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ORIGINAL ARTICLE

IN SILICO CHARACTERIZATION OF STRUCTURAL AND FUNCTIONAL ELEMENTS OF PLA2

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Abstract:

Natural inhibitors occupy an important place in the potential to neutralize the toxic effects caused by snake venom proteins and enzymes. Snake venom is highly modified saliva containing zootoxins . "Snake venom are highly complex chemical mixture that contains many proteins, mainly particular enzymes, and strongly basic polypeptides". Neurotoxic venomis fast acting and is rapidly absorbed, attacking the central nervous system. Peptide toxins are usually found inanimal venom associated with specialized envenomationapparatus that allows their delivery into the soft tissue of animals via subcutaneous, intramuscular or intravenous routes. Most venom comprises a highly complex mixture of peptides, often with diverse and selective pharmacologies. Phospholipase A2 causes hemolysis by lysing the phospholipid cell membranes of red blood cells.Amino acid oxidases and proteases are used for digestion. The Therapeutic uses of cobra venom Neurotoxins in ancient China, cobra venom saw its primary use in the treatment of cancer and arthritis. Reportedly the venom was used to treat liver cancer, lung cancer, esophageal cancer, skin cancer, and leukemia. In the Indian Unani system of medicine, cobra venom has been used as a tonic, hepatic stimulant. In India and China, such venoms are administered at specialized medical centers and for an extensive array of applications.SWISS-MODEL is a structural bioinformatics web-serverdedicated to homology modeling of protein 3D structures. Homology modeling is currently the most accurate method to generate reliable three-dimensional protein structure models and is routinely used in many practical applications.

KEYWORDS:

Snake venom, Envenomation, Neurotoxin, Phospholipase A2, Therapeutic uses.

INTRODUCTION

Various animals produce venom in specialized glands, for efficient hunting of prey and for defense. Venomous animals are diverse an include species of jellyfish, cone snails, bees and wasps, spiders, scorpions, fish, snakes and even platypuses. Snakes use their venom principally for hunting though they do not hesitate to employ it defensively. Venomous snake bites may cause a variety of symptoms, including pain, swelling, tissue necrosis, low blood pressure, convulsions, hemorrhage (varying by species of snake), respiratory paralysis, kidney failure, coma and death. Envenomation is the process by which venom is injected into some animal by the bite (or sting) of a venomous animal (Bauchot and Roland, 1994).

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VENOM

Venom is highly modified salivacontaining zootoxins used by snakes to immobilize and digest prey or to serve as a defense mechanism against a potential predator or other threat. The venom produced by the snake's venom gland apparatus is delivered by an injection system of modified fangs that enable the venom to penetrate into the target (Bauchot and Roland, 1994). Venom composition

Venoms contain more than 20 different compounds, mostly proteins and polypeptides (Hallidayet al., 2002). A complex mixture of proteins, enzymes, and various other substances with toxic and lethal properties serves to immobilize the prey animal (Mattison and Chris, 2007). Enzymes play an important role in the digestion of prey (Bottrall and Joshua L., December 2011) and various other substances are responsible for important but non-lethal biological effetcs.Some of the proteins in snake venom have very specific effects on various biological functions including blood coagulation, blood pressure regulation and transmission of the nervous or muscular impulse and have been developed for use as pharmacological or diagnostic tools or even useful drugs (Bauchot and Roland, 1994).

Lucien Bonaparte, the younger brother of Napoleon Bonaparte was the first to establish the proteinaceous nature of snake venom in 1843. Proteins constitute 90-95% of venom's dry weight and they are responsible for almost all of its biological effects. Among hundreds, even thousands of proteins found in venom, there are toxins, neurotoxins, in particular as well as nontoxic proteins (which also have pharmacological properties) and many enzymes, especially hydrolithic ones (Bauchot and Roland 1994).

VENOM TOXINS

Snake venom is a mixture of different enzymes includes phospholipase A2, L-amino acid oxidase, nucleosidase, ribonuclease and having toxic (cardiotoxin, myotoxin, neurotoxin, cytotoxin) and non-toxic activities (antimicrobial) produced by snake venom glands. Pharmacological and biochemical properties of those components in venoms are studied in many snakes, however not yet every toxic ingredient in Indian snake venoms have been isolated and characterized for pharmacological perspectives.

Enzymes (molecular weight 13-150 KDa) make-up 80-90% of snake venoms: digestive hydrolases, L-amino acid oxidase, phospholipases, thrombin-like pro-coagulant and kallikrein-like serine proteases and metalloproteinases (hemorrhagins), which damage vascular endothelium. Polypeptide toxins (molecular weight 5-10 KDa) include cytotoxins, cardiotoxins and postsynaptic neurotoxins (such as α-bungarotoxin and α-Cobratoxin) which bind to acetylcholine receptors at neuromuscular junctions. Compounds with low molecular weight (up to 1.5 KDa) include metals, peptides, lipids, nucleosides, carbohydrates, amines and oligopeptides which inhibit angiotensin converting enzyme (ACE) and potentiate bradykinin (BPP). Inter- and intra-species variation in venom chemical composition is geographical and ontogenic (Halliday et al.,2002).

NEUROTOXIN

Neurotoxin is derived from the (nevron / neuron) meaning "nerve" (derived from neuro: "cord")and Latin toxicum meaning "poison". They are an extensive class of exogenous chemical neurological insults (Spencer, 2000) which can adversely affect function in both developing and mature nervous tissue (Olney, 2002). The term can also be used to classify endogenous compounds which when abnormally concentrated can prove neurologically toxic. Though neurotoxins are often neurologically destructive, their ability to specifically target neural components is important in the study of nervous systems. Common examples of neurotoxins include lead, ethanol,glutamate, nitric oxide (NO) (Zhang, 1994),botulinum toxin, (Rosales, 1996)tetanus toxin and tetrodotoxin.

The venoms of four common poisonous Indian snakes like cobra (Najanaja), krait (BangarusCaeruleus), Russell's viper (Daboiarusselli) and saw scaled viper (EchisCarinatus) [designated as Big Four] are neurotoxic in nature, considered to attack victim's central nervous system and result in severe damage leading to death.Neurotoxin activity can be characterized by the ability to inhibit neuron control over ion concentrations across the cell membrane or communication between neurons across a synapse (Arnon, 2001).

Neurotoxic proteins isolated from various snake venoms because of their high affinity for a particular target site are used extensively as pharmacological tools to gain insights into the function of the nervous system. The potency of these molecules lies in their affinities towards the biomolecules involved in the functioning of neuromuscular transmission. Neuromuscular and patho physiological effects of neurotoxic proteins result from their interaction with various micro compartments based on their similarities in mass and conformation to the types of amino acids and disulfide bridges in the normal

ligands.

