Insilico Structural 3D Modelling of Novel Cry1Ib9 and Cry3A Toxins from Local Isolates of *Bacillus thuringiensis*

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Abstract Three-dimensional (3D) models for the 79.2 kDa activated Cry1Ib9 and 77.4 kDa activated Cry3A δ -endotoxins from *Bacillus thuringiensis* (*Bt*) native isolates that are specifically toxic to Coleopteran insect pests were constructed by utilizing homology modeling online tool. Evidences presented here, based on the identification of structural equivalent residues of Cry1Ib9 and Cry3A toxin through homology modelling indicate that, they share a common *Bt* toxin tridimensional structure. The main differences observed in Cry1I9 domain I at positions α 2b (S56-I60), α 4 (F78-I93) and additionally β 0 (Q10-L12), α 8a (T280-V282) were observed, in domain II at positions α 9b (P333-L339), β 6 (T390-Q393), β 7 (V398-W404), β 8 (V418-W425), β 9 (E453-N454), β 10 (S470-I479) where as in domain III the changes were observed at positions β 19 (R601-F607), β 20 (609-L613), β 21 (S618-F627) and α 11a (K655-F664), α 13, α 14 components present at downstream sites, where as in Cry3A main differences observed in domain I is at the position of α 4 (P105-I152), α 5 (Q163-A185), β 1A (E190-L192), α 6 (F193-Y217), Domain II is not conservered and main variations were observed at β 2

(E292-L295), β 3 (V299-L308), β 4 (I340-F347), β 5 (D356-P368), β 6 (I375-T377), β 7 (V389-F394), β 8 (K398-N405), β 9 (Y416-Y427), β 10 (T436-Y439), β 12 (G476-H495), β 12A (M503-I504) where as in domain III main variations observed at positions of β 18 (P583-I593), β 19 (F604-S610), β 20 (P611-L615), β 21 (N619-G626). Cry1Ib9 and Cry3A contain the most variable regions in the loops of domain II, which determine the specificity of these toxins. These are the first models of Coleopteran-active protein from native isolates of *Bt* and its importance can be perceived since members of this group of toxins are potentially important candidates for coleoptera insect pest control programs.

Keywords *Bacillus thuringiensis* · Coleopteran-active · δ -Endotoxin · Disulfide bond · Homology modeling · 3D Model

Introduction

Bacillus thuringiensis, commonly known as *Bt*, is a gram-positive bacterium that occurs naturally in the soil around the world. For decades, bacteriologists have known that some strains of *Bt* kill certain insects and that the toxic substance responsible for the insects death is a protein. When certain insects ingest either the bacterium or the protein produced by the bacterium (the protein is called δ -endotoxin), the function of their digestive systems is disrupted, eventually resulting in death. *Bt* has the potential to impact significantly in the near term on productivity and sustainability and represents a replacement for a significant quantity of the conventional pesticides currently applied to crops. Yet the long-term impact can only be corroborated if efficacious and responsible deployment strategies can maintain the durability of the *Bt* genes.

