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Understanding the Structures and Functions of Snake Venom Cardiotoxins.

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ABSTRACT

Cardiotoxins belonging to three-finger toxin (TFT) superfamily of snake venoms are basic, monomeric and simple β-sheet fold proteins consisting of 59 - 62 amino acids with four conserved disulfide bridges. The protein toxins are highly abundant among the TFTs and exhibit a wide array of biological activities such as cytolysis, haemolysis, cardiac muscle damages and membrane depolarization. The main focus of the present study is to clearly summarize the salient structural features and classifications of the cardiotoxins and as well systematically analyse the multiple functions of unique topology of the protein toxins.

Keywords: Anti-venom, Cardiotoxin, Three-finger fold, Snake venom.

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INTRODUCTION

Snake venoms are rich of complex mixture of over 100 pharmacological active polypeptides and enzymes [1]. A wide diversity of biological activities exists in snake venoms and each of these activities is generally accounted by a family of proteins present in the venom. Therefore, snake venoms contain an extraordinary source of 'proteic lancets' ideal for identifying critical receptors and target molecules in biological processes [2]. Most of the biomacromolecules have been grouped into any one of the six super-families: i) Three-finger toxin (TFT) family ii) Proteinase inhibitor family iii) Lectin family iv) Phospholipase A2 family v) Serine proteinase family and vi) Metalloproteinase family [3, 4]. However, the lethal actions of the snake venoms are primarily due to the presence of basic polypeptides belonging to three-finger toxin (TFT) superfamily. Protein toxins belonging to the TFT superfamily have been reported to exist in all families of snakes [5]. Most of the TFTs are single polypeptide chain consisting of 59 - 74 standard amino acids and all β-sheet proteins. Of the many groups of the TFT superfamily, cardiotoxins (CTXs) are most abundant and one of main principal toxic components of snake venoms. Moreover, the CTXs are well-characterized proteins among the TFT superfamily, in terms of 3D structures, stabilities and functions, to date [6, 7]. The CTXs exhibit cytolysis, haemolysis, cardiac muscle damages and membrane depolarization. Notwithstanding many hypotheses proposed to facilitate for understanding the mechanism by which the CTXs may act on their target molecules, no clear-cut experimental evidences are yet available to authenticate any of these hypotheses [8-11].

In the present study, all CTXs reported to date have been critically analyzed in terms of primary structures and 3D folds. Moreover, classifications of CTXs, evolution of the protein toxins as probed by phylogenetic methods and various hypotheses proposed to describe the biological functions of the CTXs have also been brought into fore.

Structures of snake venom cardiotoxins

Cardiotoxins are the most abundant and principal toxic component of three-finger toxin superfamily, which is present in all venomous snakes. To date, 83 authentically annotated primary structures of the CTXs purified from various species of snakes have been reported in the literature. The CTXs are from elapid snake species such as *Naja atra*, *Naja naja*, *Hemachatus haemachatus*, *Naja sputatrix*, *Naja sagittifera*, *Naja pallida*, *Naja oxiana*, *Naja nivea*, *Naja mossambica*, *Naja melanoleuca*, *Naja kaouthia*, *Naja haje annulifera* and *Naja haje haje*. The CTXs exhibit several salient amino acids composition as explained herein. The primary structures of the CTXs are constituted by 59 – 62 amino acids: of 83 sequences, 69 sequences have 60 residues, 7 sequences have 61 residues, 6 sequences have 62 residues and only one sequence (Q9PS33) is characterized to have 59 residues. Similarly, of 83 CTX sequences known to date, 81 CTXs begin with 'Leucine' residue and only 2 CTXs (P01443, Q9W6W9) begin with 'Arginine' residue. Interestingly, all CTXs invariably end with a pair of Cys - Asn ('Cysteine' and 'Asparagine') residues as illustrated from multiple sequence alignments of the CTXs (Figure 1).

In addition to the above sequence features, the CTXs depict unique patterns for most conserved and as well for most variable residues in the primary structures. Residues such as Cys3, Pro8, Cys14, Gly17, Leu20, Cys21, Arg36, Cys38, Pro43, Ser46, Cys53, Cys54 and Cys59 are conserved in all CTXs (Figure 1). In contrast, most variable positions of CTXs are found to be 9, 10, 16, 28, 29, 30 and 31 residue positions as illustrated in multiple sequence alignments of the protein toxins (Figure 1). Most of CTXs (65 out of 83 sequences) consists of I-D-V signature tri-peptide at positions 39 – 41 and other 18 CTXs are comprising of different types of tri-peptide sequences such as I-D-A or I-N-V or A-D-N or A-A-T or A-D-A or T-D-A or T-D-T in the counter positions [12]. In addition, a few distinct CTXs differing from common characteristic features of CTXs could also be noticed. For instance, a CTX bearing UniProt ID O93472 differs from other CTXs by having a 'Tyrosine' residue at position of 42 in the sequence. In this context, it should be pointed out that all CTXs invariable have 2 'Tyrosine' residues at 22 and 51 positions. Similarly, presence of single 'Histidine' and single 'Glutamic acid' residues were found only in 28 and 34 of total 83 CTX sequences known to date, respectively (Figure 1).