Snake venom toxins can be broadly classified depending on whether their site of action is at the skeletal neuromuscular junction or at sites other than the skeletal neuromuscular junction. Skeletal neuromuscular junction specific neurotoxins include the following: postsynaptic toxins, presynaptic toxins with musculotropic or myonecrotic actions, presynaptic and postsynaptic, etc.

MECHANISM OF ACTION OF NEUROTOXINS

Snake venoms contain high levels of neurotoxins that target nicotinic acetylcholine receptors (nAChRs) (Liu YL et al., 2009). Neurotoxins are one of the largest families of proteins and have shown analgesic effects in animal and human pain models (Koh DC, andArmugam A., 2006). Snake venoms contain α- and β-neurotoxins which act through different mechanisms to block the transmission of Ach. (Koh DC et al.,2006; Liu YL, Lin HM et al.,2009; Zhang HL et al., 2006). nAChRs have been shown to modulate pain transmission and antinociceptive responses in the central nervous system. The activation of cholinergic pathways by nicotinic receptor antagonists decreases the perception of pain in a variety of species (Damaj MI et al., 1998).

α-Neurotoxins

α-Neurotoxins act postsynaptically and bind to nAChRs. (Koh DC, and Armugam A , 2006; Zhang HL et al.,2006) They act as nicotinic receptor antagonists(Catassi A et al., 2008) by competitively binding to the nAChR at the postsynaptic membrane of skeletal muscles and neurons, thereby reversibly blocking nerve transmission. The major groups of α-neurotoxins include short-chain neurotoxins, long-chain neurotoxins, and weak neurotoxins.(Koh DC, and Armugam A, 2006) Short-chain neurotoxins have between 60 and 62 amino acid residues and 4 disulfide bonds; long-chain neurotoxins have between 66 and 74 residues and 5 disulfide bonds.(Zhang HL et al.,2006) Weak neurotoxins are isolated from cobra venom and, as the name suggests, have very weak affinity for skeletal and neuronal nAChRs.(Koh DC et al.,2006).

β-Neurotoxins

β-neurotoxins isolated from cobra venoms exhibit phospholipase activity.(Zhang HL et al., 2006)The β-neurotoxins act presynaptically and affect the release of ACh through a variety of mechanisms including causing the disappearance of ACh-containing vesicles, which prevents the release of ACh and blocks impulse transmission. In nature, β-neurotoxins demonstrate high toxicity and are responsible for respiratory paralysis after envenomation.(Koh DC, and Armugam A, 2006).

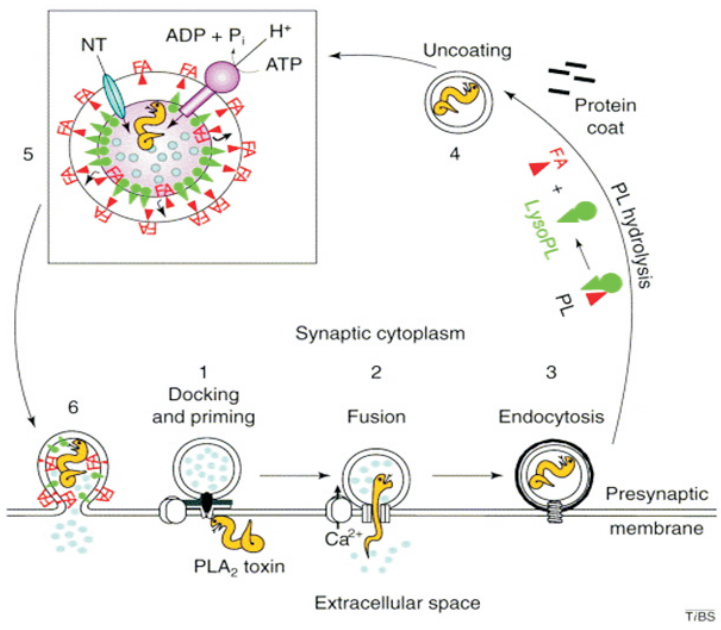

Figure 1. Recycling of small synaptic vesicles at nerve terminals and possible mode of action of presynaptic PLA2 snake neurotoxins. Synaptic vesicles release their neurotransmitter content (light-blue dots) following binding to the presynaptic membrane and priming by ATP (step 1), and fusion (step 2) triggered by local calcium entry. At this stage, the lumen of the vesicle communicates with the extracellular space. It is suggested that the neurotoxin bound to the external face of the membrane gains access to the lumen (step 2) and that it is then endocytosed inside the small synaptic vesicle (step 3). The toxin hydrolyses phospholipids of the inner membrane leaflet generating fatty acids (FA, red cone) and lysophospholipids (LysoPL, green inverted cone). After shedding the proteins that have mediated endocytosis (step 4), the operation of the vacuolar-type of ATPase proton pump rapidly generates a pH gradient across the synaptic vesicle membrane. This provides the driving force for the uptake and refilling of neurotransmitter (step 5) and causes FA protonation and translocation to the cytosolic layer of the vesicle membrane (enlarged squared panel), leaving LysoPL on the lumenal side. Thus, the combined PLA2 and ATPase pump activities create a largely asymmetric distribution of FA and LysoPL. Cone-shaped lipids (unsaturated FA) on the contacting lipid monolayer and inverted-cone-shaped lipids (LysoPL) on the distal monolayer actively promote membrane fusion. Thus, toxin-containing vesicles are highly fusogenic and vesicle fusion (step 6) can take place anywahere on the plsma membrane of the nerve terminal, including areas where neuro exocytosis does not normally take place, with discharge of neurotransmitter (lower left side of the figure), corresponding to the second phase (green trace). At this point, there is no longer a pH gradient across the membrane and FA redistribute among the two lipid monolayers (CesareMontecucco and OrnellaRossetto, 2000).

Phospholipase A2

More than a century ago, human pancreatic juice and cobra venom were reported to contain a phospholipase A2 (PLA2) activity that hydrolyzes egg yolk lecithin to produce a hemolytic product that was called lysolecithin. The subsequent isolation of this secreted PLA2 activity from a variety of snake venoms and mammalian exocrine glands revealed structurally-conserved enzymes that were small (14 kDa) and extensively cross-linked by cysteine disulphide bonds, and whose most obvious function was a digestive one. Later, the various sPLA2s found in snake venoms were found to exert diverse toxic effects.

Phospholipase A2 forms a diverse class of the enzyme with regard to structure, function, localization, and the regulation (Arunmozhiarasi, et al.,2009).Phospholipase A2 causes hemolysis by lysing the phospholipidcell membranes of red blood cells. Amino acid oxidases and proteases are used for digestion. The snake venom PLA2 belongs to the Ca2+-dependant secretory PLA2. Venom phospholipases A2 possess an enzymic activity and a wide variety of (patho) pharmacological activities such as antiplatelet, anticoagulant, hemolytic, neurotoxic (presynaptic), myotoxic, edemainducing, hemorrhagic, cytolytic, cardiotoxic as well as an ability to bind antagonisticallyto muscarinic acetylcholine receptor (mAChR).

