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REVIEW ARTICLE

Protein engineering of δ-endotoxins of *Bacillus thuringiensis*

Nachimuthu Saraswathy
National Research Centre for Plant Biotechnology
Indian Agricultural Research Institute
New Delhi 110012
India
E-mail: sarasiari@yahoo.com

Polumetla Ananda Kumar*
National Research Centre for Plant Biotechnology
Indian Agricultural Research Institute
New Delhi 110012
India
Tel: 91 11 25841787
Fax: 91 11 25766420
E-mail: polumetla@hotmail.com

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Abbreviations:
BBMV: Brush Border Membrane Vesicles;
Bt: *Bacillus thuringiensis;*
PLB: Planar Lipid Bilayers.

Bacillus thuringiensis **(Bt) is a valuable environment-friendly biopesticide, which occupies 90% of the world biopesticide market. Its insecticidal properties are attributed to the presence of δ-endotoxins which are synthesized during the sporulation phase of the bacterium. δ-endotoxin or crystal toxin is a multi-domain protein molecule comprising of three distinct domains. Domain I is made of seven α-helices, domain II comprises three antiparallel β sheets, which are folded into loops and domain III is made of a β sandwich of two antiparallel β strands. Molecular studies on the structure and functional properties of different δ-endotoxins revealed that the domain I by virtue of its membrane spanning hydrophobic and amphipathic α-helices is capable of forming pores in the cell membranes of the larval midgut. Domain II being hyper variable in nature determines the insecticidal specificity of a toxin and domain III is involved in varied functions like structural stability, ion channel gating, binding to Brush Border Membrane Vesicles and insecticidal specificity. Recent studies on toxin aggregation and interaction revealed that the three domains interact closely to bring about the insecticidal activity of Bt. In this review we describe the protein engineering studies conducted on different δ-endotoxins which led to an understanding of their molecular mode of action and construction of novel toxins with enhanced insecticidal activity and specificity.**

In the present era of transgenic technology, insecticidal proteins of *Bacillus thuringiensis* (Bt) assume considerable significance in the production of insect resistant crops such as Maize, Cotton, Potato, Rice, etc (Kumar, 2002). Bt is a Gram positive spore forming aerobic bacterium isolated from a wide variety of environments like soil, insect cadavers, stored grain products and phylloplane (Martin and Travers, 1989). The importance of Bt is that it accumulates certain proteins in crystalline form during sporulation phase (Kumar and Bambawale, 2002). These proteins are known to be toxic towards larvae of different orders of insect pests (Lepidoptera, Diptera, Coleoptera, Hymenoptera, and Homoptera) with different efficacies (Schnepf et al. 1998). The toxicity of a Bt toxin is highly specific and it is non-toxic to mammals and beneficial insects.

More than 150 different Cry toxins have been cloned and tested for their toxicity on various insect species till date. In an attempt to accommodate the growing list of new toxin genes/proteins, a new nomenclature has been formulated, wherein each toxin gene/protein will be having four-letter code, according to their amino acid sequence identity among them (Crickmore et al. 1998). Updated list of the Bt toxin genes can be accessed at http://www.biols.susx.ac.uk/Home/Neil_Crickmore/BT/. Bt and its δ-endotoxins have been extensively studied for their molecular mechanism of action and toxin structure-function relationships. In this review we focus on how protein engineering of different Domains of δ-endotoxins helped in understanding the structure-function relationships and.

*Corresponding author

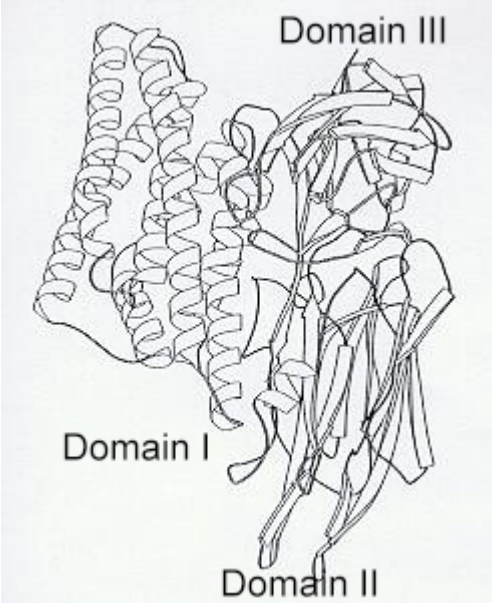

Figure 1. Structure of Bt δ-endotoxin Cry3Aa depicting the three domains (Based on Li et al. 1991)

