.6. NK-PLA_2-I-induced swelling and phospholipid hydrolysis of outer plasma membranes of intact mitochondria in the presence or absence of 2 mM Ca^{2+}. Measurements of the degree of mitochondrial swelling and phospholipid hydrolysis, and the extraction, separation and analysis of methylated fatty acids by GC-MS are described in section 3.2.6.9. Data are the mean ± S.D of triplicate determinations.

Incubation time (min)	**Mitochondrial swelling (Unit)**		**Free fatty acid released (μg FFA/20 μg PLA_2)**		**Ratio of (16:0+18:0) / (18:1+18:2)**
	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	
5	$5X10^2$ ±50	$1.1X10^3$ ±100	N.D	N.D	N.D
15	$1X10^3$ ±121	$2.3X10^3$ ±143	17.5±2	40.3±10	1.23±0.01
30	$14.5X10^3$ ±100	$18.5X10^3$±175	140±10	178.6±21	0.867±0.02
60	$43.5X10^3$ ±201	$55.5X10^{3*}$±231	408±21	520.6±23	0.859±0.01

Unit = decrease in 0.01 OD at 520 nm after 30 min per mg of mitochondria

5.1.5.7 Direct and indirect hemolytic activity

In contrast to the crude venom, purified NK-PLA_2-I hardly exhibited direct hemolytic activity on washed human erythrocytes, but showed appreciable indirect hemolytic activity in presence of egg yolk phospholipids (Table 5.5). Erythrocytes pre-incubated with neutral phospholipids PC and 1.5 mM Ca^{2+} were highly susceptible to the lysis induced by NK-PLA_2-I, but pre-incubation with PS and PE had no influence on hemolytic activity of NK-PLA_2-I, irrespective of the presence or absence of 1.5 mM Ca^{2+} in the reaction medium (Fig. 5.20).

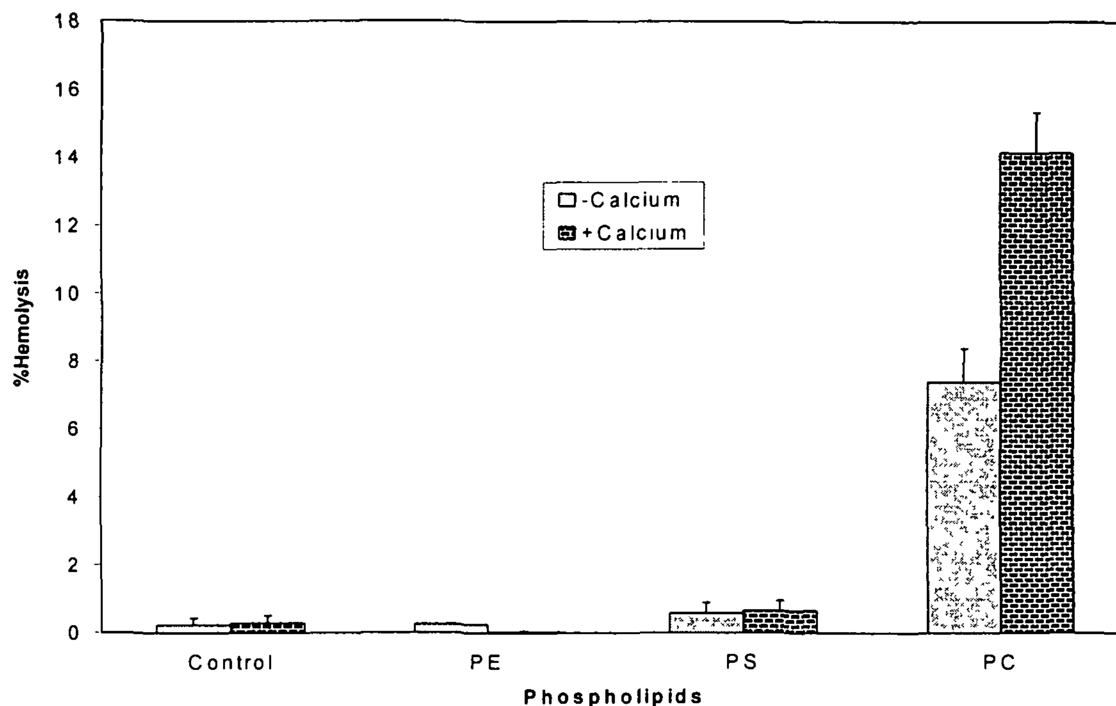

Fig. 5.20. Effect of NK-PLA_2-I on erythrocytes enriched with different phospholipids in presence or absence of 2 mM calcium. Experiment was done as described in section 3.2.6.3. Lysis is expressed as percentage, taking 100% the absorbance of erythrocyte suspension incubated with MQ water. Erythrocyte suspension without phospholipid served as control. Each result represents mean ± S.D of three individual experiments.

5.1.5.8 Myotoxicity

Intravenous injections of NK-PLA_2-I to BALB/C albino mice results significant increase in the LDH activity in serum as compared to control (Table 5.7). Figure 5.21 A&B show light micrographs of thigh muscle of mice injected with 40 μg of NK-PLA_2-I and normal saline. Histological observation of the thigh muscle of mice after injection of NK-PLA_2-I reveals several ruptured segments, separated by spaces apparently devoid of cellular materials (indicate in Fig 5.21 B. by arrow indicating signs of rupture) whereas in normal muscle the tissue are seen to be intact (Fig. 5.21 A).

Table 5.7. LDH activity in the serum of mice after NK-PLA_2-I treatment.

Results are mean ± S.D of three determinations.

	Control	Treated	%increase
LDH	$1.04 \times 10^{-3} \pm 0.01$	$1.64 \times 10^{-3} \pm 0.02$	58

Unit = μmol of LDH release/min/ml.

A B

Fig. 5.21. Photomicrograph of thigh muscle of mice. A: Normal thigh muscle (Control) mice received only 50 μl of normal saline. B: Thigh muscle treated with 40 μg of NK-PLA_2-I dissolved in 50 μl of PBS. Sections of the thigh muscles were stained with hematoxylin-eosin stain and viewed under (400X magnification) Lica ATC 2000 microscope. Photographs were taken with Kodak film (Max 200).

5.1.5.9 Antibacterial activity

Even at a concentration of 20 μg/ml, NK-PLA$_2$-I did not exhibit any antibacterial activity against Gram positive or Gram negative bacteria. Crude venom inhibited significant growth of Gram positive bacteria (*B. subtilis*) when tested by time course experiment (Fig. 5.22) and displayed clear zone of inhibition on Gram negative bacteria (*E. coli*, DH5α strain) (Fig. 5.23).

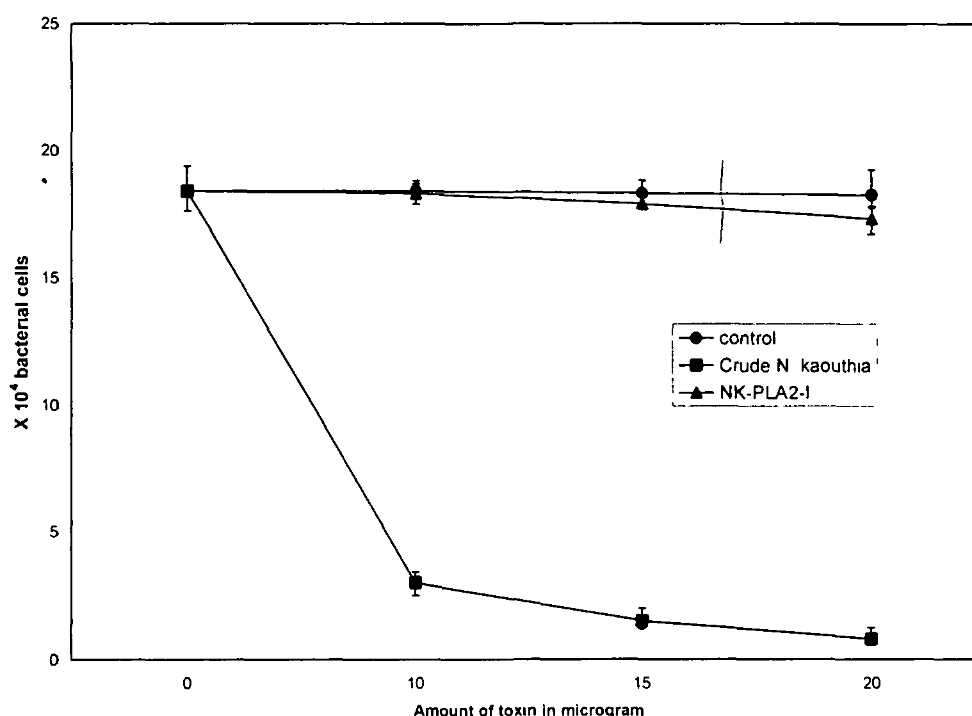

Fig. 5.22. Bacterial activity of *N. kaouthia* crude venom and NK-PLA$_2$-I on *B. subtilis*. Varying amount of crude venom or NK-PLA$_2$-I was incubated with 18.4 X 10^4 cells for 4 h at 37 °C and change in the optical density was recorded at 630 nm. 1 OD at 630 nm = 10^6 cells. Each point represents mean ± S.D. of four determinations.

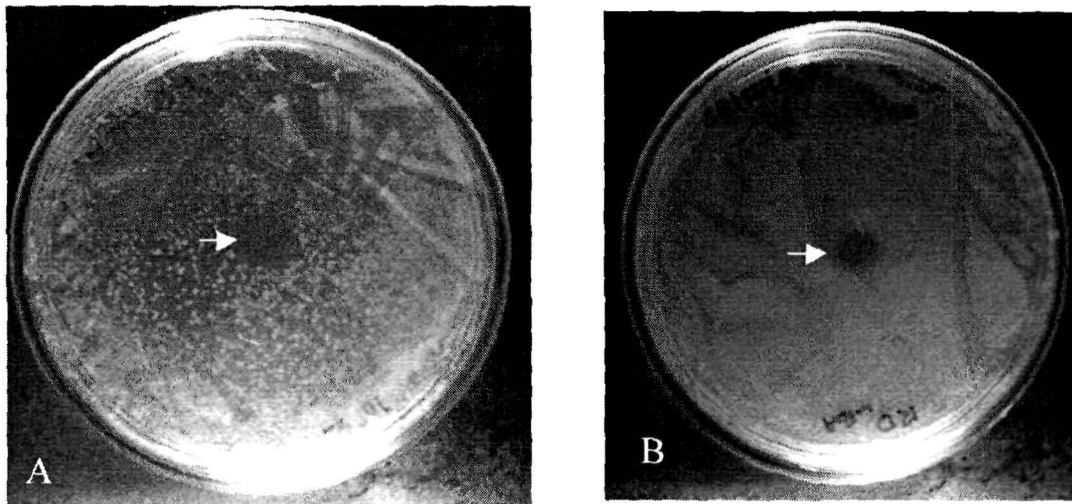

Fig. 5.23. Bactericidal activity of *N. kaouthia* crude venom on DH5α. Arrow mark indicates the zone of inhibition of bacterial growth by crude venom (10μg). Plates were prepared after serial dilution A= 10^{-3}, B=10^{-2} dilution.

5.1.6 Immunological Characterization

5.1.6.1 Western blotting

NK-PLA$_2$-I was separated by SDS-PAGE and blotted on PVDF membrane to check the cross-reactivity with that of *Naja naja* antiserum. The band in the PVDF membrane corresponds to the NK-PLA$_2$-I band on SDS-PAGE indicating that NK-PLA$_2$-I was recognized by *Naja naja* antiserum raised in horse at 1:4000 dilution (Fig. 5.24).

5.1.6.2 Isolation of Immunoglobin

The total protein content of the serum before separation of the IgG was 50 mg/ml. By ammonium sulfate precipitation 35 mg/ml of IgG protein was recovered. The presence of anti-NK-PLA$_2$-I IgG antibodies was tested by immunodiffusion and immunoelectrophoresis techniques.

Fig. 5.24. Immunoblotting experiment showing cross-reactivity of NK-PLA$_2$-I against commercial *N. naja* antiserum. About 50 μg of protein was separated by 10% SDS-PAGE and transferred to Immobilon-P membrane. Detection was done with horse redish peroxidase conjugate rabbit anti-horse IgG and TMB/H_2O_2 as substrate.

5.1.6.3. Immunological cross-reactivity

5.1.6.3.1 Immunodiffusion

Presence of antibodies against NK-PLA$_2$-I in rabbit serum was determined by immunodiffusion technique. The single precipitin band observed in the immunodiffusion plate indicates the presence of anti-NK-PLA$_2$-I antibodies in the rabbit serum (Fig. 5.25). As a control, rabbit serum before immunization was collected and immunodiffusion was performed which showed no precipitin band.

5.1.6.3.2 Immunoelectrophoresis

The immunoelectrophoresis experiment performed in the present study also indicates the presence of antibodies against NK-PLA$_2$-I in the serum of rabbit post immunization against NK-PLA$_2$-I. The presence of single precipitin band shows the cross reactivity between the antibodies and the purified protein (Fig. 5.26).

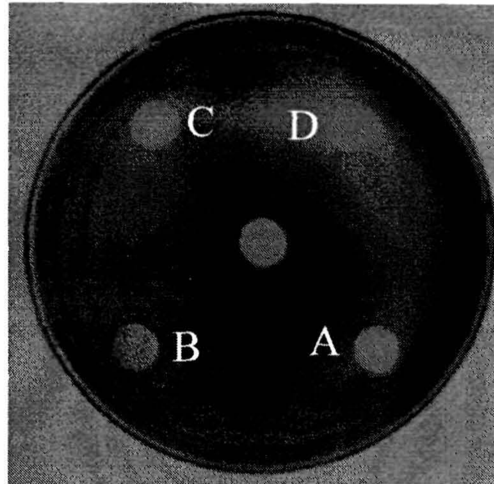

Fig. 5.25. Immunodiffusion. Precipitin line formed by the reaction between NK-PLA$_2$-I and antibodies against NK-PLA$_2$-I raised in rabbit. The peripheral wells contain NK-PLA$_2$-I (A=1μg, B=4 μg, C=8 μg, D=10 μg) while the central well contains 20 μl antibody (anti-NK-PLA$_2$-I antibodies).

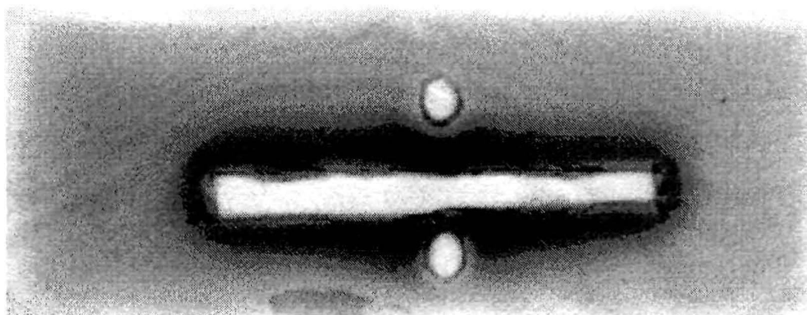

Fig. 5.26. Immunoelectrophoresis of NK-PLA$_2$-I. 10 μg NK-PLA$_2$-I was electrophoresed in a horizontal electrophoresis chamber at 20 mA for 3 h. 20 μl of antibody against NK-PLA$_2$-I (1 mg/ml) was added to the trough and incubated in humid chamber at 37 °C till the precipitin line was visible. The gel was stained in 0.1% coomassie blue solution.

5.1.7 Neutralization of catalytic activity

5.1.7.1 Effect of ρ-bromophenacyl bromide

After modification of enzyme with ρ-bromophenacyl bromide (3.3 mM), only 4.5 % of the original catalytic activity of NK-PLA$_2$-I was retained. But Triton X-100 and Triton X-100 plus Ca^{2+} significantly reversed this inactivation. Triton X-100 at 1 mM concentration had little protective effect against the inhibition while at higher concentration (30 mM) in presence of 10 mM Ca^{2+} displayed higher protection against the inactivation of PLA$_2$ activity by ρ-bromophenacyl bromide (Table 5.8).

Table 5.8. Effect of ligands on inactivation of PLA$_2$ activity of NK-PLA$_2$-I with ρ-bromophenacyl bromide. Inactivation conditions were 3.3 mM ρ-bromophenacyl bromide at pH 8.0. Values represent mean ± S.D of four determinations.

Ligands	% of activity remaining after treatment
Control (no inhibitor)	100
ρ-bromophenacyl bromide	4.52 ± 1.2
ρ-bromophenacyl bromide +Triton X 100 (1 mM)	19.7 ± 1.8
ρ-bromophenacyl bromide +Triton X 100 (10 mM)	25.1± 2.1
ρ-bromophenacyl bromide +Triton X 100 (30 mM)	34.6 ± 2.5
ρ-bromophenacyl bromide +Triton X 100 (30 mM), Ca^{2+}10 mM)	56.5 ± 3.8

5.1.7.2 Effect of other inhibitors

As shown in Table 5.9, serine protease inhibitors like PMSF, TLCK and TPCK did not affect PLA_2 activity of NK-PLA_2-I. Treatment with DTT (reducing agent for disulphide bridge) at a final concentration of 2 mM inhibited the PLA_2 activity up to 80%, whereas EDTA at the same concentration inhibited up to 92 % of the enzymatic activity. On the other hand, α-bromo-2-acetophenone and α-chloroacetophenone, at a final concentration of 2 mM, inhibited 2.5% and 9% of PLA_2 activity respectively.

Table 5.9. Effect of various inhibitors on PLA_2 activity of NK-PLA_2-I. Values represent mean ± S.D of four experiments.

Reagents/condition	% activity remaining
Control	100
TLCK (2 mM)	99 ± 1.0
TPCK (2 mM)	91.4 ± 2.3
Phenylmethylsulfonyl flouride (2 mM)	100
Diiopropyl flurophosphate (2 mM)	100
Diothiothreitol (1 mM)	33.4 ± 3.4
Diothiothreitol (2 mM)	20.0 ± 2.8
Ethylenediamine tetra-acetic acid (1 mM)	18.7 ± 2.1
Ethylenediamine tetra-acetic acid (2 mM)	8.0 ± 1.9
α-bromo-2-acetophenone (2 mM)	97.5 ± 2.1
α-chloroacetophenone (2 mM)	91.5 ± 6.0

5.1.7.3 Effect of anti-NK-PLA$_2$-I antibodies

With the increase in concentration of anti-NK-PLA$_2$-I antibodies the inhibition of catalytic activity of NK-PLA$_2$-I was found to be increasing. At 1:100 (w/w) antigen:antibody ratio it could neutralize up to 68.34% of the original catalytic activity contained in NK-PLA$_2$-I whereas commercially available polyvalent antivenom at same ratio could inhibit only 48.12% of the catalytic activity of NK-PLA$_2$-I (Table 5.10).

Table 5.10. Neutralization of phospholipase A$_2$ activity of NK-PLA$_2$-I by polyvalent antivenom (Bharat Serums & vaccines Ltd, Thane) and anti-NK-PLA$_2$-I IgG (raised in rabbit). Results are mean ± S.D of three determinations.

Ratio (antigen:antibodies)	% inhibition by	
	Commercial *N. naja* antivenom	Anti-NK-PLA$_2$-I antibodies
1:1	3.85 ± 0.12	14.69 ± 2.13
1:5	8.17 ± 1.1	30.84 ± 2.45
1:10	19.34 ± 1.32	40.27 ± 2.31
1:50	35.21 ± 2.01	45.55 ± 3.01
1:100	48.12 ± 2.3	68.34 ± 3.21

5.1.8 Neutralization of pharmacological activities of NK-PLA_2-I

5.1.8.1 Effect of ρ-bromophenacyl bromide

Alkylation of NK-PLA_2-I by ρ-bromophenacyl bromide (2 mM) significantly inhibited its pharmacological activities. Anticoagulant, direct and indirect hemolytic and edema inducing activities were inhibited within a range of 0-8.3% (Table 5.11). *In-vitro* liver and heart tissue damaging activities were reduced to 31 ± 2.9% (mean ± S.D.) and 26.8 ± 3.8% (mean ± S.D.) of the original activity respectively (Table 5.11).

5.1.8.2 Effect of other inhibitors

As shown in table 5.11, treatment of NK-PLA_2-I with 2 mM TLCK, was ineffective in modulating any of the tested pharmacological properties of NK-PLA_2-I. TPCK and PMSF could significantly reduce the *in-vitro* tissue damaging activity, but failed to inhibit other tested pharmacological properties of NK-PLA_2-I. Treatment with 2 mM of either EDTA or DTT resulted significant inhibition of the tested pharmacological properties of the purified protein. Interestingly, anticoagulant activity of NK-PLA_2-I was increased in the presence of EDTA. (Table 5.11)

Table 5.11. Effect of chemical modification on pharmacological properties of NK-PLA$_2$-I. Experiment was done as described in section 3.2.5.13 and 3.2.5.14. Values are mean ± S.D. of four individual experiments.

Properties	% of activity remaining after treatment (Control 100%)					
	ρBPB (3.3 mM)	TLCK (0.1 mM)	TPCK (0.1 mm)	PMSF (1 mM)	EDTA (1 mM)	DTT (2 mM)
Anticoagulant	5.3±1.9	99±8	95±2	100	114±3.1	0
Indirect hemolysis	8.66±1.8	88.6±2.8	90±1.0	99±1.0	6.9±1.1	0
Edema	8.32±1.3	99±1	93±3	100	0	0
Liver tissue damage	31±2.9	100	55.1±6.2	35.9±3.2	0	0
Heart tissue damage	26.8±3.8	98.6±1.1	61.8±6.9	38.9±2.1	0	0
Lung tissue damage	29.8±3.2	100	46.8±2.8	36.8±1.8	0	0
Mitochondrial swelling	4.21±3.8	26.4±1.1	26.4±6.9	93±2.1	0	0

5.1.8.3 Effect of anti-NK-PLA$_2$-I IgG

Anti-NK-PLA$_2$-I IgG was very much not effective in neutralizing the tested pharmacological properties of NK-PLA$_2$-I (Table 5.12). *In-vitro* tissue damaging activities and mitochondrial swelling were not neutralized at 1:100 (mol:mol) antigen:antibody ratio. 90% of anticoagulant activity was found even after treatment of NK-PLA$_2$-I with anti-NK-PLA$_2$-I IgG at 1:100 ratio (antigen:antibodies). Whereas indirect hemolytic activities and edema were neutralized upto 13% and 10% respectively under same ratio.

5.1.8.4 Effect of heating on pharmacological effects of NK-PLA_2-I

Heating the NK-PLA_2-I (in 0.1 M Tris-HCl, pH 8.0) at 100 °C for 20 min at pH 8.0 had no influence in the pharmacological properties, except release of hemoglobin from NK-PLA_2-I –treated tissues (Table 5.12). About 16% reduction in release of hemoglobin from the tissues was observed post-heating NK-PLA_2-I for 20 min at 100 °C was observed. However, when the heating time was increased up to 45 min, anticoagulant, mitochondrial swelling and *in-vitro* heart tissue damaging activities were completely abolished, whereas direct and indirect hemolytic activities were retained upto 2.8% and 7% respectively of the original activity. Edema and *in-vitro* liver and lung tissue damage were reduced to 6%, 6.5% and 2.8% respectively under the identical heating condition (Table 5.12).

Table 5.12. Effect of polyvalent antivenom and heating on the pharmacological properties of NK-PLA_2-I. Values are mean ± S.D. of four individual experiments.

Properties	% of activity remaining after treatment (Control 100%)		
	Anti-NK-PLA_2-I IgG (1:100) (antigen: antibody)	Heating at 100 °C	
		20 min	45 min
Anticoagulant	90±2.1	98.3±1.1	0
Indirect hemolysis	87±1.1	96.9±1.3	7±1.06
Edema	90±2.0	98.2±1.2	6±1.04
Liver tissue damage	94±2.3	84.2 ± 2.1	6.5±1.32
Heart tissue damage	93 ±1.2	85.6 ± 2.8	0
Lung tissue damage	96±2.3	86.8±1.8	2.8±0.6
Mitochondrial swelling	95± 1.1	96.29 ± 0.8	0

5.1.8.5 Screening of Indian medicinal plants for anti-PLA_2 activity

Out of the 13 plants selected for screening of anti-PLA_2 activity, none of them were found to be positive except the leaves of *Azadiracta indica* locally known as 'Mahaneem' (Table 5.13). The water extract of *Azadiracta indica* leaves at 1:5 (w/w) could not inhibit the enzymatic activity significantly however, at 1:100 (w/w) ratio it could neutralize the PLA_2 activity of crude venom up to 27% only. While the PLA_2 activity of NK-PLA_2-I was inhibited up to 15.67%. The methanol extract shows highest inhibitory effect against PLA_2 activity of crude venom up to 49.05% whereas the PLA_2 activity of NK-PLA_2-I was inhibited up to 17%. However, the chloroform extract was not so effective in neutralizing the PLA_2 activity of crude as well as NK-PLA_2-I (Table 5.14)

5.1.8.6. Neutralization by *Azadiracta indica*

The effect of water, chloroform and methanol extract of *A. indica* leaves on catalytic as well as indirect hemolytic activity of both crude and NK-PLA_2-I is shown in Table 5.14. The methanol extract at 1:100 (w/w) could neutralized the catalytic activity of crude venom and NK-PLA_2-I up to 49.05% and 27.12% while the water extract could inhibit up to 27% and 15.67% respectively. The indirect hemolytic activity of crude venom and NK-PLA_2-I was inhibited up to 36.7% and 32.8% respectively at 1:100 (w/w), water extract at same concentration could neutralized only 7% and 5% of indirect hemolytic activity whereas chloroform extract did not displayed any inhibitory activity (Table 5:14).

Table 5.13. Screening of medicinal plants for anti-PLA_2 activity against crude *N. kaouthia* venom and NK-PLA_2-I. Preparation of plant extract and screening was done as described in section 3.2.8 and 3.2.9.2.

Medicinal plants	Plant part used	Anti PLA_2 activity		
		Water extract	Methanol extract	Chloroform extract
Agyl marmolos	Leaves	-	-	-
Alostonia scholaris	Bark	-	-	-
Aristolochia indica	Leaves	-	-	-
Azadiracta indica	Leaves	+	+	+
Calamus rotang	Root	-	-	-
Carica papaya	Fruit	-	-	-
Cronton aromatica	Rhizome	-	-	-
Curcuma longa	Rhizome	-	-	-
Leucas lavendulaefolia	Leaves	-	-	-
Murrya koenigii	Leaves		-	-
Piper longum	Spike	-	-	-
Piper nigrum	Fruit	-	-	-
Terminalia arjuna	Bark	-	-	-
Zingiber officinali	Rhizome	-	-	-

+ sign indicates able to neutralization of PLA_2 activity

- sign indicates unable to neutralization of PLA_2 activity

Table 5.14. Percent inhibition of PLA_2 activity and indirect hemolytic activities of crude venom and NK-PLA_2-I (1 µg protein) by different extract of *Azadiracta indica.* Preparation of plant extract and inhibition was done as described in section 3.2.9.2. PLA_2 activity without plant extract was treated as control (100%). Results are ± S.D of four determinations.

Amount of Plant extract (µg)		% inhibition of			
		PLA_2 activity		Indirect hemolytic activity	
		Crude venom	NK-PLA_2-I	Crude venom	NK-PLA_2-I
Water extract	5	3.34 ± 0.1	0.5 ± 0.03	0	0
	10	7.75 ± 1.0	2 ± 0.1	0	0
	50	12.1 ± 0.2	7 ± 1	0	0
	100	27 ± 1.2	15.67	7±2	5±1
Chloroform extract	5	1.06 ± 0.05	0	0	0
	10	3.12 ± 1.01	0	0	0
	50	8.23 ± 2.0	3 ± 0.03	0	0
	100	18.15 ± 2.01	9 ± 2.1	0	0
Methanol extract	5	9.28 ± 1.1	2 ± 0.01	7.4±1	6.1±1.1
	10	15.23 ± 2.2	7 ± 0.5	13.8±0.2	11.47±1
	50	26.89 ± 2.1	11 ± 1.1	23.2±1.01	20±0.1
	100	49.05 ± 2.3	27 ± 1.2	36.7±2	32.8±1.21

CHAPTER VI

ISOLATION, PURIFICATION AND CHARACTERIZATION OF NK-PLA$_2$-II

6.1 Results

6.1.1 Fractionation of CM-II on RP-HPLC

Fractionation of CM II (see section 5.1.1) on a Nova pack C_{18} RP-HPLC column resulted separation of proteins into eight peaks (HP I to HP VIII) (Fig 6.1). Screening of peaks for PLA$_2$ activity showed that maximum enzymatic activity was associated with peak HP VI. Peak HP VI was collected manually and rechromatographed on the same column under the identical condition. This purified enzyme designated as NK-PLA$_2$-II, constitutes 3.5% of the total venom protein. It was lyophilized and stored in –20 °C for further characterization. The summary of purification of NK-PLA$_2$-II is shown in Table 6.1.

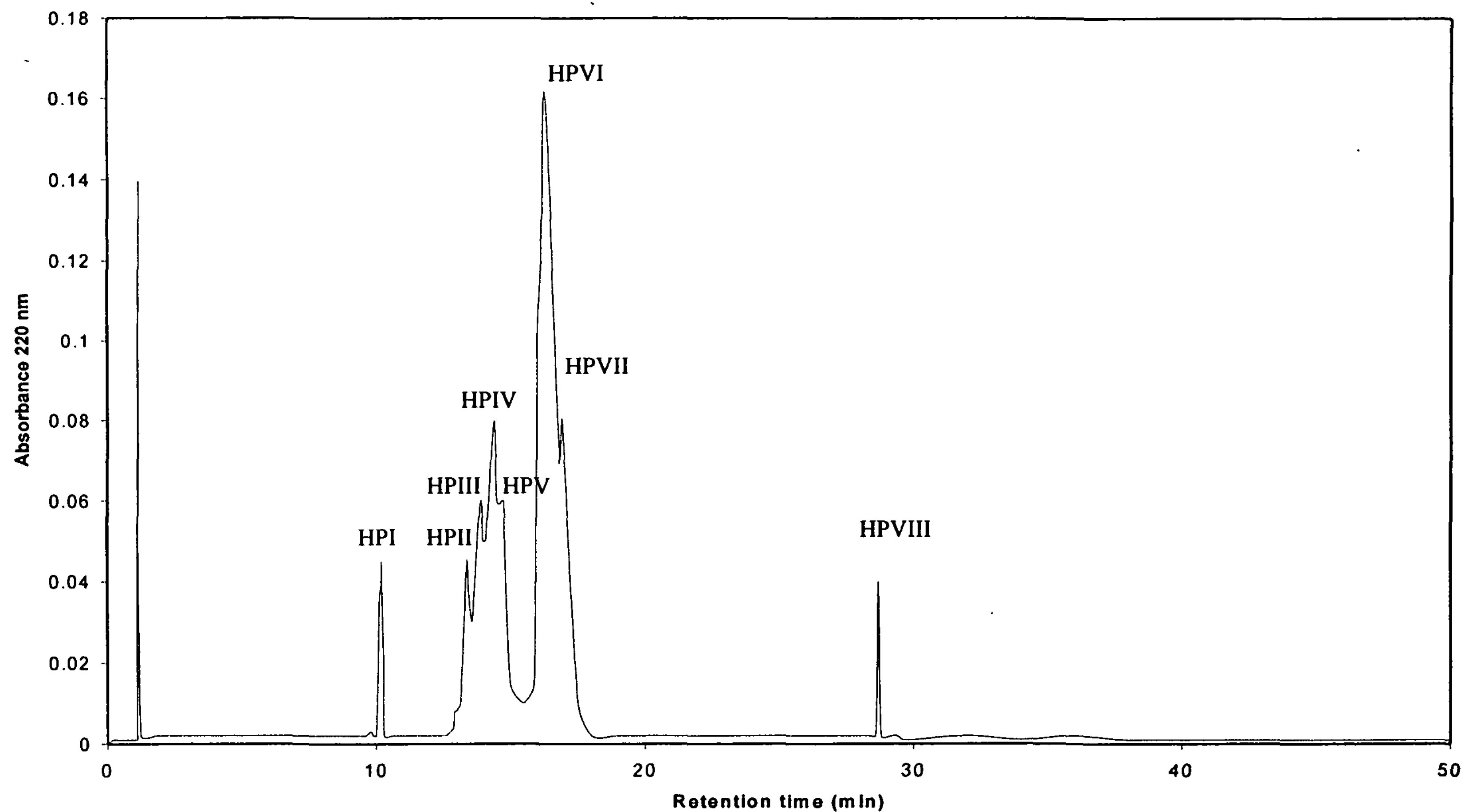

Fig. 6.1. RP-HPLC of CM-II using C_{18}-μ-Nova pack column (Waters). Buffer A was 0.1% (v/v) TFA and buffer B was 0.1% (v/v) TFA in CH_3CN. Elution was performed at a flow rate of 1 ml/min using gradient as described in section 3.2.3.1. The elution was monitored at 220 nm.

