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FUNCTIONAL ANALYSIS OF Bacillus thuringiensis Cry1A TYPE 8-ENDOTOXINS AT THE MOLECULAR LEVEL

DISSERTATION

Presented in partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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*****

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1996

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Substitution of Arg for His168 in domain 1 of Cry1Ac δ-endotoxin causes a two fold increase in toxicity for *Manduca sexta* larvae. H168R also showed greater thermostability than Cry1Ac. Furthermore, H168R toxin had a greater inhibition of the short circuit current than the wild type toxin consistent with higher toxicity. Computational analysis suggested that H168R toxin could form additional hydrogen bonds and a salt bridge to account for its greater thermal stability. This electrostatic interaction may account for the increased ion flux and toxicity.

Substitution of a positively charged residue (R93F) or addition of a negatively charged residue (A92D) on the proximal surface of domain I in Cry1A toxins results in a substantial reduction in toxicity. The loss of toxicity was not caused by a decrease in the initial binding but rather on reduced irreversible binding. On the other hand, replacing A119 in a loop on the distal side of the helices with negatively charged residues did not affect toxicity or irreversible binding.
The role of surface-exposed loops 1 and 2 in the insecticidal specificity of Cry1Aa for *Bombyx mori* was studied. The most pronounced effect on toxicity for *B. mori* was with double mutant, DLS (substitution of Cry1Ab loop 2 and loop3 with Cry1Aa residues) showing 70% greater toxicity than Cry1Ab toxin. The mutant had a better ion-channel function than the wild type Cry1Ab. However, DLS did not completely regained the binding and toxicity of Cry1Aa suggesting that loops 2 and 3 of Cry1Aa are important, but not sufficient, to cause toxicity and specificity for *B. mori*.

The loop 2 of domain II, in Cry1Aa toxin, was mutated at amino acid residue 371 to generate L371K. Mutant was cleaved and separated into 35 kDa (domain I and partial domain II) and 30 kDa fragment (domain III and II) by trypsin. Incubation of L371K with of *M. sexta* gut juice proteases resulted in almost complete degradation of 30 kDa fragment however, it was not degraded in the presence of *M. sexta* BBMV. Similarly Cry1Aa toxin showed 65% more resistance to gut juice proteases in the presence of BBMV. This indicates that membrane insertion may involve all three domains of the toxin.
To my *late* Friend **MUSTAFKA HART**

*For his unfulfilled Ambition*
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_In the name of ALLAH The Most Beneficent The Most Merciful_

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The rod-shaped, gram-positive bacterium, *Bacillus thuringiensis* (*B.t.*), has attracted the attention of both microbiologists and entomologists since the early 20th century. *B.t.* was first isolated from the silkworm in 1901 by Ishawata, a Japanese bacteriologist. Later, in 1911, E. Berliner isolated a similar bacterium from the diseased larvae of the Mediterranean flour moth (*Anagasta kuehniella*) and named it *Bacillus thuringiensis* after the city of Thuringen (Frankenhuyzen, 1993). Earlier, this insect pathogen was considered to be a simple spore-forming, infectious bacterium. Later, the proteinaceous parasporal crystalline inclusion bodies were recognized to be associated with the spores (Angus, 1954). It was also found that the inclusion bodies formed by any *B.t.* strain may consist of one, or more than one, type of protein called either δ-endotoxins, Cry proteins, *B.t.* toxins, or ICPs (*insecticidal crystal proteins*). All insecticidal *B.t.* strains carry δ-endotoxin genes on large plasmids (Stahly *et al.*, 1978; Gonzalez *et al.*, 1981), which express the crystal protein gene during sporulation.
Application and Manipulation of Toxicity.