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Currently, the Cry δ -endotoxins have been shown to be highly active against a wide variety of insect larvae in the Diptera (mosquitoes and flies), Lepidoptera (moths and butterflies), Coleoptera (beetles and weevils), and Hymenoptera (wasps and bees) orders [1, 2]. Coleopteran insects became increasingly difficult pests over worldwide. Their plague has been reported throughout the world and magnitude of the problem has been widespread over the past years. In majority of the farming situation, control of these pests are largely abandoning because of the lack of control over their damages. In general, the management strategy depends primarily on the use of highly poisonous poor graded chemical pesticides. The use of bio-control agents in general and bacterial based *Bt* in particular are or ignored largely. Intensive screening programs are leading to a broader activity spectrum of toxins as the result of isolation and characterization of new strains with different combinations of crystal proteins, as well as the discovery of new toxins. δ -endotoxins are of great interest for development of new bioinsecticides and in the control of agriculturally important insect pests. Strains containing novel *cryII* genes have been evaluated as a source of new proteins with a broad host range [3, 4]. CryII proteins were initially characterized by their dual activity towards Lepidoptera and Coleoptera [5]. The Cry3A toxin, produced by *Bt* var. *tenebrionis* [6, 7] and other strains [8, 9], is toxic to coleopteran species including the yellow mealworm, *Tenebrio molitor* [6, 8], the Colorado potato beetle, *Leptinotarsa decemlineata* [9, 10] and the cottonwood leaf beetle, *Chrysomela scripta* [11]. The comparatively simple structure of the Cry3A toxin [12], which is remarkably similar to Cry1Aa [13], makes it a useful model for exploring the structure and function relationship between ligand and receptor. The three-dimensional structure of the Cry toxins consists of three functional domains: (I) a cluster of seven K-helices predicted to be involved in membrane interaction [12]; (II) three antiparallel L-sheets involved in receptor binding [12–14]; and (III) a L-sandwich implicated in receptor binding [15–17] and ion channel activity [18–20] in related Cry toxins.

Homology modelling is the most reliable method to predict the 3D structure of a protein with accuracy comparable with a lower resolution, experimentally determined structure. Even models with errors can be useful because some aspects of function can be predicted from coarse structural features [21]. Currently, the precise mechanism of toxicity of the *Bt* Cry δ -endotoxins is still not completely understood, although the understanding of how these larvicidal proteins work at the molecular level has substantially increased over the last decade [22]. To gain molecular structural knowledge of the two closely related *Bt* Coleopteran-active insecticidal crystal proteins, CryII and Cry3A, we generated plausible 3D models by homology modeling online tool. Entailments for the

molecular basis of proteolytic activation, membrane-pore formation, and specificity determination of these Coleopteran-active insecticidal crystal proteins are discussed.

Materials and Methods

Bacterial Strains

Bacillus thuringiensis of unknown strain was obtained from the Andaman and Nicobar Island using sodium acetate selection method. The putative *Bt* isolates were characterized using microscopic and molecular approach [23]. Nucleotide and deduced amino acid sequences were analyzed with the Blast tools (www.ncbi.nlm.nih.gov/BLAST) (Data not shown). Signal peptide sequence was analyzed using SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP>). Related sequences obtained from databases using the software GENSCAN online tool (www.genes.mit.edu/GENSCAN.html) were used for identification of gene features such as exon and splice sites in genomic DNA. BioEdit (version 7.0.4.1) was used for sequence editing and analysis (Figs. 1, 2). The 3D structures was predicted using phyre2 server (<http://www.sbg.bio.ic.ac.uk/phyre2/>), Conserved Domains and Protein Classification (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>, version CDDv2.32-40526 PSSMs) (Data not shown) and the predicted structure was validated using protein structure validation software suite (PSVS) tool. Determination of protein functional analysis obtained from databases using InterProScan (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>), ProFunc (<http://www.ebi.ac.uk/thornton-srv/databases/profunc/index.html>), Protein peeling (http://www.dsmb.inserm.fr/dsmb_tools/peeling/) and Superpose and (<http://wishart.biology.ualberta.ca/SuperPose/>). The final models were submitted to the PMDB database (<http://www.caspar.it/PMDB/>), and the PMDB identifier was PM0078393 (CryIIb9) and PM0078392 (Cry3A).

Results and Discussion

Functional characterization of a protein sequence is one of the most frequent problems in biology. This task is usually facilitated by accurate three-dimensional (3D) structure of the studied protein. In the absence of an experimentally determined structure, comparative or homology modeling can sometimes provide a useful 3D model for a protein that is related to at least one known protein structure. Comparative modeling predicts the 3D structure of a given protein sequence (target) based primarily on its alignment to one or more proteins of known structure (templates). The prediction process consists of fold assignment, target-template alignment, model building, and model evaluation. The number of protein sequences that can be modeled and