The snake venom phospholipases are divided into two main groups, group I and group II, based on their primary structures. The group I PLA2 is found in abundance in the venom of cobras, kraits and sea snakes, while group II PLA2 is common in vipers and pit vipers. Venom of Najasputatrtix, a Malayan spitting cobra, comprises of three isoforms (2 neutral and 1 acidic) of group 1A PLA2. One of the neutral forms, nPLA-1 (nPLA) is a highly potent anticoagulant protein that exhibits relatively high enzymatic activity (Braud, S. et al., 2000).

Therapeutics of Venom toxins and peptides

Snake venom elicits multiple biological responses, including the following: neurotoxic, myotoxic, cardiotoxic, coagulant, hemostatic, edema-inducing, and hemorrhagic (Koh DC and Armugam A. et al., 2006; Markland FS Jr.s., 1997). Snake bites are normally used to immobilize prey (Pal SK, et al., 2002). The signs and symptoms of snake bites include paralysis, myolysis, coagulopathy and hemorrhage, renal damage and failure, cardiotoxicity, and local tissue injury at the bite site (Koh DC et al., 2006). Specifically, snake venoms contain a variety of biologically active proteins and peptides, which act on the human hemostatic system and affect the blood coagulation pathway, endothelial cells, and platelets (Pal SK, and Gomes A., 2002; Markland FS Jr. s. 1997). Also neuroactive peptides, or the neurotoxins derived from snake venom, block neuromuscular transmission by reducing acetylcholine (ACh) release (Pal SK, and Gomes A., 2002).

Symptoms of a snakebite or envenomation suggest that venoms affect multiple organ systems, but the most severe reactions occur in the central nervous system, the muscular system, and the vascular system. In addition to the harmful effects, snake venoms contain potentially therapeutic compounds,

including proteins, nucleotides, and inorganic ions. Snake venoms have been used in Ayurveda and folk medicine, as well as homeopathy, for centuries as treatments for a variety of conditions (Koh DC et al., 2006). Snake venoms have also been used in allopathic medicine for more than a century to treat thrombosis, arthritis, cancer (Pal SK and Gomes A et al., 2002) immune dysfunction, viral infections (Chen ZX et al., 2006) delirium, hallucinations, chorea, and melancholia (Hayman M and Macht DI, 1940). At this time, most of the therapeutic use of snake venoms is based on observations or anecdotal reports (Cheng BC, et al., 2009).

In the mid-1930s, David I. Macht was among the pioneers of research involving the use of snake venoms for medicinal purposes. Macht, a researcher for a pharmaceutical company, outlined the use of cobra venom extracts as an analgesic; his results proved that minute doses of cobra venom were superior to morphine in terms of pain relief. Macht reported that snake venom showed a longer onset of action, but possessed a longer duration of activity, than traditional morphine (Macht DI., 1936).Similarly, case reports published more than 50 years ago detail the use of small amounts of cobra venom for the treatment of pain related to trigeminal neuralgia (Williams EY., 1960).

Today there are numerous medications and diagnostic tools derived from snake venoms, developed on the basis of the variety of organ systems snake venoms affect, including antihypertensive agents, platelet aggregation inhibitors, fibrinogen inhibitors, and anticoagulants (Koh DC and A, Jeyaseelan K., 2006; Pal SK, and Gomes A, A., 2002). The first drug derived from snake venom, and one of a widely used group of drugs for treating high blood pressure. Homeopathy is an independent segment of CAM practices Complementary and alternative medicine (CAM) is comprised of a diverse group of medical systems, therapies, and products (Tan G et al., 2007).

OBJECTIVES

Keeping in view all the above basic information, the therapeutic, pharmacological importance of PLA2 neurotoxin and available related literature the present study on “Insilico characterization of structural and functional elements of PLA2” was undertaken with following major objectives.

Retrieval of PLA2 protein sequence Data of all the four species
Identification of possible templates for 3D structure prediction.
Prediction of 3D structures of all the four PLA2 proteins by Homology modeling.
Visualization of predicted structures using molecular viewer tool.
Validation and quality assessment of structures.
Identification of major structural and functional elements in PLA2
Characterization of structural and functional elements Insilico.

REVIEW OF LITERATURE

The snake toxins are known to be complex mixtures of organic compounds, mainly proteins, a number of which have enzymatic activities (Russell, 1984). The snake venoms are reported to containsatleast twenty five such enzymes including proteolytic enzymes, phospholipases, oxidases and the enzymes effecting circulatory system (Russell, 1983). The identification of those enzymes in past three decades not only lead to the better understanding of clinical manifestations produced by snake envenomation in human victims but also the production of drugs, antivenom and antidotes against the venom and its components.

Protein Sequence/Structure Similarity Relationships

Proteins display diverse sequence/structure similarity relationships. Understanding protein similarity relationships is vital for the annotation of genome sequences (Andrade et al., 1999; Pearl et al., 2000; Wilson et al., 2000; Todd et al., 2001). Proteins with high sequence identity and high structural similarity tend to possess functional similarity and evolutionary relationships, yet examples of proteins deviating from this general relationship of sequence/structure/ function homology are well-recognized. For example, high sequence identity but low structure similarity can occur due to conformational plasticity, mutations, solvent effects, and ligand binding. Despite this protein diversity most current surveys have focused on the expected similarity relationship where the proteins have significant sequence and structural similarity (Wilson et al., 2000; Chothia and Lesk, 1986; Russell et al., 1997; Levitt and Gerstein, 1998; Wood and Pearson, 1999). Furthermore, the physical basis of the expected sequence/structure similarity relationship remains unexplored.

Knowledge of the three-dimensional structure of a protein can provide invaluable hints about its functional and evolutionary features and in addition the structural information is useful in drug design efforts. Genome-scale sequencing projects have already produced more than 108 million individual sequences (Benson et al., 2010) but due to the inherently time consuming and complicated nature of structure determination techniques only around 53,000 of these have their 3D structures solved experimentally (Dutta et al., 2009).

Phospholipases A2 of Asian Snake Venoms

The structural and functional information of various phospholipase A2 (PLA2) isoforms purified from Asian snake venoms. A phylogenic tree of group I PLA2s was constructed herein based on many recently resolved amino acid sequences of the venom enzymes (Inn-Ho Tsai1997). The venom PLA2s in each of the subgroup show more or less functional similarity specific for the subgroup: the Glu6-PLA2s are usually antiplatelet, the Asn6-PLA2s are neurotoxic and/or myotoxic and many Arg6-PLA2s are anticoagulating, while the Lys49-PLA2s are myotoxic and edema-inducing. Mechanisms for the pharmacological actions of venom PLA2s have beendiscussed, including neurotoxicity, myotoxicity, antiplatelet activity, anticoagulating activity, heparin-binding, protein-acylation and deacylation.