A whole family of unique crystalline δ-endotoxins are known to attack various insects. Earlier use of B.t. toxin took place in the 1920's and 1930's against the European corn borer. The first commercial product for the control of flour moth was available in 1938 in France (Weiser, 1986). Through the development of genetic engineering in the past decade and the use of non-recombinant methods, today B.t. toxin is one of the emerging commercial microbial insecticides with worldwide applications for protection of crops, forests, and human health; it is also starting to replace conventional chemical insecticides in several areas of application. B.t.-based bioinsecticides are usually formulations of spores and crystalline inclusions that are released upon lysis of bacteria during the stationary phase of growth (Feitelson et al., 1992). Since naturally occurring B.t. carry a number of plasmids encoding different toxins, commercial B.t. preparations contain toxins from strains of B.t. with widely divergent activities. This is achieved by either plasmid curing, to eliminate undesirable genes, or conjugal plasmid transfer, to bring in genes encoding toxins with desirable insecticidal activities (Carlton et al., 1990). These products show improved activity against target insects because of the production of more potent proteins.
Larger increases in toxicity by reciprocal recombination or by mutating specific amino acid residues have not been well-documented, with a few exceptions reported recently. One such study involved a chimeric protein obtained by reciprocal recombination between cry1Aa and cry1Ac genes. The resultant toxin was found to be 30-times more toxic against *Heliothis virescens* than native parental toxin (Ge *et al.*, 1991). Another study, involving block alanine mutation, indicates a 2.5-fold greater toxicity against *Tenebrio molitor* than the wild type Cry3A toxin (Wu & Dean, 1996).

Recently, a different approach involving transduction and transformation was used to construct novel *B.t.* strains with different insecticidal activities. In one study, a shuttle vector, pHT3101, bearing a copy of cry1Aa δ-endotoxin gene, was transferred into several *B.t.* species through a phage CB-54Ber-mediated transduction. One such transduction displayed dual specificity when a *B.t.* strain, having activity against Coleoptera, was a recipient of pHT3101 (Lecadet *et al.*, 1992). Another study showed that when cry1C gene was transformed into *B. thuringiensis* subsp. *kurstaki* HD73 by electroporation, the resultant *B.t.* strain showed enhanced toxicity against *Spodoptera exigua* than the parental strain (Kalman *et al.*, 1995).
These observations suggest that it is possible to improve the insecticidal activity spectrum and toxicity of \textit{B.t.} toxins through gene rearrangement, site-directed mutagenesis, and transduction.

\textbf{Diversity of \textit{B.t.} Strains and Classification of Toxins.}

The entomopathogenic properties of \textit{B.t.} are greatly variable with approximately 40,000 natural strains being isolated from various regions of the world and from different sources such as soil, insects, and plants (Martin & Travers, 1989; Smith & Couche, 1991; Lambert & Peferoen, 1992). One explanation of the enormous natural diversity of \textit{B.t.} strains might be that as an insect becomes resistant to one strain of \textit{B.t.}, a selective pressure for a different microbial strain may be created. Thus, bacterial genes encoding \textit{B.t.} toxin and the toxin target gene in the insect may evolve in tandem-coevolution (Feitelson \textit{et al.}, 1992).

Another reason for the natural diversity in \textit{B.t.} populations is due to the reason that a vast majority of toxin genes are on self-transmissible (conjugal) plasmids that allow their transfer between related cells to produce novel combinations of toxins (Gonzalez \textit{et al.}, 1982; Reddy \textit{et al.}, 1987).
A large number of *B.t.* toxin genes have now been cloned and sequenced. Höfte and Whiteley (1989) have proposed a nomenclature and classification system for Cry toxins based on the primary amino acid sequence and host range. The classes are: CryI (lepidopteran-[caterpillars] specific), CryII (lepidopteran- and dipteran- [mosquitoes and blackflies] specific), CryIII (coleopteran- [beetles] specific), and CryIV (dipteran-specific). Several new genes have been sequenced and, after analysis, have also been added to the previously defined classes. Furthermore, two new nematode-active classes, CryV and CryVI, have been added by Feitelson et al. (1992) to the Höfte and Whiteley classification. Although this nomenclature is still being used, it has several inconsistencies, such as CryIVA, CryIVB, and CryIVC have 44% sequence similarity, but CryIVD is more closely related to CryI proteins. Therefore, a new nomenclature, based solely on the primary sequence of Cry toxin, is being proposed (Crickmore et al., 1995). In the revised nomenclature, roman numerals have been exchanged for Arabic numerals in the primary rank (e.g., Cry1Aa → Cry1Aa) to better accommodate new genes (Fig. 0.1).
Figure 0.1. Dendrogram showing sequence relatedness among δ-endotoxins. Four levels of ranks: Arabic, upper and lower case letters, and allele numbers, based on sequence identity, are demarcated by vertical bars. Boxed (Arabic numerals) indicate the first level grouping of proteins, eg Cry1, Cry2, Cry3 etc. Individual proteins are designated by their revised nomenclature and data base accession numbers (Crickmore et al., 1995).
[Figure 0.1]
Among the various δ-endotoxins, the Cry1A-type ICPs are the best studied. These toxins are active against various species of Lepidoptera with different toxicities (Höfte & Whiteley, 1989). For example, Cry1Aa, although 85% homologous to Cry1Ac, is 200 times more toxic against *Bombyx mori* (silkworm) than Cry1Ac (Ge *et al.*, 1989).