Structure of Bt δ–endotoxins

designing of novel toxins with wider host spectrum and high potency in a rational and directed way Bt δ-endotoxins are globular protein molecules, which accumulate as protoxins in crystalline form during late stage of the sporulation. Protoxins are liberated in the midgut after solubilization and is cleaved off at C-terminal part to release ~66 kDa active N-terminal toxic molecule. The protoxin contains well-conserved cysteine residues (as many as 16 in Cry1Ac), which helps in bridging the protoxin molecules through intermolecular disulphide bonds and thereby crystal formation. Currently, 3-dimensional protein structures have been determined for three Bt toxins through X-ray crystallography. Among them two are crystals forming (Cry) proteins or δ-endotoxins viz. Cry1Aa (Lepidoptera-specific; Grochulski et al. 1995) and Cry3A (Coleoptera-specific; Li et al. 1991). Since primary amino acid composition determines the final structure of a protein, closely related proteins, Cry1Aa and Cry3A, with 36% amino acid sequence identity showed superimposable structure with similar mode of action, whereas Cyt2A protein, which shares less than 20% amino acid sequence identity, is made of single domain with different functional properties (Schnepf et al. 1998). The tertiary structure of δ-endotoxins is comprised of three distinct functional domains connected by a short conserved sequence. Each domain of δ-endotoxin has independent and inter-related functions in the larval midgut, which brings out colloid osmotic lysis (Knowles, 1994). The nature of each domain predicted from X-ray crystallography is given in Figure 1 and Table 1 (Li et al. 1991; Grochulski et al. 1995). Phylogenetic analysis on the domains of δ-endotoxins revealed that domain I is the most conserved and domain II is hyper variable among all δ-endotoxins. (Bravo, 1997).

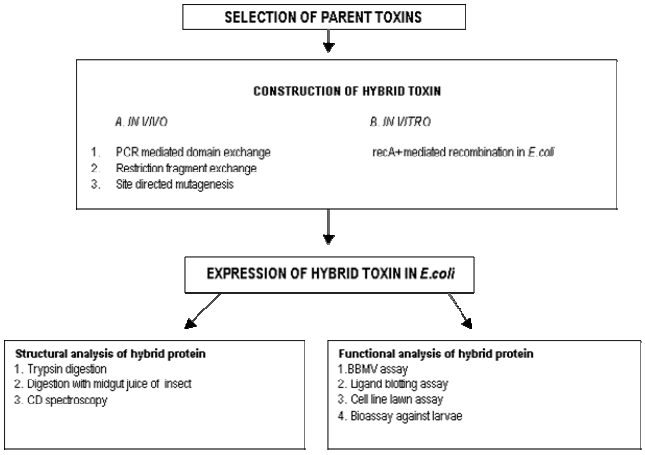

Figure 2. Flow chart showing engineering of novel insecticidal proteins of *Bacillus thuringiensis.*

Structure-function analysis of δ–endotoxins

In addition to traditional bioassays on larvae of insect species, advancements in molecular biology, protein chemistry and biophysics paved the way for a thorough understanding of structure-function relationships of δ-endotoxins. Protein engineering studies are based on methods for introducing mutations in the genes that encode proteins of interest and for producing the proteins in large amount in bacteria for further analysis. In the last decade numerous modifications were created in δ-endotoxins using various protein engineering techniques such as (a) single or multiple amino acid change in variable and conserved regions through site directed mutageneis (b) restriction fragment exchange between closely related cry toxin genes or with other bacterial toxin genes and (c) exchange of domains between toxin genes through PCR mediated cloning or *in vivo* recombination in recA+ (recombinant proficient) *Eschericha coli* Strains. The effects of mutations or modifications in δ-endotoxins have been studied on various aspects like:

1) Pore forming capacity in artificial membranes and measuring ion channel conductance using voltage patch clamping, light scattering and light quenching techniques.