Homology Modeling

Comparison of three Classes of Snake Neurotoxins by Homology Modeling and Computer Simulation Graphics (Hsueh-Fen Juan Biochemical and Biophysical Research Communications 257, 500–510, 1999).Systematic structure comparison of three major classes of postsynaptic snake toxins, which include short and long chain a-type neurotoxins plus one angusticeps-type toxin of black mamba snake family, two novel a-type neurotoxins isolated from Taiwan cobra (Najanajaatra) possessing distinct primary sequences and different postsynaptic neurotoxicities were taken as exemplars for short and long chain neurotoxins and compared with the major lethal short-chain neurotoxin in the same venom, i.e., cobrotoxin, based on the derived three-dimensional structure of this toxin in solution by NMR spectroscopy (Chiou, S. H. and Hung, 1995).

A structure comparison among these two a-neurotoxins and angusticeps-type toxin (denoted asFS2) was carried out by the secondary-structure prediction together with computer homology-modeling based on multiple sequence alignment of their primary sequences and established NMR structures of cobrotoxin and FS2 (Albrand J. P. et al., 1995).It was further found that elapid neurotoxins comprised two subclasses of neurotoxins based on their molecular sizes:i.e., short chain toxins of 60–62 residues and 4 pairs of disulfide bonds versus long chain toxins of 65–74 residues and 5 pairs of disulfide bonds (Karlsson E., 1979 and Dufton et al., 1983).

UniProtKb/Swiss-Prot

UniProtKB/Swiss-Prot contains high-quality manually annotated and non- redundant protein sequence records. Manual annotation consists of analysis, comparison and merging of all available sequences for a given protein, as well as a critical review of associated experimental and predicted data (www.uniprot.org/help/uniprotkb).

BlastP

In bioinformatics, Basic Local Alignment Search Tool, or BLAST, is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of different proteins. BLAST is a heuristic method to find the highest locally optimal Alignments (Barbera van Schaik). A BLAST search enables a researcher to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold (S. Karlin and S. F. Altschul (NCBI). The BLAST program was designed by Stephen Altschul Warren Gish, Webb Miller, Eugene Myers, and David J. Lipman at the NIH and was published in the Journal of Molecular Biology in 1990 (Altschulet et al., 1990).

PDB

There are currently over 6000 three-dimensional structures of biological macromolecule primary protein & nucleic acid in the Brookhaven protein Data Bank (Bernstein et al., 1977). The World Wide Web

provides an ideal tool for making such data readily accessible to the scientific community (Stampf.D.R.etal.,1995). PDB has its own web based search engine called PDB Browse, which allows complete text search of the entries in the PDB (http://www.Pdb.bnl.gov).

SWISS-MODEL

SWISS-MODEL is a structural bioinformatics web-serverdedicated to homology modeling of protein 3D structures. Homology modeling is currently the most accurate method to generate reliable three-dimensional protein structure models and is routinely used in many practical applications. Homology (or comparative) modeling methods make use of experimental protein structures ("templates") to build models for evolutionary related proteins ("targets").

Successful model building requires at least one experimentally determined 3D structure (template) that shows significant amino acid sequence similarity with the target sequence. Building a homology model comprises four main steps: identification of structural template(s), alignment of target sequence and template structure(s), model building, and model quality evaluation. These steps can be repeated until a satisfying modeling result is achieved. Each of the four steps requires specialized software and access to up-to-date protein sequence and structure databases.

SWISS-MODEL is a fully automated protein structure homology-modeling server, accessible via the ExPASy web server, or from the program DeepView (Swiss Pdb-Viewer). The purpose of this server is to make Protein Modelling accessible to all biochemists and molecular biologists worldwide (www.swissmodel.expasy.org).

EASY MODELLER

The present version EasyModeller 4.0 is modification over the previous version Easy Modeller 2.0. This version is simpler, fast, accurate and straightforward providing a clear view of all the steps involved in homology modeling. Homology modeling has become a key component in structural bioinformatics for prediction of the three-dimensional structure of proteins from their sequences due to availability of huge amount of protein sequence data and the growing number of known structures. It becomes even more useful when constraints from X-ray diffraction or NMR are not yet available. Many tools have been developed for homology modeling.

The aim of this tool is to help inexperienced users as well as regular users to perform modeling, assessment, visualization, and optimization of protein models in a simple and straightforward way. EasyModeller can produce 3D structural models of proteins from sequence and given templates information using MODELLER in backend.

PDB Sum

A web-based database which to complement the data already available on protein and nucleic acid structure from the various sources. The database provides a summary of the molecule in each pdb file (that is protein, nucleic acid, ligands, water molecule and metal) together with various analysis of their structural features (S. C. Alom, 1993). (http://www.ebi.ac.uk/pdbsum/).

Cysteine Motifs and Protein Stability

The formation of covalent links among cysteine (Cys) residues with disulphide bridges is an important and unique feature of protein folding and structure. Simulations (V.I. Abkevich and E.I. Shankhnovich, 2000), experiments in protein engineering (M. Matsumura et al., 1989; J. Clarke and A.R. Fersht, 2000; T.A. Klink et al.,1993), theoretical studies(S. Betz, 1993; W.J. Wedemeyer et al., 2000) and even evolutionary models (L. Demetrius, 2000) stress the importance of disulphide bonds in stabilizing the native state of proteins. Disulphide bridges may link distant portions of a protein sequence, providing strong structural constraints in the form of long-range interactions. Thus prediction/knowledge of the disulphide connectivity of a protein is important and provides essential insights into its structure and possibly also into its function and evolution.

Existing approaches to connectivity prediction use stochastic global optimization (P. Fariselli and R. Casadio, 2001), combinatorial optimization (J.L. Klepeis and C.A. Floudas, 2003) and machine learning techniques (P. Fariselli, et al., 2002; A. Vullo and P. Frasconi, 2004). The method in (P. Fariselli and R. Casadio, 2001) represents the set of potential disulphide bridges in a sequence as a complete weighted undirected graph.