**General Mode of Action and δ-endotoxin Structure.**

The mega plasmids (30-200 mDa) carrying cry genes encode crystalline inclusions which are dissolved in the alkaline environment of the insect gut, releasing one or more proteins of 130-140 kDa (Tojo *et al.*, 1983; Dow, 1986) and 70 kDa δ-endotoxins. The inclusion bodies can also be dissolved *in vitro* with high pH buffers (>9.5). The Cry1- and Cry4-type (130-140 kDa) solubilized crystals are in the inactive protoxin form which are converted into 65 kDa-active toxin form by proteolytic cleavage of 28 residues from the N-terminal (Nagamatsu *et al.*, 1984; Yoshida *et al.*, 1989) and highly conserved C-terminal (Höfte and Whiteley, 1989; Choma *et al.*, 1990). On the other hand, Cry2-, Cry3-, and Cry4D-type (70 kDa) proteins are considered to be naturally truncated.

The activated toxin interacts with the cell membrane lining the gut of the susceptible insect. Several immunological methods have identified the luminal
brush border as the site of toxin association (Heimpel & Angus, 1989; Hofmann & Lüthy, 1986). This has been further shown by binding assays using $^{125}$I-labeled toxin and brush border membrane vesicles (BBMV) isolated from the midgut of the susceptible insect (Hofmann et al., 1988a, 1988b). In vitro binding studies with BBMV and $^{125}$I-labeled toxin have shown the existence of specific binding sites on the membrane. These high affinity toxin binding sites, in most cases, are correlated with the insecticidal activity of B.t. toxin (Hofmann et al., 1988a; Van Rie et al., 1989).

Putative receptors (toxin binding sites) have been identified in Manduca sexta, Lymantria dispar (gypsy moth), and Heliothis virescens (Sangadala et al., 1994; Knight et al., 1995; Gill et al., 1995). The amino acid sequences have shown that these binding proteins belong to aminopeptidase-N family of proteins with a glycosyl phosphatidyl inositol anchor (Lu & Adang, 1996).

Subsequent to specific binding, there is likely to be a conformational change in the toxin that facilitates insertion of toxin into the apical cell membrane of the insect midgut columnal cells (Gill et al., 1992; Walter et al., 1994). This leads to the formation of either pores (Knowles & Ellar, 1987) or ion channels (Schwartz et al., 1993).
Thus, the broad outlines of the basis of toxin specificity and the mode of action are known. However, the knowledge of the nature of specific receptor interaction and how the toxin inserts into the membrane is still in its infancy. Further studies are needed on the post-binding events, such as whether or not oligomerization is required for membrane insertion. Moreover, if oligomerization is required, is it a pre- or post-insertion phenomenon? However, from the general mode of action, it can be inferred that for the function of δ-endotoxins, four parameters are involved: conversion of protoxin to toxin, binding of toxin to specific membrane receptor(s), and finally pore/ion channel formation leading to insect death (Fig. 0.2).
Mode of Action of *B.t.* Toxin on the Insect Gut