Table 6.1. Summary of fractionation of CM-II on RP-HPLC column.

Datas are from a typical experiment.

Purification steps	Total protein (mg)	yield of protein (%)	PLA_2 activity[a]	
			Total activity (Units)	Specific activity (Units/mg)
Whole venom	50	100	418.5 X 10^4	83.7 X 10^3
Ion-exchange (CM II)	2.5	5.0	106.5 X 10^3	42.6 X 10^3
RP-HPLC Peak (HP VI)	1.75	3.5	154.17 X 10^3	88.1 X 10^3

[a]One unit of PLA_2 activity is defined as decrease of 0.01 absorbency at 740 nm per 10 minute.

6.1.2 Homogeneity and Molecular Weight of NK-PLA_2-II

6.1.2.1 SDS-PAGE

Under both reduced and non-reduced condition, NK-PLA_2-II showed a major band corresponding to a subunit with apparent molecular weight of 13.1 kDa indicating the presence of single peptide in the preparation. On the other hand, the whole venom displayed several sharp and diffused bands (Fig. 6.2a). The molecular weight was determined from calibration curve (Fig.6.2b).

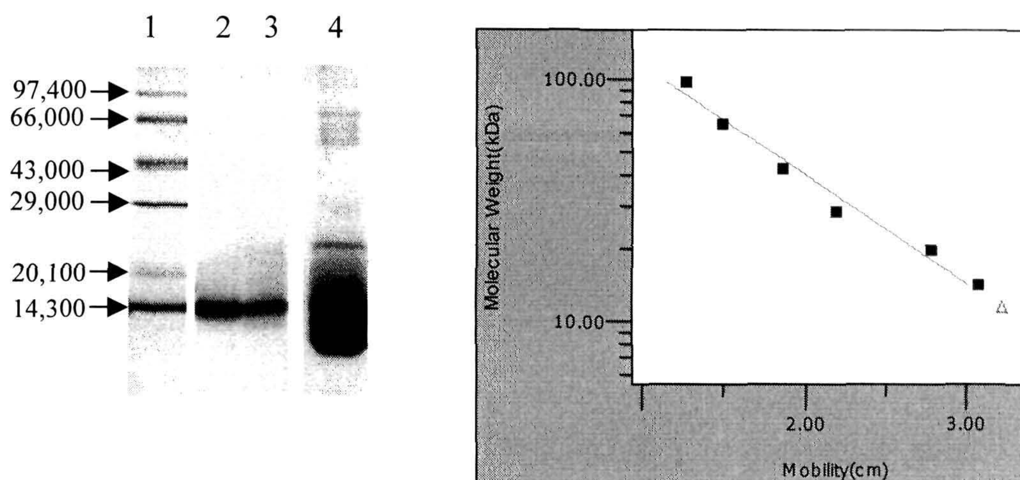

Fig. 6.2. a. 10% SDS-PAGE. Lane 1: molecular weight marker, Phosphorylase b (97,400), BSA (66,000), Ovalbumin (43,000), Carbonic anhydrase (29,000), Soyabean trypsin inhibitor (20,100) and Lysozyme (14,300). Lane 2: Reduced NK-PLA_2-II (30 μg); Lane 3: Non-reduced NK-PLA_2-II (30 μg) and Lane 4: Crude *N. kaouthia* venom (80 μg).

b. Calibration curve of 10% SDS-PAGE. Graph was obtained using Bio-Rad Multi-Analyst^{TM}/PC version 1.1 software (Bio-Rad).■ Sign Indicates the molecular weight marker whereas ▲ sign represents the molecular weight of NK-PLA_2-II (13.1 kDa)

6.1.2.2 Gel filtration

The native molecular weight and purity of the preparation was also judged by running the NK-PLA_2-II on a Sephadex G-50 column (1 X 64 cm^2) calibrated with molecular weight marker proteins. NK-PLA_2-II was eluted as a sharp, symmetrical peak, indicating purity of the preparation (Fig. 6.3). Molecular mass of NK-PLA_2-II, was determined as 12.4 kDa from the calibration curve (Fig. 5.4b),

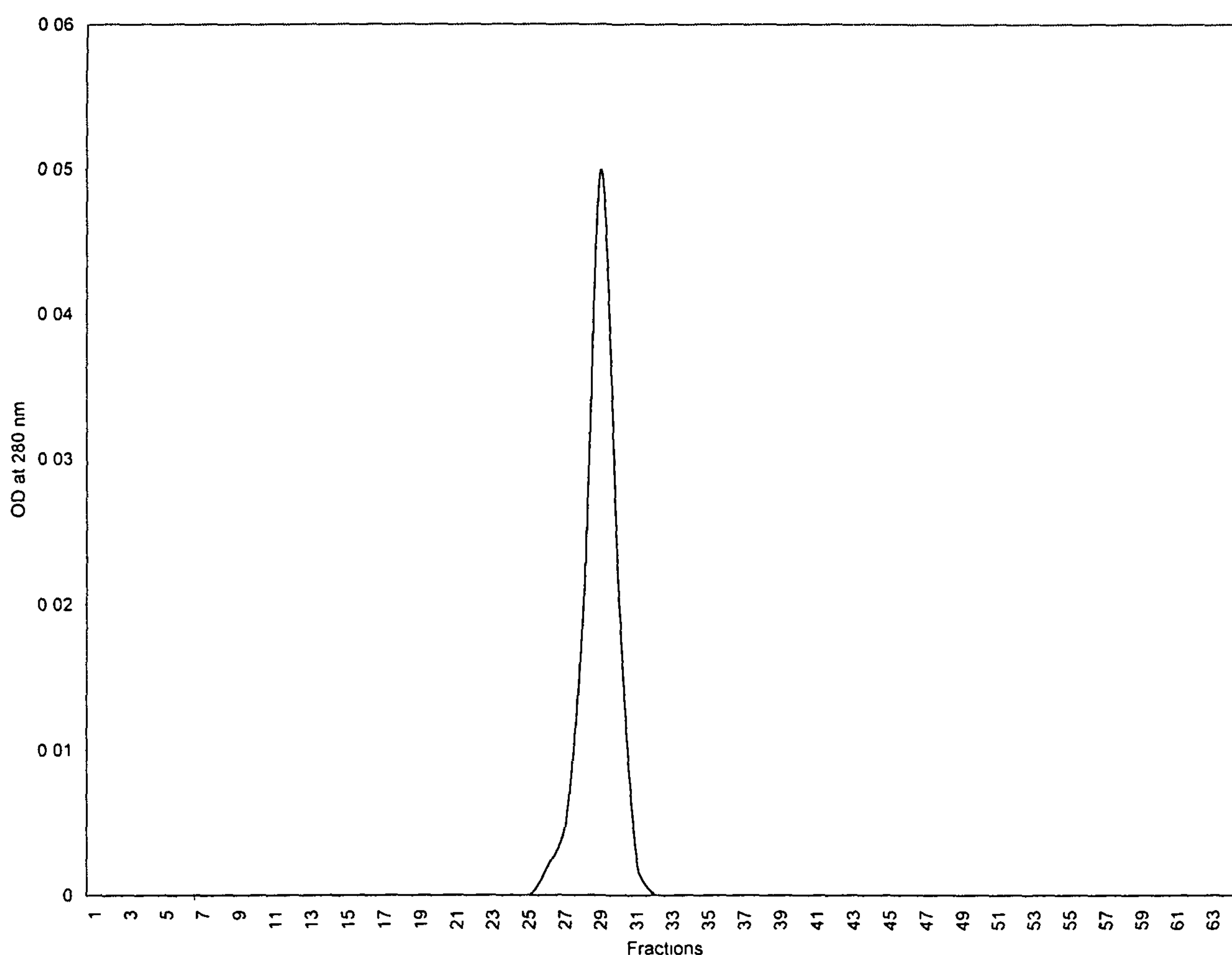

Fig. 6.3. Elution of NK-PLA_2-II in Sephadex G-50 column. Elution was carried out with equilibration buffer as described in section 3.2.4.2. and protein elution was monitored at 280 nm.

6.1.2.3 RP-HPLC

Rechromatography of 30 µg NK-PLA_2-II on a RP-HPLC C_{18} column resulted separation into two peaks with a retention time of 1.152 min and 16.15 min respectively (Fig. 6.4). The former peak was that of solvent, which was confirmed by a blank run (only solvent) while the later peak was that of NK-PLA_2-II.

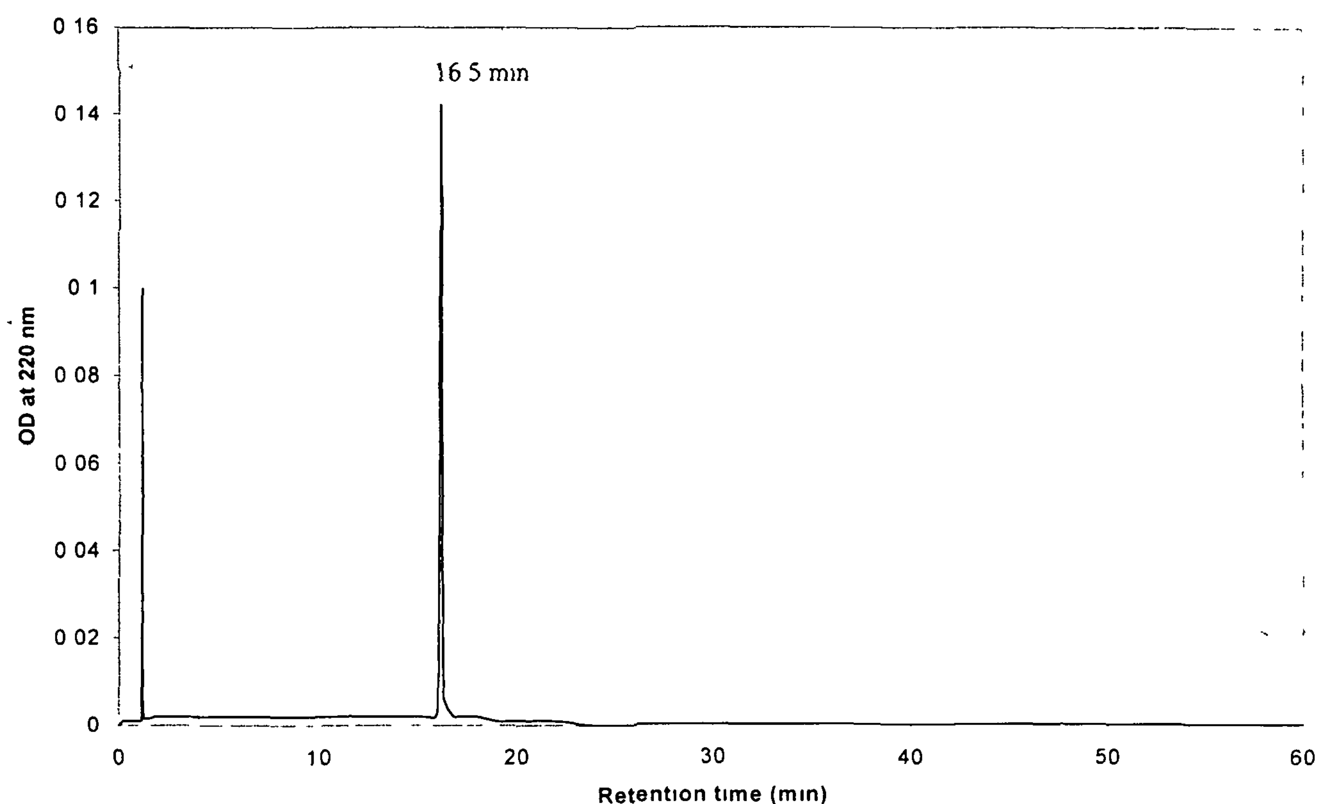

Fig. 6.4. RP-HPLC of NK-PLA_2-II using C_{18}-µ-Nova pack column (Waters) Buffer A was 0.1% (v/v) TFA and buffer B was 0 1% (v/v) TFA in CH_3CN Elution was performed at a flow rate of 1ml/min (0- 60% B) and protein elution was monitored at 214 nm

6.1.2.4 MALDI-MS

The purity and molecular mass of NK-PLA_2-II was further confirmed by MALDI-MS, which revealed protonated molecular ion [MH^+] at m/z 13,346.19 Da, indicating molecular mass of this protein is 13.34 kDa which was quite close to the mass determined by SDS-PAGE. MALDI-MS normally yields single charged states but a lower intensity doubly charged [MH^{2+}] peak at m/z 6673 Da was also observed (Fig. 6.5).

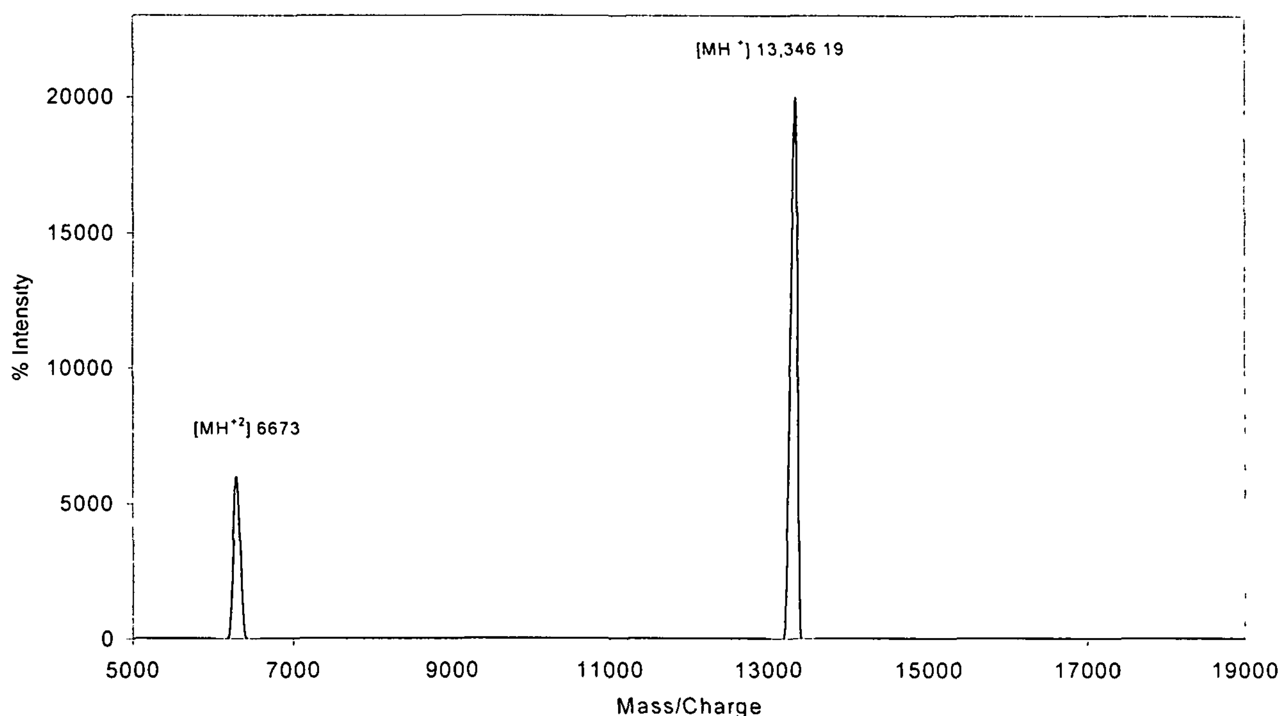

Fig. 6.5. MALDI-MS spectra of NK-PLA_2-II. Matrix: Sinapinic acid; number of shots 50; Laser power 30-40%. The spectra show both single [MH^+ = 13,346.19] and doubly charged [MH^{+2} = 6673] molecules, giving the molecular weight of 13.34 kDa.

6.1.3 Biochemical Characterization of NK-PLA$_2$-II

6.1.3.1 Partial N-terminal amino acid sequencing

The partial NH_2 –terminal amino acid sequencing of NK-PLA$_2$-II up to 10 amino acid residue reveals the sequence NIYQFKNNIQ. This sequence was identical to the NK-PLA$_2$-I isoenzyme. The homology search using ExPASy BLAST search indicated that NK-PLA$_2$-II sequences share substantial homology with sequences of some of the already described snake venom phospholipase A_2 enzymes (Fig. 6.6).

PLA$_2$ enzyme	Amino acid sequence *	% identity
NK-PLA$_2$-II	**NI YQFKNMIQ**	
PA2_NAJSP	**NLYQFKNMIQ**CTVPNR	90
PA21_MICNI	**NLYQFKNMIQ**CTTKRSVLEFMEYGCYC	90
PA22_NAJKA	**NLYQFKNMIQ**CTVPNRSWWHFADYGCFCGYGGSGT	90
PA2X_BUNFA	**NLYQFKNMIQ**CAGTRLWVAYVNYGCYCGKGGSGT	90
PA2T_AUSSU	**NLYQFKNMIQ**CANHGRRATWHYLDYGCYCGPGGLGT	90
PA2A_PSEPO	**NLYQFKNMIQ**CANKGSRSWLDYVNYGCYCGWGGSGT	90
PA22_NAJME	**NLYQFKNMIQ**CTVPNRSWWHFANYGCYCGRGGSGT	90

Fig. 6.6. N-terminal amino acid sequence of NK-PLA$_2$-II in comparison to other PLA$_2$s from other snake venom PA2_NAJSP, *N. sputatrix*, (Miyoshi et al., 1996); PA21_MICNI, *M. nigrocinctus*, (Mochca-Morales et al., 1990); PA22_NAJKA, *N. siamensis*, (Chuman et al., 2000); PA2X_BUNFA, *B. fassciatus*, (Liu et al., 1988); PA2T_AUSSU, *A. superbus*, (Singh et al., 2000); PA2A_PSEPO, *P. porphyriacus*, (Schmidt and Middlebrook, 1989) and PA22_NAJME, *N. melanoleuca*, (Joubert, 1975) amino acid residues in bold indicates the total homology among the PLA$_2$s and an asterisk (*) indicates the difference in amino acid residue.

6.1.3.2 Other enzymatic activity

NK-PLA_2-II displayed 88.1 X 10^3 units/mg of phospholipase A_2 activity when egg yolk phospholipid was used as a source of substrate. Even at a concentration of 25 μg/ml of NK-PLA_2-II, no detectable protease, acetylcholinesterase and adenosine monophosphatase activity was observed with this protein (Table 6.2).

Table 6.2. Other enzymatic activities of NK-PLA_2-II in comparison to crude venom.

Results are mean ± S.D of six determinations.

Properties	Crude venom	NK-PLA_2-II
PLA_2 specific activity[a]	83.7 X 10^3 (n = 6)	88.1 X 10^3 (n = 6)
Caseinolytic activity[b]	5.2 ± 0.04	Absent
Acetylcholinesterase activity[c]	26 ± 1.5	Absent
Adenosine monophosphate activity[d]	118 ± 9.0	Absent
Adenosine triphosphate activity[d]	100 ± 5.5	Absent

a= Units/mg protein

b= nmole equivalent to tyrosine released

c= μmole of thiocholine formed per minute.

d= μg Pi liberated per minute at 37 °C

6.1.3.3 Substrate specificity

To determine the substrate specificity of NK-PLA_2-II, phospholipids having different head groups were used as source of substrate. The order of hydrolysis of purified phospholipid by NK-PLA_2-II was PC>> PS >PE, clearly indicating that NK-PLA_2-II has a distinct preference for the hydrolysis of PC over PS or PE (Table 6.3).

Table 6.3. Substrate specificity of NK-PLA_2-II. Enzymatic activity of NK-PLA_2-II using various substrates was determined as described in section 3.2.5.10.1.
Results are expressed as mean ± S.D of four determinations.

Substrate (Phospholipid) (1 mM)	PLA_2 activity Specific activity (Units/min/mg)
Phosphatidylcholine	200.5 ± 14
Phosphatidylserine	90.2 ± 9.0
Phosphatidylethanolamine	41 ± 7.0

One unit of PLA_2 activity is defined as one μl of 0.1 N NaOH required to neutralize the liberated fatty acids from the phospholipid substrates.

6.1.3.4 Effect of substrate concentration

Effect of substrate concentration on catalytic activity of NK-PLA_2-II using PC as a source of substrate is depicted in the Fig. 6.7. With an increase in the substrate concentration, hydrolysis increased until saturation was reached at 1.5 mM substrate concentration.

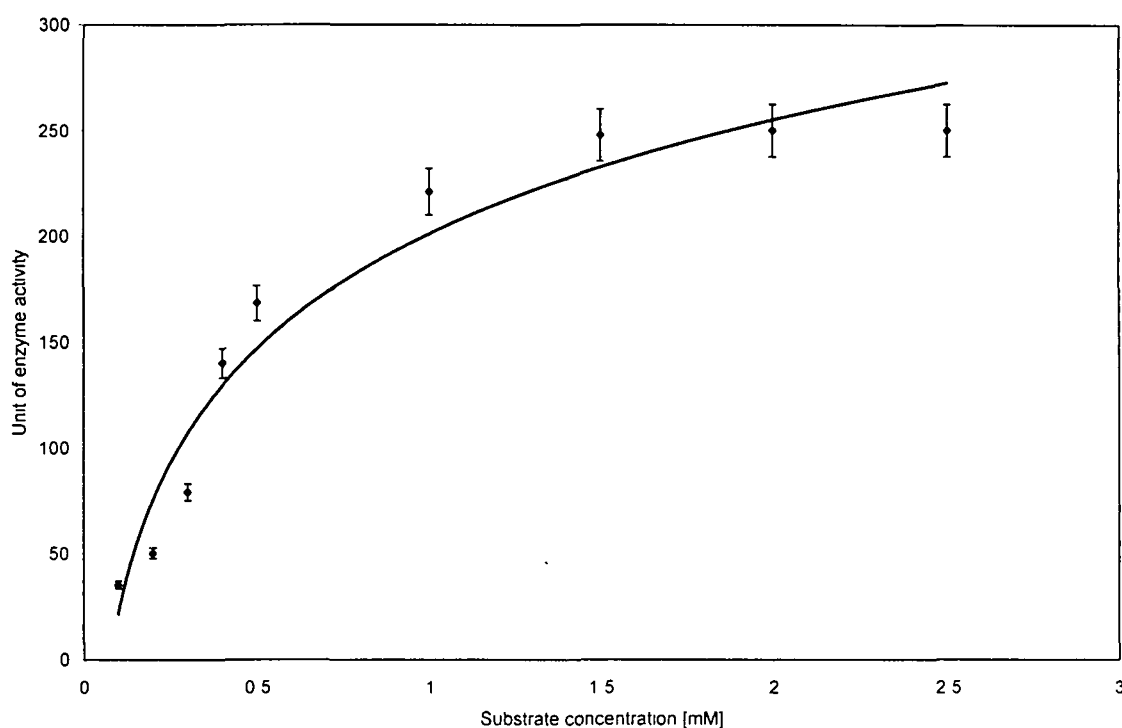

Fig. 6.7. Effect of substrate concentration on PLA_2 activity of NK-PLA_2-II. Effect of substrate concentration on PLA_2 activity of NK-PLA_2-II was determined as described in section 3.2.5.10.2. Results are expressed as mean ± S.D of four determinations.

6.1.3.5 Effect of enzyme concentration

With the increase in enzyme concentration, the hydrolyzing capacity of NK-PLA_2-II on purified phospholipid (PC) increases. The enzymatic activity shows maximum hydrolysis on purified phospholipid at 8 μg/ml enzyme concentration. With further increase in enzyme concentration it shows a saturation level (Fig. 6.8).

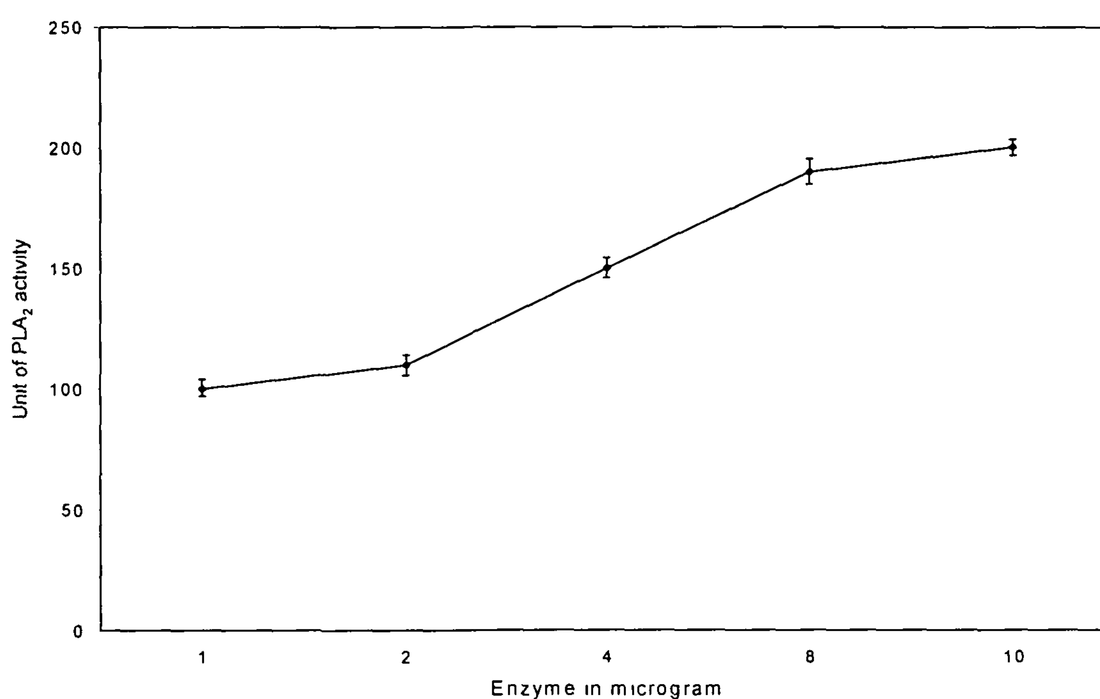

Fig. 6.8. Effect of enzyme concentration on PLA_2 activity of NK-PLA_2-II. Effect of enzyme concentration on PLA_2 activity of NK-PLA_2-II was determined as described in section 3.2.5.10.3. Results are expressed as mean ± of four determinations.

6.1.3.6 Determination of K_m and V_{max}

Using Lineweaver-Burk representation, the Michaelis constant (K_m) and maximum velocity (V_{max}) of the NK-PLA$_2$-II catalyzed reaction was determined. By plotting the values of 1/v as a function of 1/[S] (Fig. 6.9), a straight line was obtain, that intersect the vertical line at a point which is the $1/V_{max}$ (since 1/[S] =0, we have $1/v=1/V_{max}$). If we extent the straight line then the horizontal axis (1/[S]) intersect at the point which is $-1/K_m$.

From the graph we have the equation y= 1.114x +10.322

When y=0

Then x = -10.322/1.114 or -9.265

i.e $-1/K_m$ = -9.265, K_m = 0.1079 mM or 1.079 X 10^{-4} M or 0.926 X 10^{-4}M
$1/V_{max}$ = 10.322, V_{max} = 0.09688 X 10^{-3} mM μg^{-1} or 96.88 X 10^{-3} $\mu mol\ \mu g^{-1}$ or 96.88 $\mu mol\ mg^{-1}$ or 1.032 X 10^{-2} $\mu mol\ mg^{-1}$
Hence the K_m and V_{max} of NK-PLA$_2$-II is 0.926 X 10^{-4}M and 1.032 X 10^{-2} $\mu mol\ mg^{-1}$.

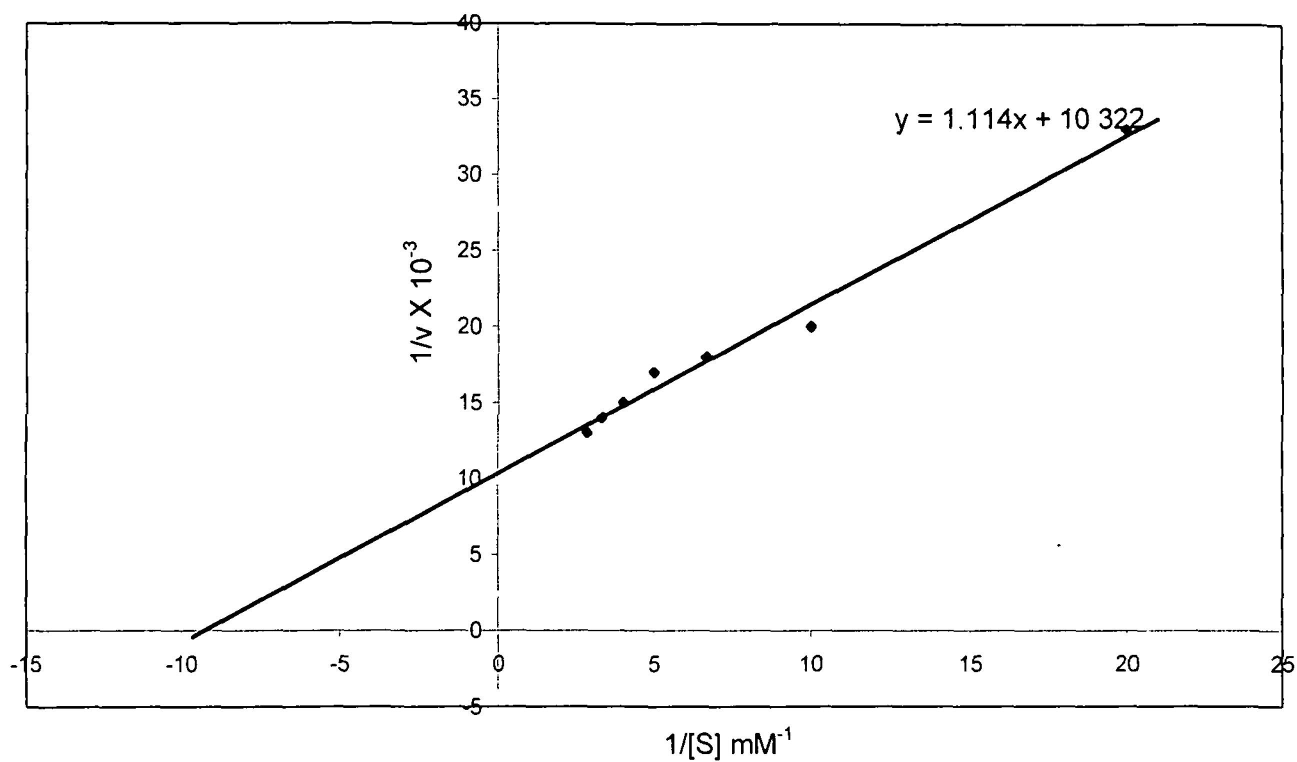

Fig. 6.9. Determination of K_m and V_{max} of NK-PLA$_2$-II using Lineweaver-Burk plot. Substrate used was purified PC. Values are of four determinations. One unit of PLA$_2$ activity is defined as one μl of 0.1 N NaOH required to neutralize the reaction mixture.

6.1.3.8 Optimum temperature

Effect of temperature on the catalytic activity of NK-PLA_2-II is shown in figure 6.10. NK-PLA_2-II exhibited maximum catalytic activity at 37 °C and either with an increase or decrease in the temperature, the catalytic activity diminishes gradually. For example, at 30 °C and at 50 °C temperature, enzyme displays 67 and 50 units of the catalytic activity.