The cysteine knot motif in toxins and implications for drug design

The term ``cysteine knot' was first introduced in 1993 (McDonald and Hendrickson 1993; Murray-Rust et al., 1993) in reviews describing recently determined structures for several growth factors including nerve growth factor (NGF), transforming growth factor b2 (TGFb2), and platelet derived growth factor BB (PDGF-BB). It was noted that these proteins contained an unusual motif comprising an embedded disulphide ring and a penetrating disulphide bond. The apparently knotted arrangement of disulphide bonds was associated with several b-strands in these structures. While the first published examples of cysteine knots were associated with growth factors it soon became clear that the motif is also relatively common in a range of smaller peptides. In the early 1990's the structures of several small disulphide rich proteins, including trypsin inhibitors, cone snail toxins and a novel uterotonic agent from plants called kalataB1 were determined. It turned out that the structures of these small molecules contained a knot motif very similar to that present in the growth factors. All of these molecules shared the disulphide connectivity Cys(I-IV),Cys(II-V) and Cys(III-VI), but when protein topologies were taken into account the motifs were not directly superimposable with those of the growth factors. This led to the proposal in 1994 that the family of small disulphide rich molecules, all of which adopted some inhibitory role, be referred to as inhibitor cysteine knots (Pallaghy et al., 1994) to distinguish them from the growth factor knots. This included a family of small disulfide rich proteins that had earlier been referred to as knottins (Le Nguyen et al., 1990). A review in 1995 (Isaacs, 1995) formalized the separation of cysteine knots into inhibitory or growth factor families. Several other reviews relating to cysteine knots have since been published (Narasim- han et al., 1994; Harrison and Sternberg, 1996; Norton and Pallaghy, 1998; Tamaoki et al., 1998). The main feature which has been used in the past to distinguish the two types of cysteine knots, apart from the different protein families from which they are de- rived, is that in the growth factor family the Cys(I-IV) disulfide bond penetrates the embedded ring formed by the other two disulfide bonds, while in the inhibitor family Cys(III-VI) is the penetrating disulphide bond.

Toxins containing the cysteine knot motif exhibit a range of biological activities from antimicrobial to anti-HIV to ion-channel blockade. This wide range of activities opens the possibility that some of these molecules may be useful leads in drug design applications. The synthesis and folding of conotoxins have been extensively studied because of their potential for therapeutic applications. It was originally thought that mature conotoxin sequences may not contain sufficient information to produce correctly folded material because of their small size and limited sequence conservation (Olivera et al., 1990, 1991).

The foundation for such applications has been laid by protein engineering studies designed to establish that cysteine-knot-containing peptides can accommodate sequence modifications. For example, an early study on two squash peptides showed that it was possible to generate a chimeric protein which retains activities of both proteins by grafting functional residues from one sequence onto another (Chiche et al., 1993).

The venoms of Conussnails are a complex mixture of pharmacologically active peptides. These disulphide-rich, compact peptides called conotoxins are classified into several different families based on their structure and biological activity (Jones, R. M., and Bulaj, G., 2000). Structurally, the disulphide bonds are intrinsic to the stable three-dimensional conformation and play an important role in their function. Hence, a change in the disulphide bonding pattern leads to a significant change in the overall conformation and function of the toxin.

Venominformatics approach

Cysteine Motif and Prediction Database (CMPD) is a database of cysteine flanking motifs. It entails all the Cysteine flanking and pairing motif Cysteinesextracted from Protein Databank (PDB) and UniProt. Computational approaches to disulfide bonding state and its connectivity pattern prediction are based on various descriptors. One generated descriptor is based on the sequence's amino acid composition and flanking residues around the cysteine residue. These immediate residues have been shown to influence the cysteine redox potential and the cysteine's steric accessibility (Ferrè F, Clote P., July 2006).

Since its proposal as a descriptor in 1990, these sequence motifs have been fed into various prediction methods such as machine learning approaches (i.e statistical methods, neural networks (NNs), support vector machine(SVM) and has been the basis of various prediction tools such as DiaNNA (Ferrè F, Clote P., July 2006), DISULFIND (Ceroni A. et al., July 2006). DCON (Vullo A. and Frasconi P., March 2004) and CysView (Lenffer J. et al., July 2004).

The expansion of RCSB and the increase of PDB files have significantly increased the number of motif beyond what has been utilized in prior research. Over 8,78,000 cysteine motifs were extracted from

which the users can now query more than 77,000 unique cysteine motifs and cysteine pairing motifs generated from PDB and UniProt files. CMPD query types include PDB ID, UniProt ID, sequence and motifs. These datasets are downloadable and parseable using web service and API.

3. METHODS

3.1 Retrieval of PLA2 protein sequence Data

The complete protein sequence data of neurotoxin phospholipase-A2 of all the four species were retrieved from the major protein sequence database Uniprotkb cited at (www.uniprot.org/help/uniprotkb) with their uniprot IDs recorded. UniProtkbcontains high-quality manually annotated and non-redundant protein sequence records.

3.2 Template identification and Homology

The identification of template for the structure prediction of all the four PLA2 was performed using Blastp alignment tool which is cited at NCBI. The PDB IDs of the templates with maximum percentage of similarity were identified for each PlA2 and tabulated.
(URL: http://www.ncbi.nlm.nih.gov/blastp)

3.3 Prediction of tertiary structures of PLA2

The tertiary structures of all the four PLA2proteins were predicted by homology modeling using Swiss model cited at (http://swissmodel.expasy.org/), which is fully automated online tool for structure prediction. The tool makes use of the templates with maximum percentage similarity above 90% for the structure

prediction. The prediction was carried out by setting default parameters and the predicted structures of all the PLA2 were stored in PDB format.

3.4 Visualization of 3D structures of PLA2

The predicted 3D structures of all the four PLA2 were visualized using RasMol molecular visualization tool, which is an offline structure visualization tool, developed to give detailed insights into structural elements and features of a molecule under study. All the four PLA2 structures were visualized with major structural elements being highlighted.

3.5 Structure validation and quality assessment of PLA2

The efficiency of the tool and the validation of the predicted structures of all the four PLA2 proteins were carried out by Ramachandran plot and statistics using ProCheck. The plot usually assess the quality of a structure by showinh the possible conformations of dihedrals in a polypeptide.

3.6 Analysis of Cysteine Motifs of PLA-2

The predicted 3D structures of all the four PLA2 proteins were subjected to PDB Sum and their subsequent PDB IDs were generated. The detailed structural analysis of PLA2 proteins was performed by subjecting the PDB IDs generated to PDBSum database and the major structural and functional elements of PLA2 involved in the interaction were deciphered. (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/GetPage.pl).

RESULTS AND DISCUSSION

4.1 Retrieval of PLA2 protein Sequence Data

The pure protein sequence of neurotoxin phospholipase-A2 of all the four Indian snake species were retrieved from UniProtKB protein sequence database and stored in fasta format. The Uniprot Ids of all the sequences are as mentioned in table1.

4.2 Identification of Template for tertiary structure prediction

The template identification for structure prediction of all the four PLA2 proteins was carried out by subjecting the sequences to Blastp alignment against PDB. The hits with maximum homology obtained for the corresponding toxin sequences were identified and selected as the best templates for structure prediction. The identified templates are tabulated as shown in table 2.

4.3 Prediction of Tertiary Structures of PLA2

The three dimensional structures of PLA2 of all the four species were predicted by using swiss model which is an automated structure prediction tool. The PDB templates searched were used respectively for building the structural models of all the four PLA2 proteins. The structures were obtained by setting default parameters, saved in pdb format and visualized further.