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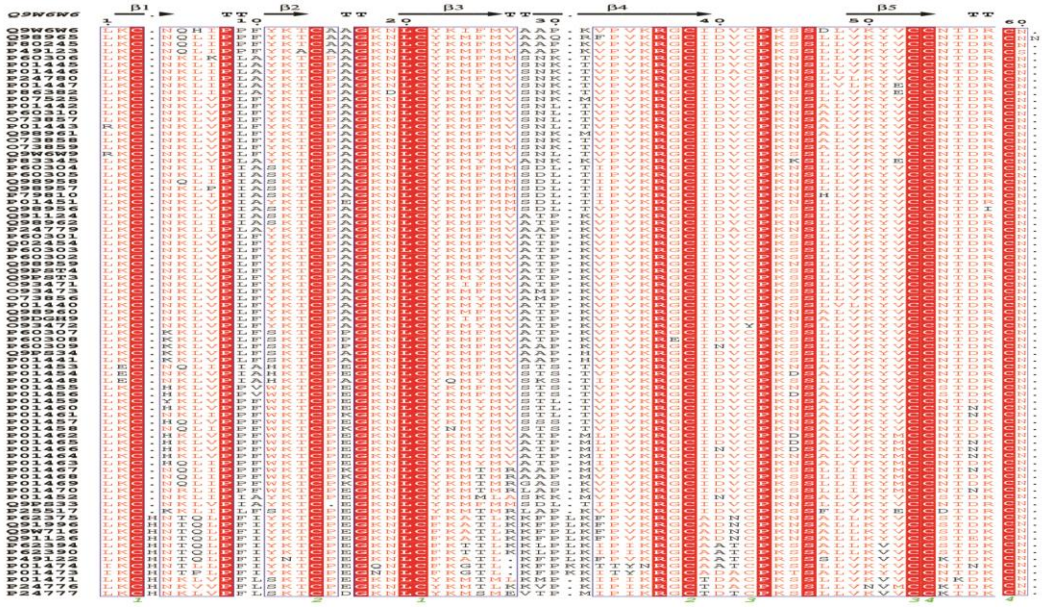

Figure 1: Multiple sequence alignments of the 83 CTXs reported in the literature to date as determined by using Multalin.

As on Dec 2015, 20 three-dimensional (3D) structures of the CTXs determined by experimental methods have been deposited in the 'protein data bank' (3VTS, 1TGX, 1CDT, 1CVO, 1CXN, 2CCX, 1CRE, 2CDX, 2CRT, 1KBS, 1KXI, 1CCQ, 1CHV, 1FFJ, 1I02, 1H0J, 1UG4, 1XT3, 1ZAD and 2BHI). The CTXs depict simple β-sheet folds: five anti-parallel strands, three loops, a globular head and an unstructured C-terminal segment [13]. The CTXs are popularly referred as 'three-finger proteins' as the backbones of the toxins folds into three loops emerging from a globular head (Figure 2). In general, the three loops of the CTXs denoted as 'Loop I', 'Loop II' and 'Loop III' were composed of residues from 1 to 15, 20 to 39 and 44 to 54, respectively and rest of amino acids constitute globular heads of the protein toxins (Figure 2). The secondary structures of the CTXs are formed of 5 anti-parallel β-stands, which align such a way into a double-stranded (comprising of stand I & II) and a triple stranded domain (comprising of strand III, IV & V). However, a CTX bearing PDB ID of 1CHV has been reported to have only three β-strands forming a triple stranded domain of the protein. The two β-sheet domains of the CTXs are tightly tied by 4 disulfide bonds (Cys3 - Cys21, Cys14 - Cys20, Cys42 - Cys53 and Cys54 - Cys59) in a compact manner and the pattern of the disulfide bonds is one of main structural forces responsible for extraordinary structural rigidity and unfolding stabilities of the protein toxins. It is also worthy of mentioning that structural stabilities and folding pathways of a few CTXs have been characterized at molecular and as well residue level resolution by using various biophysical methods including multi-dimensional NMR techniques [14-19].