2) Binding of toxins to Brush Border Membrane vesicles (BBMV) isolated from susceptible larval midguts using labeled toxins.

3) Stability of proteins using proteases like trypsin and larval midgut juice.

4) *In vivo* toxicity analysis on larvae.

5) *In vitro* toxicity analysis in insect cell lines – lawn assay.

6) Prediction of secondary structural changes using CD spectrometry.

Role of Domain I in ion channel formation

N-terminal part of the toxic fragment comprising of six amphipathic helices (a- 1, 2, 3, 4, 6, 7) with a central hydrophobic helix (a -5) makes the domain I of δ-endotoxins (Li et al. 1991; Grochulski et al. 1995). Although domain I of δ-endotoxins shares little sequence homology with other bacterial toxins like Colicin A, Diphtheria B subunit and *Pseudomonas* exotoxin, it works similar to other bacterial toxins by forming pores in the cell membranes (Parker and Pattus, 1993). pH dependent ion channel formation is a common feature of bacterial toxins such as colicin, botulinum toxin and diphtheria toxin (Slatin et al. 1990). pH dependent conformational change in δ-endotoxins provided evidence for assuming a similar mode of action for Bt toxins (Convents et al. 1990). Recently it has been observed that pore forming capacity of the δ-endotoxin is affected by pH and is directly correlated with toxin potency (Tran et al. 2001). Two alternative models viz. "penknife model" (Hodgman and Ellar, 1990) and "umbrella model" (Li et al. 1991) were proposed to explain the pore forming mechanism of domain I of δ-endotoxins, both of them previously proposed for the colicin toxin (Parker and Pattus, 1993). Ion channels formed by the δ-endotoxins appear to have more than one channel due to aggregation of toxins. Co-operative gating of more than one identical channel is observed lending support to the co-operative gating hypothesis (Slatin et al. 1990). Model artificial membranes like liposomes, planar lipid bilayers (PLB) and phosphotidylcholine vesicles are used to assess the pore forming ability of toxins under *in vitro* conditions. Insect cell lines like SF9 (*Spodoptera fugiperda*) and CF-1 (*Choristonecera fumiferana*) are also used to assess the lysis of the cell by δ-endotoxins.

It was clearly demonstrated that Cry1Ac and Cry3A toxins are involved in ion channel formation in lipid bilayers (Slatin et al. 1990). Isolated N-terminal fragments from Cry1Ac retained the ability for pore formation in lipid bilayer membranes (Walters et al. 1993). Domain I of Cry3B2 toxin was isolated and analysed for its pore forming capacity. Although domain I could induce ion channel conductance on PLB but it was not sufficient, because the native toxin showed better conductance than the domain I fragment alone (Von Tersch et al. 1994). It could be assumed that interaction of other domains is also important for the proper conductance. Disulphide bridging of Cry1Aa domain I showed reduced conductance on PLB, but under reducing conditions the activity was recovered. Therefore, the reduction in conductance under oxidized state may be due to restricted movement of domain I, which alters the channel mouth environment (Schwartz et al. 1997). Mutations were created in different helices of domain I and analyzed for their role in ion channel formation and toxicity (Wu and Aronson, 1992; Gazit and Shai, 1993; Aronson et al. 1995). Except α-5 mutants, all other helix mutants did not show any significant reduction in toxicity, whereas α-5 mutants showed no or low level of toxicity on tested insects, without concomitant reduction in receptor binding, implying its major role in ion channel formation (Aronson et al. 1995). Synthetic peptide of Cry1Aa-α-5 helix showed its ability to form ion channels and toxin aggregation in PLB (Gazit and Shai, 1993). Mutations in a5 helix and α4-α5 helix loops resulted in loss of toxin aggregation and loss of toxicity. Recently it has been found that oligomerization of toxin is important step and correlated with activity of δ–endotoxin (Aronson et al. 1999). It is consistent with possible oligomerisation and co-operative gating of toxin in ion channel formation.

