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CONTENTS

MOLECULAR MODEL OF CYTOTOXIN-1 FROM *NAJA MOSSAMBICA MOSSAMBICA* VENOM IN COMPLEX WITH CHYMOTRYPSIN

Aisha Munawar[1] · Ahmed Akrem[2] · Ashiq Hussain[3]
Patrick Spencer[4] · Christian Betzel[5]

1. *Department of Chemistry, University of Engineering and Technology, G. T. Road, PK-54890 Lahore (Pakistan)*
2. *Botany Division, Institute of Pure and Applied Biology, Bahauddin Zakariya University, PK-60800 Multan (Pakistan). Email: ahmedakrem@hotmail.com*
3. *Ashiq Hussain, Max-Planck-Institute for Neurobiology, Am Klopferspitz 18, D-82152, Martinsried (Germany). Email: ahussain@neuro.mpg.de*
4. *Biotechnology Centre, Institute of Nuclear Energy Research, Linnaeus Prestes Avenue, 2242 São Paulo (Brazil). E-mail: pspencer@IPEN.br*
5. *University of Hamburg, Department of Chemistry, Laboratory for Structural Biology of Infection and Inflammation, c/o DESY. Bldg. 22a, Notkestrasse 85, D-22607 Hamburg (Germany). Email: Christian.Betzel@uni-hamburg.de*
Corresponding author: Aisha Munawar. E-mail: aisha.munawar@uet.edu.pk

Keywords: Antitumor activity, Chymotrypsin, Cytotoxin-1, *Naja mossambica mossambica*, 20S Proteasome.

Abstract: *Snake venom is a myriad of biologically active proteins and peptides. Three finger toxins are highly conserved in their molecular structure, but interestingly possess diverse biological functions. During the course of evolution the introduction of subtle mutations in loop regions and slight variations in the three dimensional structure, has resulted in their functional versatility. Cytotoxin-1 (UniProt ID: P01467), isolated from Naja mossambica mossambica, showed the potential to inhibit chymotrypsin and the chymotryptic activity of the 20S proteasome. In the present work we describe a molecular model of cytotoxin-1 in complex with chymotrypsin, prepared by the online server ClusPro. Analysis of the molecular model shows that Cytotoxin-1 (P01467) binds to chymotrypsin through its loop I located near the N-terminus. The concave side of loop I of the toxin fits well in the substrate binding pocket of the protease. We propose Phe[10] as the dedicated P1 site of the ligand. Being a potent inhibitor of the 20S proteasome, cytotoxin-1 (P01467) can serve as a potential antitumor agent. Already snake venom cytotoxins have been investigated for their ability as an anticancer agent. The molecular model of cytotoxin-1 in complex with chymotrypsin provides important information towards understanding the complex formation.*

1. Introduction

Snake venom is a complex mixture of biologically active proteins and peptides. Among the non-enzymatic components of the snake venom, three finger tox-