Fig. 1 Alignment of toxic region (N-terminal domain) of CryII of Bt sub spp. Kurstaki, Andaman and Nicobar isolate SITA and Andaman and Nicobar isolate AND8. The sequences have been compared with corresponding region of CryII protein of positive Control—CryII proteins, CryII Kurstaki from NCBI. A total of 720 amino acids were found in CryII protein profile. The amino acids sequences of toxic

region of CryII protein of two Bt isolates was aligned with CryII protein of positive control Bt sub spp. Kurstaki and CryV gene. 43 mutations were found in CryII SITA Bt isolate. A total of 44 mutations were found in CryII of AND8A isolate. The amino acid residues ranged between 17.08 % (Cys) and 33.98 % (Ala) of the total content

the accuracy of the predictions are increasing steadily because of the growth in the number of known protein structures and because of the improvements in the modeling software. It is currently possible to model with useful accuracy significant parts of approximately one half of all known protein sequences [24]. The theoretical models of Cry1Ib9 and Cry3A toxin that were obtained correspond to residues 1–720 and 1–662 respectively of the primary structure (Data not shown).

3D Structure and Functional Prediction of CryII and Cry3A Protein

The structure and function of a new CryII and Cry3A protein of native isolates of *Bt* is determined by using conserved

domain database search service at the NCBI (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>) Fig. 3 and the protein homology/analogy recognition engine (Phyre²) computational tool (<http://www.sbg.bio.ic.ac.uk/phyre2>) Fig. 3a, b. These results shows that the query protein sequence is indeed belongs to *Bt*. Three-dimensional (3D) structure is developed with the given a high confidence match (>90 % confidence) and descriptors of the fold and superfamily of the template used, the overall fold of the model will be almost certainly correct and the central core of the model will be tend to be accurate. Consensus prediction score to obtain a confidence value for the secondary structure predicted (0 = low confidence, 9 = high confidence) to ascertain whether regions of the query are structurally ordered (O) or disordered (d). Such disordered regions have

Fig. 2 Comparison of the amino acid sequence from new *cry3A* gene produced with the *cry3A* gene specific primers and that of a known Coleopteran-active gene, *cry3A* (GenBank accession number U10965). A “dot” is placed at those positions in new *Bt cry3A* where the amino acid composition is the same as that in U10965 represented in blocks. * Indicates the 5' region of the *cry3A* transcript.

The sequence of the cloned crystal protein gene showed almost complete homology with a Coleopteran-active Cry3A toxin gene with 117 mutations scattered in different domain regions encoding a protein of 645 amino acid residues in length, with a predicted molecular mass of 77.4 kDa

often been found to be involved in protein function and should be taken into account when analyzing predicted functional sites. The practical applications of protein structure prediction are many and varied, including improving phasing signals in crystallography, selecting sites for mutagenesis and the rational design of hybrid toxins/domain swapping [25]. A better understanding of the 3-D structure of Cry1I and Cry3A will be helpful in designing the domain swapping experiments to improve its insecticidal toxicity.

The reported structural models corresponds with residues 720 (Cry1I) and 645 (Cry3A) of the primary structure using the structural based alignment of the amino acid sequence of the CryV (X62821) and with Cry3A (U10985). Alignment of Domain I was straightforward and the highly conserved nature of helix 5 in the Cry1Ib9 and Cry3A toxin made the placement of the other residues in this domain possible. Alignment of Domain II was also reliable.