Mutations in the helix region along with synthetic peptide mimicking studies gave clear evidence for the ion channel formation by domain I. Studies on orientation of the membrane-bound state of the seven a helices comprising of the pore forming domain of Cry3A δ-endotoxin showed that a4 -5 helix loop inserts into the membrane in a hairpin-like manner, leaving all other helices on the surface of the membrane in a bound state (Gazit et al. 1998). This observation supports the "umbrella model" proposed by Li et al. (1991). Following insertion of the toxin, helix 1 is removed due to protease digestion and it is the only helix, which did not bind to BBMV vesicles. Therefore, it is clear that the N-terminal fragment comprising of domain I alone is enough to form ion channel in PLB. Synthetic peptide mimicking studies showed that a5 helix and a4-a5-helix loop is important for toxin aggregation and ion channel formation (Gerber and Shai, 2000). Specific mutations within the a4-a5 loop of Cry4B toxin reveal a crucial role for Asn-166 and Tyr-170 (Kanintrokul et al. 2003). Mutations in the other helices of the domain I did not affect the toxicity. List of mutations created in domain I of δ-endotoxins and their effects is given in Table 2.

Role of domain II in receptor binding and insect specificity

δ-endotoxins are characterized by their narrow range of specificity towards selected group of insects and the specificity is attributed to domain II. Domain II is made of three antiparallel β-sheets, oriented parallel to the α–helices of domain I. Apex of domain II is formed by three surface exposed loops of variable length and the tips of these hairpins are comprised of residues 310 to 313, 367 to 379 and 438 to 456 from sheets 1, 2 and 3 respectively (Grochulski et al. 1995). These surface exposed loops located in the hypervariable blocks of domain II of the δ-endotoxins are identified as specificity determining regions. After ingestion, the crystals are solubilised to release protoxins in the alkaline midgut environment of the larvae. Protoxins (~130 kDa) are converted into toxic fragment (~ 66 kDa) by gut proteases. *In vitro* experiments like BBMV binding and immunoblotting with labelled toxins showed the direct correlation between toxicity and binding of toxins to the midgut receptors. Binding site heterogeneity (binding of different toxins to different receptor sites or partially overlapping sites in different insects) was identified as a major specificity-determining factor for insecticidal activity of δ-endotoxins.

Table. 1 Structural-functional features of δ-endotoxin domains.

S.no	Domain	Amino acid sequence		Secondary structures	Function(s
		Cry1Aa	Cry3A		
1.	Domain I	33-252	58-290	Seven α-helices	Pore formation
2.	Domain II	265-461	291-500	Three antiparallel 'β' sheets folded into three loops	Receptor binding and specificity determination
3.	Domain III	463-609	501-644	'β' sandwich of two antiparallel 'β' sheets	Receptor binding, Ion channe regulation and insect specificity

Ligand blot analysis of SDS-PAGE separated *Heliothis virescens* BBMV proteins with labeled δ-endotoxins sharing high homology in the domain II loops revealed that the receptor A (170 kDa) was recognized by the all the tested toxins (Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, and Cry1Ja), whereas receptor B (130 kDa) was recognized by Cry1Ab and Cry1Ac and the third receptor C (110 kDa) was recognized only by Cry1Ac (Jurat-Fuentes and Adang, 2001). Three toxins (Bt2, Bt3 and Bt73) with varying toxicity levels towards *H. virescens* were tested for their kinetic properties. Concentration of binding sites and equilibrium dissociation constants of these toxins showed no significant differences in binding affinity, rather considerable differences in the concentration of binding sites were observed (Van Rie et al. 1989). Hence, there is a complexity in toxin-membrane interactions in the insect midgut environment. Binding of toxin to the receptor is mediated by a two-step mechanism involving initial reversible binding followed by irreversible binding and membrane insertion. Several studies showed that toxicity did not depend on initial reversible binding rather it was correlated well with irreversible binding (Garczynski et al. 1991). These results support the earlier idea that the post-binding events seem to be integration of δ-endotoxins into the membrane and formation of pores. Receptor-bound toxin molecule could facilitate additional toxin-toxin interactions. Thus, the toxins insert themselves into the membrane as oligomers. This gives the idea that domain II is involved in other processes like toxin-toxin interactions apart from receptor binding.