A. Ingestion

↓

B. Solubilization

↓

C. Proteolytic activation

↓

D. Receptor binding

↓

E. Formation of ion channels

↓

1. Breakdown of the permeability barrier of the membrane
2. Cell lysis
3. Disruption of gut integrity

↓

Death of the insect

Figure 0.2 (obtained from Sheng-Jiun Wu)
**Structural Features of ICPs.**

Earlier studies, to determine the structure of Cry proteins, were made at the primary structure and higher order level. At the primary level, amino acid sequence analysis showed that Cry-type crystal proteins show >90% sequence homology at the C-terminal tail, ranging from residues 600-1150. On the other hand, the N-terminal part of the toxin is less conserved with homology varying from 40-90%. The C-terminal tail, having 12-16 cysteine residues in the intact protoxin, is considered to be involved in crystal formation through intermolecular disulfide bridges (Lüthy & Ebersold, 1981; Bietlot et al., 1990). The toxic segment located in the N-terminal half of the protoxin is much less conserved (Honée & Visser, 1993).

Studies in the protoxin and toxin conformation have been carried out involving biochemical and biophysical approaches. Unfolding of Cry1Ac protoxin in the presence of urea or guanidium hydrochloride was measured by fluorescence emission and sensitivity to proteolysis. These experiments indicated that, *in vitro*, the N-terminal toxic fragment folds and unfolds independently of the C-terminal protoxin region (Choma et al., 1991). Other studies involving proteolysis of the toxic fragments of Cry1-type δ-endotoxins,
under mild denaturing conditions, generated two fragments of the toxin molecule by cleaving at residues 371-373. Circular dichroism (CD) studies revealed that the N-terminal half of these toxin fragments is rich in alpha helices, while C-terminal contains beta sheets and coils. In addition, the α-helical part is more hydrophobic than the C-terminal fragment (Convent et al., 1990, 1991).

Comparative analysis of the amino acid sequence of the δ-endotoxins, with the exception of Cry2 proteins, shows the presence of five conserved blocks (Fig. 0.3), separated by highly variable sequences of variable lengths (Höfte & Whiteley, 1989; Crickmore et al., 1995). Conserved blocks 1 and 2 are in the N-terminal the toxin molecule. The other three conserved blocks are in the C-terminal. Cry2 proteins only show homology to the first conserved block (Fig. 0.4).
Figure 0.3. Conserved amino acid blocks 1 to 5 among Cry proteins. For each block shaded area indicates the amino acids are conserved among at least 75% of the proteins. Lower case letters (a = AGPST; d = DENQ; f = FTY; i = ILMV; k = KR), indicates positions where an amino acid from that group appears in at least 75% of the sequences. Amino acids are indicated by single-letter amino acid code (Crickmore et al., 1995).
[Figure 0.3]
Figure 0.4. Amino acid sequence comparison of various δ-endotoxins.

Dark boxes indicate the highly conserved blocks of amino acid. Block 1 to 5 are within the toxin molecule. The functional significance of blocks 6 to 8 is not known. Double arrows at the top figure show the location of Cry3A domains. Figures are drawn to scale given by horizontal bar (Crickmore et al., 1996)
3-D Crystal Structure.

X-ray crystal structure of Cry3A shows that the core of this toxin molecule is built on the five conserved amino acid blocks (Li et al., 1991). Conservation of blocks 1 through 5 are consistent with the notion that all the δ-endotoxin groups (Cry1, 4, 7, 8, 9, and 10) might adopt the tertiary structure similar to Cry3A. This has been further proven by the recently elucidated 3-D structure of Cry1Aa (Grochulski et al., 1995). Both Cry3A and Cry1Aa have a three domain structure (Fig. 0.5).