Common Name	**Scientific Name**	**Uniprot Ids**
Indian cobra	*Najanaja*	**P15445**
Indian krait	*Bungaruscaeruleus*	**Q6SLM1**
Russel's viper	***Daboiarusselii***	A8CG86
Saw-scaled viper	*Echiscarinatus*	P48650

Table: 1. UniProtKB Protein Ids of PLA-2 of four species

Name of Snake	**PDB Id**	**Maximum Identity in %**
Najanaja	1PSH A	100
Bungaruscaeruleus	1G2X A	100
Daboiarusselii	2H4C A	99
Echiscarinatus	2QHD A	100

Table: 2. Templates identified for structure prediction with PDB Ids

4.4 Visualization of 3D structures of PLA2

The modeled structures of PLA2 were visualized using RasMol molecular visualization tool. The position of cystiene residues involved in the formation of disulphide bridges are highlighted with yellow color in all the four structures respectively as shown in figures 1, 2, 3 and 4. The number of cystiene residues and their positions in proteins determines the structural stability and functional capability of proteins in protein - protein interaction.

4.5 Analysis of structure quality and validation

The quality of each structure of PLA2 generated by swiss model tool was analysed by PROCHECK tool using Ramachandra plot and statistics as shown in figures 5, 6, 7 and 8 respectively. According to the Ramachandran plot and statistics, it's obvious that the of PLA2 structure of Najanaja has 88.1% of residues in most favored regions, Bungaruscaeruleus has 91.3%, Daboiarusseliihas 69.4% and Echiscarinatushas 88.5% of residues in most favored regions respectively. It thereby depicts that the PLA2 structure of Bungaruscaeruleusis more efficient with respect to its quality followed by structures of Echiscarinatus, Najanajaand Daboiarusseliiwith least determined structure quality.