Extensive mutagenesis followed by real time receptor binding analysis using an optical biosensor (BIAcore) on wild type and Cry1Ac toxins revealed the sequential binding of toxins through domain III and II to site 1 and site 2 of gypsy moth APN receptor. Based on this study bivalent sequential binding model is proposed to δ-endotoxin binding (Jinkins et al. 2000). Receptor binding models constructed based on the BBMV binding studies showed that toxins sharing high homology in the loops of domain II recognize same receptor molecules in larval midgut, which results in cross resistance. Thus, selecting toxins with less homology in the domain II will be a better alternative to delay the resistance development against Bt toxins. Difference in activity of the toxins is due to difference in affinity for a single binding site and also difference in the concentration of binding sites.

δ-endotoxin binding receptors in the larval midgut are identified as glycoprotein molecules. It has been supported by X-ray crystallographic data that domain II loops showed immunoglobulin like structural folds (Li et al. 1991). Structural similarity is observed between δ-endotoxin folds and other known carbohydrate binding proteins like plant lectin jacalin *(Machura pomifora),* outer layer protein I from hen's egg. Carbohydrates are used as recognition epitopes by these folds. So far, three kinds of glycoproteins (Amino peptidase N, Cadherin–like proteins and anionic glycoconjugates) have been identified as receptor molecules for different insect species (Agarwal et al. 2002).

Genetic engineering studies involving exchange of fragments of C-terminal region of toxic fragment between closely related toxins but having different specificity to tested insects showed that the specificity determining regions are essentially located in domain II (Widner and Whiteley, 1989; Caramori et al. 1991; Schnepf et al. 1990; Ge et al. 1991). Hydrophobic interactions between δ-endotoxin loops and insect midgut receptor molecules were tested by substituting hydrophobic residues with Alanine residue or replacing positively charged residues with negatively charged residues. Mutations were created in the domain II-loop 2 residues of Cry1Ab toxin. Alanine substitution in ^{368} RRP ^{370} residues abolished the toxicity toward *Manduca sexta* and *H. virescens* due to reduced binding affinity to BBMV. Positively charged residues in the domain II might help in orientation of the toxin to midgut receptor molecules. Hydrophobic aromatic side chain residue at the position 371 is important for the irreversible binding. When phenylalanine at 371 position was replaced by hydrophilic aliphatic and smaller side chain residues such as Cysteine, Valine, and Serine amino acids toxicity was reduced but not by Tyrosine or Trytophan amino acid substitution (Rajamohan et al. 1995). Alanine substitution in loop 3 residues in Cry1Ab toxin (G439A and F440A) substantially reduced the toxicity toward M. s*exta* and *H. virescens.* The loss of toxicity was correlated with reduced initial binding (Rajamohan et al. 1996a). Mutants generated using site-directed mutagenesis in the loops of Cry3A toxin were tested against *Tenebrio molitor*. Alanine substitution in the loop 1 (Y350A, Y351A, N353A and D354A) residues resulted in loss of toxicity due to reduced receptor binding. Loop 2 mutants (P412A and S413A) did not show any effect on receptor binding and toxicity. Thus, loop 2 is not involved in toxicity

Table 2. Domain-I mutations related to ion channel formation /conductance.