ins belong to the well characterized polypeptides. There are two main types of three finger toxins, neurotoxins and cytotoxins, particularly found in elapid venoms [1]. Apart from that they have also been reported to be present in viperid venoms [2]. These two toxins are structurally similar, but differ significantly in their functions [3, 4], leading to structure-function complexities. The dedicated receptors for neurotoxins have been deorphanized e.g., nicotinic acetylcholine receptors [5]. The neurotoxins specifically antagonize these receptors, inhibiting the propagation of neurotransmission across the neuromuscular junction [5, 6]. On the other hand, snake venom cytotoxins (CTs) do not have specific receptors, but rather form ion channels in the cell membrane [4]. These cells, including certain type of tumour cells, may undergo necrosis, in which they lose membrane integrity and rapidly die as a result of cell lysis [7, 8]. Studies have shown that cytotoxins also manifest complex activities within the cell like interaction with intracellular organelles e.g., mitochondrial lysosomes [9, 10]. Cytotoxins from *Naja atra* and *Naja kaouthia* have been reported to inhibit the protein kinase C and this activity is considered of much importance to stop the proliferation of several cancer cell types [11-13]. The cytotoxic and haemophilic properties of CTs help in cell membrane diffusion. Numerous studies have been performed to delineate the molecular mechanism involving the CTs binding with biological membranes [9]. Cytotoxins have been classified into two types, P-type and S-type [14]. The P-type CTs are classified on the basis of the Pro-31 residue and S-type is based on the S-32 residue. The P-type CTs bind to the membrane through all three loops and have haemolytic activity, while the S-type CTs bind to the membrane through only one of the three loops and show higher cytolytic activity [15, 7]. These CTs possess a similar backbone of a globular core with four disulphide bridges and three fingers emerging from the core, yet they exhibit different biological activities [8]. Minor changes in the loop region and subtle differences in the molecular structure are responsible for the variation in biological activities of these CTs [16, 3]. Numerous studies have been made to analyse the potential of these cytotoxins as anti-cancerous agents [9, 17-20]. Furthermore a cytotoxin, NN-32, isolated from *Naja naja* venom, was reported to have antioxidant and anticancer activity and it was shown that the anti-cancerous activity in mice model was mediated through its apoptogenic and antioxidant properties [21]. Most of the reports have indicated that both membrane proteins and phospholipids of the cell membrane are receptors of these CTs [22]. Despite these findings, the mechanism for the intracellular diffusion of CTs leading to anti-cancerous potential remains to be fully understood. In this regard, the present study was planned to understand the molecular interactions between chymotrypsin and its inhibitor cytotoxin-1.

In a previous study we have shown the inhibitory activity of cytotoxin-1 (P01467), isolated from *Naja m. mossambica*, towards chymotrypsin and the 20S-proteasome [23]. Here we report the molecular model of cytotoxins-1 in complex with chymotrypsin, in order to analyze the protein-ligand binding site.

2. Methods

The NMR structure of cytotoxin-1 (PDB ID: 2CCX) was used to model the complex with chymotrypsin. ClusPro (http://cluspro.bu.edu) [24-28], a fully automated pro-

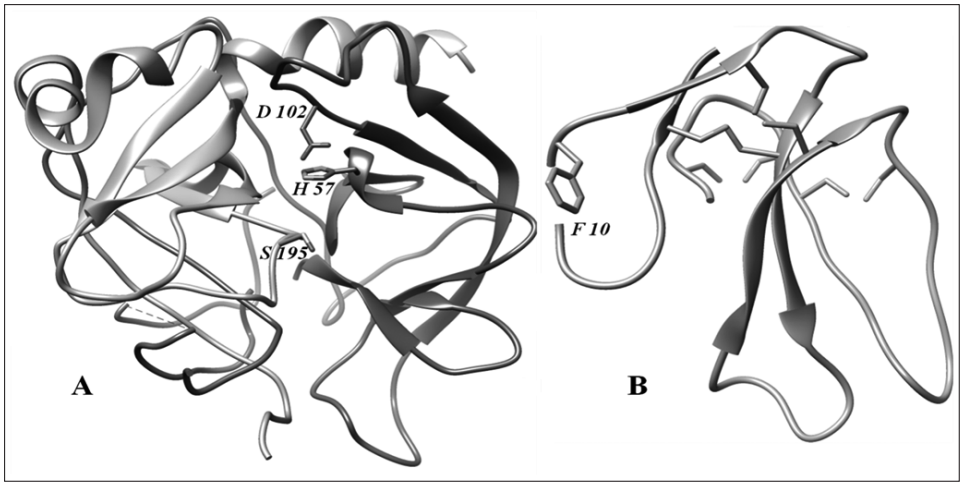

FIG. 1. a: Chymotrypsin showing the catalytic triad (His 57, Asp 102 and Ser 195) embedded between two beta barrels; b: Structure of cytotoxin-1. Cysteines forming the four disulfide bridges and the key residue phenylalanine (F 10) are shown as sticks.