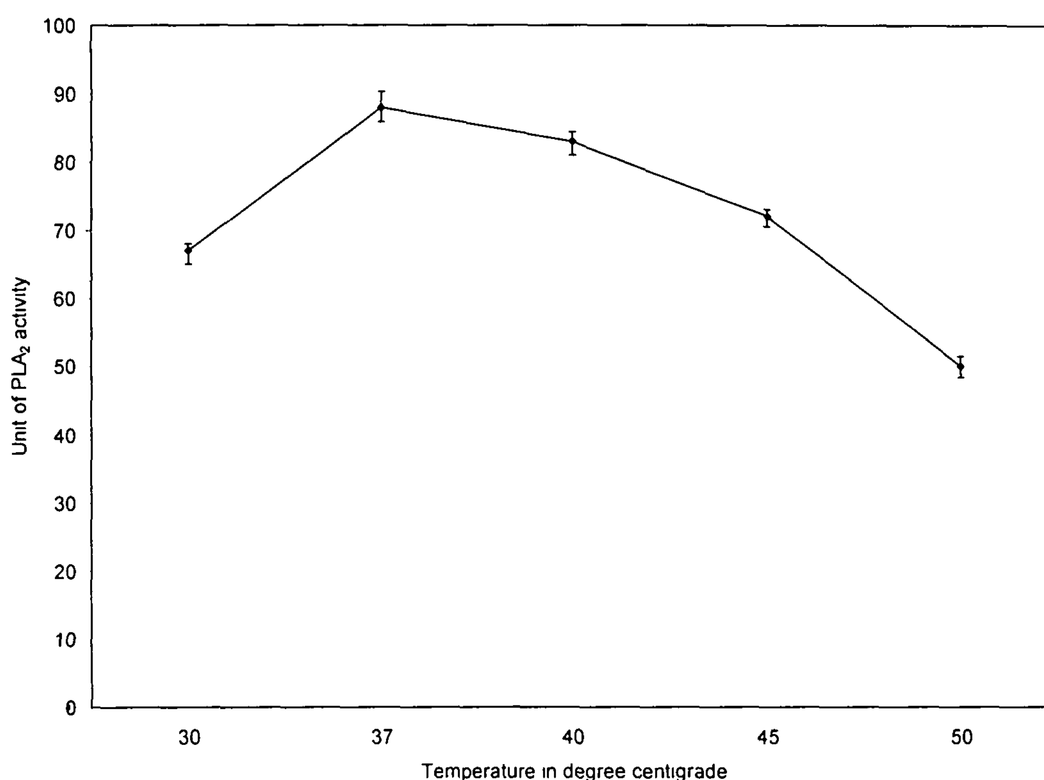

Fig. 6.10. Effect of temperature on PLA_2 activity of NK-PLA_2-II. PLA_2 activity of NK-PLA_2-II at different temperature was determined as described in section 3.2.5.10.5. Results are expressed as mean ± S.D. of three determinations. One unit of PLA_2 activity is defined as decrease in 0.01 absorbance at 740 nm per 10 min.

6.1.3.7 Optimum pH

The influence of pH on the enzymatic activity of NK-PLA_2-II was assayed at a pH range from 6.0-9.0 using egg yolk phospholipids a source of substrate. The enzyme at pH 9.0 shows maximum activity (117.65 units) whereas the lowest activity of 6.25 unit was displayed at pH 6.5 (Fig. 6.11).

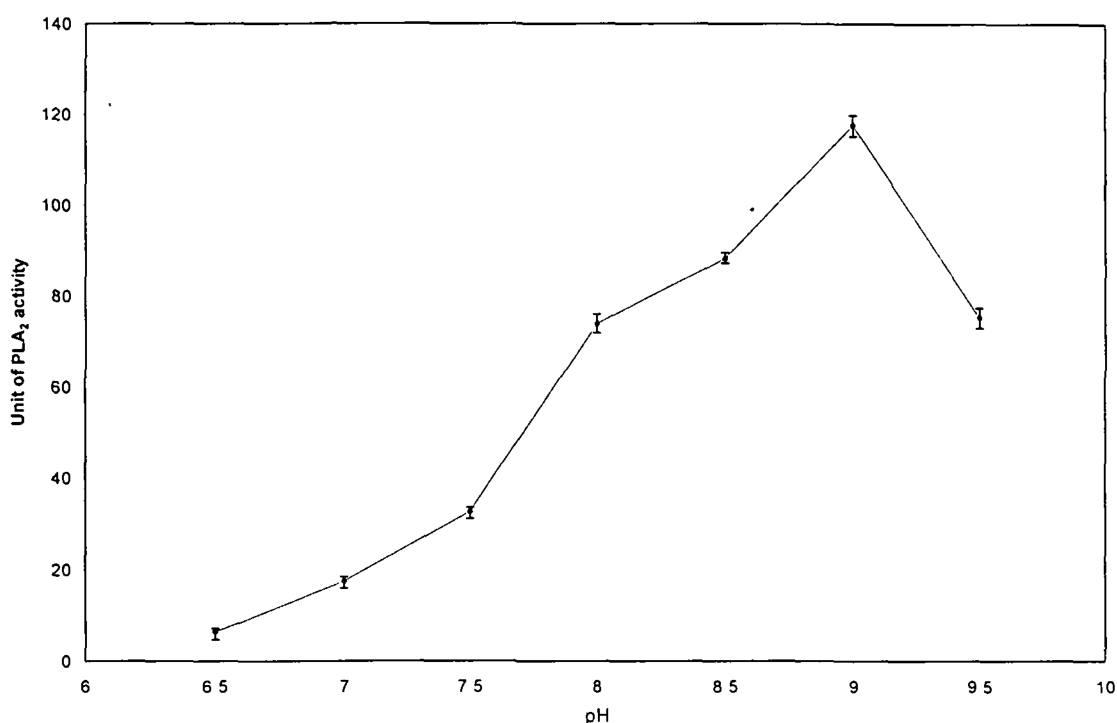

Fig. 6.11. Effect of pH on PLA_2 activity of NK-PLA_2-II. PLA_2 activity of NK-PLA_2-II at different pH was determined as described in section 3.2.5.10.6. The various pH values were obtained as follows; 0.1M Sodium acetate, pH 6.5, 0.1M K-Phosphate pH 7.0-7 and 0.1 M Tris-HCl pH 8.0-9.5. Results are expressed as mean ± of three determinations.

6.1.3.9 Heat-inactivation study

Heating the NK-PLA_2-II at 100 °C for 10 min has no effect on its catalytic activity while heating for 20 min and 45 min at 100 °C resulted in reduction of 14% and 57% respectively of the enzymatic activity (Fig. 6.12).

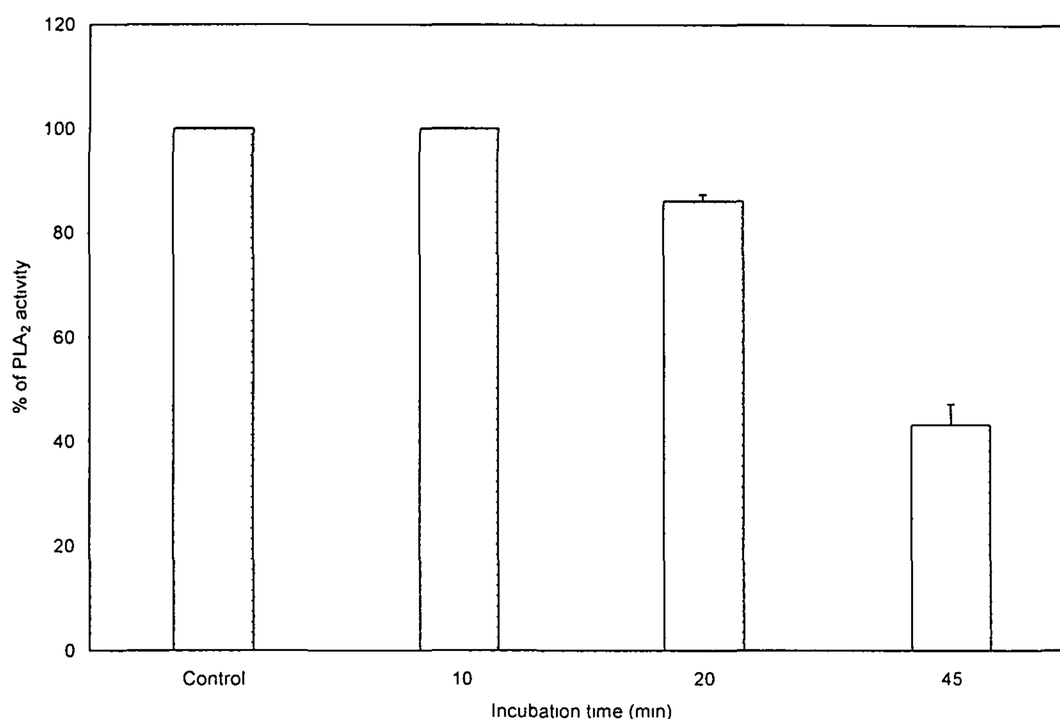

Fig. 6.12. Heat-inactivation of NK-PLA_2-II. PLA_2 activity of heat-inactivated NK-PLA_2-II was determined as described in section 3.2.5.11. PLA_2 activity of NK-PLA_2-II without heating was considered as control. Results are expressed as mean ± S.D. of three determinations.

6.1.3.10 Circular dichroism

CD spectrum of native NK-PLA_2-II demonstrated defined minima at 210 and 222.5 nm, indicating a strong α–helical contribution to the CD signal (Fig.6.13).

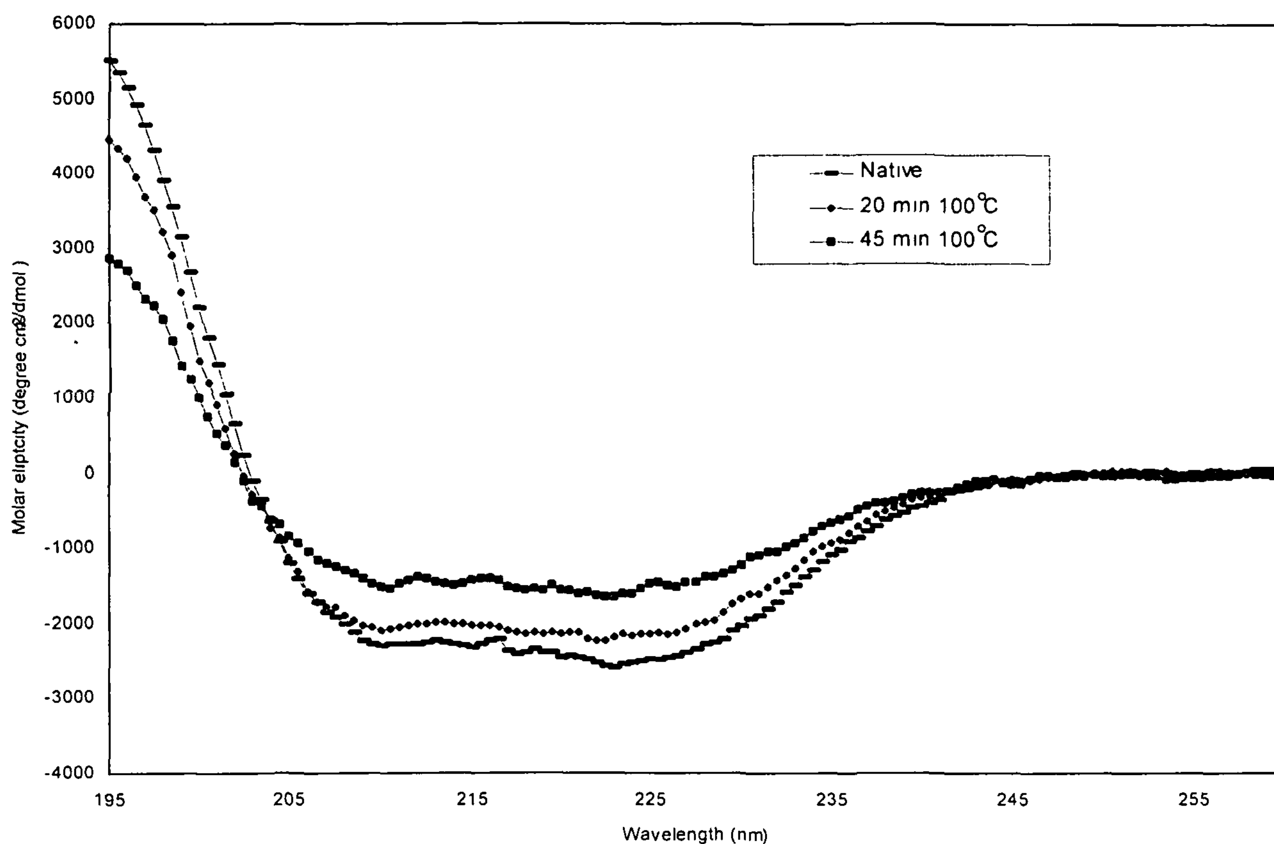

Fig. 6.13. CD spectra of NK-PLA_2-II. (▬) Native PLA_2, (▲) PLA_2 after heating for 20 min at 100 °C and (■) PLA_2 after heating for 45 min at 100 °C. CD measurements were performed as described in section 3.2.4.6. The CD signal is expressed as mean residue ellipticity [θ] (degrees cm^2 $dmol^{-1}$), using 113 as the mean residue molecular weight.

6.1.4 Pharmacological Characterization of $NK\text{-}PLA_2\text{-}II$

6.1.4.1 Lethality and neurotoxicity

The purified $NK\text{-}PLA_2\text{-}II$ at a dose of 10 mg/kg body weight was non-lethal to mice, because no death was observed post 72 hrs of protein injection. Further, neurotoxic symptoms like muscle paralysis in hind limb, gradual decrease in movement, excessive restlessness, urination etc. were not observed 72 hr post injection of $NK\text{-}PLA_2\text{-}II$ at a dose as high as 10 mg/kg body weight of mice (Table 6.4).

6.1.4.2 Edema-inducing activity

The minimum edema dose (MED) of $NK\text{-}PLA_2\text{-}II$ was found to be 4.14 μg when injected into footpad of mice (Table 6.4). However, $NK\text{-}PLA_2\text{-}II$ was more potent than $NK\text{-}PLA_2\text{-}I$ in inducing edema, MED of $NK\text{-}PLA_2\text{-}I$ was only 2.52 μg (Table 5.5).

6.1.4.8 *In-vitro* tissue damaging activity

In-vitro tissue damaging activity of $NK\text{-}PLA_2\text{-}II$ is shown in table 6.4. Heart and liver tissue damaging activity of $NK\text{-}PLA_2\text{-}II$ was lower as compared to $NK\text{-}PLA_2\text{-}I$ but lung tissue damaging activity was same as that of $NK\text{-}PLA_2\text{-}I$.

Table 6.4. Pharmacological properties exhibited by NK-PLA$_2$-II. Results are expressed as mean ± S.D of four experiments.

Properties	NK-PLA$_2$-II
LD_{50} (i.p mg / kg)	Absent
Neurotoxic symptoms	Absent
Direct hemolytic activity (%Hb released by 10μg of protein)	0
Indirect hemolytic activity (% of Hb released by 10μg of protein)	20.77 ± 1.07
Minimum edema inducing dose (MED) (μg)	4.14± 0.4
Anticoagulant activity (Ca clotting time in sec by 1 μg protein)*	98 ± 2.0
Prothrombin time test (Clotting time in sec by 1 μg protein)**	20.25 ± 0.15
In-vitro mice tissue damaging activity (% of Hb released by 25 μg protein)	
a. Liver	5.85 ± 1.34
b. Heart	15.67 ± 2.1
c. Lung	10.27 ± 1.4

* Ca-clotting time of normal plasma = 87± 1 sec.

** PT of normal plasma =19.2 ± 24 sec

6.1.4.2 Anticoagulant activity

In-vitro condition, NK-PLA$_2$-II increases the ca-clotting time of platelet poor goat or human plasma in a dose dependent manner (Fig. 6.14), but does not effect the prothrombin time of platelet poor goat plasma (Table 6.4). The anticoagulant activity of NK-PLA$_2$-II was higher as compared to that of the crude venom (Table 5.5). Further, with an increase in the pre-incubation time of plasma with NK-PLA$_2$-II clotting time was concomitantly increased (Fig. 6.15).

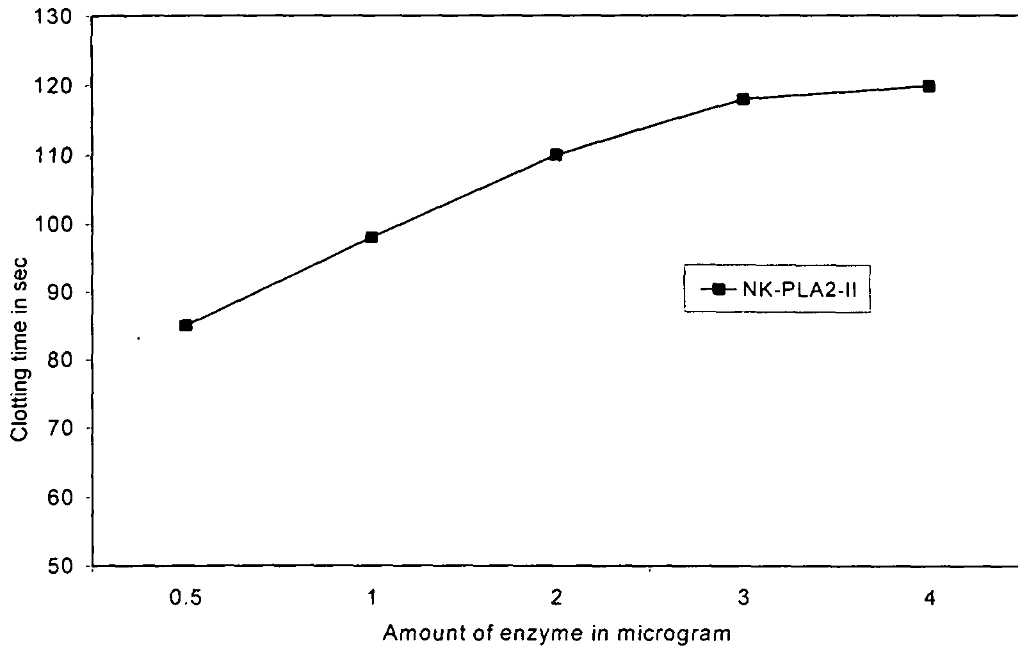

Fig. 6.14. Dose dependent anticoagulant activity of NK-PLA_2-II. Values represent mean ± S.D of four individual experiments. Ca-clotting time of control human plasma is 87±1 sec.

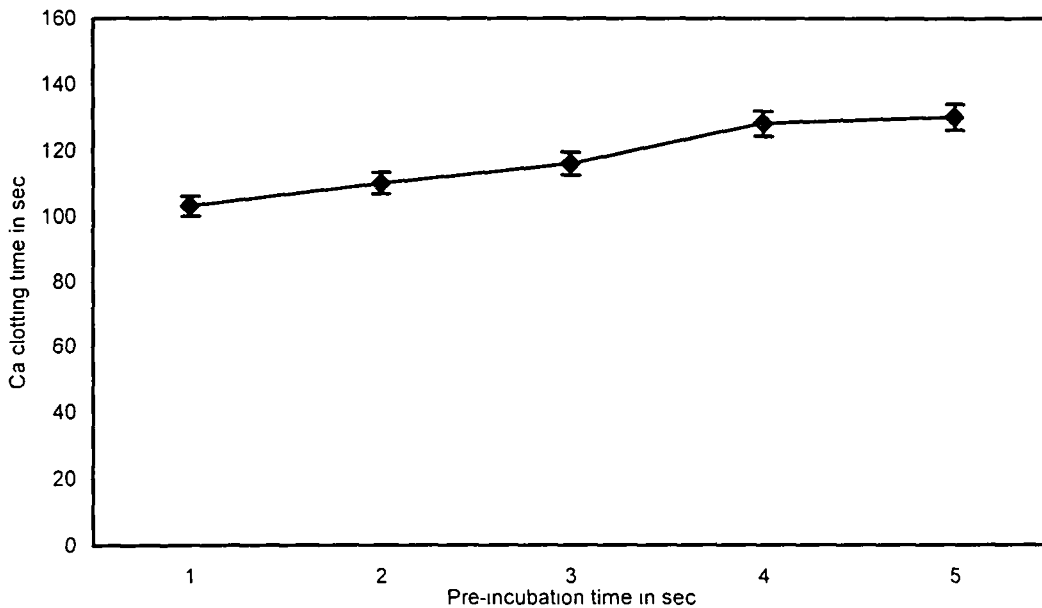

Fig. 6.15. Effect of pre-incubation time on anticoagulant activity of NK-PLA_2-II. Values represent mean ± S.D of four experiments. Ca-clotting time of control human plasma is 87±1 sec.

6.1.4.3 Mitochondrial swelling

The effect of NK-PLA$_2$-II on change in the mitochondrial matrix volume (an indicator of mitochondrial sweling) was measured at 520 nm (Fig. 6.17). NK-PLA$_2$-II hydrolyzes the mitochondrial phospholipid membrane in a very characteristic manner without any lag phase of initiation of reaction. Although the presence of millimolar Ca^{2+} could enhance the mitochondrial damage by NK-PLA$_2$-II however, its presence was not an absolute requirement for the PLA$_2$-induced mitochondrial swelling to occur (Table 6.5). In order to gain further insight into the mode of attack on the mitochondrial membrane by NK-PLA$_2$-II, enzyme-induced fatty acids release patterns were analyzed by GC-MS (Fig. 6.18). Within the initial 15 min of attack, fatty acids of carbon chain length C16 and C18 were the major FFA released by NK-PLA$_2$-II (Table 6.5).

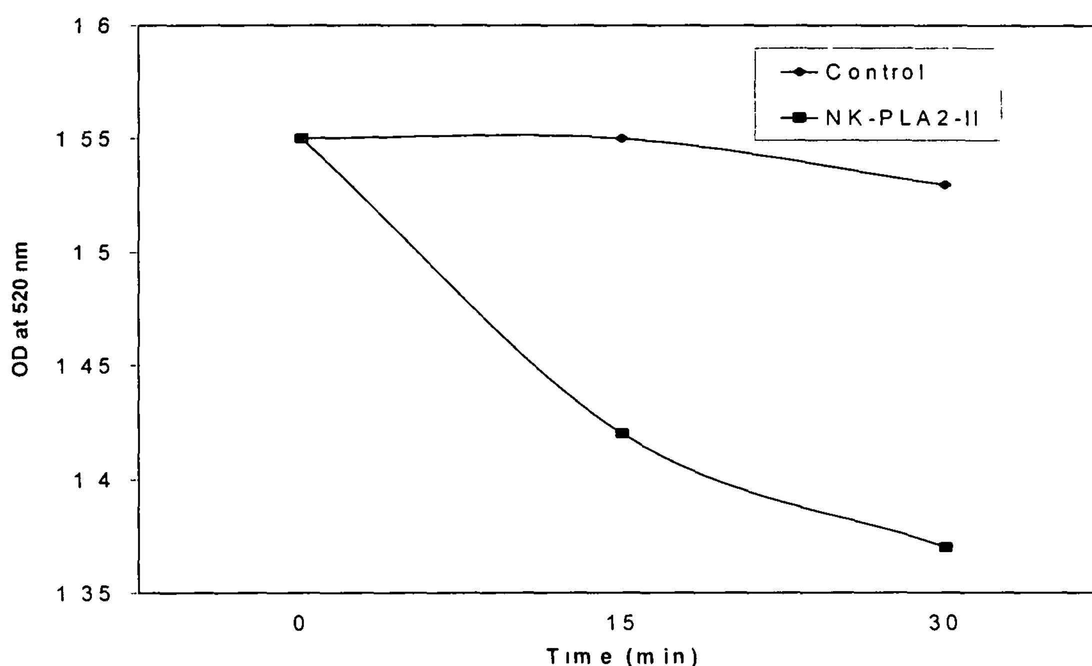

Fig. 6.17. Mitochondrial swelling caused by NK-PLA$_2$-II. About 100 mg equivalent mitochondria from chicken liver were previously incubated with 100nM NK-PLA$_2$-II and decrease in absorbance at 520 nm was recorded from 0 to 30 minute. Results represent mean of at least three determinations.

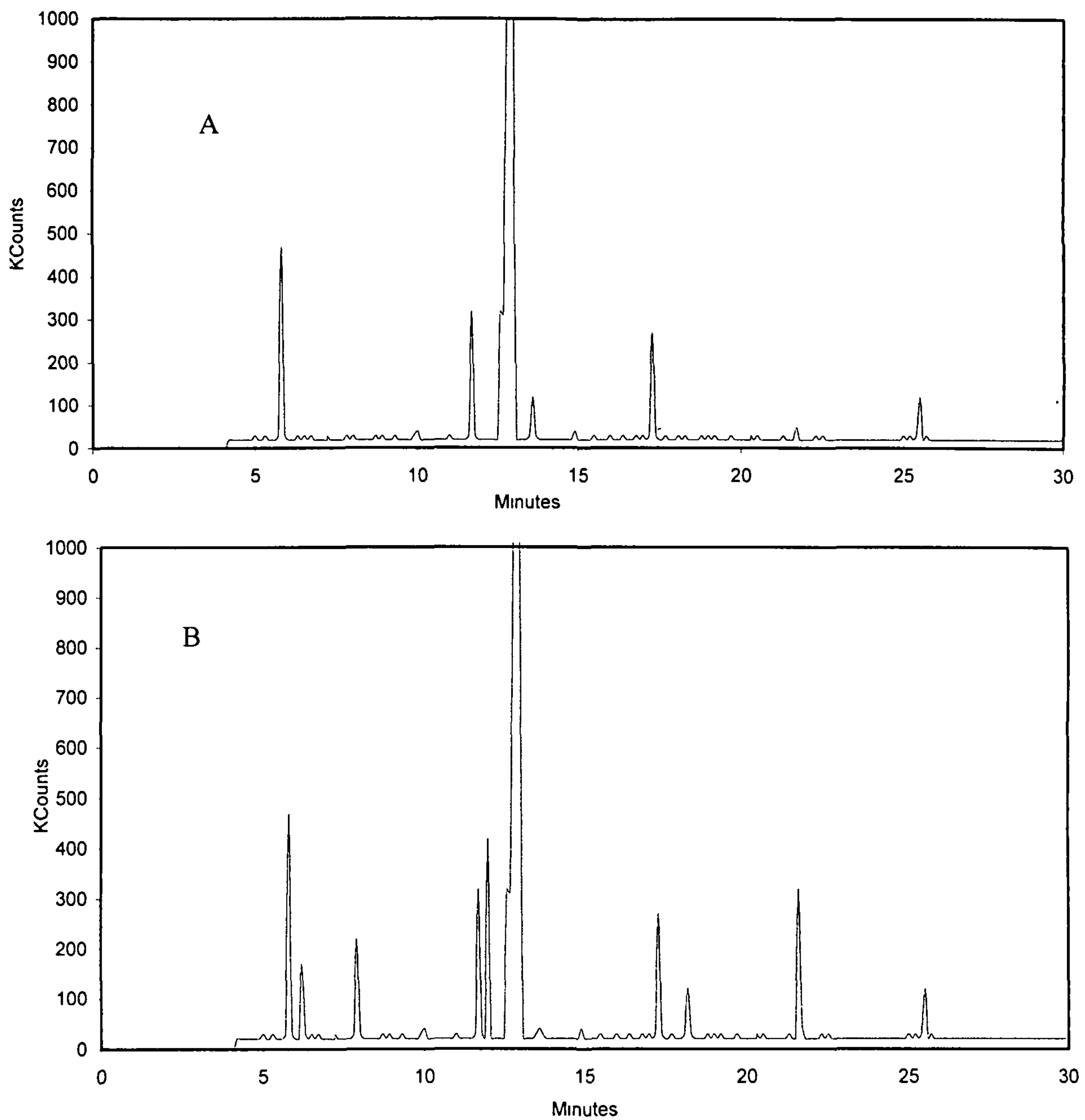

Fig. 6.18. Gas chromatography of methylated free fatty acid. Liberated free fatty acids were extracted from 100 mg equivalent mitochondria (mitochondria obtained from 100 mg wet weight liver tissue), methylated and analyzed by GC-MS as described in section 3.2.6.9.2. A: without enzyme treatment (Control); B: incubated with 100nM NK-PLA$_2$-II. Results are from a typical experiment.

Table 6.5. NK-PLA_2-II-induced swelling and phospholipid hydrolysis of outer plasma membranes of intact mitochondria in the presence or absence of 2 mM Ca^{2+}. Measurements of the degree of mitochondrial swelling and phospholipid hydrolysis, and the extraction, separation and analysis of methylated fatty acids by GC-MS are described in section 3.2.6.9. Data are the mean ± S.D of triplicate determinations.

Incubation time (min)	Mitochondrial swelling (Unit)		Free fatty acid released (μg FFA/20 μg PLA_2)		Ratio of (16:0+18:0)/ (18:1+18:2)
	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	
5	$1.0X10^2±10$	$0.58X10^3±60$	N.D	N.D	N.D
15	$7.0X10^2±53$	$1.3X10^3±80$	2.85±1	5.29±2.1	0.50±0.01
30	$7.7X10^3±100$	$12.7X10^3±121$	27.2±3	44.9±3	0.43±0.012
60	$21.66X10^3±213$	$38.9X10^3±221$	68.1±4	122.64±10	0.31±0.03

Unit = decrease in 0.01 OD at 520 nm after 30 min per mg of mitochondria

6.1.4.4 Direct and indirect hemolytic activity

In contrast to the crude venom, purified NK-PLA_2-II did not exhibit direct hemolytic activity on washed human erythrocytes, but showed appreciable indirect *hemolytic activity in presence of egg yolk phospholipids (Table 6.4).* Erythrocytes pre-incubated with neutral phospholipid PC and 1.5 mM Ca^{2+} were highly susceptible to the lysis induced by NK-PLA_2-II. Pre-incubation with PS and PE had virtually no influence on the hemolytic activity of NK-PLA_2-II, irrespective of the presence or absence of 1.5 mM Ca^{2+} in the reaction medium (Fig. 6.16).

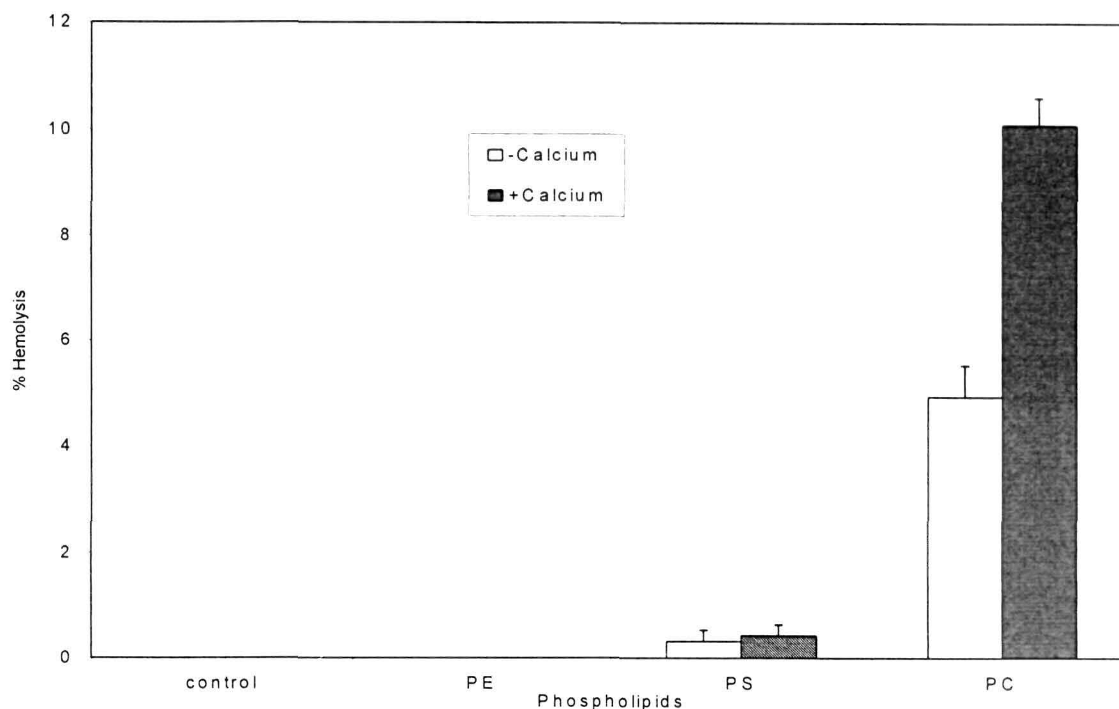

Fig. 6.16. Effect of NK-PLA_2-II on erythrocytes enriched with different phospholipids in presence or absence of 2 mM calcium. Experiment was done as described in section 3.2.6.3. Lysis is expressed as percentage, taking 100% the absorbance of erythrocyte suspension incubated with MQ water. Erythrocyte suspension without phospholipid served as control. Each result represents mean ± S.D of three individual experiments.