S.no	Toxin	Residue(s)	Mutation(s)	Effect	Reference
1.	Cry1Ab	50	F-K	Loss of toxicity due to impaired pore formation	Ahmed and Ellar, 1990
2.	Cry1Ac	'N' terminal fragment of toxin	-	Forms ion channel in PLB	Walters et al. 1993
3.	Cry3B2	Domain I alone	-	Forms ion channel on PLB bu less than that of native toxin	Von Tersch et al. 1994
4.	Cry1Ac	92	A-D	Except Negatively charged substitutions all changes of Ala-92 were fully toxic	Wu and Aronson, 1992
	Cry1Ac	93	R-H R-G R-A R-S	3-10 folds reduction in toxicity due to loss of positive charge.	Wu and Aronson, 1992
5.	Cry1Ac	206-215	R209A, P T213A W210L V218N Y211N, R, D	No change in toxicity	Aronson et al. 1995
6.	Cry1Ac	α Helix-7	Substituted with Diphtheria Toxin Fragment	Enhanced toxicity due to large pore formation	Chandra et al. 1999
7.	Cry1Ac	Helix 7 amino acids 210 211 211 214	 W-C Y-D Y-C G-E	No alteration in toxicity on tested insects	Aronson et al. 1995
8.	Cry1Aa	-	Disulphide bridging of Domain I region	Reduced ion channe conductance in oxidized condition	Schwartz et al. 1997
9.	Cry1Aa	521	R-K R-Q R-H R-E R-K	Reverse the ion conductance Reverse the ion conductance No change in conductance No change in conductance No change in conductance	Schwartz et al. 1997
10.	Cry1Aa	526 532	R-K R-K	Specifically reduces the toxin's inhibition of I_{SC}	Chen et al. 1993
11.	Cry1Ac1	135	N-Q	Binding to BBMV membrane not affected, pore formation affected	Tigue et al. 2001
12.	Cry1Ac	132 130 131	I-S, L, V, N M-T M-I	Reduced the toxicity No change in toxicity No change in toxicity	Manoj Kumar and Aronson, 1999

determination against *Tenebrio molitor*. However, loop 3-block mutant (Alanine substitution of ^{481}QGSRG ^{486} residues) showed enhanced toxicity due to increased irreversible binding (Wu and Dean, 1996). Recently Gomez et al. (2003) have demonstrated that loops α–8 and 2 of Cry1Ab domain II interact with *Manduca sexta* Bt-R1 receptor.

From these studies, it is clear that all loops of domain II are not involved in binding of toxins to the receptor molecules of single insect species. Therefore, a toxin, which became ineffective due to loss of receptor recognition need not be ineffective on other susceptible insects. High homology in the domain II of toxins results in cross-resistance due to

Table 3. Mutants with enhanced or novel toxicity.

S.no.	Toxin	Fold increase in toxicity than wild type toxin -Insect tested	Effect	Reference
1.	Cry1Ac H168R	+2 *M. Sexta*	High irreversible binding	Wu and Aronson, 1992
2.	Cry4B domain I	+3 Mosquito	Removing site of proteolytic instability	Angsuthanasombat et al. 1993
3.	CryA Loop3 451MOGSRG 486	+2.4 *Tenebrio molitor*	Improvement in irreversible binding	Lee et al. 1995
4.	Cry1Ac N327A N372G	+8, *Lymantrea dispar*	Increased initial binding	Rajamohan et al. 1996a
5.	N372A A282G L283S	+36, *Lymantrea dispar*	Higher binding affinity and binding site concentration	Rajamohan et al. 1996b
6.	Cry1E and Cry1C Domain III exchange	*Spodoptera exigiua*	IE-IE-IC hybrid showed toxicity with different receptor binding	Bosch et al. 1994
7.	Cry1Ab and Cry1C hybrid	+2.5 that of Cry1Ab and +19.5 that of Cry1C, *Plutella xylostella*	High affinity binding due to domain III exchange	Ballester et al. 1999
8.	Cry3A loop1	+11.4 *Tenebrio molisor*	--	Schnepf et al. 1998
9.	Mutation in α helix7 of Cry1Ac with that of Diphtheria toxin hydrophobic domain	+8 fold on *Heliothis armigera*	Large pore and increased conductance	Chandra et al. 1999
10.	Cry1Ba and Cry1Ia hybrid	+42, *Tenebrio molisor*	Enhanced toxicity due optimum combination of Domains	Samir et al. 2001
11.	Transnational fusion of Cry1Ab-Cry1C	*Heliothis virescence* and *spodoptera litura*	Enlarged toxicity	Honee et al. 1990
12.	Cry1Ac F134L	+3 *Manduca Sexta* and *Heliothis virescence*	-	Manoj Kumar and Aronson, 1999
13.	Cry4Ba loop 3 domain II amino acid substitution	+700, *Culex quinquesfaciatus* +285*, Culex pipiens*	-	Abdullah et al. 2003