tein-protein docking online server was used to prepare the complex. The coordinates of the two molecules *i.e.*, bovine alpha-chymotrypsin (PDB ID: 1MTN) and cytotoxin-1 (PDB ID: 2CCX), were uploaded to this server. Chymotrypsin was uploaded as receptor while cytotoxin-1 as ligand. As a result of protein-protein docking the model of the complex was generated. In total 20 different models were generated. The 10 top-ranked complexes from ClusPro were further analyzed, based on prior knowledge of active site interactions. The model illustrating interactions of the ligand with the active site of protein was selected. The server *PDBsum* (www.ebi.ac.uk/pdbsum/) [29] was used to study the interactions across the protein-protein interface.

3. RESULTS

Chymotrypsin, a serine protease, is initially synthesized as a 245 amino acid inactive precursor (a zymogen) termed chymotrypsinogen. This zymogen has two six stranded beta barrels with the active site located between them [30]. The residues His^{57}, Asp^{102} and Ser^{195} form the catalytic triad as shown in Figure 1a. The structure of cytotoxin-1 has been described previously [31]. This polypeptide is made up of 60 amino acids and the secondary structure consists of five β-sheets, stabilized by four disulfide bonds as shown in Figure 1b. The inhibitor-protease complex of cytotoxin-1 in complex with chymotrypsin is shown in Figure 2. Here we show that cytotoxin-1 binds to the chymotrypsin through its N-terminus via loop 1. This loop of cytotoxin-1 interacts with the active site of chymotrypsin. Literature studies have shown that, if any of the residues, such as Leu, Met, Phe, Tyr, Trp or Asn is present at P1 site of the peptide the inhibitor tends to inhibit chymotrypsin [30]. The main

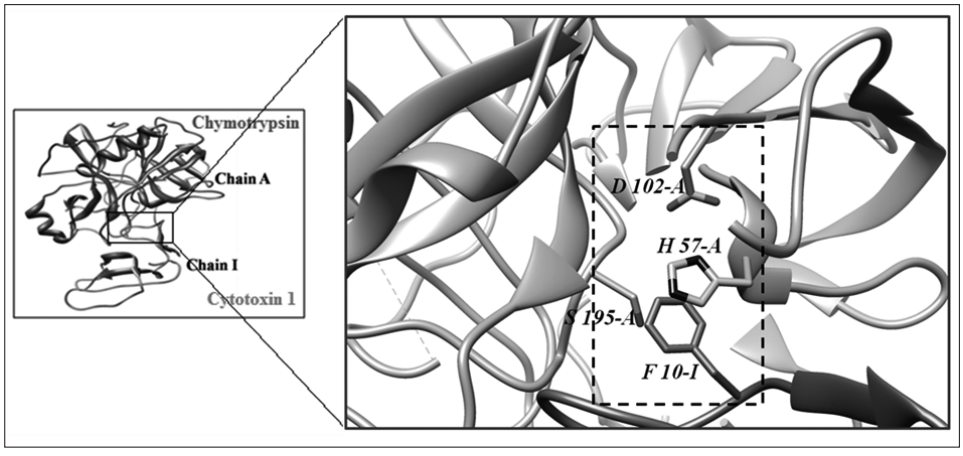

FIG. 2. Molecular model of bovine alpha-chymotrypsin in complex with snake venom cytotoxin-1. Zoom, side chains of residues involved in interactions are shown in ball and stick mode.