Domain III of the protein is quite well conserved on the N- and C-terminal sides. Domain I was composed of N-terminal 267 (60–282) amino acid residues folded into a bundle of nine amphipathic α -helices and two small -strands (Table 1). These features are considered highly conserved among the Cry toxins and have been proposed to be involved in ‘pore formation’ by analogy with the helical bundle pore forming structures of Colicin A toxin [26] and diphtheria toxin [27]. Evidence from several studies has shown that the central helix (α 5) is specifically involved in pore formation [[14], [28, 29]]. All the helices in the Cry1Ib9 model were similar than those in CryV. Helix α 1 probably does not play an important part in toxin activity after the protoxin has been cleaved. It is possible that mutations aimed to increase the amphiphilicity in these helices are anticipated to improve the pore forming activity of Cry1 type toxins. Cry3A domain I was composed of

Fig. 3 Ribbon representation of the crystal structure of Cry1Ib9 and Cry3A toxins. The *colored* boxes denote the positions of the different domains. The *arrows* indicate the positions of the loop regions of domain II

N-terminal 284 amino acid residues folded into a bundle of nine amphipathic α -helices and two small β -strands (Table 2). As with other Cry toxins, Domain II of Cry1Ib9 and Cry3A consist of three Greek key β sheets arranged in β prism topology. It comprises residues 387–487, with one helix and 11 β strands in Cry1Ib9 and 12 β strands in Cry3A. Domain II consists of three anti-parallel β sheets, each ending with exposed loop regions. These loops are thought to participate in receptor binding and hence in determining the specificity of the toxin for attachment on insect receptors. Domain III is composed of highly conserved residues 507–644 with reference to Cry1Ib9 whereas scattered variation was observed in Cry3A.

The loops (β 2– β 3 and β 4– β 5) probably interact with the receptor through both hydrophobic and electrostatic interactions. This probably helps in receptor binding by providing more mobility to glycine and other similar residues that may interact through salt bridges with the receptor. Loop β 4– β 5 is mostly hydrophilic and the charged residues at the tip of the loop are probably important determinants for insect specificity. Aromatic amino acids within and adjoining the vicinity of apical loops two and three of Domain II have been postulated for protein–protein, protein–ligand interactions and have been reported to interact specifically with the outer envelope of the lipid membrane. It has been proposed that these residues interact with hydrophobic lipids tails. The exposed loop architecture has

structural affinity for binding to glycoprotein receptors of the target insect membrane. The absence of α 12a and presence of additional α 9b components in comparison with CryV. A few of the components α 2b, α 9a, β 2, α 10b, β 3, β 4, β 5, β 6, β 7, β 8, β 9, β 11, β 17, β 18 and β 20 differ in their locations (Cry1Ib9). Whereas in case of Cry3A the absence of β 3, β 24 and presence of β 12a components. A few of the components α 2a, α 3, α 4, α 5, β 1a, α 6, α 8a, α 8b, β 2, β 4, β 5, β 6, β 7, β 8, β 9, β 10, β 11, β 12, β 13a, β 13b, β 14, β 15, β 16, β 17, β 18, β 19, β 20, β 21 and β 23 differ in their locations. Like all the known Cry structures, the core and domain interfaces of both model molecules are built from sequence blocks that are a highly conserved feature of all Cry toxins [30, 31].

The prediction of the secondary structure of proteins has been applied to many areas of protein chemistry. The experiments on proteins folding demonstrate that a protein could be denatured and refolded without loss of biological activity. This implies that the amino acid sequence contains all sufficient and necessary information to define a 3D structure of a protein. During the analysis, we needed a quick and effective way to build automatic alignments that would still give good predictions is needed such as Phyre2. The validation of the 3D structure of protein using bioinformatics tools is necessary to evaluate the Structure–function association. A structural comparison of Cry1Ib9 and Cry3A toxin with the theoretical models protein

Table 1 Comparison among three domain structural components of CryV and CryIIb9 new toxin molecules