Domain I is a cluster of several α-helices. Helix 2 has a small loop, so it can be divided into α2a and α2b. A cross-section of domain I shows that α5 is a central helix surrounded by the other helices. The outer helices, especially the long α1 and α7, are amphipathic in character. α5 is also amphipathic, while the surfaces of the helices (α2, α3, α4, and α6) facing the solvent are relatively hydrophilic (Yamamoto & Powell, 1993; Grochulski et al., 1995). Helices α4 and α5, along with an interhelical loop, form a hairpin that contains the most hydrophobic segment of domain I (Li et al., 1991; Grochulski et al., 1995).
Figure 0.5. Comparison of X-ray crystal structures of δ-endotoxins. A: Cry3A (coleopteran active) and B: Cry1Aa (lepidopteran active) toxins showing the level of similarity in their folds. Domain I is on the left, domain III is on the right and domain II is in front. The loops at the bottom of domain II shows the greatest diversity (Schematic drawing courtesy of Dr. F. Rajamohan).
Domain II consists of three structurally homologous β-sheets which appear like a triangular column. Sheets 1 and 2 are each formed by four antiparallel β-strands, β2 to β5 and β6 to β9, respectively, which assume a "Greek-key" topology. Sheet 3 contains three β-strands: one from the N-terminal of domain II (β1), and two from the C-terminal part of domain II. There is also a short helix (α8) coming from the N-terminal of domain II. Two middle strands of each of the three β-sheets form long β-hairpin extensions and the apexes of these extensions are in the form of loops. In Cry1Aa, these loops are comprised of residues 310-313 (loop 1), 367-379 (loop 2), and 438-446 (loop 3). Domain II has the axis of pseudo-symmetry being nearly parallel to the axis of α5 helix of domain I (Grochulski et al., 1995).

Domain III is a "jelly-roll" sandwich, formed by two sheets of anti-parallel β-strands. One surface of the outer sheet is exposed to the solvent and comprised of five β-strands (β13, β16, β22, β18, and β19), with β13 being at the N-terminus. The inner sheet, facing domain I and II, also contains five strands with β23 being at the C-terminus.

The 3-D structure comparison of Cry3A with Cry1Aa shows a high, overall similarity (Grochulski et al., 1995). Domain I and III show the highest structural
homology, whereas domain II, although similar, shows large differences in the position and length of connecting loops (Fig. 0.5).

Furthermore comparison of the crystal structure of Cry3A and Cry1Aa shows that the five conserved amino acid blocks are present in the same regions of the two toxins. Block 1 is present in $\alpha_5$ helix of domain I. Block 2 is extended from the latter half of $\alpha_6$ to $\beta_1$ in domain II. Block 3 overlaps in the latter part of $\beta_{11}$ and extends from the loop between domains II and III into domain III. Block 4 and 5 constitute the three inner strands overlapping $\beta_{17}$ and $\beta_{23}$, respectively (Fig. 0.6).
Figure 0.6. Schematic ribbon diagram of Cry3A 3D-structure. Three domains of the toxin and their proposed functions are indicated. Five conserved blocks are shown as shaded segments (Adopted from Schnepf, 1995).
Structure-Function Relationship of δ-endotoxins.

Several studies were carried out prior to the 3-D structure of Cry3A and Cry1Aa to determine the role of various regions of δ-endotoxins. Most of these studies involved only the knowledge of the primary structure of various toxins and the general mode of action.

Specificity and Receptor Binding.

Pioneering work that was carried out prior to the crystal structure elucidation involved homolog-scanning mutagenesis to determine the specificity-determining regions of closely related Cry1-type toxins of differing specificities (Ge et al., 1989, 1991; Widner & Whiteley, 1989; Schnepf et al., 1990). High insect specificity of the B.t. toxins suggests the presence of receptor(s) on the insect gut epithelial cells (Van Rie et al., 1989).

In order to determine the binding affinity, binding assays, reported by Höfmann et al. (1988a), involving 125I-labeled toxin and the BBMV of susceptible insects, are routinely used. In one such experiment, involving Cry1B toxin active against Pieris brassicae and Cry1Ab toxin, active against both P. brassicae and M. sexta were labeled with 125I. Saturation and competition binding assays
showed that Cry1Ab binds with high affinity to both *P. brassicae* and *M. sexta* BBMV, whereas Cry1B showed binding only to the BBMV from *P. brassicae*.