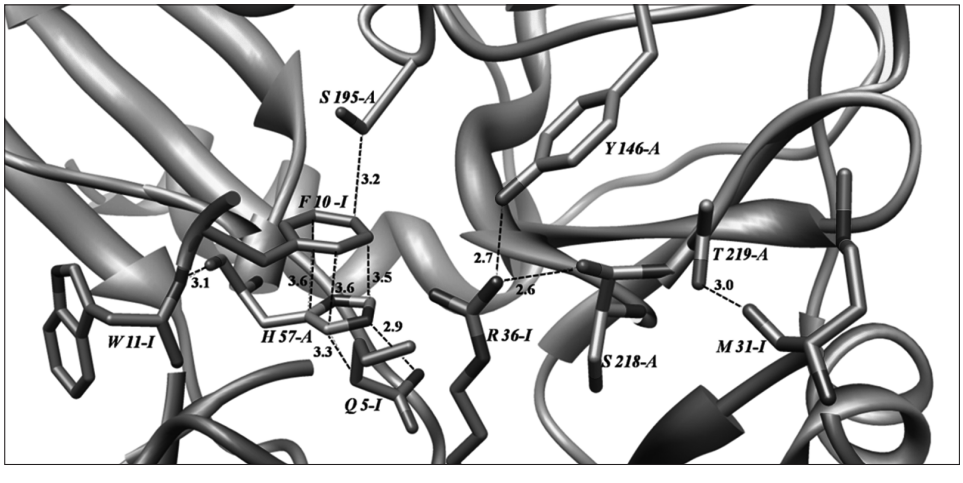

FIG. 3. Proposed key interactions of the cytotoxin-1 and chymotrypsin model complex are highlighted.

interactions at the protease-inhibitor interface are illustrated in Figure 3 and are summarized in Table 1 and 2.

The model of the protease-inhibitor complex shows the possible sites of interaction and illustrates the insertion of the cytotoxins-1's hydrophobic residue Phe^{10} in the catalytic triad of chymotrypsin as shown in Figure 2. The S1 pocket neighbours the catalytic triad. Phe^{10} of cytotoxins 1 forms a pi stacked interaction with His^{57} in a parallel orientation. This pi stacking of aromatic rings is known to reduce the energy of the system and thereby further stabilize the complex [32]. In addition to this, Phe^{10} also makes non covalent bond contacts with Ser^{195}. 19 amino acids from chy-

motrypsin and 14 from cytotoxin-1 are involved in non covalent bond interactions. The side chain of Gln^{5} (cytotoxins 1) forms a hydrogen bond with the side chain of His^{57} of chymotrypsin. Trp^{11} forms a hydrogen bond with main chain of His^{57} through its backbone nitrogen. Other residues of cytotoxin-1 are also forming hydrogen bonds and Van der Waals contacts with surface residues of chymotrypsin, further stabilizing the complex (FIG. 3; TABS 1, 2). Table 2 summarizes Van der Waals contacts at the interface of the complex, which play an indispensible role to stabilize the complex. The LIGPLOT diagram of the modelled complex interface also illustrates the interactions of residues near the N-terminus of the CT1 with the active site of chymotrypsin (FIG. 4). In this diagram the hydrogen bonding is shown by green dotted lines. The LIGPLOT (FIG. 4) also demonstrates non covalent bond contacts between protein residues and atoms of the CT1. Protein residues located in the interface region are represented by arcs with spokes pointing towards the ligand atoms, while the ligand atoms involved in these contacts are shown with spokes radiating back. The non covalent contacts play an important role in stabilizing the three dimensional structure of the protein-ligand complex [33, 34]. From the model, Phe^{10} can be proposed to be the P1 site, as it interacts with side chain (The aromatic ring) of His^{57} present adjacent to the S1 pocket. All these interactions of the cytotoxin-1 with bovine alpha chymotrypsin result in a deactivation of the enzyme, as water molecules cannot approach the active site of the enzyme to support the hydrolytic action of the enzyme. Furthermore, as Phe^{10} forms a pi stacking interaction with the side chain of His^{57}, the Og of Ser^{195} cannot form the native H-bond with the side chain of His57, essential to facilitate the hydrolytic process.