	Domain I			Domain II			Domain III	
	CryV	CryII		CryV	CryII		CryV	CryII
$\beta 0$	H10-F13	H10-L13	$\alpha 8a$	L184-W209	L184-W209	$\beta 13a$	A522-R526	A522-R526
$\alpha 1$	E33-Q35	E33-K35	$\alpha 8b$	S214-S245	A214-246	$\beta 13b$	G533-R538	G533-R538
$\alpha 2a$	H39-S46	N39-S46	$\alpha 9a$	A251-P276	A251-F275	$\beta 14$	F544-N552	F544-N552
$\alpha 2b$	A57-I60	S56-I60	$\alpha 9b$	—	T280-Y283	$\beta 15$	Q557-S567	Q557-S567
$\alpha 3$	Q61-T73	Q61-T73	$\beta 2$	L291-T297	L292-T297	$\beta 16$	L571-I577	V571-I577
$\alpha 4$	P77-L93	F77-L93	$\alpha 10a$	F322-V329	F322-A329	$\beta 17$	A581-N592	A581-S588
$\alpha 5$	K98-V146	K98-V146	$\alpha 10b$	L335-L338	P333-L339	$\beta 18$	F601-F613	T601-F607
$\alpha 6$	T152-F177	T152-F177	$\beta 3$	V342-S346	V342-L348	$\beta 19$	T619-F627	T609-L613
$\beta 1a$	E182-P184	E182-P184	$\beta 4$	Q358-T369	M359-P369	$\beta 20$	E632-P642	S618-F627
			$\beta 5$	L374-Q379	L374-N375	$\beta 21$	—	E632-V643
			$\beta 6$	T390-F394	T390-Q393	$\alpha 11a$	E654-F664	<i>*K655-F664</i>
			$\beta 7$	V398-S403	V398-W404	$\alpha 12a$	G671-K673	—
			$\beta 8$	N416-K426	V417-W425	$\alpha 13$	D678-S690	<i>*E678-S690</i>
			$\beta 9$	H471-L478	S470-I479	$\alpha 14$	L697-R718	<i>*L697-R718</i>
			$\beta 10$	A486-H493	A486-H493			
			$\beta 11$	T502-E504	T501-I502			
			$\beta 12$	I507-L518	I507-L518			

— Similar component not present

* Components in italics are present at downstream sites

Table 2 Comparison among three domain structural components of CryIIIA (NCBI accession number U10985) and Cry3A new toxin molecules

	Domain I			Domain II			Domain III	
	CryIIIA	Cry3A		CryIIIA	Cry3A		CryIIIA	Cry3A
$\beta 0$	D10-K13	D10-K13	$\alpha 8a$	K222-K253	L223-K253	$\beta 13a$	T487-H495	M503-I504
$\alpha 1$	T36-D39	T36-D39	$\alpha 8b$	Y259-Y286	Y259-Y284	$\beta 13b$	M403-I404	I509-L520
$\alpha 2a$	K43-T49	K43-M48	$\beta 2$	R301-T305	E292-L295	$\beta 14$	Q511-L520	A524-A528
$\alpha 2b$	T61-V79	T61-V79	$\beta 3$	F334-F346	—	$\beta 15$	A524-A528	I537-C540
$\alpha 3$	P83-L96	P83-I99	$\beta 4$	F356-P368	V299-L308	$\beta 16$	I537-C540	A546-P553
$\alpha 4$	D104-K152	P105-I152	$\beta 5$	I376-T377	I340-D347	$\beta 17$	A546-P553	Q559-S569
$\alpha 5$	P160-F184	Q163-A185	$\beta 6$	F380-K384	S356-P368	$\beta 18$	Q559-S569	I573-L579
$\beta 1a$	E190-F194	E190-L192	$\beta 7$	V389-E393	I375-T377	$\beta 19$	I573-L579	P583-I593
			$\beta 8$	K398-N400	V389-F394	$\beta 20$	P583-D590	F604-S610
$\alpha 6$	L194-W218	F193-Y217	$\beta 9$	Y416-Q426	K398-N405	$\beta 21$	T592-I593	P612-L615
			$\beta 10$	T436-T438	Y416-Y427	$\beta 22$	T600-E614	N619-G626
			$\beta 11$	I453-Q455	T436-Y439	$\beta 23$	N619-L627	K632-P642
			$\beta 12$	S470-L480	H471-L480	$\beta 24$	K632-P642	—
			$\beta 12a$	—	G486-H495			

— Similar component not present

indicates correspondence with the general model for Cry proteins.