6.1.4.5 Myotoxicity

Administration of NK-PLA_2-II BABL/C albino mice results significant increase in the LDH activity in serum as compared to control (Table 6.6). Figure 6.19 A&B show light micrographs of thigh muscle of mice injected with 40 μg of NK-PLA_2-II and normal saline. Histological observation of the thigh muscle of mice after injection of NK-PLA_2-II reveals several ruptured segments, separated by spaces apparently devoid of cellular materials (indicated by arrow in Fig. 6.19) whereas in normal muscle the tissue are seen to be intact (Fig. 6.19A).

Table 6.6. LDH activity in serum of mice post NK-PLA$_2$-II injection. Results are mean ± S.D of three determinations.

	Control	Treated	%increase
LDH	1.04 x 10^{-3}±0.01	2.34 x10^{-3}±0.1	125

Unit = μmol of LDH release/min/ml

A B

Fig. 6.19. Photomicrograph of thigh muscle of Mice. A: Normal thigh muscle (Control) mice received only 50 μl of normal saline. B: Thigh muscle treated with 40 μg of NK-PLA$_2$-II dissolved in 50 μl of PBS. Sections of the thigh muscles were stained with hematoxylin-eosin stain and viewed under (400X magnification) Lica ATC 2000 microscope. Photographs were taken with Kodak film (Max 200).

6.1.4.6 Antibacterial activity

Even at a concentration of 20 μg/ml, NK-PLA$_2$-II did not exhibit any antibacterial activity against Gram positive or Gram negative bacteria (Fig. 6.20). Crude venom inhibited growth of Gram positive bacteria significantly when tested by time course experiment (Fig. 6.19) and displayed clear zone of inhibition on Gram negative bacteria, when crude venom was applied as a single point on the plate (Fig. 5.23).

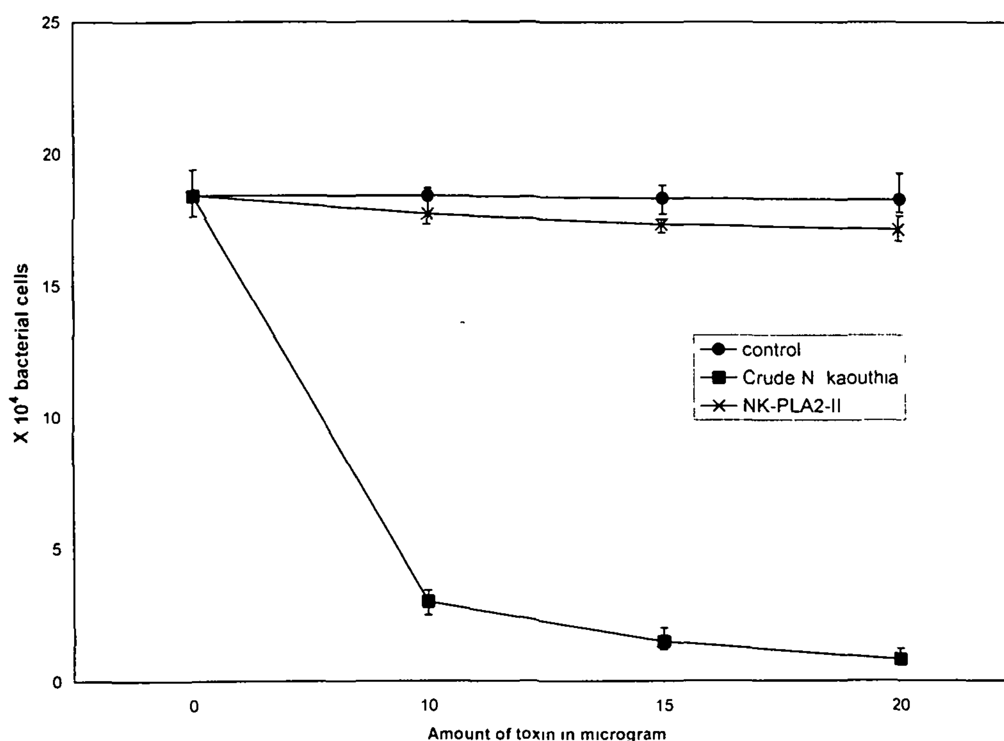

Fig. 6.20. Effect of crude *N. kaouthia* crude venom and NK-PLA$_2$-II on *B. subtilis*. Varying amount of crude venom or either enzyme was incubated with 18.4 X 10^4 cells for 4 h at 37 °C and change in the optical density was recorded at 630 nm. 1 OD at 630 nm = 10^6 cells. Each point represents the mean ± S.D. of four determinations.

6.1.5 Immunological Characterization of NK-PLA$_2$-II

6.1.5.1 Western blotting

The cross-reactivity between anti-NK-PLA$_2$-I antibodies and NK-PLA$_2$-II was checked by western blotting. NK-PLA$_2$-II was separated by SDS-PAGE and blotted on PVDF membrane. The dark band in the PVDF membrane corresponds to the NK-PLA$_2$-II band on SDS-PAGE indicating that NK-PLA$_2$-II was recognized by anti-NK-PLA$_2$-I antiserum raised in rabbit at 1:4000 dilution (Fig. 6.21).

Fig: 6.21. Immunoblotting experiment showing cross-reactivity of NK-PLA$_2$-II against anti-NK-PLA$_2$-I IgG. About 50 μg of protein was separated by 10%SDS-PAGE and transferred to Immobilon-P membrane. Detection was done with horse redish peroxidase conjugate rabbit anti-horse IgG and TMB/H_2O_2 as substrate.

6.1.6 Neutralization of catalytic activity of NK-PLA_2-II

6.1.6.1 Effect of ρ-bromophenacyl bromide

ρ-bromophenacyl bromide specifically inhibits the catalytic activity of phospholipase A_2 enzyme by alkalyting the histidine residue of the enzyme. NK-PLA_2-II after treatment with ρ-bromophenacyl bromide (3.3 mM), retained only 3.67 % of its original PLA_2 activity. However, Triton X-100 and Triton X-100 plus Ca^{2+} significantly reversed this inactivation. Triton X-100 at 1 mM concentration had little protective effect against the inhibition while at higher concentration (30 mM) in presence of 10 mM Ca^{2+} displayed higher protection against the inactivation of PLA_2 activity by ρ-bromophenacyl bromide (Table 6.7). This inactivation result is nearly identical to that shown by NK-PLA_2-I (Table 5.8).

Table 6.7. Effect of ligands on inactivation of PLA_2 activity of NK-PLA_2-II with ρ-bromophenacyl bromide. Inactivation condition were 3.3 mM ρ-bromophenacyl bromide at pH 9.0. Values represent ± S.D of four determinations.

Ligands	% of activity remaining after treatment
Control (no inhibitor)	100
ρ-bromophenacyl bromide (No ligand)	3.67 ± 1.2
ρ-bromophenacyl bromide + Triton X 100 (1 mM)	15.99 ± 1.8
ρ-bromophenacyl bromide + Triton X 100 (10 mM)	21.1± 2.1
ρ-bromophenacyl bromide + Triton X 100 (30 mM)	30.3 ± 2.5
ρ-bromophenacyl bromide + Triton X 100 (30 mM), Ca^{2+}10 mM)	50.05 ± 3.8

6.1.6.2 Effect of other inhibitors

Treatment of NK-PLA$_2$-II by serine inhibitors like PMSF, TLCK, and TPCK inhibited 5.0, 0.39 and 0.26% respectively. But DTT (reducing agent for disulfide bridge) at a final concentration of 2 mM inhibited the PLA$_2$ activity up to 41.49% and treatment of the enzyme with EDTA totally inhibited the PLA$_2$ activity. While α-bromo-2-acetophenone and α-chloroacetophenone could inhibit 3.77% and 9.84% of phospholipase A$_2$ activity of NK-PLA$_2$-II respectively (Table 6.8).

Table 6.8. Effect of various inhibitors on PLA$_2$ activity of NK-PLA$_2$-II. Values represent mean ± S.D of four experiments.

Reagents/condition	% of activity remaining after treatment
Control	100
ρ-BPB	3.67 ± 1.80
TLCK (2 mM)	99.61 ± 1.7
TPCK (2 mM)	99.74 ± 3.6
Phenylmethylsulfonyl flouride (2 mM)	95 ± 2.1
Diothiothreitol (1 mM)	93.37 ± 1.67
Diothiothreitol (2 mM)	58.51 ± 2.3
Ethylenediamine tetra-acetic acid (1 mM)	0
Ethylenediamine tetra-acetic acid (2 mM)	0
α-bromo-2-acetophenone (2 mM)	96.23 ± 3.2
α-chloroacetophenone (2 mM)	90.16 ± 2.1

6.1.6.3 Effect of polyvalent antivenom

Polyvalent antivenom raised against cobra, Common krait, Russell's viper and Saw-scaled viper in horse (Bharat Serums & vaccines Ltd, Thane Lot No. 06/01) could inhibit up to 50% of the PLA$_2$ activity of NK-PLA$_2$-II at 1:100 ratio (antigen:antibody) (Table 6.9).

6.1.6.4 Effect of anti-NK-PLA$_2$-I antibodies

With the increase in concentration of anti-NK-PLA$_2$-I IgG the catalytic activity of NK-PLA$_2$-II was neutralized up to 48% at 1:100 antigen:antibody ratio (Table 6.9) as compared to 68% inhibition of catalytic activity of NK-PLA$_2$-I (Table 5.10).

Table 6.9. Neutralization of PLA$_2$ activity of NK-PLA$_2$-II by polyvalent antivenom (Bharat Serums & vaccines Ltd, Thane) and anti-NK-PLA$_2$-I IgG (raised in rabbit). Results are mean ± S.D of three determinations.

Ratio (antigen:antibodies)	% inhibition by	
	Commercial *N. naja* antivenom	**Anti-NK-PLA$_2$-I antivenom**
1:1	5.85 ± 0.12	4.69 ± 1.1
1:5	11.17 ± 0.1	20.84 ± 2
1:10	20.24 ± 1.3	30.27 ± 0.31
1:50	35.11 ± 1.01	35.55 ± 1.01
1:100	50.10 ± 1.3	48.34 ± 0.21

6.1.7 Neutralization of pharmacological activities of NK-PLA$_2$-II

6.1.7.1 Effect of ρ-bromophenacyl bromide

ρ-bromophenacyl bromide specifically modifies the histidine residue of phospholipase A_2 enzyme which is responsible for the enzymatic activity. Modification of NK-PLA$_2$-II with ρ-bromophenacyl bromide (3.3 mM) could neutralize most of the tested pharmacological activities (Table 6.10). However, *in-vitro* liver and heart tissue damaging activities were neutralize up to 46% and 37% respectively. Inhibition of anticoagulant, edema, indirect hemolytic and mitochondrial swelling by ρBPB, indicates that these properties are dependent on the catalytic activity of NK-PLA$_2$-II (Table 6.10).

6.1.7.2 Effect of other inhibitors

None of the serine inhibitors like PMSF, TPCK and TLCK could neutralize the tested pharmacological activities significantly. EDTA and DTT too were unable to neutralize the pharmacological activities of NK-PLA$_2$-II (Table 6.10).

Table 6.10. Effect of chemical inhibitors on the pharmacological properties of $NK-PLA_2$-II. Values are mean ± S.D. of four individual experiments.

Properties	% of activity remaining after treatment					
	ρBPB (3.3 mM)	TLCK (0.1 mM)	TPCK (0.1 mm)	PMSF (1 mM)	EDTA (1 mM)	DTT (2 mM)
Anticoagulant	11±1.0	15± 3.0	100	100	97.6±0.2	0
Edema	5±1.01	100	100	94.1±2.1	0	0
Indirect hemolytic	13±0.22	100	100	100	0	0
Heart tissue damaging	46±4.0	74±1	60±1.4	40±1.1	0	0
Liver tissue damaging	37±2.3	92±2.3	72±2.1	41±1.0	0	0
Mitochondrial swelling	14.97±2.2	20 ±1.1	16.8±6.9	95±3.0	0	0

6.1.7.3 Effect of anti-$NK-PLA_2$-I antibodies

Anti-$NK-PLA_2$-I IgG was found to be ineffective in neutralizing the tested pharmacological activities of $NK-PLA_2$-II (Table 6.11). 90% of the tested pharmacological activities were retained at 1:100 (mol:mol ratio). Anticoagulant activity was inhibited up to 9%. While the indirect hemolytic and edema was neutralized up to 6.8 and 3.7% respectively. 100% of *in-vitro* liver and heart tissue damaging activities were retained. Moreover the 95% of mitochondrial swelling activity was observed at 1:100 ratio (Table 6.11).

6.1.7.4 Heat-inactivation

As shown in table 6.11 heating the enzyme at 100 °C for 20 min was hardly effective in neutralizing the tested pharmacological properties of $NK-PLA_2$-II, however heating at the same temperature, but for longer duration (45 min) resulted in significant inhibition of pharmacological properties of $NK-PLA_2$-II. For example anticoagulant activity and mitochondrial swelling properties of $NK-PLA_2$-I were

inhibited up to 15% and 20% after heating the enzyme at 100 °C for 20 min but these properties were completely abolished post heating the enzyme at 100 °C for 45 min (Table 6.11).

Table 6.11. Effect of polyvalent anti-NK-PLA_2-I IgG and heating on the pharmacological properties of NK-PLA_2-II. Values are mean ± S.D. of four individual experiments.

Properties	% of activity remaining after treatment (Control 100%)		
	Anti-NK-PLA_2-I IgG (1:100) (mol:mol)	Heating at 100 °C	
		20 min	45 min
Anticoagulant	91±3.1	85.65±3.21	0
Indirect hemolysis	93.2±0.21	80.2±2.1	1.8±0.1
Edema	96.3±2.1	85.4±2.1	7±1.06
Liver tissue damage	100	83±3.0	3±1.04
Heart tissue damage	100	93±2.1	4±0.04
Lung tissue damage	100	82±2.01	5±1.32
Mitochondrial swelling	95±2.1	80.65±3.01	0

6.1.7.5 Neutralization by *Azadiracta indica*

The methanol extract of leaves of *A. indica* at 1:100 (protein/extract) could neutralize the PLA_2 activity of crude venom and NK-PLA_2-II up to 49.05% and 30% respectively, whereas the water extract could inhibit up to 27% and 14% respectively (Table 6.12). The indirect hemolytic activity of crude venom and NK-PLA_2-II was inhibited up to 36.7% and 30% respectively by methanol extract at 1:100 ratio. Whereas at the same concentration, water extract of could neutralize

only 7% and 6% of indirect hemolytic activity as compared to control but the chloroform extract did not displayed any inhibitory activity (Table 6.12).

Table 6.12. Percent inhibition of catalytic and indirect hemolytic activities of crude venom and NK-PLA_2-II (1 μg protein) by leaf extract of *Azadiracta indica*. Results are mean ± S.D of four determinations.

Amount of Plant extract (μg)		% inhibition of			
		PLA_2 activity		Indirect hemolytic activity	
		Crude venom	NK-PLA_2-II	Crude venom	NK-PLA_2-II
Water extract	5	3.34 ± 0.1	0.4 ± 0.01	0	0
	10	7.75 ± 1.0	3 ± 0.2	0	0
	50	12.1 ± 0.2	8±1. 1	0	0
	100	27 ± 1.2	14±0.6	7±2	6±0.1
Chloroform extract	5	1.06 ± 0.05	0	0	0
	10	3.12 ± 1.01	0	0	0
	50	8.23 ± 2.0	4 ± 0.01	0	0
	100	18.15 ± 2.01	10 ±0.2	0	0
Methanol extract	5	9.28 ± 1.1	3 ± 1.1	7.4±1	8±1.1
	10	15.23 ± 2.2	8 ± 0.5	13.8±0.2	10±1
	50	26.89 ± 2.1	12 ± 1.1	23.2±1.01	21±0.2
	100	49.05 ± 2.3	30 ± 1.2	36.7±2	30±1.01

CHAPTER VII

DISCUSSION

Phospholipase A_2 enzymes are biologically active components of snake venom which have been extensively studied because of their pivotal role in various biological activities and to elucidate the structure-function relationship (Kini, 1997). During the last decade, there are numerous reports published on the biochemical and pharmacological characterization including structure-function relationship of PLA_2 isoenzymes from *N. naja* venom of Indian origin (Achyutan et al., 1980; Rudrammaji and Gowda, 1998; Bhat and Gowda, 1989, Mukherjee and Maity, 1998). However, there is a dearth of knowledge on the PLA_2 enzymes from the venom of *N. kaouthia*, particularly of Indian origin. Although, Mukherjee and Maity (2002) reported the difference in biochemical properties, lethality and pathophysiology in the venom samples of *Naja naja* and *Naja kaouthia* of the same geographical origin, however, they did not mention in detail about the number of PLA_2 isoenzymes present in the venom of these two closely related snakes, biochemical and pharmacological properties of purified PLA_2 enzymes, particularly from *N. kaouthia* venom. Therefore, the purpose of the present study is three fold. First, an effort has been given to compare and characterize the PLA_2 isoenzyme present in the venom of adult male *N. naja* and *N. kaouthia* snakes of eastern India origin to exclude the influence of age, sex and geographical variation in the venom composition. Secondly, two novel, most catalytically active phospholipase A_2 isoenzymes viz. NK-PLA_2-I and NK-PLA_2-II have been isolated, purified and characterized from venom sample of *N. kaouthia* of Indian origin. Finally, the pharmacological reassessment of medicinal plants of north-east India for their inhibitory activity against the PLA_2 enzyme(s) of *N. kaouthia* venom has been done in an effort to provide for an alternative to antivenom therapy for snakebite.

To compare and identify the number of PLA_2 isoenzymes present in *N. naja* and *N. kaouthia* venom samples, elution pattern of PLA_2 isoenzymes through a cation and anion exchangers under the identical condition, was studied. Fractionation of both the venom samples on a cation exchanger viz. CM-Sephadex C-50 reveal presence of seven basic PLA_2 isoenzymes in both the venom samples. Since the basic PLA_2 isoenzymes are positively charged, therefore they are retained by the cation exchanger and eluted with buffers of increasing molarity and pH values. On the other hand, neutral or acidic PLA_2 isoenzymes are not retained by the cation exchanger and are eluted in a single peak with the equilibration buffer. However, it is worthy to be mentioned here that although both of the venom samples possess identical number of basic PLA_2 isoenzymes, but they differ in their net positive charge on the enzyme molecule resulting in differences in elution profile from a cation exchanger. Further basic PLA_2 isoenzymes of *N. naja* and *N. kaouthia* venom samples also differ quantitatively. The total protein content as well as the catalytic activity of the basic PLA_2 enzymes from the *N.naja* venom sample are higher than the basic enzymes from *N.kaouthia* venom sample, which may explain the observed higher lethality of *N. naja* venom (LD_{50} 0.4 $\pm$ 0.08 mg/kg body weight of mice) than that of *N. kaouthia* venom (LD_{50} 0.7 $\pm$ 0.09 mg/kg body weight of mice) (Mukherjee and Maity, 2002). Since, basic PLA_2 enzymes are more toxic than the acidic or neutral PLA_2 enzymes, therefore, the former group of enzymes shows higher potency in inducing pharmacological effects (Boffa et al., 1980; Verheij et al., 1980; Kini and Evans, 1989; Kanashiro et al., 2002). The higher toxicity of the basic PLA_2s are due to the higher penetrability, probably due to abundance of positive charges on the PLA_2 molecule (Kini, 2003).

To investigate the number of acidic PLA_2 isoenzymes present in either venom sample, both the venom samples were fractionated on a DEAE Sephadex A-50 anion exchanger. Because of net negative charge on acidic PLA_2 isoenzymes, therefore, they are retained by the anion exchanger and are eluted only with the buffers of increasing molarity and decreasing pH values. Fractionation reveals the

presence of three acidic PLA_2 isoenzymes in *N. naja* and two acidic PLA_2 isoenzymes in *N. kaouthia* venom samples. Assay of enzymatic activity shows that acidic PLA_2 isoenzymes are catalytically more active in hydrolyzing phospholipids than those of the basic PLA_2 enzymes from the same venom, which is in close agreement with other findings (Rosenberg, 1986; Rudrammaji and Gowda, 1998; Ketelhut et al., 2003).

Therefore, the present study documents that venom of *N. naja* of eastern India origin possesses a total of 10 PLA_2 isoenzymes-seven basic and three acidic, whereas *nine* PLA_2 isoenzymes including seven basic and two acidic PLA_2 isoenzymes are present in the venom sample of *N. kaouthia* from the same geographical origin. Differences in the quantitative and qualitative distribution of PLA_2 isoenzymes in *N. naja* and *N. kaouthia* venom samples reinforces the difference in venom composition between these two species of Indian cobra (Mukherjee and Maity, 2002). Further, since the commercial polyvalent antivenom available in India contain antibodies against venom of *N. naja* and not against *N. kaouthia*; this prompts us to give proper consideration for preparing appropriate antivenom for treating the cobra bite patients.

Till date, amino acid sequence of about 280 PLA_2 enzymes have been determined (Tan et al., 2003) and they are found to share common homology. But these isoenzymes show different potencies in inducing pharmacological activities. Neither the mechanism by which venom phospholipase A_2 enzymes show their pharmacological effects, nor the role of enzymatic activity in inducing these effects is clearly understood (Rosenberg, 1986; Fletcher et al., 1981; Kini and Evans, 1988; 1989). Therefore, a complicated and subtle interrelationship exists between these two properties of PLA_2 enzymes. To investigate this relationship further, in the present study, two major phospholipase A_2 isoenzyme from *N. kaouthia* venom were isolated, purified and characterized.

Fractionation of crude *N. Kaouthia* venom on CM-Sephadex C-50 ion-exchanger column resulted in separation of venom components into eight fractions, based on the net surface charge on the PLA_2 enzyme. Screening of these fractions for phospholipase A_2 activity reveals that highest enzymatic activity was associated with the unbound acidic fraction CM I, followed by basic fraction CM II. Therefore, these two fractions were selected for further purification and characterization. CM I was further fractionated by gel filtration chromatography followed by RP-HPLC for the purification of NK-PLA_2-I, while NK-PLA_2-II was purified from the basic fraction CM II by RP-HPLC. NK-PLA_2-I and NK-PLA_2-II constitute about 6% and 3.5% of the total venom proteins respectively.

Both the purified PLA_2 enzymes at a concentration of 30 μg showed a single band in SDS-PAGE indicating the purity of the preparation. NK-PLA_2-I displayed an apparent subunit molecular mass of 13.6 kDa and 19.26 kDa under reduced and non-reduced conditions respectively showing it is dimer in nature. The molecular weight of NK-PLA_2-II under both reduced and non-reduced condition was determined as 13.1 kDa documenting this protein's existence as a monomer (Selistre et al., 1996). The molecular weight of native NK-PLA_2-I and NK-PLA_2-II, as determined by gel filtration, were 12.9 kDa and 12.4 kDa respectively, which are very close to the molecular weight of these proteins determined by SDS-PAGE. The purity and molecular mass of NK-PLA_2-I and NK-PLA_2-II were further confirmed by MALDI-MS which revealed a protonated molecular ion [MH^+] at m/z 13,786.205 and 13,346.19 Da respectively, which is similar to the mass determined by SDS-PAGE. MALDI-MS normally yields single charged states, but a lower intensity, doubly charged [MH^{2+}] peak at m/z 7193.1025 and 6673 Da were also noticed. PLA_2s also displayed such doubly charged peaks as seen in case of PLA_2 enzyme from the venom of the sea snake *Hydrophis cyanocinctus* (Ali et al., 1999). The molecular mass of NK-PLA_2-I and NK-PLA_2-II from the venom of *N. kaouthia* are quite consistent with the molecular masses of PLA_2 enzymes from many other snake venoms such as H1 and H2 PLA_2s (from Sea snake *Hydrophis cyanocinctus*

venom) having molecular mass of 13.588 and 13.247 kDa respectively (Ali et al., 1999); NN-I_{2c}-PLA_2, NN-I_{2d}-PLA_2, and NN-I_{2e}-PLA_2 having molecular mass of 14.5, 13.3 and 13.7 kDa respectively isolated from *Naja naja naja* venom (Rudrammaji and Gowda, 1998); MiPLA_2 2,3,4 having molecular mass of 13.95, 13.88 and 14.01 kDa respectively isolated from *Micropechis ikaheka* venom (Gao et al., 2001); Notechis II-1 having molecular mass of 13.233 kDa; Notechis II-2 having molecular mass of 13.013 kDa; and Notechis II-5 having molecular mass of 13.676 kDa isolated from *Notechis scutatus* venom (Halpert et al., 1976). Further, absence of protease, 5′-nucleotidase, ATPase, and acetylcholiesterase activities in these two enzymes clearly indicate that these purified proteins are not contaminated with other venom enzymes.

Biochemical characterization of either enzyme reveals that NK-PLA_2-I is glycoprotein in nature while NK-PLA_2-II is devoid of any carbohydrate moiety. The specific activity of NK-PLA_2-I and NK-PLA_2-II are 94,700 ± 1500 (mean ± S.D.) and 88,100 ± 1100 (mean ± S.D.) (Units/mg protein) respectively when egg yolk phospholipid was used as source of substrate. NK-PLA_2-I displays maximum catalytic activity in the pH range of 7.5-8.5 whereas, NK-PLA_2-II shows sharp pH optima at 9.0, demonstrating that these two enzymes require different pH optima for exerting maximum catalytic activity. The pH optima of *N. kaouthia* PLA_2s are higher than the reported pH optima for acidic PLA_2s (pH 6.9), isolated from the Indian cobra *N. n. naja* venom (Rudrammaji and Gowda, 1998), but in close agreement with the pH optima of many other snake venom PLA_2s, such as VRV-PL-VIIIa, optimum pH 7.2 (Kasturi and Gowda, 1989) and Bj IV, optimum pH 8.2 (Bonfim et al., 2001).

Determination of *in-vitro* head-group specific phospholipid hydrolyzing capacity of phospholipases has great relevance in elucidating the catalytic efficiency of the enzyme and to explain some of their pharmacological effects on the target cell membranes (Kini, 1997; Fletcher and Rosenberg, 1997). Present experiments

showed preferential hydrolysis of PC over PS or PE by both NK-PLA_2-I and NK-PLA_2-II; however, the rate of hydrolysis of PC by NK-PLA_2-I is much higher than NK-PLA_2-II. This is in contrast to the earlier reports demonstrating enhanced hydrolysis of PE over PC or PS by PLA_2s from *N. n. kaouthia, N. n. atra* (Fletcher and Rosenberg, 1997) and *D. russelli* venom samples (Vishwanath et al., 1988). This may be due to geographical and/ or species specific variation in the substrate specificity of PLA_2 isoenzymes. The K_m and V_{max} values for NK-PLA_2-I is 0.988 X 10^{-4} M and 1.4 X 10^{-2} μmol mg^{-1} while the same values for NK-PLA_2-II is 0.926 X 10^{-4} M and 1.032 X 10^{-2} μmol mg^{-1} when PC is used as a source of substrate. The K_m values of either enzyme is quite lower than the reported Km value of NN-I_{2c}-PLA_2, NN-I_{2d}-PLA_2 and NN-I_{2e}-PLA_2 (3.2 X 10^{-4}, 4.2 X 10^{-4} and 3.8 X 10^{-4} M respectively) from venom of Indian cobra *N. n. naja* (Rudrammaji and Gowda, 1998). NH_2 – terminal amino acid sequence (determined up to 10 amino acid residue) of both the *N. kaouthia* PLA_2s are identical (NIYQFKNNIQ). A comparison of the PLA_2 enzyme sequence listed in the Gene bank protein database indicates that both PLA_2 sequences share substantial homology with sequences of some of the already described snake venom phopholipase A_2s. The similarity of N-terminal amino acid sequence with that of already sequenced snake venom PLA_2s reinforces that the purified proteins from the venom of *N. kaouthia* belong to PLA_2 family.

The optimum temperature range for exerting maximum catalytic activity of NK-PLA_2-I is 37-40 °C while that for NK-PLA_2-II is 37±1 °C. Heat-inactivation study shows that heating the PLA_2 enzymes at 100 °C for 20 min has hardly any effect on their catalytic activity or secondary structure, documenting that like many other venom PLA_2s, *N. kaouthia* PLA_2s are highly thermostable in nature (Vishwanath et al., 1988; Francis et al., 1995). The CD spectra gives further evidence of its thermostability. The CD spectra of NK-PLA_2-I and NK-PLA_2-II are quite similar. The far-UV CD spectrum of native NK-PLA_2-I demonstrated defined minima at 210 and 222 nm, while NK-PLA_2-II displayed almost identical spectra having a minima at 210 nm and 222.5 nm, indicating a strong α–helical contribution to the CD signal. α–

helix is the major secondary structure element for a large number of PLA_2s from snake venoms (Ali et al., 1999; Dufton et al., 1983). NK-PLA_2-I and NK-PLA_2-II were both highly thermostable and did not begin to lose their secondary structure until heating at 100 °C for 45 min, which is in close agreement with the earlier reports (Dufton et al., 1983; Vishwanath et al., 1988; Francis et al., 1995; Soares et al., 2001b). The thermostability is due to compact folding of the enzyme, which is based on core structure of the disulfide bridges (Dufton et al., 1983; Dufton and Hider, 1983).

It has been reported that a single histidine residue, present at position 48 is conserved among cobra venom PLA_2 enzymes and plays a significant role in phospholipid hydrolysis (Kini, 1997; Ali et al., 1999; Fuly et al., 2000; Gao et al., 2001). However, exception is noticed for a dimer toxic PLA_2 from venom of *Vipera ammodytes meridionalis*, where His-48 residue is replaced by Gln-48 (Komori et al., 1996; Perbandt et al., 1997). Treatment of *N. kaouthia* PLA_2 enzymes with ρ-BPB results in a dramatic loss of enzymatic activity, presumably due to alkylation of active site histidine residue. Although ρ-BPB bears little resemblance to a phospholipid molecule, but the hydrophobic tail of this reagent could mimic a fatty acid chain which helps this inhibitor to bind at the active-site histidine residue of PLA_2s and preventing the binding of physiological substrate at the active site of the enzyme (Roberts et al., 1977). A significant protection against this activation by Triton X-100 (30 mM) and Ca^{2+} (10 mM) might be evidenced for either a surface dilution effect of reagents or by sequestering ρ-BPB into the apolar micellar core and thus lowering the reagent concentration available for reaction with enzyme (Roberts et al., 1977). Although Roberts et al. (1977) reported the almost complete inhibition of PLA_2 enzyme from *N. n. naja* venom by α-bromo-2-acetophenone and α-chloroacetophenone, but the catalytic activity of NK-PLA_2-I and NK-PLA_2-II were least affected by these reagents suggesting absence of a hydrophobic site on the *N kaouthia* PLA_2 enzymes. Further, phospholipid hydrolytic activity of the purified enzymes are not inhibited by serine-protease inhibitors like PMSF, TLCK and

TPCK, indicating absence of active serine group in the catalysis process (Roberts et al., 1977). EDTA on the other hand, inhibits the phospholipid hydrolysis by chelating the metal ions required for enzymatic activity (Kini, 1997). But inhibitory effect of DTT on PLA_2 activity of either enzymes might be due to reduction of intramolecular disulfide bridges required for maintaining the three dimensional structure of the active enzyme.

Although NK-PLA_2-I and NK-PLA_2-II have shown significant phospholipid hydrolysis activity, but in contrast to crude venom, they do not display lethality in rodents, a fact which is in close agreement with many of the previous reports describing non-toxic but catalytically active PLA_2 molecules from snake venom (Bouchier et al., 1991; Yang et al., 1991). In general, basic PLA_2 are more toxic as compared to acidic or neutral PLA_2 and have a significant contribution in the overall toxicity, membrane hydrolysis and edema-inducing activity of the venom (Bhat et al., 1991; Mukherjee et al., 1998a,b; Mukherjee and Maity, 1998). Therefore, presence of higher amount of basic PLA_2, including other toxic enzymes, low molecular weight membrane active polypeptides as well as non-enzymatic toxins in crude venom may be responsible for its higher lethality as compared to NK-PLA_2-I or NK-PLA_2-II (Mukherjee and Maity, 2002).