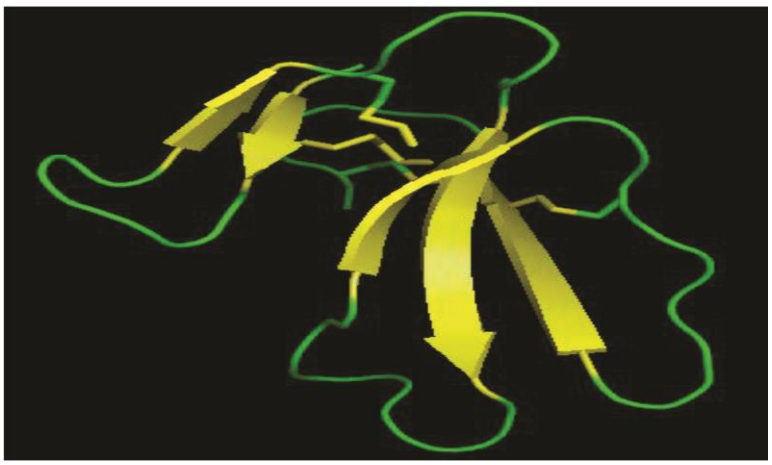

Figure 2: Three-dimensional structure of CTX VI (1UG4) from *Naja atra* is depicted with strands and unstructured regions in yellow and green colors, respectively.

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Classifications of the CTXs and phylogenetic analyses

Cardiotoxins are classified into different groups on the basis of structural architectures and biological interactions of the proteins with lipid bilayers. There are two types of CTXs on the basis of 'Loop I' orientations of the proteins: Group I & Group II [20]. The CTXs belonging to Group I have two proline residues with *cis*-peptide bond conformation in 'Loop I', whereas CTXs of Group II consist of only one proline residue in the 'Loop I'. The CTXs belonging to the two Groups are characterized to have distinct circular dichroism spectra from each other. The Group I CTXs exhibit intense positive band at 192 nm and negative minima at around 218 nm. Contrary, CTXs of Group II exhibit a broad positive band near 225 nm and negative minima at around 212 nm in Far-UV CD experiments. From structural standpoints, it has been represented that the 'Loop I' of the Group I CTXs has a 'banana-twist' shape, whereas the counter region of Group II CTXs has more extended structure. In addition, existence of two different conformations of CTXs due to *cis-trans* proline isomerizations, especially at a peptide bond between Xaa – Pro8, has also been documented in the literature [21].

On the basis of differential binding interactions of the CTXs with the bilayers, the CTXs could be classified into P-type CTXs and S-type CTXs. The P-type and S-type CTXs have invariably 'proline' and 'serine' residues at positions 30 and 28 in their primary structures, respectively [22]. It has also been demonstrated that while both types of CTXs strongly interacted with anionic phospholipids, P-type CTXs only showed strong perturbation with the zwitterionic phospholipids. However, specific structural contacts rationalizing differential affinities between the CTXs and phospholipids have not yet been understood at high resolutions. Interestingly, S-type CTXs have further classified into SK-type and SL-type having invariably 'lysine' and 'leucine' residues at 30th positions in their primary structures [23]. The SK-type CTXs have been demonstrated to exhibit higher cytolytic activity than that of P-type CTXs. The differential activities of the CTXs could be attributed to the presence of positively charged amino acid, Lys30, at the loop II of S-type CTXs suggesting prerequisite electrostatic interactions between the polar heads of lipid moieties and 'Loop II' of the CTXs for exhibiting stronger membrane lytic activities.