In earlier studies, receptor binding was considered to be the deciding factor in toxicity. VanRie *et al.* (1990) identified that resistance of a lepidopteran strain, *Plodia interpunctella*, to Cry1Ab was due to reduction in affinity of Cry1Ab toxin binding.

Homologous recombination between Cry1Aa and Cry1Ac has determined that a region between 332 and 450 is responsible for the toxicity of Cry1Aa towards *Bombyx mori* (Ge *et al.*, 1989). Recently, deletion and alanine-scanning mutagenesis of Cry1Aa have determined residues 367-373 to be essential for binding to *B. mori* BBMV and toxicity (Lu *et al.*, 1994; Hussain *et al.*, 1995). This segment of the toxin has now been shown to be in the loop 2 of domain II. Similarly, mutations in the Cry1C region, corresponding to loop 1 and loop 2 of Cry1Aa, modulate both toxicity and specificity (Smith & Ellar, 1994). Other studies involving alanine-scanning and block mutation in Cry3A δ-endotoxin indicate that loop 1 and loop 3 of domain II are involved in receptor binding (Wu and Dean, 1996).

These studies suggest that loops of domain II are the primary determinants of specificity. However, recent studies involving hybrid protein
indicate a role of domain III in receptor binding and specificity (Bosch et al., 1994; Lee et al., 1995).

Extensive receptor binding studies have shown that binding is not always correlated with toxicity. Wolfersberger (1990) observed an inverse relationship between binding affinity and toxicity for Cry1Ab and Cry1Ac toxins against gypsy moth. Gould et al. (1992) found similar binding of Cry1Ac toxin to resistant and sensitive strains of Heliothis virescens. Recent studies with Cry1Ab toxins have shown that certain mutations in loop 2 of domain II do not affect the initial receptor binding. However, toxicity was affected significantly (Rajamohan et al., 1995, 1996). These observations suggest that initial binding alone cannot account for the discrepancy in binding and toxicity.

Recently a two-step interaction scheme has been described for toxin-BBMV (Brush border membrane vesicle) interaction (Liang et al., 1995), which include initial reversible binding and irreversible binding.

\[
\text{Toxin + Receptor } \leftrightarrow \text{ Reversible Binding } \rightarrow \text{ Irreversible Binding}
\]

Irreversible binding has been observed in several cases (Wolfersberger et al., 1986; VanRie et al., 1989) and indirectly corresponds to the membrane integration process.
Besides understanding the role of different residues involved in receptor binding, studies have now been directed towards identifying and isolating membrane receptors. Knight et al. (1994) has identified a 120 kDa \textit{M. sexta} BBMV protein which shows homology to aminopeptidase-N and is claimed to be the receptor for Cry1Ac toxin. A 210 kDa protein is also isolated from \textit{M. sexta} BBMV which is shown to bind Cry1Ab toxin (Vadlamudi et al., 1993, 1995). Furthermore, Oddou et al. (1991) studied the binding of Cry1Aa, Cry1Ab, and Cry1Ac toxin to solubilized \textit{H. virescens} BBMV. Their ligand blots showed a 170 kDa binding protein to Cry1Aa and Cry1Ab, while Cry1Ac binds to two proteins of 140 kDa and 120 kDa. Most of these receptor proteins are found to be glycoprotein (Honeé & Visser, 1992; Vadlamudi et al., 1995) with a glycosylphosphatidylinositol (GPI) anchor (Lu & Adang, 1996).