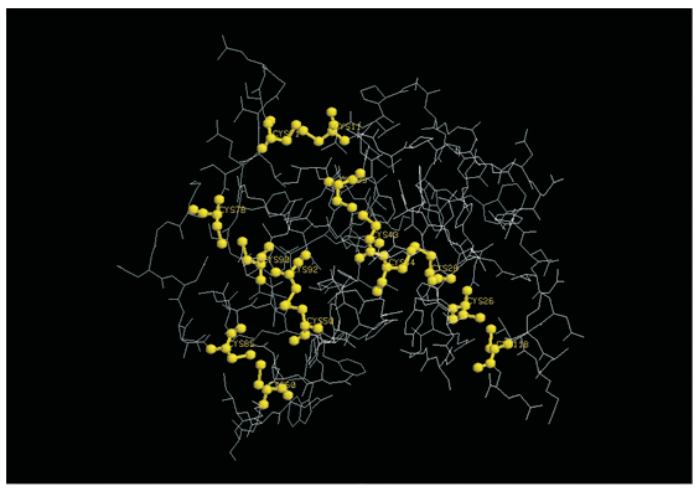

Fig. 2 3D structure of PLA2 of Najanaja

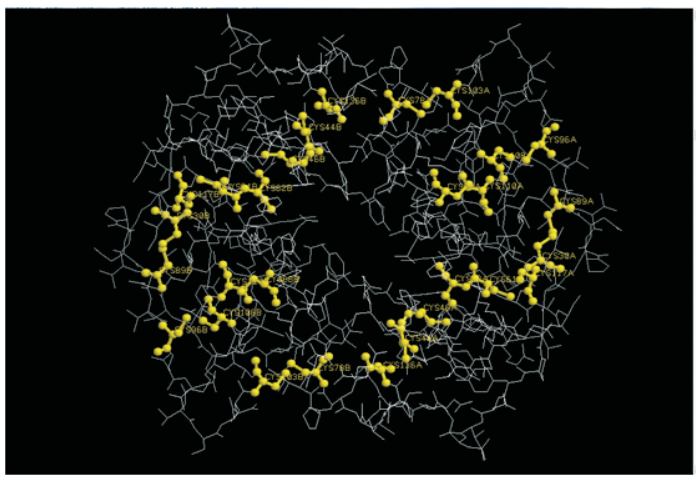

Fig. 3 3D structure of PLA2 of Bungaruscaeruleus

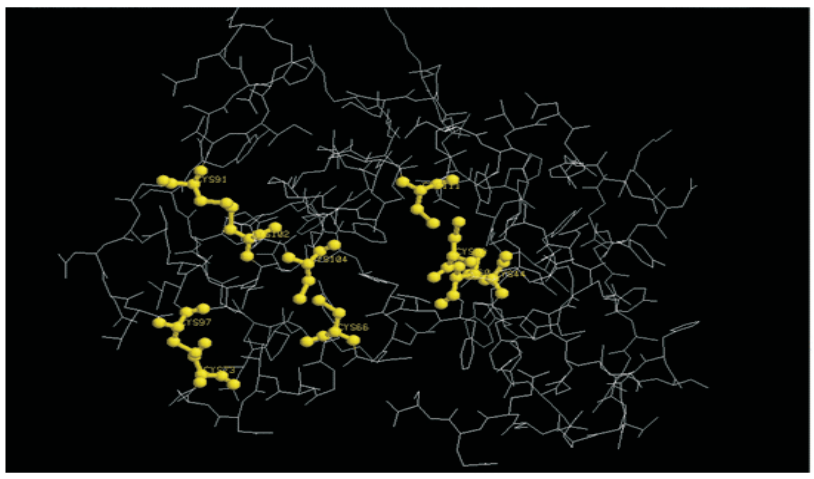

Fig. 4 3D structure of PLA2 of Daboiarusselii

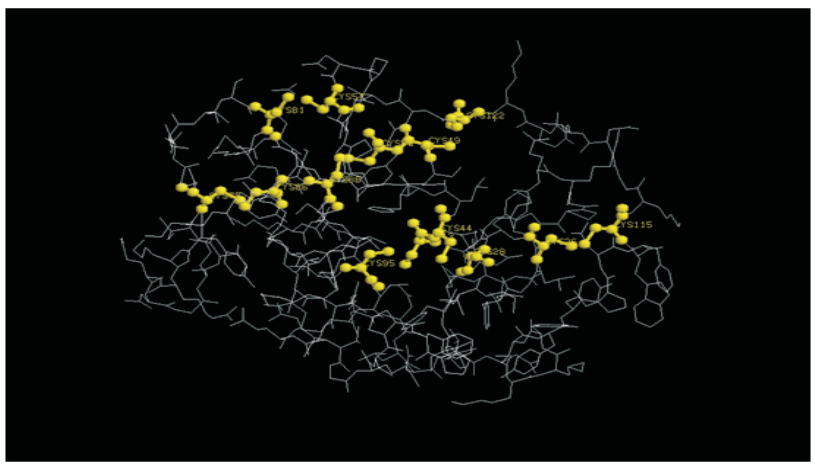

Fig. 5 3D structure of PLA2 of Echiscarinatus

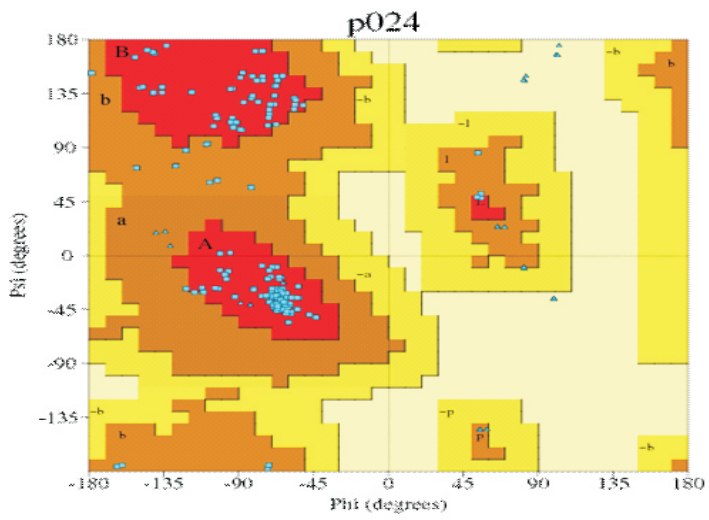

Fig. 6 Ramachandran plot of PLA2 (NajaNaja)

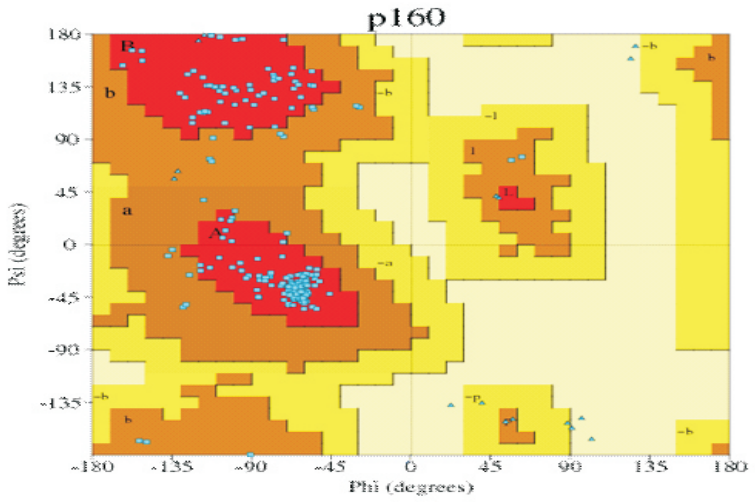

Fig. 7 Ramachandran plot of PLA2 (Bungaruscaeruleus)

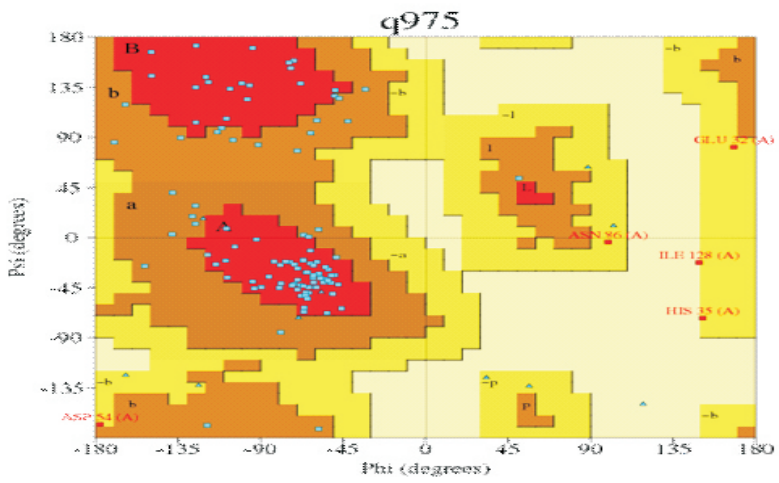

Fig. 8 Ramachandran plot of PLA2 (Daboiarusselii)

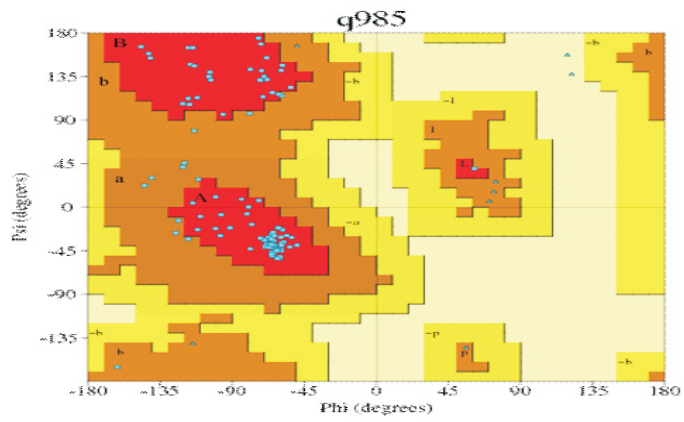

Fig. 9 Ramachandran plot of PLA2 (Echiscarinatus)

Ramachandran plot statistics

Parameters	Naja Naja		Bungarus caeruleus		Daboia russelii		Echis carinatus	
	No. of Residues	%	No. of Residues	%	No. of Residues	%	No. of Residues	%
Most favoured regions	275	88.10%	188	91.30%	75	69.40%	92	88.50%
Additional allowed regions	37	11.9%	18	8.7%	28	25.9%	12	11.5%
Generously allowed regions	0	0.0%	0	0.0%	4	3.7%	0	0.0%
Disallowed regions	0	0.0%	0	0.0%	1	0.9%	0	0.0%
Non-glycine and non-proline residues	312	100.0%	206	100.0%	108	100.0%	104	100.0%
End-residues (excl. Gly and Pro)	6		4		2		2	
Glycine residues	27		16		10		9	
Proline residues	12		10		2		7	
Total number of residues	357		236		122		122	

6 Prediction and analysis of Cysteine motifs

The prediction and arrangement of cystiene motifs in all the four PLA2 was carried out by using PDBsum and represented as shown in figure 9, 10, 11 and 12 respectively. The analysis of all the cysteine motifs in all the four PLA2 like position of each motif, length, number of charged residues and hydrophobic residues found within the motif was determined and tabulated as shown in table 3, 4, 5 and 6. The analysis depicts that in PLA2 of Najanaja, out of 7 cysteine motifs, the motif positioned from 43 to 99 possess most number of charged and hydrophobic residues, while in PLA2 of Bungaruscaeruleus, Daboiarusseliiand Echiscarinatusthe motifs positioned at 61-117, 66-104 and 49-122 possess most number of charged and hydrophobic residues respectively.