Anticoagulant PLA_2 were isolated from elapidae and other snake venoms (Evans et al., 1980; Angulo et al., 1997). It may be reasonable to assume that hydrolysis of essential phospholipids of coagulation complex by PLA_2 inhibits the coagulation process (Evans and Kini, 1997). However, there is enough controversy concerning the role of enzymatic activity in the anticoagulant and other pharmacological effects of PLA_2 (Kini and Evans, 1988, 1998; Chwetzott, 1989; Gao et al., 2001). It has been suggested that both enzymatic and non-enzymatic process have contributed in the pharmacological process (Kini and Evans, 1988; Kini, 1997). Present study documents the significant role of catalytic activity of *N.*

kaouthia PLA_2s on the anticoagulant effect. Our hypothesis can be supported by the following observations:-

1. The anticoagulant activity was enhanced either with the increase in the amount of PLA_2s or with an increase in the pre-incubation time of PLA_2 enzymes with the platelet poor plasma supporting that anticoagulant potency is parallel with the catalytic activity of PLA_2 enzymes.

2. Further, parallel inhibition of anticoagulant activity along with catalytic activity of either enzyme by ρ-BPB clearly indicates that the catalytic activity of *N. kaouthia* PLA_2s has a role to play in the anticoagulant process.

3. However, for unexplained reason, EDTA not only failed to inhibit, but apparently enhanced the anticoagulant potency of NK-PLA_2-I. This can be correlated with the effect of EDTA on the anticoagulant activity of *Vipera berus* PLA_2 (Boffa et al., 1972). The catalytic activity of NK-PLA_2-I being more active than NK-PLA_2-II, therefore the anticoagulant potency of former is higher under identical condition.

Majority of the PLA_2 enzymes are reported to be devoid of direct hemolytic activity (Jiang et al., 1989a, 1989b; Fletcher et al., 1991; Mukherjee and Maity, 1998, 2002). In contrast to the crude venom, neither PLA_2 demonstrated significant hemolytic activity on washed human erythrocytes; NK-PLA_2-I could induce only 0.23% hemolysis whereas NK-PLA_2-II fails to induce direct hemolysis. It seems that large number of low molecular weight membrane active polypeptides of crude venom along with PLA_2 may be responsible for hydrolysis of erythrocyte phospholipids and subsequent hemolysis (Harvey et al., 1982; Jiang et al., 1989a; Fletcher et al., 1991; Mukherjee and Maity, 1998, 2002). However, significant hemolytic activity shown by either PLA_2 in the presence of exogenously added PC and 1.5 mM Ca^{2+} is due to hydrolysis of PC resulting formation of phospholipid hydrolysis products, like lysophospholipids and free fatty acids which are lytic by

themselves (Condrea et al., 1964). Since NK-PLA_2-I hydrolyze PC to a much higher extent than NK-PLA_2-II, therefore, it may be assumed that the indirect hemolytic activity of the former PLA_2 is higher as compared to the latterPLA_2. Since treatment of either enzyme with ρBPB, polyvalent antivenom and heat-inactivation have the same affect on their catalytic as well as indirect hemolytic activities, therefore, it is suggested that indirect hemolytic activity of *N. kaouthia* PLA_2s are dependent on catalytic activity (Kini and Evans, 1988). It is to be noted here that for this reason indirect hemolytic activity is also used as a semiquantitative method of PLA_2 assay.

Unlike specific PLA_2 enzymes, non-specific enzymes lack specific pharmacological sites and therefore fail to bind specifically to the target site or target cell. However, when incubated with organ or tissue in vitro condition, these nonspecific enzymes could induce pharmacological effects similar to that of the specific PLA_2 enzymes (Kini and Evans, 1989). Present study indicates that *in-vitro* condition, tissue damaging property of NK-PLA_2-I and NK-PLA_2-II is specific in nature because, nanomolar concentration of the either enzyme used in the current investigation completely eliminated the possibility of non-specific binding to the target tissue membrane and subsequent hydrolysis of membrane phospholipids at high enzyme concentration. However, it is difficult to distinguish between *N. kaouthia* PLA_2 induced release of hemoglobin from the tissues due to haemolysis and haemolysis followed by RBC release. But from the present data, it seems that *in-vitro* tissue damaging activity of NK-PLA_2-I and NK-PLA_2-II are independent of their haemolytic property because these two pharmacological properties are inhibited to a different extent by inhibitors and antivenoms under the identical test conditions. But rupturing of blood capillaries of tissues was necessary for the release of haemoglobin and it may be contributed by phospholipid hydrolysis property of PLA_2 enzymes (Datta and Bhattacharyya, 1999). Although, NK-PLA_2-I and NK-PLA_2-II even at a concentration of 25 μg/ml failed to show any proteolytic activity towards conventional substrates like casein, BSA, plasma albumin, globulin and fibrinogen, but it might require to investigate some other natural substrates of

$NK-PLA_2-I$ and $NK-PLA_2-II$ for protease activity because, *in-vitro* release of haemoglobin from tissues was partially inhibited by serine and chymotrypsin like serine protease inhibitors e.g PMSF and TPCK respectively. ρBPB also could inhibit the *in-vitro* tissue damaging activity partially suggesting that the catalytic activity may be partially responsible.

Although the total phospholipid compositions of tissues like heart, lung and liver are similar (Fletcher et al., 1981), however, percent hydrolysis of heart tissue by either PLA_2 enzyme is significantly higher as compared to other tissues. This observation is well consistent with our previous reports that phospholipid constituents of microsomal membranes are less hydrolyzed as compared to lysosomal membranes by the action of *Viperal russelli* basic PLA_2 (Mukherjee et al., 1997; Mukherjee et al., 1998a,b). The reason for the organ/subcellular organelle preference of the purified PLA_2s is not clearly understood (Warrell, 1989). It has been suggested that PLA_2 may be particularly active at domain interfaces that are sites of structural defects and hence good point of attack by PLA_2 enzyme (Jorgensen et al., 2002). Further, it may be assumed that differences in the biochemical nature of the vascular wall in different organs/tissues like phospholipid/cholesterol ratio, presence of specific phospholipids, vitamin E content of that membranes etc, may have some relevance to differential membrane hydrolysis (Simionescu, 1983; Kini, 1997; Mukherjee et al., 1997, 1998). Moreover, membrane specificity of PLA_2 enzymes may also be explained on the basis of mutational theory, which states that mutation in the surface residues could alter the function of the protein as the surface residues play a critical role in protein-protein interaction with receptor/acceptor proteins present in the membrane (Kini and Chan, 1999).

Edema inducing activity of purified PLA_2 enzyme was first reported by Vishwanath et al. (1985) and till date, several snake venom PLA_2 enzymes are reported to induce edema in experimental animals. Edema induced by $NK-PLA_2-I$ and $NK-PLA_2-II$ was rapid and a saturation level was reached after 45 min followed

by a gradual diminishing of edematous condition, which is in contrast to the edema induced by *B. schlehelii* myotoxin I, that lasted for 6 h (Angulo et al., 1997). However, the minimum edema-inducing dose of either *N. kaouthia* PLA_2 enzyme (MED 2.21 μg and 4.14 μg for NK-PLA_2-I and NK-PLA_2-II respectively) was quite similar to those of acidic PLA_2s from Indian cobra *N .n. naja* venom (MED in the range of 2.5 to 2.7μg) (Rudrammaji and Gowda, 1998), but significantly higher than that of Daboiatoxin (DbTx) from venom of *Daboia russelli siamensis* (0.05 μg) (Maung-Muang-Thwin et al., 1995). The edema induced by PLA_2 may be due to processes which promotes cell membrane dysfunction by- (i) loss of membrane phospholipid and/or (ii) the generation of phospholipid break down products such lysophospholipid and free fatty acids including arachidonic acid (Vishwanath et al., 1988). In the secondary phase, transformation of the released arachidonic acid into prostagladins and leukoterines leads to increase in vascular permeability and subsequent edema formation (Ali et al., 1999). The role of catalytic activity of PLA_2 enzyme in inducing edema is still controversial. Basavarajappa et al. (1993) reported the presence of separate edema-inducing site on the PLA_2 molecule distinct from the catalytic site. For example, *B. asper* myotoxin II possesses low PLA_2 activity but induces severe edema in the footpad of mice (Angulo et al., 1997). In a sharp contrast to these findings, various other researchers, for example Vishwanath et al. (1987), Bhat et al. (1991), Fuly et al. (2002) have demonstrated a direct relationship between the catalytic activity and edema-inducing property of snake venom PLA_2s. Our current study supports the latter hypothesis because alklyation of active site histidine residue or treatment of either *N. kaouthia* PLA_2 enzyme with polyvalent antivenom has equal inhibitory effect on the catalytic activity as well as edema inducing potency of the enzymes.

NK-PLA_2-I and NK-PLA_2-II hydrolyze the mitochondrial membrane phospholipids PC–pools in a dose dependent and highly characteristics manner. The reaction reached a steady level after 45 min, and the extent of phospholipid splitting by NK-PLA_2-I after 60 min was about 6 fold more than that induced by NK-PLA_2-II under

the identical condition. The presence of mM amount of Ca^{2+} in the reaction mixture did not enhance the PLA_2-induced membrane hydrolysis to a much higher extent, indicating that presence of Ca^{2+} is not an absolute requirement for the *N. kaouthia* PLA_2 induced mitochondrial membrane phospholipid hyrolysis reactions. It has been reported that lysis of artificial membranes by PLA_2s from various snake venom e.g. *Bothrops pirajai*, *Crotalus durissus terrificus* have shown to be independent of their enzymatic activity, since both native as well as catalytically inactivated enzymes are fully able to disrupt the membranes (Soares et al., 2001a; 2001b). In contrary to this observation, present study provide strong evidence that membrane hydrolyzing properties of *N. kaouthia* PLA_2s are dependent on their catalytic activity and there exists distinct catalytic and membrane binding sites in PLA_2s. The following observations support our hypothesis-

1. With an increase in the incubation time of membranes with PLA_2s, degree of phospholipid hydrolysis increases concomitantly; documenting membrane hydrolysis is dependent on the catalytic activity of the enzyme.

2 Chemical modification of histidine residue, which is most conserved amino acid among all PLA_2s, results significant inhibition of catalytic as well as membrane hydrolyzing activities without interfering the affinity of the modified PLA_2s towards the membranes. In this respect, *N. kaouthia* PLA_2s differ from *N. nigricollis* and *Vipera berus* PLA_2s, because the *N. nigricollis* PLA_2, inactivated at the histidine residue has a lower affinity for the membrane phospholipids compared to native enzyme and that *V. berus* PLA_2 has a higher affinity for phospholipids (Dachary et al., 1980).

3 Modification of serine or cysteine residue results drastic reduction of membrane binding as well as phospholipid hydrolysis action, but does not interfere catalytic activity of either PLA_2. $sPLA_2$ have a common interfacial-binding surface that is located on the flat external surface surrounding the

active site slot. It has already been shown that presence of Trp on the putative interfacial binding surface of human Group V and *N. n. naja* PLA_2s plays an important role in the binding of PLA_2s to PC vesicles and the outer plasma membrane (Han et al., 1999; Demel et al., 1975). Present study document that serine and cysteine also forms a part of putative interfacial binding surface in *N. kaouthia* PLA_2 molecules and hence modification of these two residues might result significant loss of affinity as well as penetrability of the PLA_2 into the phospholipid bilayer of the membranes.

4 Heating PLA_2s at 100 °C for different time periods or incubation with anti-NK-PLA_2-I IgG results differential inhibition of their catalytic and membrane-hydrolyzing properties reinforcing catalytic site is separated from the membrane-binding region in the *N. kaouthia* PLA_2s.

However, one may argue that chemical modification of PLA_2s or their interaction with antibodies may induce structural changes in the PLA_2 molecule, resulting in different properties of modified enzymes. CD spectrum and electrophoretic analyses of native and chemically modified enzymes and recognition of modified PLA_2s by anti-NK-PLA_2-I antibodies eliminate the chances of structural changes as a consequence of these treatments (Doley et al, 2004). Further advance studies are required for a deeper understanding of the relationships between the structural and dynamical properties of different membrane phospholipids and mechanism of activation of phospholipase A_2 at the interfaces for their application in liposome based drug delivery.

There are several reports on snake venom PLA_2 enzymes possessing antibacterial activity against Gram positive and Gram negative bacteria (Diaz et al., 1991; Paramo et al., 1998; Lomonte et al., 1999; Soares et al., 2001b). In case of human $sPLA_2$, the presence of cationic charge allows the enzyme to penetrate the anionic cell wall of Gram positive bacteria (Beers et al., 2002). Identically,

antibacterial peptides isolated from snake venom contain cationic site(s), flanked by hydrophobic residues, which might disturb the phospholipid bilayer integrity, initiating a rapid cell death process (Kini and Evans, 1989; Calderon and Lomonte, 1989). Further, it was observed that the membrane damaging activity in case of Myotoxin II, isolated from *Bothrops asper* snake venom, depends on its amphiphilic character (Lomonte et al., 1994). It has been suggested that bactericidal activity of PLA_2 are dependent on their interaction with essential membrane components common to both eukaryotes and prokaryotes; for example, in case of Gram-negative bacteria, these membrane components are identified as lipopolysaccharide moieties (Paramo et al., 1998). In the present study, none of the PLA_2 up to a dose of 10 μg/ ml shows bactericidal activity against Gram positive or Gram negative bacteria. Since NK-PLA_2-II was base eluted from the cation exchanger, therefore it might be assumed that this pLA_2 molecule bears positive charge on its surface and should show antibacterial property. However, it might be inferred from our observation that not the overall positive charge but the specific distribution of the positive charge in the phospholipase A_2 molecules may be responsible for their penetrability in the bacterial cell wall (Kini, 1997). Moreover, further studies are essential to decipher the antibacterial properties of PLA_2 molecules.

Acute skeletal muscle damage is a frequent manifestation in envenomations induced by snakes of the families Elapidae and Viperidae (White, 1995; Warrell, 1996). Histological and ultrastructural studies of the effect of venom PLA_2s on skeletal muscle reveal a common series of pathological alterations which include: (1) plasma membrane disruption, (2) formation of 'delta-lesions', wedge shaped areas of degeneration located at the periphery of muscle fibers, (3) hypercontraction of myofilaments, (4) mitochondrial swelling together with formation of flocculent densities and rupture of mitochondrial membranes, (5) disruption of intracellular membrane systems, i.e. sarcoplasmic reticulum and T tubules, and (6) pycnosis of nuclei (Harris and Maltin, 1982; Gutierrez et al., 1984a; Harris and Cullen, 1990;

Mebs and Ownby, 1990). Acute muscle degenerative events are followed by an acute inflammatory reaction associated with pain, edema and recruitment of polymorphonuclear leucocytes and macrophages (Harris and Maltin, 1982; Gutierrez et al., 1984a, 1990; Chacur et al., 2003). Finally, a regenerative process ensues, with activation of myogenic satellite cells and fusion of myoblasts to form myotubes within the space delimited by the remaining basal lamina of necrotic muscle cells (Gutierrez et al., 1984b; Gutierrez and Ownby, 2003). The present study reveals that both NK-PLA_2-I and NK-PLA_2-II induce degeneration of the muscle cells when injected to the thigh muscle of mice. The rise in the activity of intracellular enzymes in the serum is a clear indication of cell damage, that results in leakage of cytosolic and membrane bound enzymes of the organ in circulation (Mukherjee and Maity, 1998; Sahu et al., 1991). Thus the myotoxicity of *N. kaouthia* PLA_2s may be related to their membrane damaging activity of muscle tissues (Fletcher and Jiang, 1993; Fletcher et al., 1991).

Comparison of the charge density distribution has demonstrated that presence of hydrophobic and cationic regions in PLA_2 molecules are responsible for displaying their myotoxicity (Kini and Iwanaga, 1986). It has been seen in case of myotoxic K49 PLA_2 that the cationic regions interacts with negatively charged lipids, forming a cytolytic motif (Lomonte et al., 1994; Gutierrez and Lomonte, 1997) which penetrates and disorganizes membranes (Murakami and Arni, 2004). Further, it has been observed that myotoxic PLA_2s from crotaline snake venoms and crotoxin isolated from *Crotalus durissus terrificus* have a set of Try residue located in the C-terminal region of molecule. This Try may contribute to the hydrophobic-cationic combination proposed to play a role in myotoxicity (Francis, et al 1991; Soares, et al., 2001a). A multidomain membrane protein of 180 kDa, named M-type receptor, having a tandem repeat of eight domains homologous to C-type lectin carbohydrate recognition regions, was characterized in the plasma membrane of myocytes and other cell types that binds to PLA_2 enzyme (Lambeau et al., 1990). On the other hand, the demonstration of the existence of lipid domains and rafts within plasma

membranes suggest that regions enriched in particular glycerophospholipids or glycolipids may function as acceptors of PLA_2s (Gutierrez and Ownby, 2003). However, further studies are essential to explore the role of cationic site or involvement of specific amino acid residue(s) of *N. kaouthia* PLA_2 in inducing myotoxicity.

Formation of single precipitin band between anti-NK-PLA_2-I antibodies raised in rabbits and NK-PLA_2-I clearly indicates the polyvalent nature of the antibodies (Basavarajappa et al., 1993). Further, western blotting experiment demonstrated that anti-NK-PLA_2-I antibodies strongly cross-reacted with both NK-PLA_2-I as well as NK-PLA_2-II, documenting these enzymes are immunogenic in nature and are closely related (Calderon, et al., 1993). Anti-NK-PLA_2-I IgG at 1:100 (w/w) antigen:antibody ratio, could neutralize the catalytic activity of NK-PLA_2-I and NK-PLA_2-II up to 68.34% and 48.34% respectively of their original catalytic activity. The differential inhibition of two enzymes may be explained by the fact that the antibodies were raised against the NK-PLA_2-I enzyme; therefore, the extent of inhibition of catalytic activity and other pharmacological properties of NK-PLA_2-I enzyme by anti-NK-PLA_2-I antibodies was higher as compared to the inhibition against NK-PLA_2-II enzyme. The separation of the catalytic site from the pharmacological sites in snake venom PLA_2 molecules using polyclonal/monoclonal antibodies and plant alkaloids have been reported (Jayanthi et al., 1989; Kasturi and Gowda, 1991; Bhat et al., 1991). Poor inhibition of catalytic activity as compared to pharmacological properties of either enzyme by anti-NK-PLA_2-I IgG suggests that *N. kaouthia* PLA_2 enzymes possess distinct, perhaps overlapping sites for catalytic activity and pharmacological effects and this hypothesis is in close agreement with those proposed by Rosenberg, (1986) and Kini and Iwanaga (1986). Differential inhibition of catalytic activity and pharmacological effects of PLA_2s by anti-PLA_2 antibodies might be due to the fact that the pharmacological site of NK-PLA_2-I and NK-PLA_2-II is a poor epitope for antibody production and recognition. Furthermore, heat-inactivation study in the current investigation

reinforces our hypothesis because, nearly half of the original catalytic activity of either enzyme was lost when heated for 45 min at 100 °C, but the tested pharmacological properties of the enzymes were completely abolished under the identical heating condition.

Snake venom is a collection of many enzymes (Stocker, 1990; Tsai et al., 1996; Mukherjee and Maity, 1998). Some of which are very toxic in nature (Mukherjee and Maity, 1998, Mukherjee, 1998) and responsible for the most pharmacological effects of the envenomation. Neutralization of these enzyme activities by the plant extracts reflects the presence of anti-snake venom compounds in those plants. Isolation and purification of the active compound(s) responsible for inhibition of enzyme activities might be useful, because they may have a potential application in future as therapeutic agents against snake envenomation. Further, since majority of these natural compounds are stable at room temperature (Finar, 1989), therefore they can be stored even at room temperature and can easily be supplied to rural areas, where maximum snakebites take place. It should be pointed out that antivenom vials (currently available for snakebite therapy) have to be stored under refrigerated condition (at 4 ^{0}C to 8 ^{0}C). Since majority of rural hospitals or health centers in India or other developing countries lack proper refrigerated storage condition (personal observation), therefore antivenom vials are not kept in these centres. As a result of which, in the rural tropics, it is often late when snakebite patients arrive at district (town) hospitals for treatment hours after bite travelling long exhaustive journey; and late antivenom therapy may not be useful to save the life of the patients (Mukherjee, 1998; Mukherjee et al., 2000).

In the present investigation, out of the 13 medicinal plants tested for anti-PLA_2 activity, only the leave extract of *Azadiracta indica* was found to possess anti-PLA_2 activity. The methanol extract of the leaves could neutralize the catalytic activity of crude venom as well as the purified enzymes significantly as compared to water or chloroform extracts. *Azadiracta indica* has been extensively used for anti-

inflammatory, antipyretic, analgesic, immunostimulant, diuretic, hypoglycaemic, cardiovascular, antimicrobial, antiviral, antimalarial and anthelmintic (Dhawan and Patnaik, 1993; Sivarajan and Balachandran, 1994). Many constituents from this plant, for example, diterpenoids, triterpenoids, polyphenolics, sulphurous compounds, and polyacetate derivatives are responsible for showing many of these medicinal properties of *A. indica* (Dev Kumar and Sukh Dev, 1993). Several plant constituents like flavonoids, quinonoid, xanthene, polyphenols and terpenoids posses protein binding and enzyme inhibiting properties (Havsteen, 1983; Selvanayagam et al., 1996). Which also inhibit snake venom phospholipase A_2 activities of both viper and cobra venom (Alcaraz and Hoult, 1985). Although *A. indica* has been used as antidote (folk medicine) to snakebite in the rural tropics of Indian subcontinent, but till date, neither the plant was scientifically screened to explore the antivenom property nor a single antivenom compound has been isolated and characterized from this plant. Result of the present investigation documents that methanol soluble active compounds like triterpenoids and others, present in the leave of *A. indica*, may be responsible for neutralization of PLA_2 activity (Siddiqui et al., 2001; Alam and Gomes, 2003). Interestingly, all the tested plant extracts except *A. indica,* used as folk medicine to treat snakebite patients, failed to show inhibitory activity against the *N. kaouthia* PLA_2 enzymes. However, their role in inhibiting the toxic effects of other components of snake venom cannot be ruled out. It should be mentioned here that folk medicines are administered in the form of mixture, comprising of different plant constituents, which may act synergistically in neutralizing the toxic effects of snake venom (A.K. Mukherjee, personal communication). Therefore, whatever every plant used by local people as antidote against snakebite, must be examined systematically, carefully and critically to evaluate their actual antivenom potency. This will further assist us in overcoming the limitations of currently available polyvalent antivenom therapy, particularly in the developing countries.

Conclusion

In the present investigation, PLA_2 isoenzyme pattern of two Indian cobras, *N. naja* and *N. kaouthia* venom samples of eastern India origin were compared and two major phosppholipase A_2 isoenzymes were purified to homogeneity from the venom of Indian monocled cobra (*Naja kaouthia*). Further, some of the Indian medicinal plants were screened for anti-PLA_2 activity.

Comparison of the PLA_2 isoenzymes of *Naja kaouthia* and *Naja naja* venom samples reveals that 10 PLA_2 isoenzymes are present in *Naja naja* whereas 9 PLA_2 isoenzymes are present in *Naja kaouthia* venom. Therefore, these two venom samples differ with respect to qualitative and quantitative distribution of PLA_2 isoenzymes.

The two major phospholipase A_2 isoenzymes viz. NK-PLA_2-I and NK-PLA_2-II from *Naja kaouthia* venom were purified to homogeneity by combination of ion-exchange, gel filtration and RP-HPLC. NK-PLA_2-I had a molecular weight of 13.6 kDa and 19.26 kDa under reduced and non-reduced conditions whereas NK-PLA_2-II displayed sharp band of 13.1 kDa under both reduced and non-reduced conditions in SDS-PAGE indicating NK-PLA_2-I is dimmer and NK-PLA_2-II is monomer in nature. The molecular mass of NK-PLA_2-I and NK-PLA_2-II was further confirmed by MALDI-MS, which revealed a protonated molecular ion [MH^+] at m/z 13,786.205 and 13,346.19 Da which is similar to the mass determined by SDS-PAGE respectively. NH_2–terminal amino acid sequence (determined up to 10 amino acid residue) of both the *N. kaouthia* PLA_2s are identical (NIYQFKNNIQ) and share 90% homology with that of already characterized snake venom PLA_2s. The CD spectra of NK-PLA_2-I and NK-PLA_2-II showed strong α–helical structure, which is well consistent with other snake venom PLA_2 enzymes. Further, heat inactivation study

as well CD spectra of both the enzymes revealed that they are thermostable in nature.

Both the enzymes, at a dose of 10 mg/kg body weight of mice did not display any sign of lethality. Pharmacological characterization reveals that none of PLA_2s shows any direct hemolytic activity on washed human or goat erythrocyte unless phospholipid hydrolysis products were present in the incubation medium but both the PLA_2s displayed strong anticoagulant, myotoxicity, edema-induction, damage of liver mitochondria and tissues of heart, liver and lung in *in-vitro* condition. although NK-PLA_2-I is more potent than NK-PLA_2-II in inducing mitochondrial swelling, but both these PLA_2s have displayed remarkable similarity in inducing a prompt and marked swelling of mitochondria without any lag phase. Further, it is observed that NK-PLA_2-I and NK-PLA_2-II hydrolyze the mitochondrial membrane phospholipids PC–pools in a dose dependent and highly characteristic manner. NK-PLA_2-I is more potent in exhibiting anticoagulant, indirect hemolytic and *in-vitro* liver tissue damaging activity as compared to NK-PLA_2-II, where as reverse is true for induction of edema, myotoxicity and indirect hemolytic activity. The tissue damaging activities of either enzyme is specific and heart tissue damaging activity of NK-PLA_2-I is detected as more as compared to NK-PLA_2-II.

Chemical modification study reveals that like other snake venom PLA_2 enzymes, histidine residue is also present in the active site of both the enzymes. Pharmacological activities, of *N. kaouthia* PLA_2s, e.g. like anticoagulant, edema and indirect hemolytic activities were significantly neutralized by treatment with ρBPB, indicating that catalytic activity plays a significant role in inducing these effects. However *in-vitro* tissue damaging activity was partially inhibited by ρBPB, reinforcing that PLA_2 enzymes have a separate pharmacological site for displaying tissue damaging activity other than the catalytic site. Furthermore, heat-inactivation study in the current investigation reinforces our hypothesis because, nearly half of the original catalytic activity of either enzyme was lost when heated for 45 min at

100 °C, but the tested pharmacological properties of the enzymes were completely abolished under the identical heating condition.

Immunological study reveals that both the enzymes are immunogenic in nature and are closely related. Antibodies were raised against NK-PLA_2-I in rabbit, but the catalytic activity and pharmacological effects of PLA_2s were inhibited in differential manner by anti-PLA_2 antibodies suggesting that the pharmacological site of NK-PLA_2-I and NK-PLA_2-II is a poor epitope for antibody production and recognition.

In the present study, medicinal plants used by the tribal people of NorthEast were scientifically screened for the anti-snake venom compound(s). Interestingly, all the tested plant extracts except *A. indica,* used as folk medicine to treat snakebite patients, failed to show inhibitory activity against the *N. kaouthia* PLA_2 enzymes. However, their role in inhibiting the toxic effects of other components of snake venom cannot be ruled out because folk medicine are administered in mixture of different plants. Therefore, each and every plant used as antidote must be examined critically which will help to over come the limitation of antivenom therapy.