sharing of the receptor molecules. It is established that loops in the domain II affect irreversible or reversible binding through hydrophobic interactions with receptor molecules.

Studies on domain III

Domain III is made of two antiparallel β-sheets into β-sandwich structure. Intermolecular interactions through salt bridges and hydrogen bonding between domains III and I have been identified through X-ray crystallographic studies. Initially it was proposed that maintaining the stability of the protein is the major function of this domain. From the studies on β strand structure of other protein molecules, it could be assumed that domain III β- sandwich of δ-endotoxin can take part in other functions such stability as receptor binding, specificity determination and ion channel gating (Schnepf et al. 1998). Arginine rich block in the domain III of δ-endotoxins is called “arg face”, through which domain III makes contacts with domain I and

regulates the ion channel conductance. Recent studies involving site-directed mutagenesis of conserved regions of the domain III and domain III exchange between *cry* genes demonstrated the above-mentioned functions for domain III of δ-endotoxins. Three substitution mutants were created in the "arg face" of Cry1Aa domain III with negatively charged amino acids (R528G, R530G and R530K). One mutant was poorly expressed in *E. coli* due to instability of the protein molecule. This may be due to the disturbance of salt bridges between domains III and I as predicted from X-ray crystallographic studies. Other two mutants showed reduced toxicity towards *Bombyx mori*, without any alteration in protein structure (predicted from CD spectroscopy and trypsin digestion assay on SDS-PAGE) and binding to BBMV of *B. mori*. But these mutants showed reduced conductance in PLB membranes. Mutations created in the highly conserved region of Cry1Ac toxin (R 525G or R525A and R529G or R529A) resulted in 4 to 12 fold and 3-fold reduction in toxicity, respectively. These mutants displayed one quarter of the maximum conductance recorded for the native Cry1Ac protein. This result is consistent with earlier observation that domain III "arg face" influences the ion channel formation through domain I interactions (Chen et al. 1993; Masson et al. 2002).

Binding studies using reciprocal hybrids made by exchanging a fragment between 451-623 amino acids of Cry1Aa with that of Cry1Ac on BBMV from *Lymantria dispar* showed the location of receptor binding in the third domain (Lee et al. 1995). Therefore, first direct evidence for domain III binding to receptor was established. Interestingly, hybrid with Cry1Aa third domain resulted in binding of 210-kDa receptor molecule, which is not recognized either by Cry1Aa or Cry1Ac, showing that the domain III also influences the receptor binding. Since loss of receptor binding is attributed as a major reason for the resistance development towards existing toxins, hybrid toxins with differential binding capacity can be used.

Chimeric protein constructed by exchanging domain III of Cry1E (inactive on *Spodoptera exiqua*) with that of Cry1C (most active on *Spodoptera exigua*) showed toxicity level equal to most active toxin, Cry1C. In heterlogous binding assay, it was demonstrated that hybrid toxin was bound to the receptor that is recognized by Cry1E toxin. Since Cry1E is already capable of binding to *S. exigua* BBMV without showing any appreciable toxicity, replacement of domain III with Cry1C might have helped in stabilizing the domain II interaction with the receptor (Bosch et al. 1994). Hybrid toxin comprising of domains I and II from Cry1Ab and domain III from Cry1C showed more toxicity than Cry1C towards *S. exiqua* (De Maagd et al. 1996). BBMV Binding studies showed that the chimeric toxin with domains I and II from Cry1Ab and domain III from Cry1C was failed to bind to 200-kda receptor, which is recognized by parental toxin Cry1Ab and another reciprocal hybrid with domains I and II Cry1C and domain III from Cry1Ab bound to 200-kDa proteins. This shows the involvement of the domain III in receptor binding. These studies suggest that the Cry1C domain III substitution in previously weak inactive toxins like Cry1Ab and Cry1E makes them toxic towards *S. exigua*. Therefore, domain III exchange can be followed for the other weak toxins to make them more active on agronomically important pests.