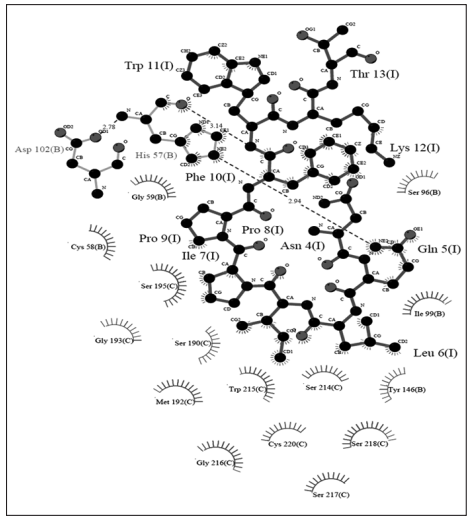

FIG. 4. LIGPLOT of the modelled cytotoxin-1 chymotrypsin complex.

4. DISCUSSION

Snake venom cytotoxins belong to the three finger toxins family. Despite having a similar overall fold structure this toxins can target different cell types and bind to different receptors. Functional variations have been attributed to modifications in the region of loops and small changes in the 3D structure. Different isoforms of three finger toxins have been identified in a single snake venom [23, 35, 36]. Numerous efforts have been made to determine the structure-function-relationship of these toxins. It has been reported that the short-chain neurotoxins contain functional residues in loop 1, which play a crucial role in binding towards the nicotinic

SR. NO	ATOM NAME	RESIDUE NUMBER	CHAIN		ATOM NAME	RESIDUE NUMBER	CHAIN	DISTANCE(Å)
1	O	HIS^{57}	A	↔	N	TRP^{11}	I	3.14
2	NE2	HIS^{57}	A	↔	NE2	GLN^{5}	I	2.94
3	OH	TYR^{146}	A	↔	NH2	ARG^{36}	I	2.68
4	O	SER^{218}	A	↔	NH2	ARG^{36}	I	2.57
5	OG1	THR^{219}	A	↔	O	MET^{31}	I	3.04

TAB. 1. Calculated intermolecular H-bonds essential for the interaction at the interface of the cytotoxin-1 chymotrypsin complex. Chain A: Chymotrypsin; Chain I: Cytotoxin-1.

SR. NO	ATOM NAME	RESIDUE NUMBER	CHAIN		ATOM NAME	RESIDUE NUMBER	CHAIN	DISTANCE(Å)
1	C	HIS^{57}	A	↔	CE1	PHE^{10}	I	3.69
2	CG	HIS^{57}	A	↔	CD1	PHE^{10}	I	3.67
3	CG	HIS^{57}	A	↔	CE1	PHE^{10}	I	3.55
4	CD2	HIS^{57}	A	↔	CD2	PHE^{10}	I	3.55
5	N	CYS^{58}	A	↔	CD1	PHE^{10}	I	3.61
6	SG	CYS^{58}	A	↔	CE1	PHE^{10}	I	3.66
7	CA	GLY^{59}	A	↔	CE2	TRP^{11}	I	3.55
8	O	SER^{96}	A	↔	CD	LYS^{12}	I	3.56
9	CD1	ILE^{99}	A	↔	CD	GLN^{5}	I	3.66
10	CD1	ILE^{99}	A	↔	NE2	GLN^{5}	I	3.55
11	CZ3	TRP^{172}	A	↔	O	VAL^{34}	I	3.58
12	N	GLY^{193}	A	↔	CD	PRO^{9}	I	3.61
13	CB	TRP^{215}	A	↔	NE2	GLN^{5}	I	3.68
14	O	SER^{217}	A	↔	CB	ILE^{7}	I	3.56

TAB. 2. Calculated Van der Waals contacts at the interface of the cytotoxin-1 chymotrypsin complex. Chain A: Chymotrypsin; Chain I: Cytotoxin-1.