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LIST OF PUBLICATIONS

1. Research papers

a. **Doley, R.** and Mukherjee, A.K. (2001). Pharmacological reassessment of medicinal plants to ascertain their anti-snake venom activity. In: **Ethnomedicine of North East India: Protection, Utilization and Conservation.** Singh, G., Singh, H.B and Mukherjee, T.K (Eds.) **National Institute of Science Communication and Information resources. CSIR New Delhi** pp: 300-304.

b. **Doley, R.** and Mukherjee, A.K. (2003). Purification and characterization of an anticoagulant phospholipase A_2 from Indian monocled cobra (*Naja kaouthia*) venom. ***Toxicon*** 41, 81-91. **Elsevier Publication, UK.**

c. **Doley, R.**, King, G.F. and Mukherjee, A.K. (2004). Differential hydrolysis of erythrocyte and mitochondrial membrane phospholipids by two phospholipase A_2 isoenzymes (NK-PLA$_2$-I and NK-PLA$_2$-II), from Indian monocled cobra *Naja kaouthia* venom. ***Archives of Biochemistry and Biophysics***, (In press) **Elsevier Publication, UK.**

2. Paper / Poster presentation

a. **Doley, R.** and Mukherjee, A.K. (2002). Biochemical and biological characterization of phospholipase A_2 enzyme from Indian cobra venom. 2nd ***National symposium on venoms and toxins***. **November 15-16, 2002. Organized by Department of Physiology, Acharya Shri Chander College of Medical Sciences and Hospital, Sidhra, Jammu, India.**

b. **Doley, R.** and Mukherjee, A.K. (2004) Characterization of an anticoagulant phospholipase A_2 isoenzyme from Indian monocled cobra *Naja kaouthia* venom. 3rd ***National symposium on venoms and toxins***. **23-25 January 2004, Organized by Department of Biochemistry, University of Mysore, Mysore, Karnataka, India.**

c. **Doley, R** and Mukherjee, A.K. (2004). Scientific exploration of medicinal plants of North-East India as an antidote to snake envenomation: A novel approach to resource management. ***National Workshop on Regional Development: Case for North East India.*** **3-6 Feb. 2004. Organized by Tezpur University, IIT Guwahati and C-MMACS, Bangalore, India at IIT Guwahati, Assam, India.**

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Purification and characterization of an anticoagulant phospholipase A_2 from Indian monocled cobra (*Naja kaouthia*) venom

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Abstract

An anticoagulant, non-toxic phospholipase A_2 was isolated from the venom of Indian monocled cobra (*Naja kaouthia*) by a combination of ion-exchange chromatography on CM-Sephadex C-50 and gel filtration on Sephadex G-50 This purified protein named NK-PLA_2-I, had a subunit molecular mass of 13 6 kDa and migrated as a dimer under non-reduced condition in SDS-PAGE NK-PLA_2-I was a highly thermostable protein requiring basic pH optima for its catalytic activity and showed preferential hydrolysis of phosphotidylcholine This protein exhibited higher anticoagulant, indirect hemolysis, liver and heart tissue damaging activity but exerted less toxicity, direct hemolysis, edema and lung tissue damaging activity as compared to whole venom Treatment of NK-PLA_2-I with p-BPB, TPCK, PMSF, antivenom and heating had almost equal effect on PLA_2, and other pharmacological properties except in vitro tissue damaging activity Current investigation provides a fairly good indication that NK-PLA_2-I induces various pharmacological effects by mechanisms, which are either dependent or independent of its catalytic activity

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Keywords *Naja kaouthia*, Snake venom, Phospholipase A_2, Haemorrhagin, Anticoagulant, Hemolysis

1. Introduction

Phospholipase A_2 (PLA_2 EC 3 1 1 4), a major component of snake venom specifically catalyzes the hydrolysis of fatty acid ester bonds at position 2 of 1,2-diacyl-sn-3-phosphoglycerides in presence of calcium (Dennis, 1983) In addition to the digestion of prey, PLA_2 exhibit wide varieties of pharmacological effects such as neurotoxicity, cardiotoxicity, myotoxicity, necrotic, anticoagulant, hypotensive, hemolytic, haemorrhage and edema etc (for review see Kini, 1997) However, the structural–functional relationships of PLA_2 have not been well understood and there may be presence of separate, perhaps overlapping sites on the PLA_2 for catalytic and pharmacological properties (Kini and Evans, 1989, Gao et al , 2001) Phospholipase A_2 from different snake venoms differ widely in their spectrum of toxin actions, although they share a high degree of homology in the amino acid sequence and enzyme active sites (Heinrikson, 1991, Scott and Sigler, 1994, Tsai et al , 2001)

Phospholipase A_2 from various Elapidae venom have been purified and characterized In 1980, Joubert and Taljaard reported the purification and some properties of PLA_2 from *Naja kaouthia* venom from Thailand Recently we reported that venom from Indian monocled cobra (*N kaouthia*) is a rich source of phospholipase A_2 enzyme (Mukherjee and Maity, 2002) But till date not a single phospholipase A_2 enzyme from this venom from Indian origin is purified and characterized This is of great importance, because venom composition from the same

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Abbreviations K-phosphate, potassium phosphate, SDS PAGE sodium dodecyl sulphate polyacrylamide gel electrophoresis, PLA_2 phospholipase A_2, p-BPB p-bromophenacyl bromide, TLCK, Nα-p-tosyl-L-lysine chloromethyl ketone, TPCK α-tosyl-L-p phenylalanine chloromethyl ketone, PMSF, phenyl methyl sufonyl fluoride, PC, phosphotidylcholine, PE, phophotidyl ethanolamine, PS phosphotidyl serine

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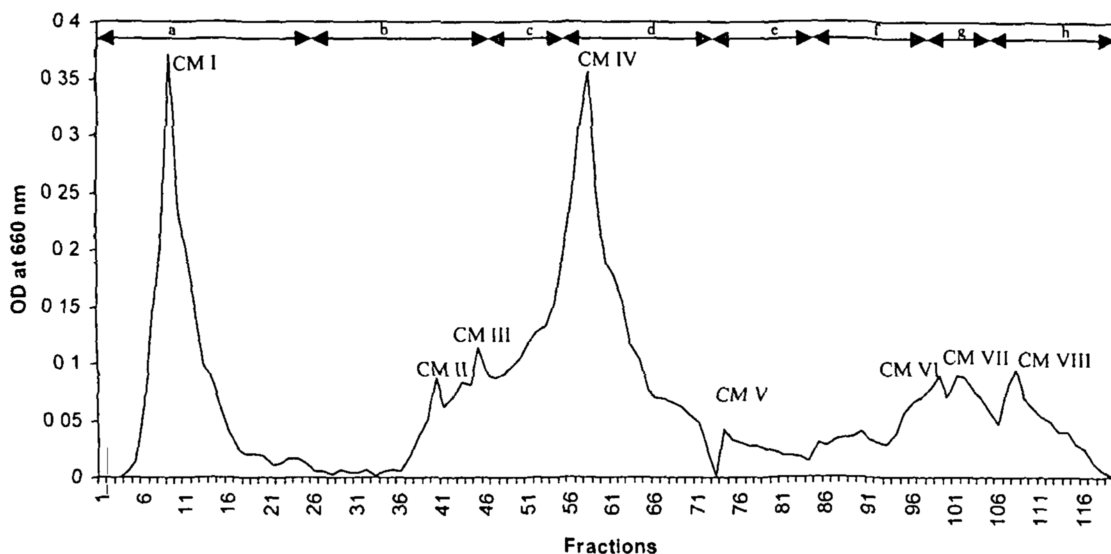

Fig 1 CM-Sephdex C-50 chromatography of *N kaouthia* venom The column ($20 \times 60\ mm^2$) was equilibrated with 20 mM K-phosphate buffer (pH 7 0) and loaded with 50 0 mg venom dissolved in 4 0 ml of the same buffer Elution was carried out stepwise with phosphate buffers of various molarities and pH values (a) 20 mM K-P pH 7 0, (b) 75 mM K-P pH 7 5, (c) 90 mM K P pH 8 0, (d) 100 mM K P pH 8 0, (e) 110 mM K-P pH 8 0 (f) 130 mM K-P pH 8 0, (g) 150 mM K-P pH 8 0 and (h) 180 mM K-P pH 8 0) at 23 °C Fraction volume was 1 ml and flow rate was 24 ml/h

species of cobra may vary depending on the geographical origin of the snakes (Mukherjee and Maity, 1998) The present study is the first report describing purification, biochemical and pharmacological characterization of an anticoagulant phospholipase A_2 from *N kaouthia* venom from India

2. Materials and methods

2 1 Materials

Venom of *N kaouthia* was obtained from Calcutta Snake Park, Kolkata Sephadex G-50 (fine grade) and CM-Sephadex C-50 were obtained from Pharmacia Fine Chemicals, Sweden All other reagents of analytical grade were purchased from Sigma (USA) Polyvalent antivenom was purchased from Serum Institute of India Ltd, Pune (Batch No #256) Albino mice of both sexes weighing 18-20 g were used

2 2 Isolation and purification of PLA_2

Crude *N kaouthia* venom (50 mg protein) was dissolved in 4 0 ml of 20 mM K-phosphate buffer, pH 7 0 and centrifuged (5000 rpm × 10 min) at 4 °C in a Sorvall RC 5B PLUS centrifuge The clear supernatant was applied to a CM-Sephadex C-50 ($20 \times 60\ mm^2$) column pre-equilibrated with 20 mM K-phosphate buffer, pH 7 0 and eluted stepwise using phosphate buffers of various molarities and pH values (as shown in Fig 1) at room temperature (~23 °C) The flow rate was adjusted to 24 ml/h and 1 ml fraction was collected in each test tube Peak CM-I was pooled, concentrated at −20 °C in a MAXI dry plus (Heto Lab Equipment, Denmark) and applied to a Sephadex G-50 gel filtration column ($1 \times 64\ cm^2$) previously equilibrated with the 20 mM K-phosphate buffer, pH 7 0 Elution was carried out with the equilibration buffer at room temperature (~23 °C) The flow rate was adjusted to 20 ml/h and 1 ml fraction was collected in each test tube Protein content of the individual fraction was estimated by the method of Lowry et al (1951) using bovine serum albumin as standard The peak GF-II showing anticoagulant activity was pooled, desalted through Sephadex G-25 column, lyophilized and stored at −18 °C temperature The purified PLA_2 was designated as NK-PLA_2-I

2 3 Homogeneity and molecular weight

The homogeneity of the preparation was checked by (a) 10% SDS-PAGE was carried out with or without reduction of protein with β-marcaptoethanol (Laemmli, 1970) About 30 μg of crude venom or purified protein was loaded into the gel containing 5% glycerol Before staining, proteins were fixed by incubating the gel in 20% TCA for 30 min followed by washing the gel several times with distilled water Protein bands were visualized by staining with 0 1% Coomassie Brilliant Blue R-250 and destained with

methanol/acetic acid/water (40 10 50) Mobility of coagulant protein was compared with the following molecular weight markers—phosphorylase b (97,400), bovine serum albumin (66,000), ovalbumin (43,000), carbonic anhydrase (29 000), soyabean trypsin inhibitor (20,100) and lysozyme (14,300) where a linear dependency of log M_w vs migration of protein bands were observed Gel was scanned in Gel Doc 1000 (Bio Rad) (b) Rechromatography of the coagulant protein on a $1\ 0 \times 90\ cm^2$ Sephadex G-50 column, pre-equilibrated with 20 mM K-phosphate buffer, pH 7 0 One milligram of the protein, dissolved in 0 5 ml of equilibration buffer was loaded into the column and eluted with the same buffer at room temperature (~23 °C) Flow rate was adjusted to 20 ml/h and 1 0 ml of fraction was collected in each test tube Elution of protein was monitored according to the method of Lowry et al (1951) Gel filtration column was equilibrated with the following molecular weight markers—aprotinin (6500), cytochrome c (12,400), carbonic anhydrase (29,0000), bovine serum albumin (66,000) and blue dextran (2,000,000) (c) Reverse phase high performance liquid chromatography (RP-HPLC) on Waters reverse phase C_{18}-μ-Nova pack column About 40 μg of NK-PLA_2-I was pre-incubated with 0 1% trifluro acetic acid (TFA) in water for 30 min at 4 °C and passed through Spartan 3 nylon filters before application to HPLC Protein was eluted with a gradient from 0 to 60% acetonitrile containing 0 1% TFA and detection was monitored at 214 nm

2 4 Phospholipase A_2 activity

Phospholipase A_2 activity of crude venom as well as purified fractions was determined by the method of Joubert and Taljaard (1980) One unit of PLA_2 activity was defined as the amount of protein, which produces a decrease of 0 01 absorbance in 10 min at 740 nm Hydrolysis of purified phospholipids viz PC, PS and PE by NK-PLA_2-I was assayed by the titrametric method using 0 1 N NaOH (Reynolds et al , 1991)

2 5 Protease assay

Digestion of casein or human plasma protein was evaluated colorimetrically (Ouyang and Teng, 1976) 1% (w/v) casein or 0 5% (w/v) plasma protein in 20 mM phosphate buffer, containing 150 mM NaCl, pH 8 0 was incubated with specified amount of venom protein for 1 h at 37 °C followed by addition of 0 5 ml of 10% TCA The digested protein in the supernatant was determined using Folin–Ciocalteau's reagent

2 6 Biochemical characterization

Total carbohydrate was estimated by phenol–sulfuric acid method (Dubois et al , 1956) Other enzymatic activity viz 5′-nucleotidase, ATPase and acetylcholinesterase were assayed as described earlier (Mukherjee and Maity, 2002)

2 7 Anticoagulant activity

Platelet poor plasma (PPP) from goat blood was prepared by centrifuging the citrated blood twice at 2500 g for 15 min at 4 °C and used within 4 h For the assay of recalcification time, specific amount of crude venom/NK-PLA_2-I (in a final volume of 30 μl) was added to 300 μl of PPP at 37 °C and the mixture was incubated for 2 min at 37 °C Then 40 μl of 250 mM $CaCl_2$ was added and the time of appearance of the first clot was recorded As a control, plasma aliquots were incubated with 30 μl of phosphate buffer saline (PBS), and the coagulation time was determined identically

2 8 Pathophysiological effects

Lethality (LD_{50}), neurotoxicity, direct and indirect hemolytic activity were determined by our previous described methods (Mukherjee and Maity, 2002) To investigate the hemolytic effects of NK-PLA_2-I on PC, PS and PE enriched human erythrocytes, procedure of Diaz et al (2001) was followed Edema-inducing activity was determined according to Angulo et al (1997) For the assay of in vitro tissue damaging activity, procedure of Datta and Bhattacharyya (1999) was followed with the following modifications Fresh chicken liver, heart and lungs were washed with 0 9% sodium chloride solution, cut into small uniform sized pieces, patted dry with tissue paper and 300 ± 10 mg pieces were weighed The tissues were pre-incubated with 1 ml of 0 2 M K-phosphate buffer, pH 7 4, for 45 min at 37 °C The tissues were then washed twice with the same buffer and incubated with 25 μg of either crude venom or purified protein in a final volume of 2 5 ml in 0 2 M K-phosphate buffer, pH 7 4 for 5 h at 37 °C After incubation, reaction mixtures were centrifuged for 5 min at 3000 rpm and the absorbance of the supernatant was read at 540 nm by an UV–VIS Spectrophotometer (Hitachi, Japan) The percentage of haemoglobin released was calculated with respect to tissues incubated with 0 1% Triton X-100 solution (100% activity)

2 9 Immunoblotting

Reactivity of the horse anti-*N naja* serum towards the NK-PLA_2-I was detected by immunoblotting experiment (Fig 5) NK-PLA_2-I was separated by 10% SDS-PAGE and transferred to 0 45 μm Immobilon-P (Millipore) membrane at 150 mA for 3 h in a Mini transblot cell (Pharmacia) using the buffer described by Towbin et al (1979) Proteins on the membrane were reversible stained with ponsceau S to check the transfer efficiency Strips were cut, blocked with fat free milk and incubated with anti-*N naja* sera Bound antibodies were detected with a horse reddish peroxidase rabbit anti-horse IgG conjugate, using tetramethyl benzidine/hydrogen peroxide (TMB/H_2O_2) as substrates

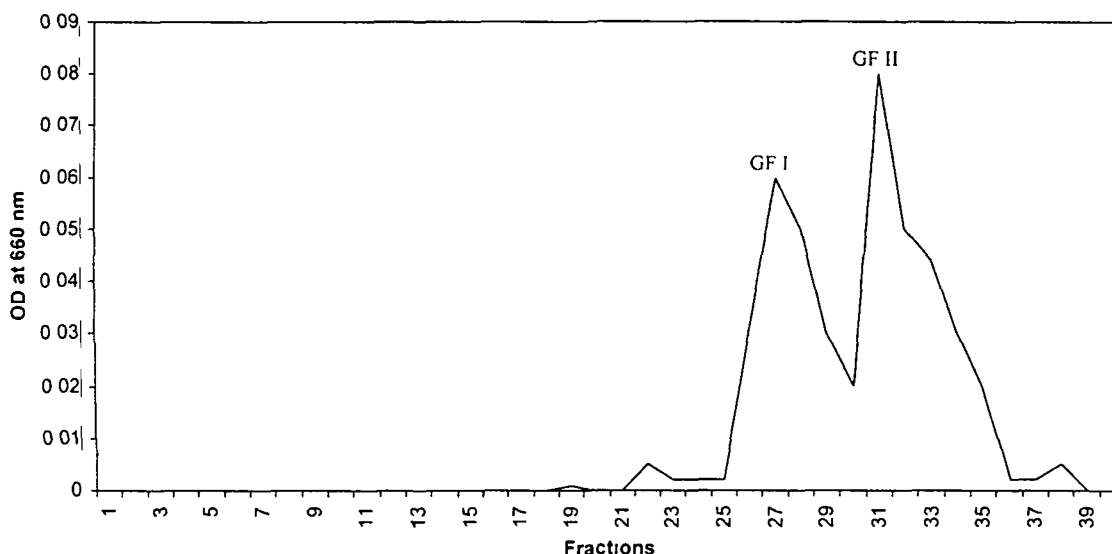

Fig 2 Gel filtration of NK-CMI on Sephadex G-50 ($1 \times 64\ cm^2$) column Fractionation was done as described in the text

2 10 Chemical modification of the purified protein

Chemical modification with p-BPB was performed by incubating protein (0 025–0 05 mg/ml) in 25 mM Tris, pH 8 0 with 3 3 mM inhibitor concentration (Roberts et al, 1977) After incubation for 24 h at 4 °C, the reaction mixture was desalted on Sephadex G-25 ($1 \times 30\ cm^2$) column to remove the excess of reagent Modified protein was assayed for the PLA_2 and other pharmacological properties To investigate the effect of other inhibitors, 100 μg of NK-PLA_2-I in 25 mM Tris, pH 8 0 was incubated with different inhibitors/antivenom at 37 °C for 30 min following assay of PLA_2 and other pharmacological activity

2 11 Heat inactivation study

NK-PLA_2-I protein (2 mg/ml) dissolved in 0 1 M Tris, pH 8 0 was heated in a boiling water bath for 10 min Immediately after heating, required volume was withdrawn at regular intervals for the assay of PLA_2 and pharmacological activity

3. Results

3 1 Isolation and purification of phospholipase A_2

When *N kaouthia* venom was fractionated in CM-Sephadex C-50 column, it was resolved into two major peaks and six minor peaks (Fig 1) One microgram from each fraction was tested for PLA_2 activity Peak CM-I showing PLA_2 activity was further subjected to gel filtration on Sephadex G-50, where it was separated into two fractions GF-I and GF-II (Fig 2) GF-II showed strong PLA_2 and anticoagulant activity Yield of this protein was 6% of the original venom load and henceforth termed as NK-PLA_2-I The summary of purification of NK-PLA_2-I is given in Table 1

Table 1
Summary of purification of PLA_2 enzyme and anticoagulant activity from *N kaouthia* venom Data are from a typical experiment

Fraction	Total protein	% yield of protein	PLA_2 activity		Anticoagulant activity	
			Total activity (unit)[a]	Specific activity (unit/mg)	Total activity (unit)[b]	Specific activity (unit/mg)
Whole venom	50 0	100 0	$418\ 5 \times 10^4$	$83\ 7 \times 10^3$	$1\ 0 \times 10^5$	$2\ 0 \times 10^3$
CM I	9 4	18 8	$876\ 1 \times 10^3$	$93\ 2 \times 10^3$	$37\ 6 \times 10^3$	$4\ 0 \times 10^3$
GF II	3 0	6 0	$568\ 2 \times 10^3$	$94\ 7 \times 10^3$	$39\ 0 \times 10^3$	$13\ 0 \times 10^3$

[a] One unit of PLA_2 activity is defined as decrease of 0 01 absorbance at 740 nm per 10 min

[b] One unit of anticoagulant activity is defined as 1 s increase in the clotting time of PPP as compared to control (without venom/fraction)

3 2 Homogeneity and molecular weight

NK-PLA_2-I was checked for homogeneity by gel filtration on Sephadex G-50, SDS-PAGE and RP-HPLC on a C_{18} column About 30 μg of NK-PLA_2-I gave a sharp, Coomassie Brilliant blue positive band on SDS-PAGE under both reduced and non-reduced conditions, but the whole venom displayed several sharp and diffused bands (Fig 3(a)) By Sephadex G-50 gel filtration chromatography, this protein was eluted as a symmetrical peak indicating the purity of the preparation (not shown) By analytical RP-HPLC, the purified toxin eluted as a single peak with a retention time of 14 min (Fig 3(b)) By SDS-PAGE, this protein appeared as a single band of 13 6 kDa after reduction with β-mercaptoethanol, but in absences of reducing agent and heating, it migrated as a single band of an apparent mass of 19 3 kDa The molecular mass estimated for NK-PLA_2-I was 12 9 kDa by gel filtration method

3 3 Biochemical characterization

Some of the biochemical properties of the purified NK-PLA_2-I are listed in Table 2 The optimum pH for the enzyme activity was 8 0–8 5 Heating this protein at 100 °C for 10 min at pH 8 0 had practically no effect on PLA_2 activity Carbohydrate content of this enzyme was 0 7% of the total protein NK-PLA_2-I hydrolyzed the purified phospholipids, viz PC, PS and PE in the decreasing order of preference NK-PLA_2-I up to 25 μg/ml had no detectable proteolytic, 5′-nucleotidase, ATPase and acetylcholinesterase activity

3 4 Anticoagulant activity

In vitro condition, NK-PLA_2-I induced dose dependent increase of Ca-clotting time of platelet poor goat plasma (Fig 4) Further, with an increase in the preincubation time of NK-PLA_2-I with plasma, clotting time was prolonged (data not shown)

3 5 Pathophysiological effects

A comparison of the different pathophysiological/pharmacological properties of the purified NK-PLA_2-I with that of the crude venom are shown in Table 3 In contrast to crude venom, NK-PLA_2-I was not lethal to albino rats and did not show any sign of neurotoxicity such as respiratory distress, paralysis of hind limbs, lacrination and profuse urination etc Purified PLA_2 did not exhibit significant direct hemolytic activity on washed human erythrocytes, but showed appreciable indirect hemolytic activity in presence of egg yolk phospholipids Erythrocytes pre-incubated with neutral phospholipids PC and 1 5 mM Ca^{2+} were highly susceptible to the lysis induced by NK-PLA_2-I, but pre-incubation with PS or PE did not increase hemolysis either in presences or absence of 1 5 mM Ca^{2+} (data not shown) NK-PLA_2-I had less edema inducing and lung tissue damaging activity as compared to crude venom But on the other hand, extent of liver and heart tissue damage by purified protein was higher than the whole venom

3 6 Effect of chemical modification on PLA_2 activity

Table 4 shows the effect of various reagents on the modulation of enzymatic activity of NK-PLA_2-I After the modification with p-BPB, NK-PLA_2-I retained 4 5% of its original PLA_2 activity But Triton X-100 and Triton plus Ca^{2+} significantly reversed this inactivation Triton X-100 at 1 mM concentration had little protective effect against inhibition while the higher concentration (30 mM) was more protective PLA_2 activity was not effected by serine reagents viz PMSF, TLCK and TPCK Treatment with α-bromo-2-acetophenone and α-chloroacetophenone at a final concentration of 2 mM inhibited 2 5 and 9% of the PLA_2 activity, respectively But treatment of NK-PLA_2-I with 2 mM of either EDTA or DTT result significant inhibition of PLA_2 activity Polyvalent antivenom at a 1 500 (mol/mol) ratio inhibited the PLA_2 activity of purified protein to 16%

3 7 Effect of chemical modification on pharmacological properties

At 2 mM concentration, p-BPB inhibited the direct and indirect hemolysis, edema and anticoagulant effect of NK-PLA_2-I within a range from 0–8 3%, but in vitro liver and heart tissue damaging activity was reduced to 31 ± 2 9% (mean ± SD) and 26 8 ± 3% (mean ± SD) of the original activity, respectively, (Table 5) TLCK was ineffective in modulating any of the tested pharmacological properties of the purified protein Although PMSF and TPCK significantly inhibited the in vitro tissue damaging activity, but failed to inhibit the other tested properties of NK-PLA_2-I Treatment with 2 mM of either EDTA or DTT resulted significant inhibition of all the tested pharmacological properties of the protein, expect anticoagulant activity, which was increased in presence of EDTA (Table 5) Polyvalent antivenom NK-PLA_2-I at 1 500 (mol/mol) ratio completely abolished the direct hemolysis and tissue damaging activity of NK-PLA_2-I, but other activities were neutralized to a much less extent

3 8 Effect of heating

Heating the NK-PLA_2-I solution at 100 °C for 10 min at pH 8 0 did not influence the catalytic and pharmacological properties except release of hemoglobin from incubated tissues There was about 16% reduction of NK-PLA_2-I induced release of hemoglobin from the tissues after heating the protein, but after cooling it for 20 min at 25 °C, it regained almost 90% of its original tissue damaging activity (Table 5)

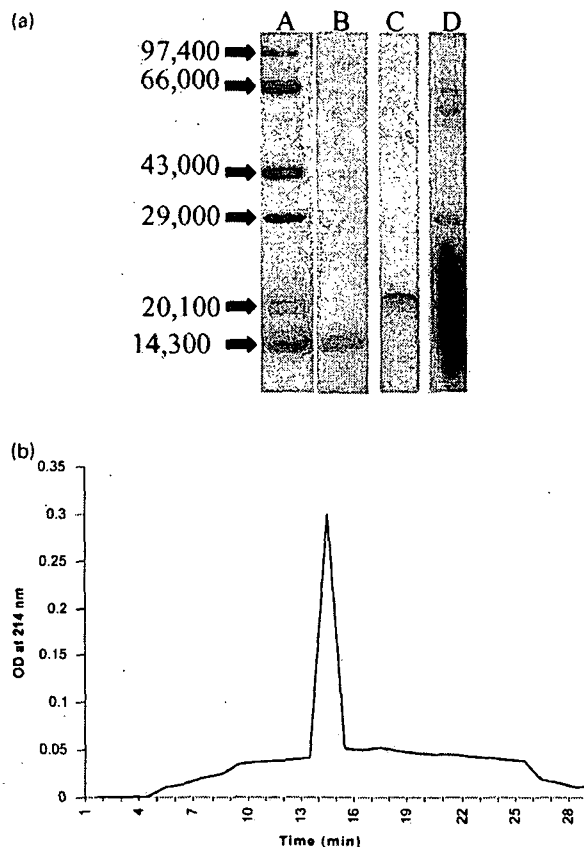

Fig. 3. (a) 10% SDS–polyacrylamide gel electrophoresis of NK-PLA_2-I. Lane A, molecular weight marker, phosphorylase b (97,000), BSA (66,000), ovalbumin (43,000), carbonic anhydrase (29,000), soyabean trypsin inhibitor (20,100) and lysozyme (14,300); lane B, reduced NK-PLA_2-I (30 μg). Lane C, non-reduced NK-PLA_2-I (30 μg) and lane D, crude *N. kaouthia* venom (30 μg). (b) RP-HPLC of NK-PLA_2-I. A waters reverse phase C_{18}-μ-Nova pack column was used. Buffer A was 0.1% TFA and buffer B was 0.1% TFA in 100% CH_3CN. Elution was performed at a flow rate of 1 ml/min (0–60% B). The elution was monitored at 214 nm.

Table 2
Biochemical characterization of NK-PLA_2-I

Properties	NK-PLA_2-I
1. Molecular mass	
i. SDS-PAGE	
a. Reduced	13,600
b. Non-reduced	19,260
ii. Gel filtration	12,940
Optimum Ph	8.0–8.5
Optimum temperature	Increased up to 100 °C
Substrate specificity	PC ≫ PS > PE
Carbohydrate content (μg glucose/mg protein)	7.14
PLA_2 specific activity (unit/mg protein)	94,700 ± 1500 (mean ± SD, $n = 6$)

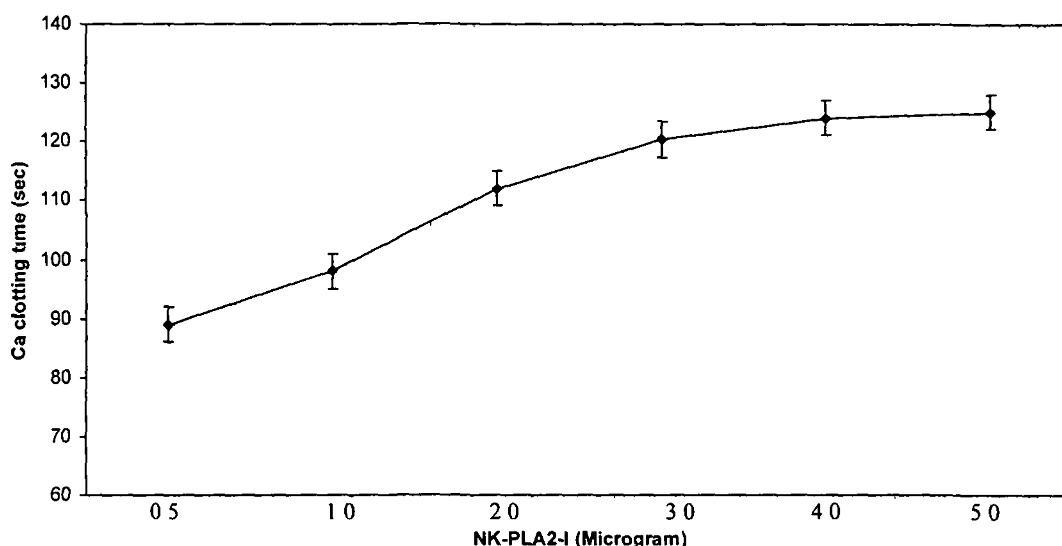

Fig 4 Dose dependent anticoagulant activity of NK-PLA_2-I Ca-clotting time of control plasma is 87 ± 1 s

3 9 Immunoblotting analyses

Immunoblotting experiment indicated that NK-PLA_2-I was recognized by *N naja* antiserum (Fig 5)

4. Discussion

Several snake venoms contain multiple PLA_2 isoenzymes, sharing high degree of identity in amino acid sequence and three dimensional structure, but exhibit different pharmacological effects (Davidson and Dennis, 1991, Scott and Sigler, 1994, Ali et al , 1999, Gao et al , 2001, Tsai et al , 2001) Neither the mechanism by which venom phospholipase A_2 enzymes show their pharmacological effects, nor the role of enzymatic activity in inducing these effects is clearly understood (Rosenberg, 1986, Fletcher et al , 1981, Kini and Evans, 1988, 1989) Therefore, a complicated and subtle interrelationship exists between these two properties of PLA_2 In the present experiments, we report the purification and characterization of a PLA_2 from the venom of *N kaouthia* from eastern India Further, we investigated the role of enzymatic activity in the pharmacological properties exhibited by the purified enzyme

NK-PLA_2-I purified by a combination of ion exchange and gel filtration chromatography, constituted about 6% of the total venom protein This protein even at a concentration as high as 30 μg showed a single band in SDS-PAGE indicating the purity of the preparation Further, a single, sharp symmetrical peak in gel filtration and reverse phase

Table 3
Pharmacological properties exhibited by NK-PLA_2-I Results are expressed as mean ± SD of four experiments

Properties	Whole venom	NK-PLA_2-I
LD_{50} (i p mg/kg)	0 7 ± 0 09	No
Direct hemolytic activity (%Hb released by 10 μg of protein)	16 85 ± 1 45	0 23 ± 0 04
Indirect hemolytic activity (% of Hb released by 10 μg of protein)	8 0 ± 0 95	25 2 ± 2 46
Edema induction (increase in foot pad thickness (mm) after 30 min by 15 μg of protein)	5 42 ± 0 34	2 52 ± 0 06
Anticoagulant activity (plasma clotting time in seconds by 1 μg protein)[a]	89 ± 1	100 ± 3
In vitro tissue damaging activity (% of Hb released by 25 μg protein)		
a Liver	3 5 ± 0 5	16 7 ± 1 09
b Heart	13 86 ± 0 98	24 43 ± 1 67
c Lung	14 87 ± 1 3	10 0 ± 1 1
Neurotoxic symptoms	Present	Absent

[a] Ca-clotting time of normal plasma is 87 ± 1 s

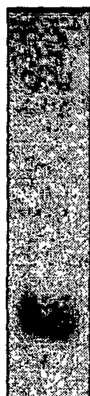

Fig. 5. Immunoblotting experiment showing cross-reactivity of NK-PLA_2-I against *N. naja* antiserum. About 50 μg of protein was separated by 10% SDS-PAGE and transferred to Immobilon-P membrane. Detection was done with horse reddish peroxidase conjugate rabbit anti-horse IgG and TMB/H_2O_2 as substrate.

HPLC eliminated the chances of contamination of NK-PLA_2-I with other proteins. An apparent subunit molecular mass of NK-PLA_2-I was determined as 13.6 kDa by SDS-PAGE and 12.9 kDa by gel filtration. This protein occurs as a dimmer. These values are well consistent with the molecular mass of PLA_2 from many other snake venoms (Sharp et al., 1989; Ali et al., 1999; Angulo et al., 2000; Gao et al., 2001; Tsai et al., 2001).

Biochemical characterization showed glycoprotein nature of NK-PLA_2-I. Interestingly, high thermostable nature of this protein was evident from the heating experiment. This is in good agreement with the earlier reports demonstrating thermostable nature of PLA_2 from other snake venoms. Presence of large number of disulphide bridges in the PLA_2 molecule may attribute to its thermostability (Vishwanath et al., 1988; Francis et al., 1995). NK-PLA_2-I showed basic pH optima for its activity, which is also in close conformity with many other PLA_2 enzymes.

Determination of in vitro head-group specific phospholipid hydrolyzing capacity of phospholipases has great relevance in elucidating the catalytic efficiency of the enzyme and to explain some of their pharmacological effects on the target cell membranes (Kini, 1997; Fletcher and Rosenberg, 1997). Present experiments showed preferential hydrolysis of PC over PS or PE by NK-PLA_2-I. This is in contrast to the earlier reports demonstrating enhanced hydrolysis of PE over PC or PS by PLA_2 from *N. n. kaouthia*, *N. n. atra* (Fleletcher and Rosenberg, 1997) and *D. russelli* venom (Vishwanath et al., 1988). This may be due to geographical and species specific variation in the substrate specificity of PLA_2 isoenzymes.

It was reported that histidine residue at position 48 is conserved in PLA_2 enzymes and played a significant role in phospholipid hydrolysis (Kini, 1997; Ali et al., 1999; Fuly et al., 2000; Gao et al., 2001). However, exception was noticed for a dimmer toxic PLA_2 from *Vipera ammodytes meridionalis* venom where His-48 residue was replaced by Gln-48 (Komori et al., 1996; Perbandt et al., 1997). Dramatic loss of enzymatic activity of NK-PLA_2-I by p-BPB treatment was due to alkylation of active site histidine.