In another experiment hybrids were constructed between the toxins Cry1B and Cry1Ia, which are not active against Colorado Potato Beetle (CPB). Chimeric protein constructed with domains I and II of Cry1I and C-terminal fragment of Cry1B showed enhanced level of toxicity towards CPB than their parental toxins. When they substituted Cry1I domain II in Cry1B background, the toxicity of chimeric protein approached the toxicity level of the most active toxin Cry3A against this insect. This shows optimum combination of domains II and III may have a role in binding the toxin to midgut receptors, there by increasing the toxicity level (Samir et al. 2001).

Recently lectin-like role for domain III of Cry3A toxin has been identified. There is a structural similarity between domain III of Cry3A toxins with that of carbohydrate binding domain of the 1,4-β glucanase from *Cellulomonas fimi*. This suggests that domain III of δ-endotoxins might have carbohydrate mediated receptor recognition regions (Burton et al. 1999). Mutants generated in the domain III (A506, G509, T513) of Cry1Ac toxin showed reduced binding to ~120 kda receptor (Cry1Ac putative receptor), But there was no significant reduction in toxicity to *M. sexta*. The function of this lectin like fold on domain III is not known under *in vivo* condition. The above-mentioned experimental results on domain III mutants of different toxins provide evidence for the functional role of domain III in channel gating regulation, receptor binding and specificity determination.

Advantages of protein engineering δ–endotoxins

Site-directed mutagenesis and domain exchange studies on different δ-endotoxins threw light on the function of each domain in binging out toxicity in susceptible insects. Protein engineering not only reveals the mechanism by which δ-endotoxins work, but it can generate toxins with enhanced toxicity with or without new BBMV binding properties. Selected list of these new toxins created through protein engineering is given in Table 3. Recent experiments with domain III replacement resulted in improved toxins that recognize different receptors. (De Maagd et al. 1999). These toxins could be used in resistant management as alternatives for the toxins already in use to which insects may become resistant by losing receptors (Figure 2).

About 40% of the currently identified Bt toxins are not active on insects, due to various reasons like low solubility in the insect gut environment, lack of binding to BBMV in the larval midgut, presence of protease cleavage sites. Knowledge of δ-endotoxins can be utilized to make these inactive toxins active by protein engineering.

CONCLUDING REMARKS

Most of the mutants created in domain I resulted in low or no toxicity on tested insects. This might be due to domain I being the most conserved among three domains and it is involved in the basic function of the δ-endotoxins viz., ion channel formation.

Domain II and III mutations resulted in altered/enhanced or decreased specificity and altered receptor binding (in case of Domain III substitutions). Variable and hyper variable regions confer differential specificity and differential receptor binding in the target cells.

Many authors reported the failure of expression of mutant proteins in *E. coli*. Instability of these proteins might be due to exposure of proteolytic cleavage sites because of conformational changes in the mutant toxins.

Properties of hybrid toxins cannot be simply predicted form their parental toxins because hybrids toxins showed altered binding property and specificity.

Mutation which alter the hydrophobicity of domain I results in complete loss of toxicity (Wu et al. 1992). Neutral or positive amino acid substitution has little effect on Domain I function than negatively charged amino acid, which completely abolishes the toxicity. Domain I inserts itself into the lipid bilayer of the membrane. Therefore, hydrophobicity in this domain is important.

For construction of hybrid toxins, parental toxins should be selected such a way that they should differ in toxicity significantly on tested insects. This will be helpful in assessing the hybrid toxins specificity clearly.

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