Ultimately, the recognition of artefacts and errors in experimental and theoretical structures remain a problem in the field of structure modelling. Web-based software

tools like PROSA have a large database and are deployed for the validation of developed models [32]. The software evaluates the model by parsing its coordinates and energy using a distance-based pair potential [33, 34] and capturing the solvent exposed protein residues [33, 34]. The results

Fig. 4 Model validation of Cry1Ib9 and Cry3A with PROSA. The result shows that the structure has features characteristic of native structures. The Z-score of -8.49 (Cry1Ib9) and -6.40 (Cry3A) is highlighted with a large dot

are displayed in form of a Z-score and a plot of residues energy. The Z-score shows overall model quality and provides deviations from the random conformation [34, 35]. The plot checks whether the Z-score of the protein is within the range of similar proteins (NMR and X-ray derived structures) as in Fig. 4. The value -8.53 (Cry1Ib9) and -6.24 (Cry3A) is among the native conformation and the overall residues energy was largely negative. Ramachandran plot analysis using RAMPAGE of the Cry3A toxin oligomer showing placement of residues in deduced model. The structure orientation residues are separately considered for angle and torsions. The Cry1Ib9 Ramachandran plot showed that most of the modelled residues (84.2 %) have ϕ and ψ angles in the core regions and 11.7 % are in allowed regions, except for some proline and glycine residues (0.3 %) that fall in the outlier region, whereas the Cry3A Ramachandran plot showed that most of the modelled residues (92.5 %; Number of residues in allowed region (~ 2.0 % expected): 37 (5.8 %; Number of residues in outlier region: 11

(1.7 %). The results for most bond lengths, bond angles, and torsion angles were among the expected values for a naturally folded protein (Fig. 5). The superimposed backbone traces show low RMS deviations between reference and generated model (Fig. 6).

To date, the X-ray crystal structures of several Cry δ -endotoxins Cry3Aa [12], Cry1Aa [13], Cry1Ac [36], Cry2Aa [37] and Cry3Bb [38] have been elucidated. Although these Cry toxins exert their specific toxicity against different target insect larvae, they display a high degree of overall 3D structural similarity, consisting of three distinct domains. Crystal structures of the active toxins have been analyzed for Cry1Aa5, Cry2A protoxin6, Cry3A7, Cry3B8, Cry1Ac9, Cry4Ba10, and Cry4A11 by X-ray diffraction crystallography and Cry1Ab17, Cry1Ib12, Cry5Aa13 and Cry5Ba by homology modelling method. These reports have shown that the toxin has three structural domains. Domain I is α -helical bundle made up of seven α helices. Domain II is comprised of antiparallel β sheets, and Domain III is made up of a β

Fig. 5 Ramachandran plot analysis of the Cry1Ib9 and Cry3A toxin oligomer showing placement of residues in deduced model. The structure orientation residues are separately considered for angle and torsions. General plot statistics are: Number of residues in favoured

region ($\sim 98.0\%$ expected): 594 (92.5 %; Number of residues in allowed region ($\sim 2.0\%$ expected): 37 (5.8 %; Number of residues in outlier region: 11 (1.7 %). Other plots are evaluated for specific residues as showed at the top left corner of each plot

sandwich. So far Cry1 toxins have been extensively used in studies of Lepidopteran insect control and has attracted less attention to their coleopteran activity either alone or in combination. In summary the models of Cry toxins

constructed in this modeling exercise will be valuable in protein engineering and these models will serve as a starting point for the design of domain swapping, mutagenesis experiments aimed to the improvement of toxicity,

Fig. 6 **A**-Superpose backbone 3d structure of Cry1Ib9 (yellow) and CryV (red) showing moderate structural deviations between the reference and the generated model, **B**-Superpose backbone 3d structure of Cry3A (yellow) and Cry3A PDB deposit (red) showing less structural deviations between the reference and the generated model

and to provide a new tool for the elucidation of the mechanism of action of these Coleopteran-active proteins.

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