Table 4

Effect of various reagents, antivenom and heating on PLA_2 activity of NK-PLA_2-I. Values represent mean ± SD of four experiments

Reagents/condition	% of activity remaining after treatment
Control (no inhibitor)	100
p-Bromophenacyl bromide (3.3 mM)	4.52 ± 1.2
p-Bromophenacyl bromide (3.3 mM), Triton X 100 (1 mM)	19.7 ± 1.8
p-Bromophenacyl bromide (3.3 mM), Triton X 100 (10 mM)	25.1 ± 2.1
p-Bromophenacyl bromide (3.3 mM), Triton X 100 (30 mM)	34.6 ± 2.5
p-Bromophenacyl bromide (3.3 mM), Triton X 100 (30 mM), Ca^{2+} (10 mM)	56.5 ± 3.8
TLCK (2 mM)	99 ± 1.0
TPCK (2 mM)	91.4 ± 2.3
Phenylmethylsulfonyl flouride (2 mM)	100
Diiopropyl flurophosphate (2 mM)	100
Diothiothreitol (1 mM)	33.4 ± 3.4
Diothiothreitol (2 mM)	20.0 ± 2.8
Ethylenediamine tetra-acetic acid (1 mM)	18.7 ± 2.1
Ethylenediamine tetra-acetic acid (2 mM)	8.0 ± 1.9
α-bromo-2-acetophenone (2 mM)	97.5 ± 2.1
α-chloroacetophenone (2 mM)	91.5 ± 6.0
Antivenom treatment (NK-PLA_2: antivenom)	
1:100	99 ± 0.2
1:200	96 ± 1.2
1:500	84 ± 2.3
Heating at 100 °C (for 10 min at pH 8.0)	99 ± 0.2

Table 5
Effect of various reagents (2 mM), polyvalent antivenom and heating on the pharmacological properties of NK-PLA_2-I Values are mean ± SD of four individual experiments except tissue damaging activity was repeated six times

Properties	% of activity remaining after treatment (control 100%)							
	p-BPB	TLCK	TPCK	PMSF	EDTA	DTT	Protein/anti-venom (1 500)	Heat
Anticoagulant	5 3 ± 1 9	99 ± 8	95 ± 2	100	114 ± 3 1	0	79 ± 3 1	98 3 ± 1 1
Direct hemolysis	0	ND	ND	100	0	0	0	97 0 ± 1 8
Indirect hemolysis	8 66 ± 1 8	ND	ND	99 ± 1 0	6 9 ± 1 1	0	83 2 ± 3 1	96 9 ± 1 3
Edema	8 32 ± 1 3	99 ± 1	93 ± 3	100	ND	ND	86 3 ± 2 1	98 2 ± 1 2
Liver tissue damage	31 ± 2 9	100	55 1 ± 6 2	35 9 ± 3 2	0	0	0	84 2 ± 2 1 91 0 ± 1 3[a]
Heart tissue damage	26 8 ± 3 8	98 6 ± 1 1	61 8 ± 6 9	38 9 ± 2 1	0	0	0	84 6 ± 2 8 90 0 ± 2 3[a]

ND not determined
[a] Assay was done after cooling for 20 min at 25 °C

A significant protection against this inactivation by Triton X-100 (30 mM) and Ca^{2+} (10 mM) might be evidenced for either a surface dilution effect of reagents or by sequestering p-BPB into the apolar micellar core and thus lowering the reagent concentration available for reaction with enzyme (Roberts et al , 1977) Although Roberts et al (1977) reported the almost complete inhibition of PLA_2 enzyme from *N naja naja* venom by α-bromo-2-acetophenone and α-chloroacetophenone, but PLA_2 activity of NK-PLA_2-I was not significantly affected by these reagents suggesting absence of a hydrophobic site on the enzyme and reflecting variation among the PLA_2 molecules Catalytic activity of the purified enzyme was not inhibited by serine inhibitors like PMSF, TLCK and TPCK indicating absence of active serine group in the catalysis process (Roberts et al , 1977) EDTA on the other hand, inhibited the phospholipid hydrolysis by chelating the metal ions required for enzymatic activity (Kini, 1997), but inhibitory effect of DTT on PLA_2 activity might be due to reduction of intramolecular disulfide bridges required for maintaining the three dimensional structure of the active enzyme

Immunoblotting experiment evidenced the immunogenic nature of NK-PLA_2-I and its cross reactivity against *N naja* antiserum But in vitro neutralization study demonstrated that at 1 500 (protein/antibody) ratio, only 16% of the PLA_2 activity were neutralized, showing site responsible for the catalytic activity of the protein may be a poor epitope for antibody production and recognition

Occurrence of PLA_2s in snake venom devoid of lethality or toxicity in experimental animals, but retaining the phospholipid hydrolytic activity are reported (Boucheir et al , 1991 Yang et al , 1991, Ali et al , 1999) In general, basic PLA_2 are more toxic as compared to acidic or neutral PLA_2 and has a significant contribution in the overall toxicity, membrane hydrolysis and edema-inducing activity of the venom (Bhat and Gowda, 1991, Mukherjee et al 1997, 1998, Mukherjee and Maity, 1998) Therefore, presence of higher amount of basic PLA_2 in crude venom makes it more toxic than the NK-PLA_2-I (Mukherjee and Maity, 2002) However, majority of PLA_2 are shown to be devoid of direct hemolytic activity This activity displayed by whole venom was due to presence of low molecular weight membrane active polypeptides (Jiang et al , 1989 Fletcher et al , 1991, Mukherjee and Maity, 1998, 2002) Indirect hemolysis exhibited by purified PLA_2 in presence of egg-yolk phospholipids was due to formation of phospholipid hydrolysis products like lysophospholipids and free fatty acids which are lytic by themselves (Condrea et al , 1964) The same hypothesis can be put forwarded to explain the enhanced susceptibility of the erythrocytes in presence of PC and Ca^{2+}, because PC is much more favored substrate of NK-PLA_2-I for hydrolysis

It is difficult to distinguish between NK-PLA_2-I induced release of hemoglobin from the tissues due to haemolysis and haemolysis followed by RBC release But from the present data, it seems that in vitro tissue damaging activity of NK-PLA_2-I was independent of its haemolytic property because these two pharmacological properties were inhibited to a different extent by inhibitors and antivenoms under the identical test conditions But rupturing of blood capillaries of tissues was necessary for the release of haemoglobin and it may be contributed by phospholipid hydrolysis and protease activity present in NK-PLA_2-I (Datta and Bhattacharyya, 1999) Although NK-PLA_2-I even at a concentration of 25 μg/ml failed to show any proteolytic activity towards conventional substrates like casein, BSA, plasma albumin, globulin and fibrinogen, but it might require to investigate some other natural substrates of NK-PLA_2-I for protease activity because in vitro release of haemoglobin from tissues was partially inhibited by serine and chymotrypsin like serine protease inhibitors, e g PMSF and TPCK, respectively However, the reason for the organ preference of the purified protein is not clearly understood (Warrell, 1989) Differences in the biochemical nature of the vascular wall in different organs like phospholipid/cholesterol ratio, presence of specific phospholipids, vitamin E

content of that membranes, etc may have some relevance to differential membrane hydrolysis (Simionescu, 1983, Kini, 1997, Mukherjee et al, 1997, 1998)

Anticoagulant PLA_2 were isolated from elapidae and other snake venoms (Evans et al, 1980, Angulo et al, 1997) It may be reasonable to assume that hydrolysis of essential phospholipids of coagulation complex by PLA_2 inhibits the coagulation process (Evans and Kini, 1997) However, there is enough controversy concerning the role of enzymatic activity in the anticoagulant and other pharmacological effects of PLA_2 (Kini and Evans, 1988, 1989, Chwetzoff et al, 1989, Gao et al, 2001) and it has been suggested that both enzymatic and non-enzymatic process have contributed in the pharmacological process (Evans and Kini, 1997, Kini, 1997) Present experiment documented the significant role of catalytic activity of NK-PLA_2-I in the anticoagulant, indirect hemolysis and edema-induction but in vitro tissue damaging activity was at least partly contributed by non-enzymatic mechanism Our hypothesis can be supported by the following observations

(a) Treatment of NK-PLA_2-I with p-BPB, TPCK, PMSF, antivenom and heating had almost equal effect on the catalytic, anticoagulant, hemolysis and edema-inducing activity, but tissue damaging activity of the protein was inhibited differently
(b) The anticoagulant activity was enhanced either with the increase in the amount of PLA_2 or with an increase in the pre-incubation time of PLA_2 enzyme with the plasma, supporting that anticoagulant potency parallel with the catalytic activity of PLA_2 enzyme However, for unexplained reason, EDTA not only failed to inhibit, but apparently enhanced the anticoagulant potency of NK-PLA_2-I This can be correlated with the effect of EDTA on the anticoagulant activity of *Vipera berus* PLA_2 (Boffa et al, 1972)

Current investigation provides a fairly good indication that NK-PLA_2-I induces various pharmacological effects by mechanisms, which are either dependent or independent of its enzymatic activity This supports the idea of the presence of separate, perhaps overlapping sites on the PLA_2 for catalytic and pharmacological properties (Kini and Evans, 1989) Currently we are investigating the mechanism of anticoagulant effect of NK-PLA_2-I

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Differential hydrolysis of erythrocyte and mitochondrial membrane phospholipids by two phospholipase A_2 isoenzymes (NK-PLA$_2$-I and NK-PLA$_2$-II) from the venom of the Indian monocled cobra *Naja kaouthia*

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Abstract

We previously demonstrated that venom from the Indian monocled cobra *Naja kaouthia* is a rich source of phospholipase A_2 enzymes, and we purified and characterized a major PLA$_2$ isoenzyme (NK-PLA$_2$-I) from *N kaouthia* venom In the present study, we report the purification and biochemical characterization of a second PLA$_2$ isoenzyme (NK-PLA$_2$-II) from the same venom A comparison of the membrane phospholipid hydrolysis patterns by these two PLA$_2$s has revealed that they cause significantly more damage to mitochondrial membranes (NK-PLA$_2$-I > NK-PLA$_2$-II) as compared to erythrocyte membranes due to more efficient binding of the enzymes to mitochondrial membranes Fatty acid release patterns by these PLA$_2$s from the membrane phospholipid PC-pools indicate that NK-PLA$_2$-I does not discriminate between saturated and unsaturated fatty acids whereas NK-PLA$_2$-II shows a preference for unsaturated fatty acids during the initial phase of attack The current investigation provides new insight into the molecular arrangement of NK-PLA$_2$-sensitive domains in erythrocyte and mitochondrial membranes and highlights the contribution of polar, but uncharged, amino acids such as serine and cysteine in NK-PLA$_2$ induced membrane damage
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Phospholipase A_2 (PLA$_2$) proteins are a diverse family of lipolytic enzymes that specifically catalyze the hydrolysis of fatty acid ester bonds at position 2 of 1,2-diacyl-*sn*-3-phosphoglycerides to produce free fatty acids and lysophospholipids [1,2] Based on the amino acid sequence, polypeptide chain length, disulfide bonding pattern, tissue damage, and functional properties, PLA$_2$ enzymes are classified into several families and subfamilies including secretory PLA$_2$ (sPLA$_2$) [3,4], arachidonyl-specific cytosolic PLA$_2$ (cPLA$_2$) [5,6], and Ca^{2+}-independent PLA$_2$[7] sPLA$_2$ with relative molecular mass of 13–16 kDa constitutes a large family of structurally related enzymes and are widespread in various mammalian cells and tissues as well as in lizard, insect, and snake venoms

There is a tremendous molecular diversity of snake venom PLA$_2$s, with both active and catalytically inactive forms, that results in a wide spectrum of toxin action, such as neurotoxicity, cardiotoxicity, myotoxicity, necrotic, anticoagulant, hypotensive, hemolytic, hemorrhagic, and edema-inducing activities [2,8] This diverse pharmacological profile has been acquired through an accelerated evolutionary process [9] A single venom may contain several PLA$_2$ isoenzymes and, in general, an individual PLA$_2$ exhibits either one or more specific pharmacological effects [2,8,10,11] Therefore, purification and biochemical and pharmacological characterization of PLA$_2$ isoenzymes from the same venom

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contributes to our understanding of their structure–function relationships.

Most of the toxic effects of snake venom PLA_2 appear to result from their promotion of membrane dysfunction by hydrolyzing phospholipids of cellular or subcellular membranes and/or generation of phospholipid hydrolysis breakdown products, namely lysophospholipid and free fatty acids, which are themselves lytic and cause considerable membrane damage [2,8,10,12–15]. PLA_2 enzymes perform phospholipid hydrolysis using a His-Asp doublet plus a conserved water molecule as a nucleophile, and a Ca^{2+} ion as cofactor. Their catalytic activity is substantially increased upon binding to the surface of phospholipid membranes or micelles [16,17]. Kinetic studies of $sPLA_2$ in the scooting mode establish that these enzymes bind to the membrane surface as a prelude to loading of the active site with a single phospholipid molecule for the lipolysis reaction [6,7]. It is becoming apparent that $sPLA_2$ from different sources can display dramatically different affinities for biomembranes, composed of different phospholipid polar head groups and fatty acyl chains [18–20]. This specificity of phospholipases has been used extensively to explore the physical structure of phospholipids in biological membranes [21].

Although our understanding of the mode of hydrolysis of different membrane phospholipids by $sPLA_2$ is increasing, there is a dearth of knowledge on the mechanism of action of *Naja kaouthia* PLA_2 on biological membranes. Recently, we purified and characterized a major PLA_2 isoenzyme (NK-PLA_2-I) from the venom of the Indian monocled cobra *N. kaouthia* [11]. In this study, we report the purification of a second catalytically active PLA_2 (NK-PLA_2-II) from the same venom. Since these PLA_2s possess significantly different biochemical properties, we decided to investigate the mode of attack by these two PLA_2s on different biological membranes. The results of this study provide new insight into the molecular arrangement of *N. kaouthia* PLA_2-susceptible phosphatidylcholine domains in mitochondrial and erythrocyte membranes, and the contribution of polar, uncharged amino acids to the PLA_2-membrane interaction.

Materials and methods

Materials

Venom of *N. kaouthia* from eastern India was purchased from Calcutta Snake Park, Kolkata. CM-Sephadex C-50 and Sephadex G-50 (fine grade) were obtained from Pharmacia Fine Chemicals, Sweden. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-palmitoyl -*sn*-glycero-3-phosphocholine (PPPC),[1] 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine (PAPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), and 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoserine (PAPS) were purchased from Avanti Polar Lipids. All other reagents of analytical grade were purchased from Sigma (USA). NK-PLA_2-I was purified from *N. kaouthia* venom as described [11].

Purification of NK-PLA_2-II

Fifty milligrams of crude *N. kaouthia* venom was fractionated on a CM-Sephadex C-50 ($20 \times 60\,mm^2$) column [11]. Peak CM-II was pooled, desalted by passing through a Sephadex G-50 column, and concentrated at −20 °C in a MAXI dry plus (Heto Lab Equipment, Denmark). About 100 µg of CM-II was dissolved in 40 µl buffer A (0.1% v/v TFA in 5.0 % v/v acetonitrile) and applied on a Waters reverse phase C_{18}-µ-Nova pack column, previously equilibrated with buffer A. Protein was eluted at a flow rate of 1 ml/min using a gradient of 5–60% buffer B (0.1% v/v TFA in 95% v/v acetonitrile) over 28 min. Detection was monitored at 220 nm and individual fractions were collected manually. Each peak was screened for protein content [22] as well as PLA_2 activity. Peak VI, showing the highest PLA_2 activity, was pooled and rechromatographed identically on the same C_{18} reverse-phase HPLC column. This fraction, designated NK-PLA_2-II, was dried in vacuo and stored at −18 °C.

[1] *Abbreviations used:* DTT, dithiothreitol, EDTA, ethylenediaminetetraacetic acid disodium salt, ELISA, enzyme-linked immunosorbent assay, GC–MS, gas chromatography–mass spectrophotometry; hGX, human group X $sPLA_2$; HPLC, high performance liquid chromatography; PAPC, palmitoyl-2-arachidonyl-*sn*-glycerol-3-phosphatidylcholine; PAPE, palmitoyl-2-arachidonyl-*sn*-glycerol-3-phosphatidylethanolamine: PAPE, palmitoyl-2-arachidonyl-*sn*-glycerol-3-phosphatidylethanolamine, PAPS, palmitoyl-2-arachidonyl-*sn*-glycerol-3-phosphatidylserine; PC, phosphatidylcholine; PDPC, palmitoyl-2-docosahexanoyl-*sn*-glycerol-3-phosphatidylcholine; PE, phosphatidylethanolamine; PLA_2, phospholipase A_2; PS, phosphatidylserine; PLPC, palmitoyl-2-linoleoyl-*sn*-glycerol-3-phosphatidylcholine; PMSF, phenylmethylsulfonyl fluoride; POPC, 1-palmitoy-2-oleoyl-*sn*-glycerol-3-phosphatidylcholine; PPPC, 1-palmitoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; TFA, trifluoroacetic acid; TLC, thin layer chromatography; TLCK, Nα-p-tosyl-L-lysine chloromethyl ketone; TPCK, α-tosyl-L-p-phenylalanine chloromethyl ketone.

Assessment of homogeneity and determination of molecular mass of NK-PLA$_2$-II

The purity of the NK-PLA$_2$-II preparation and molecular mass determination were performed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and mass spectrometric analysis. SDS–PAGE (10%) was carried out with or without reduction of about 30 µg of purified protein [23]. Molecular mass of NK-PLA$_2$-II was also determined by matrix-assisted laser desorption/ionization mass spectrophotometry (MALDI-MS). Analysis was carried out in a sinapinic acid matrix with an average of 50 shots at 30–40% laser power.

Protein sequencing

Partial N-terminal amino acid sequencing by Edman degradation was performed in a gas-phase PPSQ-10 protein sequencer (Shimadzu) connected with an on-line PTH-analyzer and a CR-7A data processor. Protein homology searches were performed using the Swiss-Prot databases.

Enzyme kinetics and substrate specificity

Unless otherwise stated, PLA$_2$ activity of crude venom as well as purified protein was determined by the turbidometric method [24] using 5% (v/v) egg yolk phospholipid as a source of substrate. One unit (U) of PLA$_2$ activity was defined as the amount of protein which produces a decrease of 0.01 absorbance units in 10 min at 740 nm.

The substrate preference was examined with commercially available phospholipids, which possess palmitic acid at the *sn*-1 position and have different fatty acids at the *sn*-2 position as well as different polar head groups. The purity of all substrate lipids was examined by thin layer chromatography (TLC) on Silica gel G and chromatograms were developed with chloroform:methanol:water (65:25:1 v/v/v). The enzyme was assayed at 25 °C in a 2 ml reaction mixture containing 0.5 mM Tris, 0.5% Triton X-100, 20 mM $CaCl_2$, 5.8 mM NaCl, 0.7 mM EDTA, 0.43 M ethanol, and 2–5 mM phospholipid substrate. Reaction mixtures were sonicated prior to initiation of the hydrolysis reaction by adding 20 nM enzyme. Following incubation for an appropriate period of time, the reaction was stopped by the addition of Dole's reagent (heptane, 2-propanol, 2 N sulfuric acid = 10:40:1 v/v/v) and the released fatty acids were quantified according to the method of Reynolds et al. [25].

Chemical modification of PLA$_2$

Chemical modification of histidine, serine, and cysteine residues was performed as described previously [11]. Modification of tryptophan residues with *N*-bromosuccinamide was performed according to Soares et al. [26]. In all cases, excess reagent was removed by ultrafiltration through an Amicon YM-3 membrane. Modified proteins were assayed for their catalytic activity as well as membrane hydrolyzing properties and compared with native proteins.

Circular dichroism

Circular dichroism (CD) measurement was performed using a Jasco J-720 spectropolarimeter (Tokyo, Japan). Protein samples (native, heated at 100 °C for up to 45 min, and chemically modified) were dissolved at room temperature (25 °C) in Millipore water at a final concentration of ~1 mg/ml. CD spectra were recorded using a quartz cuvette with an optical path length of 0.5 mm. Each CD spectrum represents an average of 5 scans in the range of 195–250 nm, collected at 0.2-nm intervals, with a spectral band width of 0.5 nm and 4 s integration time. The CD spectra were corrected by subtraction of water blank and expressed in molar ellipticity $[\theta]$ (degrees cm^2/dmol), using 113 as mean residue molecular weight [27].

Isolation of liver mitochondria

Mitochondria were isolated from fresh chicken liver. Unless otherwise stated, all operations were carried out at 4 °C. Liver tissues were homogenized in 0.25 M sucrose containing 20 mM Tris–HCl, pH 7.4, and 1 mM EDTA (isolating buffer), and centrifuged for 10 min at 460*g* to sediment the nuclei. The supernatant was transferred to another tube and centrifuged at 12,500*g* for 7 min. In appearance, the pellet has three distinct regions. The middle layer containing the mitochondria was dislodged very gently, suspended in 4 ml of isolating buffer, and re-centrifuged at 12,500g for 7 min. Finally, the pallet was re-suspended in assay buffer (20 mM Tris–HCl, pH 7.4, containing 0.3 M sucrose) to give a final protein concentration of 80–100 mg/ml. The purity of the mitochondrial preparation was assessed by determining the release of the marker enzyme succinate dehydrogenase.

Assay of mitochondrial swelling and phospholipid hydrolysis

To examine the effect of native/chemically modified PLA$_2$ on mitochondrial swelling, mitochondrial suspensions containing 100 mg equivalent of mitochondria (mitochondria obtained from 100 mg wet weight of tissues) in assay buffer were incubated with increasing doses of PLA$_2$, either in the presence or absence of 2 mM Ca^{2+}, at room temperature for the desired time period. Mitochondrial swelling was followed spectrophotometrically

by the decrease in absorbance at 520 nm [28]. One unit is defined as a decrease of 0.01 OD units at 520 nm per 100 nM of enzyme. The basic test system without PLA_2 was served as control. Qualitative and quantitative analyses of liberated free fatty acids and lysophospholipids from the mitochondrial membranes due to the action of PLA_2 were performed as described [29].

Assay of hemolysis and erythrocyte phospholipid hydrolysis

Blood, collected by venipuncture into 3.8% citrate, was obtained from normal adult volunteers of both genders, aged 21–35 years, after an overnight fast. The procedure for isolating erythrocytes has been described elsewhere [11]. For assay of hemolysis and erythrocyte phospholipid hydrolysis, the basic test system contained 3.0 ml of 5% (v/v) erythrocyte suspension in isotonic buffer with or without 1.5 mM Ca^{2+}. The reaction was initiated by addition of 100 nM PLA_2 and samples were incubated at 37 °C for the desired time period. The reaction was stopped by adding 1.0 ml ice-cold isotonic saline (0.9% NaCl), then the reaction mixture was centrifuged at 5000 rpm for 10 min and the supernatant was used for determination of hemolytic and erythrocyte phospholipid hydrolysis activity [14]. To investigate the erythrocyte phospholipid hydrolysis action of either PLA_2 in the presence of exogenously added PC, PS or PE, the procedure of Diaz et al. [30] was followed. Qualitative and quantitative analyses of total lipid were performed as described below.

Quantitation of lipids and analysis of free fatty acids by gas chromatography–mass spectrophotometry

Total lipid released from the erythrocyte or mitochondrial supernatant following treatment with PLA_2 was extracted by the method of Folch et al. [29] and quantitated by evaporating a measured amount of extract [10,14,15]. Free fatty acids, phospholipids, and lysophospholipids were separated from total lipids by thin layer chromatography on Silica Gel G plates in a solvent system of CH_2Cl_2:CH_3OH:H_2O (65:25:4 v/v/v). For separation of free fatty acids, the solvent system used was petroleum ether:ethyl ether:acetic acid (80:20:1 v/v/v). Total phospholipid was quantitated by estimating the lipid phosphorous [10,14]. Liberated lysophospholipids were estimated either by their hemolytic action on a standard system of human erythrocytes or by quantitating lipid phosphorous [14]. Total free fatty acid (FFA) was quantified by the method of Reynolds et al. [25].

For qualitative analysis, FFA separated by TLC were scraped into glass tubes and methylated as described by Shukla and Hanahan [21]. Fatty acid methyl esters were analyzed on a Varian GC–MS 3800, Saturn 2000 system. Samples were injected in split-less mode into a chrompack capillary GC column CP-Sil 8 CB low bleed (30 m × 0.25 mm × 0.25 μm) coupled with a CP-Sil 5 CB low bleed/MS (30 m × 0.25 mm × 0.25 μm) column with helium as the carrier gas. The column temperature was raised from 80 to 240 °C using an increment of 5 °C/min and held at 240 °C for 30 min. The injector temperature was 240 °C and the transfer line temperature was 300 °C. The mass spectrometric data were acquired in chemical ionization mode (70 eV). Mass spectra of unknown methylated fatty acids were identified by comparing the retention time of authenticated fatty acids, using the Saturn 2000 MS library search where 99% matching was observed.

Enzyme immunoassays for PLA_2 binding studies

Supernatants obtained from the incubation of intact mitochondria or erythrocytes with NK-PLA_2-I or NK-PLA_2-II (native or chemically modified) at 4 °C for 30 min were tested for free (unbound) toxin concentration by ELISA [30] using anti-NK-PLA_2-I IgG.

Raising polyclonal antibody against NK-PLA_2-I and immunochemical studies

Lyophilized protein (0.2 mg) in 0.2 ml phosphate-buffered saline, pH 7.4, was mixed with equal volumes of Freund's complete adjuvant and injected intradermally into an inbred male albino rabbit (2.0 kg) at several sites on the dorsal surface of the neck. Four booster doses were administered at weekly intervals at the same concentration, but with equal volumes of Freund's incomplete adjuvant. After resting the animal for 10 days, blood was drawn from the marginal ear vein and antisera were separated after allowing the blood to stand for overnight at 4 °C. γ-Globulin (anti-NK-PLA_2-I IgG) was isolated from the anti-serum using ammonium sulfate precipitation and precipitating antibodies were detected by the Ouchterlony agar gel double diffusion technique.

Cross-reactivity of NK-PLA_2-I anti-serum against NK-PLA_2-II was detected by Western immunoblotting, as described earlier [11], as well as by the Ouchterlony double diffusion test.

Neutralization studies

The degree of neutralization of enzymatic and membrane phospholipid hydrolyzing activities of both PLA_2s by NK-PLA_2-I antibodies was studied by pre-incubating a graded amount of NK-PLA_2-I antibodies with a fixed amount of either PLA_2 at 37 °C for 30 min followed by assaying their enzymatic and membrane hydrolyzing activities. The enzymatic activity and membrane damaging properties of native protein(s) served as control and were considered as 100% activity.

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Results

Purification and characterization of NK-PLA_2-II

Fractionation of crude *N. kaouthia* venom using a cation exchange column (CM-Sephadex C-50) resulted in separation of venom components into two major and six minor fractions, named CM-I to CM-VIII [11]. Peak CM-II, eluted with 90 mM K-phosphate buffer, pH 8.0, displayed the highest PLA_2 activity among all the base eluted fractions, and was subjected to further purification. Reverse-phase HPLC of CM-II proteins resulted in their separation into eight major protein peaks (Fig. 1) and maximum PLA_2 activity was shown by fraction HP-VI with a retention time of 16.5 min. Rechromatography of about 40 μg protein from the HP-VI fraction on the same RP-HPLC C_{18} column under identical conditions yielded a pure product with a protein yield of 3.5% of the original venom load and having a specific PLA_2 activity of 88,100 ± 1100 U (mean ± SD). This PLA_2 was designated NK-PLA_2-II (Fig. 1, inset).

The homogeneity and apparent molecular mass of the NK-PLA_2-II preparation was judged by SDS–PAGE analysis, where about 30 μg of protein displayed a single band of 13.1 kDa under both reducing and non-reducing conditions (Fig. 2A), indicating that NK-PLA_2-II is a monomer. The purity and molecular mass of NK-PLA_2-II was further confirmed by MALDI-MS (Fig. 2B), which revealed a protonated molecular ion [MH^+] at m/z 13346.19 Da, which is similar to the mass determined by SDS–PAGE. MALDI-MS normally yields single charged states, but a lower intensity, doubly charged [MH^{2+}] peak at m/z 6673 Da was also noticed (Fig. 2B). Such doubly charged peaks were also displayed by PLA_2s from the venom of the sea snake *Hydrophis cyanocinctus* [27]. NK-PLA_2-II up to a dose of 100 nM had no detectable proteolytic, 5′-nucleotidase, ATPase, or acetylcholinesterase activity, and was non-glycoprotein in nature.

The N-terminal amino acid sequences of NK-PLA_2-I and NK-PLA_2-II are identical (NIYQFKNNIQ) and a comparison with sequences in the GenBank database indicated that both PLA_2 sequences share substantial homology with sequences of some previously described cobra venom phospholipases such as *Naja sputatrix*, *Naja melanoleuca*, *Naja siamensis*, and *Naja atra*.