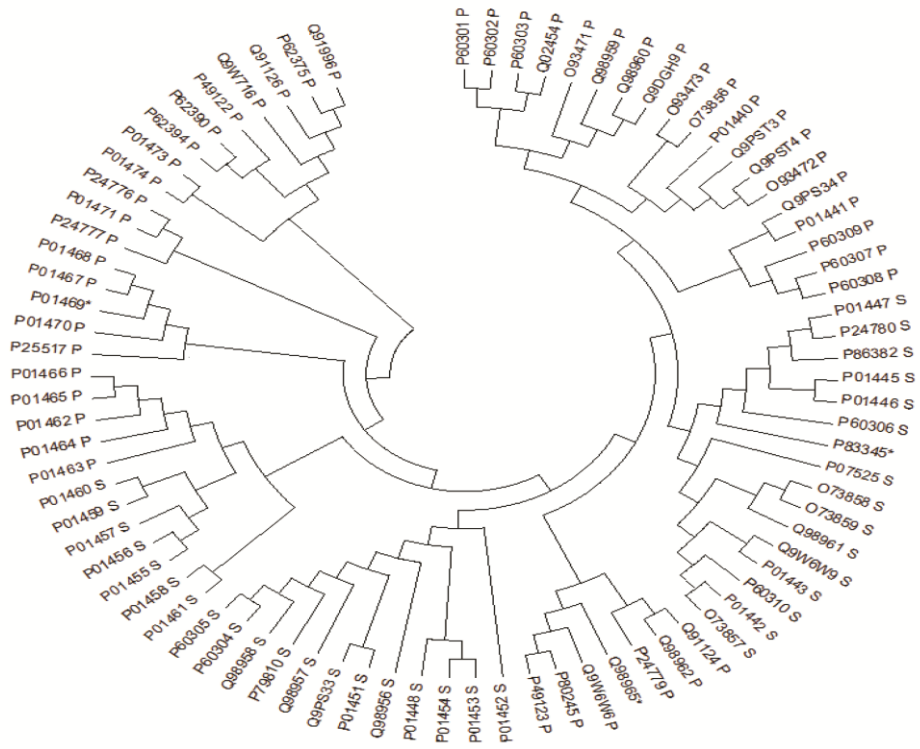

Figure 3: Phylogenetic tree of the 83 CTXs from various species of elapid snake venoms is depicted. The tree was constructed using MEGA 5.05, which employed 'NJ' method followed by 'bootstrapping' refinements.

As mentioned in the previous sections, of 83 authentically annotated primary structures of the CTXs purified from various species of snakes, there were 46 P-type cardiotoxins and 34 S-type cardiotoxins.

However, CTX2 (P01469) from *Naja mossambica,* CTX6 (Q98965) from *Naja atra* and CTX (P83345) from *Naja sagittifera* could be annotated neither as P-type nor as S-type classes since the 3 sequences show absence of the salient features essential for the P-type and S-type classifications. In these contexts, a phylogenetic tree was constructed for all the 83 CTXs by using MEGA 5.05, which employed 'Neighbour joining (NJ)' method followed by bootstrapping refinements (Figure 3). The phylogenetic tree did not differentiate the P-type and S-type CTXs into two distinct clades. However, 6 possible clades could be arbitrarily defined for the phylogenetic tree of the CTXs: of the 6 clades, three clades were exclusively for P-type CTXs, two clades were exclusively for S-type CTXs and one clade showed mixed P-type and S-type CTXs. Strikingly, of the three ambiguous CTXs, two CTXs (Q98965, P01469) were found to be branched in clades representing exclusively P-type CTXs and another CTX (P83345) was found to be branched in a clade representing exclusively S-type CTXs. However, it would be favorable to confirm the evolutionary relationships of the 3 ambiguous CTXs through experimental evidences and as well through structural standpoints.

Biological functions of the CTXs

The CTXs exhibit a wide range of biological activities such as systolic heart arrest, blindness by corneal opacification, membrane depolarisations and lysis of erythrocytes by interacting with components on the cell membranes [24-26]. Exact mechanisms by which the CTXs act on the target have not yet been clearly understood at residue level. However, many hypothesizes have been proposed on understanding the mechanism by which the CTXs act on their target molecules. For instance, according to the 'Binding-Penetration model', the CTXs bind to the negatively charged centers located on the RBC membrane surface owing to their highly basic nature (pI>10) and penetrate into membrane in an 'edgewise' orientation through hydrophobic interactions [26-30]. According to the 'Pore-formation model' the CTXs bind to the anionic centers located on the erythrocyte membrane and to promote oligomerization of certain proteins leading to formation of a pore or a channel of limited dimension permeable to both cations and anions [31]. In addition to these models, 'Calcium channel activation model' and 'Membrane permeability model' have also been proposed to explain the activities of the CTXs. However, no clear-cut experimental supports are yet available to authenticate these hypotheses on the cytolytic activities of the CTXs.

The membrane binding abilities of a few numbers of CTXs have also been characterized by using both experimental and computational methods. Binding interactions of six isoforms of CTXs from *Naja atra* on DMPA (dimyristoylphosphatidic acid) had been investigated using fluorescence and calorimetric techniques and showed that lipid-binding affinity of CTXs did not positively correlate with their membrane-damaging effect [32]. Structural interactions of CTX III from *Naja atra* with dipalmitoylphosphatidylcholine (DPPC) and dodecylphosphocholine (DPC) membrane layers have been studied using multidimensional NMR techniques and reported that hydrophobic residues of Leu6, Val7, Pro8, Leu9, Phe10 and Tyr11 from loop I, Val27, Ala28, Pro30, Lys31, Val32, Pro33, Val34 and Lys35 from loop II and Leu47, Leu48, and Val49 from loop III interacted with the hydrophobic tail of the lipids micelles [33]. Similarly, the interactions of CTX I (S-type) and CTX II (P-type) from *Naja oxiana* on negatively charged lipids (dipalmitoyl phosphatidylglycerol - DPPG) had been examined using ^{31}P-NMR methods and demonstrated that the P-type CTX II interacted with the lipid molecules much more strongly than that of the S-type CTX I [34]. However, structural segments responsible for interacting with membrane bilayers have not yet been identified unambiguously at residue level. In these backgrounds, we trust that the review will be very useful for toxicologists, bioinformatists and structural biologists to conduct exciting research on understanding the structural determinants of CTXs to elicit the membrane lytic activities in near future.

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