Representing cystiene motifs of PLA2

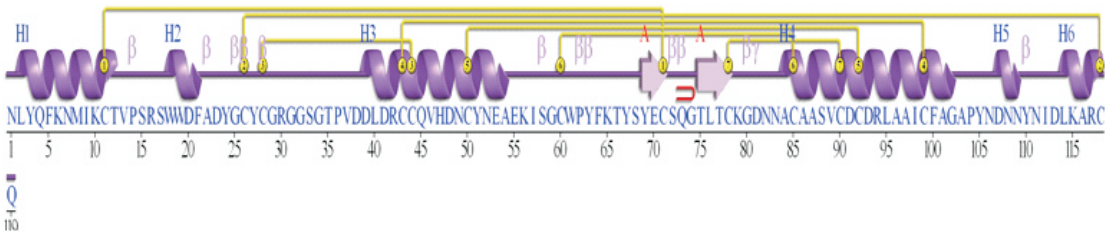

Fig. 10 Representingcystiene motifs of PLA2 of NajaNaja

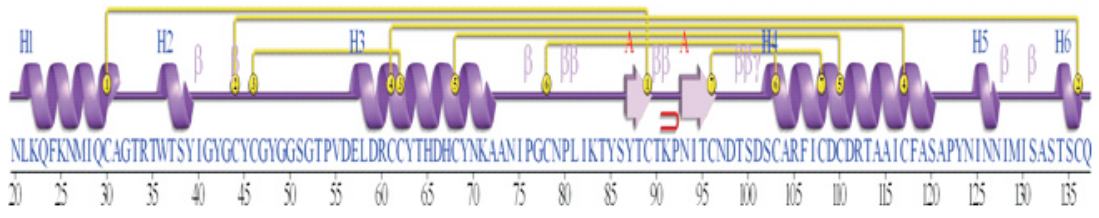

Fig. 11 Representingcystiene motifs of PLA2 of Bungaruscaeruleus

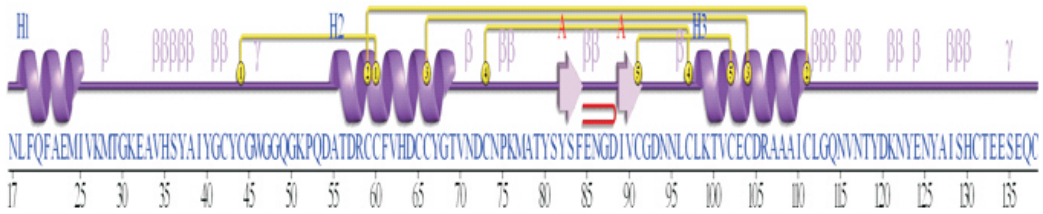

Fig. 12 Representingcystiene motifs of PLA2 of Daboiarusselii

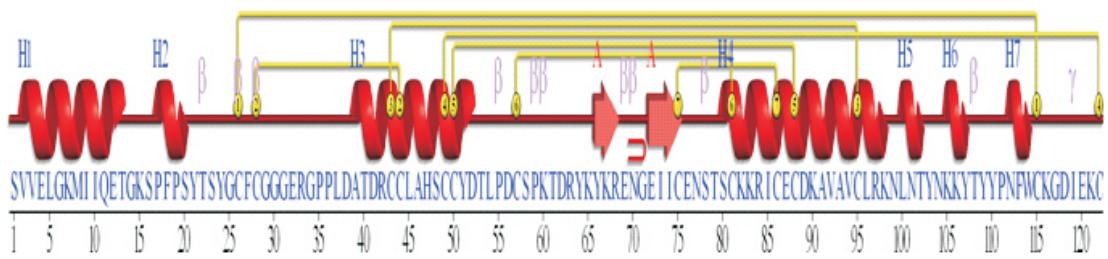

Fig. 13 Representingcystiene motifs of PLA2 of Echiscarinatus

Motif	Position	Motif Length	No. of Charged Residues	No. of Hydrophobic Residues
1	11-71	61	15	17
2	26-118	93	21	25
3	28-44	17	5	5
4	**43-99**	**57**	**12**	**18**
5	50-92	43	8	14
6	60-85	26	4	7
7	78-90	13	2	4

Table 3. Showing details of cysteine motifs of PLA2 (Najanaja)

Motif	Position	Motif Length	No. of Charged Residues	No. of Hydrophobic Residues
1	30-89	55	10	15
2	44-136	93	16	26
3	46-62	17	4	4
4	**61-117**	**57**	**12**	**17**
5	68-110	43	7	13
6	78-103	26	4	6
7	96-108	13	3	5

Table 4. Showing details of cysteine motifs of PLA2 (Bungaruscaeruleus)

Motif	Position	Motif Length	No. of Charged Residues	No. of Hydrophobic Residues
1	44-60	17	4	4
2	59-111	53	11	21
3	**66-104**	**39**	**7**	**14**
4	73-97	25	4	8
5	91-102	12	2	6

Table 5. Showing details of cysteine motifs of PLA2 (Daboiarusselii)

Motif	Position	Motif Length	No. of Charged Residues	No. of Hydrophobic Residues
1	26-115	90	26	25
2	28-44	17	5	4
3	43-95	53	18	17
4	**49-122**	**75**	**25**	**21**
5	50-88	39	15	8
6	57-81	25	9	5
7	75-86	12	4	4

Table 6. Showing details of cysteine motifs of PLA2 (Echiscarinatus)

SUMMARY AND CONCLUSION

Venom is highly modified salivacontaining zootoxins used by snakes to immobilize and digest prey or to serve as a defense mechanism against a potential predator or other threat. Venoms contain more than 20 different compounds, mostly proteins and polypeptides. The Pharmacological and biochemical properties of these components in venoms are studied in many snakes, however not yet every toxic ingredient in Indian snake venoms have been isolated and characterized for pharmacological perspectives. With this intension, an attempt was made to put the insights into structural and functional elements of snake venom neurotoxin PLA2 by retrieving the primary sequence data of all the four PLA2 proteins from the major protein sequence database. The 3D structures of all the four PLA2 proteins were predicted by homology modeling using Swiss model tool. Prior the template identification for all the four PLA2 was performed and the template with maximum percentage identity was selected for each one. The predicted 3D structures of all the four PLA2 proteins were visualized using RasMol molecular viewer tool with cystiene residues being highlighted. The validity and quality assessment of predicted structures was performed using Procheck with Ramachandran plot and statistics. The PLA2 of all the four species were characterized with respect to their structural and functional elements using PDBSum database. The major structural elements cystiene motifs and their functional elements like charged and hydrophobic residues found within them were characterized and tabulated.

The present investigations can put insights into the structural features of PLA2 as there are no single 3D structures of this venom neurotoxin experimentally determined and deposited in structural

database like PDB. As sited in the above literature, snake venoms are being exploited for their potent biological functions and employed for treatment strategies in a crude way. This insilico approach of characterizing the neurotoxin PLA2 with its cystiene motifs being most potent structural and functional element of any protein toxin with maximum number of charged and hydrophobic residues forming the stable helical conformation and highly interactive surfaces. The further detailed investigations of these cysteine motifs can lead to the discovery of some novel venom peptides with more specific and targeted functions as potent therapeutic and pharmacological agents for the treatment of various systemic diseases. This may contribute for the new era of peptide drugs.

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