Substrate specificity of PLA_2s

Among the tested phospholipids, PC was the preferred substrate for both PLA_2s, however the rate of hydrolysis of PC by NK-PLA_2-I was significantly higher

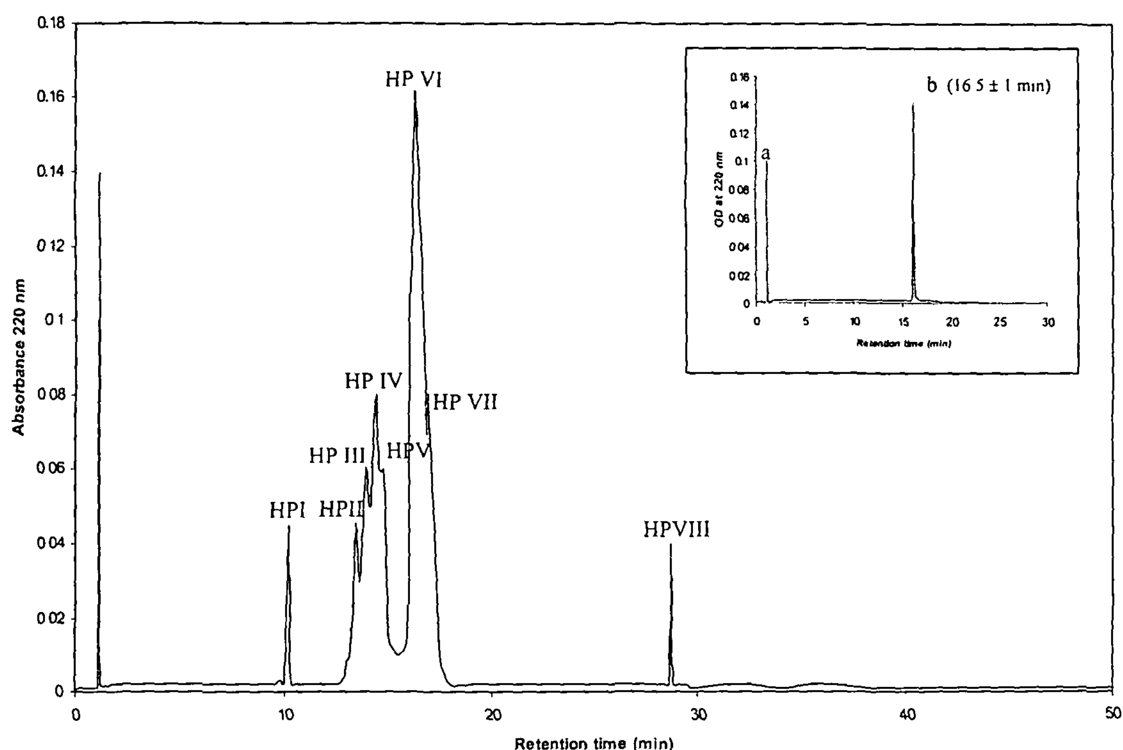

Fig 1 Reverse-phase HPLC of fraction CM II (second cation-exchange fraction of crude venom) on a Waters reverse-phase HPLC C_{18}-μNova Pack Column Buffers A and B were 0.1% (v/v) TFA in 5.0% (v/v) acetonitrile, and 0 1% TFA in 95% (v/v) acetonitrile, respectively Elution was performed at a flow rate of 1 ml/min using the gradient described in "Materials and methods " The inset shows the chromatogram resulting from rechromatography of HP-VI on the same C_{18} column under identical conditions The unabsorbed fraction was not a protein and was from the buffer as confirmed from a blank run The protein was eluted at 16 5 ± 0 1 min (mean ± SD)

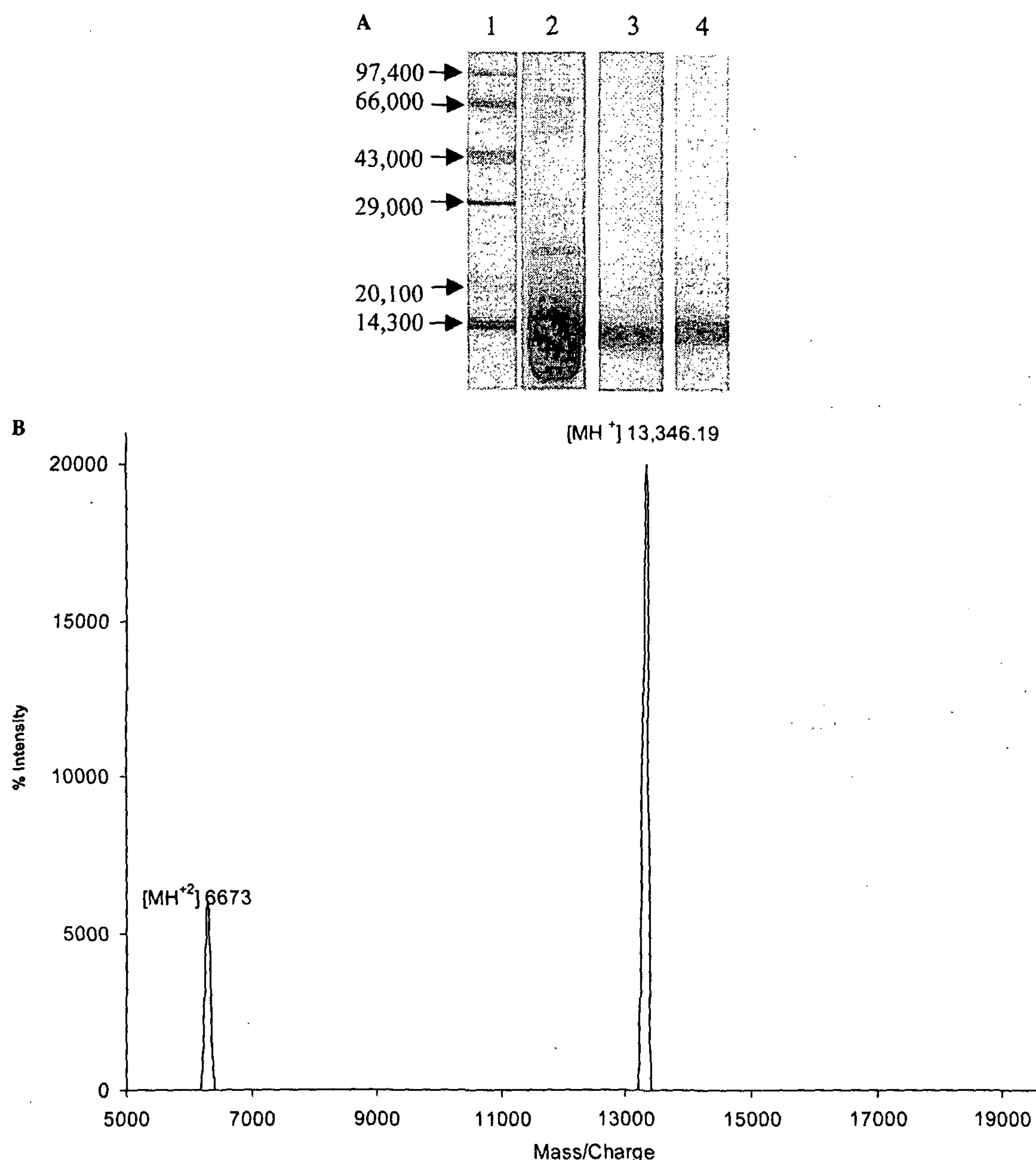

Fig. 2. (A) SDS–polyacrylamide gel electrophoresis. Purified NK-PLA_2-II (both reduced and non-reduced) and crude *N. kaouthia* venom were separated by SDS–PAGE (12.5%). Lane 1, phosphorylase b (97,400), BSA (66,000), ovalbumin (43,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (20,100), and lysozyme (14,300); lane 2, crude *N. kaouthia* venom (50 μg); lane 3, reduced NK-PLA_2-II (30 μg); and lane 4, non-reduced NK-PLA_2-II (30 μg). (B) MALDI-MS spectrum of NK-PLA_2-II. Matrix: sinapinic acid; number of shots: 50; laser power 30–40%. The spectrum shows both single [MH^+ = 13364.19] and doubly charged [MH^{+2} = 6673] molecules, corresponding to a single species with molecular weight of 13.3 kDa.

than that of NK-PLA_2-II (Table 1). Analysis of PLA_2 substrate specificity for fatty acids at the *sn*-2 position showed that NK-PLA_2-I did not discriminate between saturated (C16:0 ≈ C18:0) and unsaturated fatty acids (C18:1 > C18:2) whereas NK-PLA_2-II preferentially hydrolyzed phospholipids with unsaturated fatty acids in the *sn*-2 position (Table 1). Both enzymes could release C20:4 fatty acid present at the *sn*-2 position in PAPC, but at a significantly lower rate as compared to PC molecules containing C16:0, C18:0, C18:1, and C18:2 fatty acids at the *sn*-2 position. Both PLA_2s displayed considerably higher hydrolysis of PS than PE.

Effect on mitochondrial swelling and phospholipid hydrolysis by the action of PLA_2s

Our mitochondrial preparation was judged to be pure on the basis of the enrichment of marker enzyme succinate dehydrogenase. Cross-contamination of the mitochondrial fraction with lysosomes and microsomes

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Table 1
Substrate specificity of NK-PLA_2-I and NK-PLA_2-II

Substrate	*sn*-2 Fatty acid	Specific activity (μmol/min/mg)	
		NK-PLA_2-I	NK-PLA_2-II
PC	$C_{16 0}$	220 5	133 0
	$C_{18 0}$	243 1	153 9
	$C_{18 1}$	178 4	229 9
	$C_{18 2}$	202 3	218 4
	$C_{20 4}$	89 3	61 0
PE	$C_{18 1}$	61 0	69 2
	$C_{18 2}$	79 0	76 3
PS	$C_{18 1}$	101 2	96 3

The catalytic activity of both PLA_2s (20 nM) was measured with commercially available phospholipids containing palmitic acid at the *sn*-1 position and different fatty acids at the *sn*-2 position Fatty acids were extracted and quantified as described in "Materials and methods" Data are the average of triplicate determinations

(judged by acid phosphatase and glucose-6-phosphatase assay, respectively) ranged from 3 2 to 6 5%, which was negligible

Entry of sucrose into the mitochondrial matrix in response to an increase in membrane permeability results in a decrease in size of the organelle with a concomitant decrease in the light scattered by the mitochondrial suspension [28] This is a very useful and simple qualitative method for studying the mitochondrial membrane integrity and for observing the direct action of PLA_2 on mitochondrial membranes The effects of PLA_2s on changes in matrix volume, as measured by a decrease in OD at 520 nm, are shown in Table 2 Although NK-PLA_2-I was more potent than NK-PLA_2-II in inducing mitochondrial swelling, both PLA_2s induced a prompt and marked swelling of mitochondria without any lag phase

Both NK-PLA_2-I and NK-PLA_2-II hydrolyzed the PC pools in mitochondrial membranes in a dose-dependent and highly characteristic manner, as was evident from the TLC of released lysophospholipids (data not shown) The reaction reached a steady level after 45 min, and the extent of phospholipid hydrolysis by NK-PLA_2-I after 60 min was about 6-fold more than that induced by NK-PLA_2-II under identical conditions (Table 2) Although millimolar Ca^{2+} could significantly enhance the reaction, its presence was not an absolute requirement for PLA_2-induced phospholipid hydrolysis (Table 2) To gain further insight into the mode of attack on the mitochondrial membrane by the two-phospholipase A_2 enzymes, enzyme-induced fatty acid release patterns were analyzed by GC–MS Within the initial 15 min of attack, fatty acids of carbon chain lengths C16 and C18 were the major FFA released by NK-PLA_2-I and NK-PLA_2-II (Table 2) Release of arachidonic acid from mitochondrial membrane was not detected until 30 min of incubation with either PLA_2 and a saturation was reached after 60 min (data not shown)

The accumulated data demonstrated that these two PLA_2s behave differently towards the liver mitochondrial membrane, possibly due to recognition of different domains or populations of PC in the outer mitochondrial membrane [17,21]

Hydrolysis of erythrocyte membrane phospholipids

Table 3 shows the effect of various treatments on the hemolysis and hydrolysis of erythrocyte membrane phospholipids by *N kaouthia* PLA_2s Up to a dose of 50 nM, either PLA_2 failed to induce hydrolysis of erythrocyte phospholipids over a 30 min period However, increasing the PLA_2 dose or incubation time pro-

Table 2
Naja kaouthia PLA_2-induced swelling and phospholipid hydrolysis of outer plasma membranes of intact mitochondria in the presence or absence of 2 mM Ca^{2+}

Incubation time (min)	Mitochondrial swelling (U)		Free fatty acid released (μg FFA/20 μg PLA_2)		Ratio of (16 0 + 18 0)/(18 1 + 18 2)
	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	
	NK-PLA_2-I				
5	$5 \times 10^2 \pm 50$	$1 1 \times 10^3 \pm 100$	ND	ND	ND
15	$1 \times 10^3 \pm 121$	$2 3 \times 10^3 \pm 143$	$17 5 \pm 2$	$40 3 \pm 10$	$1 23 \pm 0 01$
30	$14 5 \times 10^3 \pm 100$	$18 5 \times 10^3 \pm 175$	140 ± 10	$178 6 \pm 21$	$0 867 \pm 0 02$
60	$43 5 \times 10^3 \pm 201$	$55 5 \times 10^{3} \pm 231$	408 ± 21	$520 6 \pm 23$	$0 859 \pm 0 01$
	NK-PLA_2-II				
5	$1 0 \times 10^2 \pm 10$	$0 58 \times 10^3 \pm 60$	ND	ND	ND
15	$7 0 \times 10^2 \pm 53$	$1 3 \times 10^3 \pm 80$	$2 85 \pm 1$	$5 29 \pm 2 1$	$0 50 \pm 0 01$
30	$7 7 \times 10^3 \pm 100$	$12 7 \times 10^3 \pm 121$	$27 2 \pm 3$	$44 9 \pm 3$	$0 43 \pm 0 012$
60	$21 66 \times 10^3 \pm 213$	$38 9 \times 10^3 \pm 221$	$68 1 \pm 4$	$122 64 \pm 10$	$0 31 \pm 0 03$

About 100 mg equivalent (mitochondria obtained from 100 mg wet weight of tissue) of mitochondria from chicken liver was incubated with 100 nM of either PLA_2 at room temperature (~25 °C) for different time periods Measurements of the degree of mitochondrial swelling and phospholipid hydrolysis, and the extraction, separation and analysis of methylated fatty acids by GC–MS are described in "Materials and methods" Data are means ± SD of triplicate determinations

ND not determined

Table 3
Naja kaouthia PLA_2-induced hemolysis and phospholipid hydrolysis of washed human erythrocytes

Incubation time (min)	% of hemolysis	% of erythrocyte phospholipid hydrolysis	Ratio of (16:0 + 18:0/18:1 + 18:2)
	NK-PLA_2-I		
30	0	0	ND
60	0.23 ± 0.02	0.34 ± 0.01	0.342 ± 0.012
90	0.6 ± 0.01	0.89 ± 0.01	0.481 ± 0.031
120	3.0 ± 0.13	4.5 ± 0.33	0.593 ± 0.022
	NK-PLA_2-II		
30	0	0	ND
60	0	0	ND
90	0	0.13 ± 0.02	0.27 ± 0.08
120	0.36 ± 0.03	0.53 ± 0.02	0.283 ± 0.06

The 5.0% (v/v) erythrocyte suspension (3 ml) was incubated with 500 nM of either PLA_2 and hemolysis was determined as described in the text. Released FFA were separated by thin layer chromatography and their methylated esters were analyzed by GC–MS. Values are means ± SD of three individual experiments.
ND, not determined.

gressively increased the extent of phospholipid hydrolysis. Interestingly, appreciable hemolysis by NK-PLA_2-II was not achieved unless egg yolk phospholipid was present in the reaction mixture. Such a phenomenon has been described for a variety of PLA_2s [2]. The fact that NK-PLA_2-I was able to hydrolyze human erythrocyte membrane phospholipids in the presence of Ca^{2+} and in the absence of BSA was corroborated with TLC (data not shown). However, erythrocytes pre-incubated with PC, but not with PS or PE, in the presence of 1.5 mM Ca^{2+}, were highly susceptible to lysis and phospholipid hydrolysis by the action of either PLA_2 (NK-PLA_2-I $\gg$ NK-PLA_2-II) over 60 min of time (Fig. 3A).

Analysis of fatty acids release patterns induced by these two PLA_2s revealed that during the initial 60 min incubation period, NK-PLA_2-I caused preferential release of unsaturated fatty acids of carbon chain lengths 18:1, 18:2, and 20:4. TLC analysis of the phospholipids and lysophospholipid released from erythrocytes by the action of PLA_2s demonstrated that more than 90% of the free fatty acids were released from PC pools. However, incubation of erythrocytes with 100 nM NK-PLA_2-I for more than 60 min resulted in hydrolysis of fatty acids of carbon chain lengths 18:1 and 18:2 from membrane PE pools (data not shown). On the other hand, even after incubating for 2 h at 37 °C, NK-PLA_2-II failed to exhibit any appreciable hydrolysis of fatty acids from membrane PE or PS pools. This reinforces the idea that the two PLA_2 isoenzymes from *N. kaouthia* have different affinities for membranes and recognize different domains or populations of phospholipids in plasma membranes.

Effect of antivenom treatment, chemical modification, and heating

In this study, we used polyclonal antibodies raised against NK-PLA_2-I to explore the relationship between antigenic site, catalytic site, and the membrane hydrolyzing properties of *N. kaouthia* PLA_2s. Purified anti-NK-PLA_2-I IgG reacted with both NK-PLA_2-I and NK-PLA_2-II when tested by Western blotting and Ouhterlony double diffusion tests (data not shown), implying antigenic cross-reactivity between these two PLA_2s. The anti-NK-PLA_2-I IgG caused significantly less inhibition of enzymatic activity as compared to the membrane damaging properties of both NK-PLA_2-I and NK-PLA_2-II (see Table 4), which indicates that the catalytic site is a poor epitope for antibody production and that the catalytic and membrane-binding regions of NK-PLA_2-I and NK-PLA_2-II are structurally distinct.

We also investigated the effect of chemical modifications or heating on the secondary structure and membrane binding properties of the *N. kaouthia* PLA_2s. The far-UV CD spectrum of native NK-PLA_2-I demonstrated defined minima at 210 and 222 nm, indicating a strong α-helical contribution to the CD signal (Fig. 3B). NK-PLA_2-II displayed almost identical spectra (data not shown). α-Helix is the major secondary structure element for a large number of PLA_2s from snake venoms [27,31]. NK-PLA_2-I and NK-PLA_2-II were both highly thermostable and did not begin to lose their secondary structure until heating at 100 °C for 45 min (Fig. 3B), which is in close agreement with the earlier reports [31,32].

Chemical modification of histidine and tryptophan residues resulted in significant inhibition of both the enzymatic and membrane damaging activities of NK-PLA_2-I and NK-PLA_2-II (Table 4). Serine-protease-specific reagents such as PMSF, even at a dose of 1 mM, failed to inhibit the catalytic activity of either PLA_2, but inhibited 7 and 85% of mitochondrial phospholipid hydrolysis activity of NK-PLA_2-I and NK-PLA_2-II, respectively. Similarly, TPCK and TLCK, which are known to modify both the serine and cysteine residues of proteins, inhibited 73 and 100% of mitochondrial phospholipid hydrolysis by NK-PLA_2-I and NK-PLA_2-II, respectively, without interfering with the catalytic

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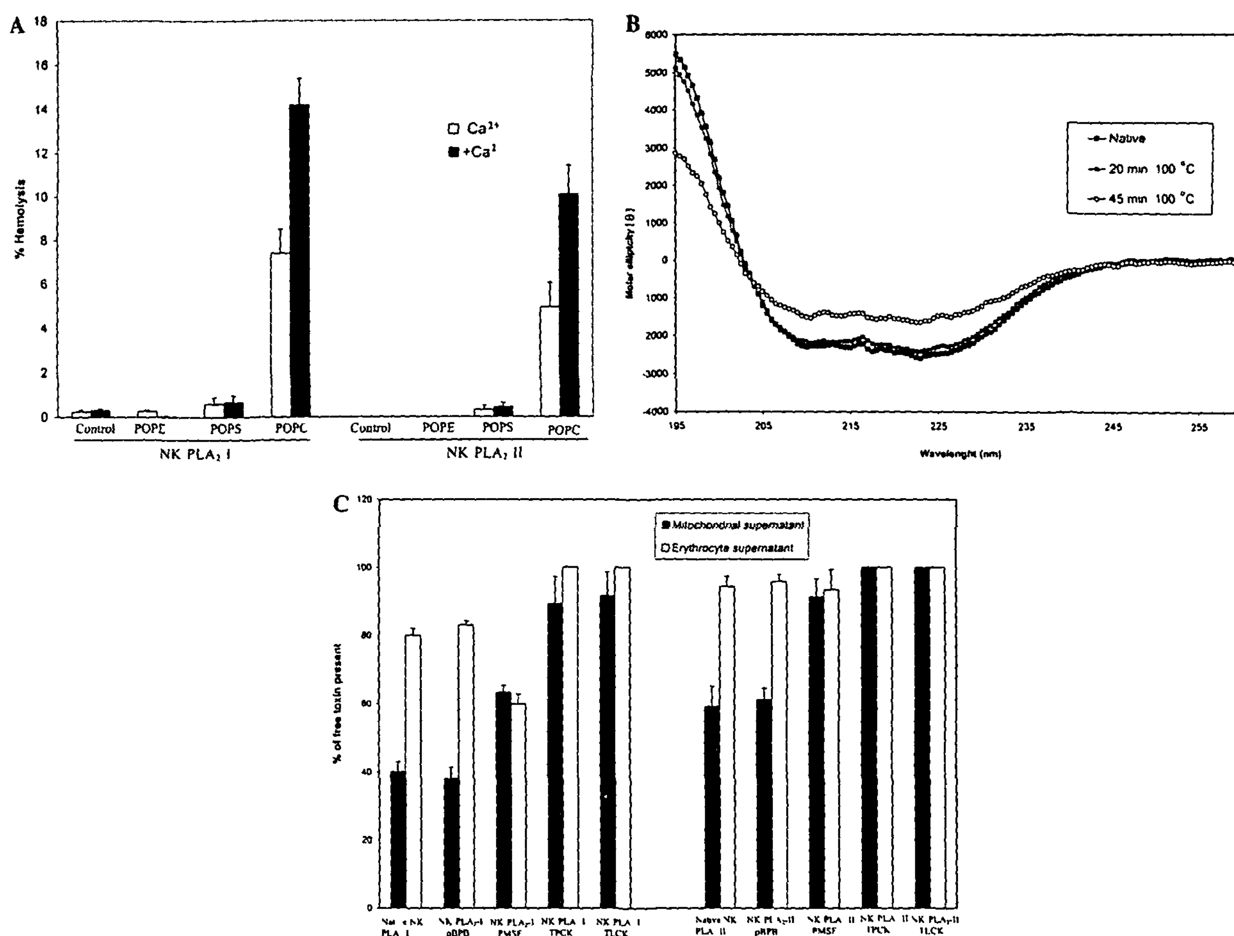

Fig 3 (A) Effect of NK-PLA_2 I and NK PLA_2 II on erythrocytes enriched with different phospholipids Human erythrocytes previously treated with PC PS or PE (5 mM) were incubated with 100 nM of either PLA_2 for a period of 60 min at 37 °C and the experiment was carried out in either the presence or absence of 1 5 mM $CaCl_2$ Lysis is expressed as a percentage taking 100% as the absorbance of an erythrocyte suspension incubated with 0 1% Triton X 100 Each result represents the mean ± SD of three independent experiments (B) CD spectra of NK-PLA_2 I (■) Native PLA_2 (□) PLA_2 after heating for 20 min at 100 °C and (O) PLA_2 after heating for 45 min at 100 °C CD measurements were performed as described in Materials and methods The CD signal is expressed as mean residue ellipticity [θ] (degrees cm^2/dmol) using 113 as the mean residue molecular weight (C) Binding of native chemically modified or heated PLA_2s to erythrocytes or mitochondria Human erythrocytes (5% v/v) or 100 mg equivalent mitochondria were incubated with 100 nM native/treated PLA_2 at 4 °C for 30 min Free (unbound) PLA_2 was quantitated by ELISA and expressed as a percentage of free toxin present in the supernatant Results correspond to means ± SD of three independent experiments

activity of either PLA_2 (see Table 4) An identical phenomenon was demonstrated for inhibition of erythrocyte phospholipid hydrolysis (Table 4) Heating PLA_2s at 100 °C for 20 min had negligible effect on their catalytic activity and secondary structure, but mitochondrial phospholipid hydrolysis by NK-PLA_2-I and NK-PLA_2-II was inhibited by 13 7 and 4 4%, respectively Heating at 100 °C for 45 min resulted in complete abrogation of the membrane-damaging action of both PLA_2s, but 50% of the catalytic activity was still present after this treatment (Table 4)

ELISA experiments revealed that more unbound toxin was present in the supernatant of erythrocytes or mitochondrial suspensions incubated with tryptophan, serine or cysteine-modified toxins, as compared to histidine-modified or native toxins, implying that the native and histidine-modified toxins were captured by intact erythrocytes and mitochondria to a greater extent than the tryptophan, serine, and cysteine-modified enzymes (Fig 3C)

Discussion

Preferential hydrolysis of phospholipid of intact mitochondrial membranes as compared to erythrocyte membranes

Secretory PLA_2 enzymes catalyze reactions at lipid–water interfaces [33,34] and various factors play a role in determining the strength and cooperativity of PLA_2–membrane interactions Results obtained from a variety

Table 4
Effect of chemical modification, neutralization with anti-NK-PLA_2-I antibodies, and heating on the catalytic and membrane phospholipid hydrolysis action of *N kaouthia* PLA_2s

Inhibitor (final concentration)	NK-PLA_2-I			NK-PLA_2-II		
	% inhibition of			% inhibition of		
	PLA_2	MPS	EPS	PLA_2	MPS	EPS
ρBPB (3 3 mM)	95 48 ± 9 0	100	80 ± 3 2	89 ± 6 1	85 03 ± 5	90 ± 4 5
PMSF (1 mM)	0	7 ± 1 3	83 ± 4 0	0	85 ± 3	94 ± 3 2
TPCK (0 1 mM)	0	73 6 ± 1 5	84 1 ± 2 0	0	100	86 ± 2 3
N-Bromosuccinamide (2 mM)	0	0	0	0	0	0
TLCK (0 1 mM)	1 2 ± 0 12	73 6 ± 2 4	100	0	100	100
Ag Ab, 1 1	3 85 ± 0 2	20 ± 1 1	23 ± 2 0	3 4 ± 0 2	15 ± 2 1	20 ± 1 1
Ag Ab, 1 5	8 17 ± 1 2	45 ± 3 0	50 ± 3 1	9 5 ± 1 1	25 ± 3 0	34 ± 2 1
Ag Ab 1 10	22 04 ± 4 0	95 ± 6 0	96 ± 3 2	22 ± 2 0	95 ± 5 0	98 ± 3 0
20 min heating at 100 °C	1 2 ± 0 1	13 71 ± 2 0	ND	1 96 ± 0 2	4 35 ± 1 0	ND
45 min heating at 100 °C	50 5 ± 5 0	100	100	57 95 ± 4 3	100	ND

Experiments were performed as described in "Materials and methods ' Values represent means ± SD of at least four individual experiments MPS, mitochondrial phospholipid hydrolysis EPS erythrocyte phospholipid hydrolysis, and ND, not determined

of experimental and theoretical studies of PLA_2 activity on lipid bilayers have suggested that membrane surface properties, including membrane fluidity, curvature, surface charge, and membrane-induced structural changes in the enzyme, determine the strength of interaction, cooperativity of membrane binding, and extent of PLA_2 activation [16,17] Therefore, both the physical properties of the membranes and the biochemical nature of PLA_2 are complementary and synergistic determinants of the activation of membrane-bound PLA_2 [16]

In the recent years, evidence has been accumulating that small-scale structure and lipid domains persist in the lipid bilayer in the nanometer range [18,20], suggesting that membranes are often laterally structured in terms of domains and so-called rafts [35,21] A number of different interactions, including membrane–cytoskeleton, lipid–protein, and lipid–lipid, contribute significantly to stabilization of these membrane domains [36] PLA_2 activity is found to be sensitive to these domains and this can be varied systematically by varying the temperature, lipid composition, and acyl-chain length of the phospholipids [18,21,37]

The preferential hydrolysis of mitochondrial outer membranes as compared to erythrocyte membranes by *N kaouthia* PLA_2s may be explained on the basis that the former membranes have a much greater % of PC on the outer leaflet (40 9%) as compared to latter membranes (19%) [38,39] It should be noted that nanomolar concentration of PLA_2s used in the current investigation completely eliminated the possibility of non-specific binding and subsequent hydrolysis of membrane phospholipids at high enzyme concentration Furthermore, it might be possible that existence of significantly greater number of *N kaouthia* phospholipase A_2 sensitive domains in mitochondrial membrane as compared to erythrocyte membrane may have relevance to differential membrane hydrolysis by two PLA_2s This hypothesis cannot be ruled out, because previously we have demonstrated that, although the total phospholipid compositions of tissues like heart, lung, liver, and kidney are similar [40], however, percent hydrolysis of heart tissue by NK-PLA_2-I is significantly higher as compared to other tissues [11] Similarly, phospholipid constituents of microsomal membranes are less hydrolyzed as compared to lysosomal membranes by the action of *Vipera russelli* basic PLA_2 [10,14] Further, it has been suggested that PLA_2 may be particularly active at domain interfaces that are sites of structural defects and hence good points of attack for the enzymes [17], although the mechanism by which this phospholipase A_2 sensitive phospholipid domain is formed in mitochondrion or erythrocyte membrane is not very clear It may be assumed that physicochemical properties of individual phospholipids, circumstantial effects of membrane proteins, phospholipid/cholesterol ratio, and vitamin E content of the membrane contribute significantly to the formation of such domains [10,14–17,21]

Preferential hydrolysis of mitochondrial and erythrocyte membrane PC pools

It has recently been shown that tryptophan on the interfacial binding surfaces of the cobra PLA_2s plays an important role in supporting interfacial binding to phosphatidylcholine [30] Efficient hydrolysis of phospholipid PC pools from outer mitochondrial and erythrocytes membranes by *N kaouthia* PLA_2s can further be explained on the basis of the fluidity of the membrane phospholipids When the fluidity of the major rabbit platelet phospholipids was assayed using the fluorescence polarization method, it was observed that, over the entire tested temperature range (4–50 °C), PC was the most fluid among the major phospholipids [41] Some membrane proteins have a tendency to be asso-

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ciated with more fluid regions of artificial membranes, and it can be assumed that a part of cellular PC constitutes the "boundary lipids" of such proteins in mitochondrial and erythrocyte membranes. These dynamic PC pools might serve as preferential substrates for exogenously added venom PLA_2 [13,42]. In support of this hypothesis is our observation that although none of the *N. kaouthia* PLA_2 enzymes preferentially hydrolyze PAPC over POPC, both PLA_2s efficiently release arachidonyl acid from intact mitochondrial and erythrocyte membranes; because arachidonyl PC would be more fluid than PC of other molecular species, this observation could be explained if arachidonyl-rich PC regions form a preferential binding site for PLA_2s [13].

Fatty acid release patterns from mitochondrial and erythrocyte membranes

The preferential release of carbon chain length C16 to C18 from the outer membrane during the initial attack by NK-PLA_2-I and NK-PLA_2-II correlates with their preference for short chain fatty acids at the *sn*-2 position (Table 1). Furthermore, it has been observed that if bilayers are composed of lipids with different fatty acid chain lengths and even if enzyme binds uniformly across solid and fluid domains enriched in the long and short chain fatty acids, respectively, enzyme shows a preference for short chain fatty acids, which are in fluid phase [18,19]. This is due to the lower surface area density of the lipid head groups composed of short chain fatty acids in fluid phase [43].

Why is there a lag phase during hydrolysis of erythrocyte membranes?

Many $sPLA_2$s, including cobra venom $sPLA_2$ and human group V and X (hGV & GhX) $sPLA_2$s, can efficiently hydrolyze mammalian cell membranes and PC vesicles without a lag in the onset of the reaction [44]. Although *N. kaouthia* PLA_2s do not show any lag phase in the onset of mitochondrial membrane hydrolysis, however erythrocyte membranes hydrolysis was only detected after a lag phase of ~30 min following addition of PLA_2s.

$sPLA_2$s display large variations in their affinities for membranes with differing surface properties. Since both NK-PLA_2-I and NK-PLA_2-II have significantly low affinity for erythrocyte membranes compared to mitochondrial membrane, as revealed by ELISA experiment, binding is extremely low during the initial phase of attack. However, the slow build-up of reaction products at the membrane interface is likely to promote higher interfacial enzyme binding, thus leading to an acceleration in the reaction progress as the reaction proceeds [16,45]. This explanation is consistent with our observation that pre-incubation of erythrocytes with reaction products or with PC not only eliminates the lag, but the extent of membrane phospholipid hydrolysis (NK-PLA_2-I > NK-PLA_2-II) increases significantly.

The catalytic and membrane-binding sites of N. kaouthia PLA_2s are structurally distinct

Lysis of artificial membranes by PLA_2s from various snake venoms (e.g., *Bothrops pirajai* and *Crotalus durissus terrificus*) has been shown to be independent of their enzymatic activity, since both native as well as catalytically inactivated enzymes are fully able to disrupt membranes [26,32]. Contrary to this observation, the present study provides strong evidence that membrane hydrolyzing property of *N. kaouthia* PLA_2s dependent on their catalytic activity and there exist distinct catalytic and membrane binding sites in PLA_2s. The following observations support our hypothesis:

1. The degree of phospholipid hydrolysis increases concomitantly, with an increase in the incubation time of membranes with PLA_2s; documenting membrane damage is dependent on the catalytic activity of the enzyme(s) [46].
2. Chemical modification of histidine residue, which is the most conserved amino acid among PLA_2s, results in significant inhibition of the catalytic as well as membrane-hydrolyzing activities of NK-PLA_2-I and NK-PLA_2-II without interfering with the affinity of the modified PLA_2s towards membranes. In this respect, *N. kaouthia* PLA_2s differ from *N. nigricollis* and *Vipera berus* PLA_2s, because *N. nigricollis* PLA_2 that was catalytically inactivated by histidine modification has a lower affinity for membrane phospholipids compared to native enzyme, whereas His-modified *V. berus* PLA_2 has a higher affinity for phospholipids [47].
3. Modification of serine or cysteine residues results in a drastic reduction of membrane binding as well as phospholipid hydrolyzing activity, but does not interfere with the catalytic activity of NK-PLA_2-I or NK-PLA_2-II. $sPLA_2$s have a common interfacial-binding surface that is located on the flat external surface surrounding the active site slot. The presence of Trp on the putative interfacial binding surface of human Group V and *N. n. naja* PLA_2s plays an important role in the binding of these PLA_2s to PC vesicles and the outer plasma membrane [48,49]. The present study documents that serine and cysteine in *N. kaouthia* PLA_2 molecules play important roles in membrane damage because modifications of these two residues result in significant loss of affinity towards the membranes as well as their hydrolysis by PLA_2. Further investigation is needed to elucidate the mechanism of involvement of serine and cysteine residues of PLA_2 in the membrane hydrolysis process.
4. Heating *N. kaouthia* PLA_2s at 100 °C for different time periods or incubation with anti-NK-PLA_2-I

IgG results in differential inhibition of their catalytic and membrane-hydrolyzing properties, reinforcing the idea that the catalytic site is separated from the membrane-binding region in these PLA_2s

It might be argued that chemical modification of PLA_2s or their interaction with antibodies induces structural changes in the PLA_2 molecule, thus explaining the different properties of the modified enzymes However, we think this explanation is unlikely for several reasons First, CD spectra of the chemically modified proteins were very similar to that of native PLA_2 Second, native and chemically modified enzymes migrated similarly in charge-shift electrophoresis (data not shown) Third, the chemically modified PLA_2s were recognized by anti-NK-PLA_2-I antibodies Thus, the present data provide new insight into the molecular arrangement of *N kaouthia* PLA_2-susceptible phosphatidylcholine domains in mitochondrial and erythrocyte membranes, and the contribution of polar, uncharged amino acids to the PLA_2–membrane interaction Further studies of the mechanism of activation of phospholipase A_2 at membrane interfaces, even in the absence of Ca^{2+}, will be required to realize their potential as therapeutic agents [17]

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