acetylcholine receptor, while the long-chain neurotoxins lack these residues in loop 1, and the long C-terminal tail contributes to the receptor recognition [37-40]. Structural analysis of hemachatoxin, a three finger toxin, suggested that its loop II is flexible, and retains its flexibility till it interacts with membrane phospholipids [1]. Studies have shown that cytotoxins have anionic binding pockets, which can accommodate low molecular weight compounds, such as head groups of phosphatidylserine. This interaction of toxin with lipids could be the first step of membrane permeabilization leading to the lysis of cell membranes [7]. Biochemical investigations and model building studies were performed to obtain further insights about diverse functions of snake venom cytotoxins. The analysis to determine the mechanism of the cell membrane lysis via these toxins types is of particular interest. Fewer studies have described the cellular internalization of these cytotoxins [22]. The internalization of CT3 from *Naja kaouthia* in promyelocytic leukameia HL 60 cells was shown by confocal spectral imagining techniques [22], and it was concluded that the internalization and lysosome-targeted action of CT3 plays an important role in CT-mediated cytotoxicity. Recently it was reported that cytotoxin-1 from *Naja atra cantor* venom manifests significant anticancer activity in a selective manner, possibly induced by programmed cell death via mitochondrial or lysosomal pathways [9]. The cytotoxic nature of the snake venom cytotoxins have been used to investigate their ability as an anticancer agent, or in the treatment of viral or bacterial infections [41, 21, 19, 17, 18, 20, 42]. In the present study the molecular model of cytotoxin-1 in complex with chymotrypsin clearly shows its interactions with the protease at the active site. As reported previously, cytotoxin-1 is a potent inhibitor of chymotrypsin and chymotrypsin like activity of the 20S proteasome [23]. Inhibition of the 20S proteasome could be a possible mechanism of cell death caused by cytotoxin-1 mediated pathways. This indicates the ability of cytotoxin-1 to translocate inside the cell and perturb other molecular or membranous species. 20S proteasome is the recycling machinery of the cell and maintains cellular homeostasis in a quality control way [43]. Inhibition of this vital machinery of the cell is currently the subject of studies to suppress tumour growth [44]. The discovery of bortezomib, a proteasome inhibitor, for effective cancer treatment concluded proteasome inhibition as an effective cancer therapeutic target [45]. Recent data published in this direction demonstrate that the development of novel proteasome inhibitors to treat various types of cancer is a important research topic [44, 46, 45]. As distinct venom components are known to be also lead drugs, like bradykinin potentiating peptides isolated from *Bothrops jararaca* venom, leading to the development of the blood pressure regulating drug captopril and its analogues [47], cytotoxin-1 can also be used to support the development of novel antitumor drugs, due to its inhibitory activity towards the 20S proteasome. Many types of cytotoxins isolated from snake venoms and other insects are under investigation as lead molecules to deal with bacterial infections and cancer [48].

5. Conclusion

Snake venom is a magnificent cocktail of medically important biomolecules, particularly proteins and peptides. Three finger toxins are the main peptidic compo-

nents of the cobra snake venom. Despite of the similar overall fold, these toxins are functionally diverse. Nicotinic acetylcholines are sensitive receptors of neurotoxins, while cytotoxins usually penetrate through the cell membrane causing cell necrosis. Experimental evidence has shown their selective nature towards different cell lines. In the present study the molecular model of cytotoxin-1 with chymotrypsin highlights the protease inhibitory potential of cytotoxin-1. The toxin molecule interacts with the protease through the concave side of its loop I and Phe^{10} can be seen to be inserted into the S1 binding pocket of chymotrypsin. Therefore Phe^{10} is proposed to be the P1 site of the toxin ligand. Furthermore being a potent inhibitor of the 20S proteasome, cytotoxin-1 (P01467) can serve and support the design of effective antitumor agents.

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Author information

The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to aisha.munawar@uet.edu.pk.

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