\textit{Ion Channel (Pore) Formation.}

Subsequent to the binding of \(\delta\)-endotoxins to receptors on the columnal epithelial cells of the insect midgut, the bound toxin induces the formation of pores, either aspecific (Knowles & Ellar, 1987) or K\(^+\)-specific (Crawford & Harvey, 1988; Slatin et al., 1990). It has also been observed that Cry1C toxin, under alkaline conditions, form K\(^+\)-selective ion channels in the lipid bilayer, but becomes non-selective and pass Na\(^+\) and anions at neutral or acidic pH (Schwartz et al., 1993).
The X-ray crystallography data on Cry3A and Cry1Aa suggest that the toxin fragment itself is able to induce pore formation via insertion into the membrane. In particular, domain I, having amphipathic α-helices, is a likely candidate for membrane insertion and involvement in ion-channel function (Li et al., 1991; Grochulski et al., 1995). Several predictive algorithms, to search for the region of δ-endotoxin that has membrane-spanning potential, have also located such regions as domain I (Bietlot et al., 1989; Haider & Ellar, 1989). However, there is no direct evidence on the portion of toxin actually spanning the membrane.

In order to clearly identify the toxicity domain and/or essential amino acids, mutants have been constructed. A double mutant in the N-terminal hydrophobic region showed reduced toxicity when Phe50 and Val51 were replaced for Asp (Ahmad & Ellar, 1990); however, the binding was not affected. Furthermore, it was observed that substituting Ala92 for Glu or Tyr153 for Asp in Cry1Ab drastically affects the toxicity and ion-channel function (Chen et al., 1995). Also the irreversible association is significantly reduced, but the initial (reversible) binding remained unaffected. Similarly, mutating Ala92 to Asp in Cry1Ac affects toxicity (Wu & Aronson, 1992) and irreversible binding, but initial binding is not altered (Hussain et al., submitted). Mutation in α5-helix, at position Ala165 to Met and Leu167 to Pro of Cry1Aa, also resulted in the loss of toxicity (Ahmad &
Ellar, 1990). Similarly, Wu & Aronson (1992) observed α5-helix mutants of Cry1A which are deficient in the inhibition of K⁺-dependent transport, but competed fully with the wild type toxin for binding to *M. sexta* BBMV. Studies with truncated fragments of Cry1Ac showed that the N-terminal fragment of the protein can serve as an ion channel in the bilipid layer (Walter *et al.*, 1993).

These studies validate the hypothesis that domain I of the δ-endotoxin plays a crucial role in pore-formation. In addition, several models suggest that domain I must be rearranged to allow hydrophobic surface out and hydrophilic side in before insertion (Knowles & Dow, 1993; Yamamoto & Powell, 1993). Recent models consider the toxic pore to be an aggregate of several toxin molecules (Gill *et al.*, 1992; Yamamoto & Powell, 1993).

The function of domain III is still obscure. Li *et al.* (1991) propose that the function of this domain is only to maintain structural stability. However, recent studies with block 4 "arginine face" in Cry1Aa have shown that conservative mutant, R521K or R527K, does not affect stability or receptor binding of the toxin, but did cause a reduction in toxicity and ion-conductance (Chen *et al.*, 1993; Wolfersberger *et al.*, 1996). This implies that the "arginine face" of domain III may be involved in channel function by serving as a voltage sensor.
SUMMARY

This review indicates that the search for new δ-endotoxins with better and diverse activities continues to progress. Advances are being made to determine the structure of toxins. The relationship between structure and function is also better understood. Significant insights are being made into the basis of insect specificity and toxicity. However, more studies need to be done to understand the salient features of toxicity of δ-endotoxin with greater impetus given to stability of structure, receptor binding, and ion-channel functions. The combination of these efforts should preserve and extend the use of this environmentally friendly bioinsecticide.
CHAPTER 1

Substituting Arg for H168 in α5 helix enhances thermal stability and insecticidal activity of Cry1Ac δ-endotoxin towards *Manduca sexta*

INTRODUCTION

*Bacillus thuringiensis* (B.t.) is a Gram-positive bacteria which produces several classes of entomocidal crystal proteins during sporulation. These crystalline inclusion bodies are called δ-endotoxin or Cry proteins that are solubilized in the alkaline and often reducing environment of the larval midgut. The solubilized Cry1 type, 130-140 kDa, protoxin is activated to 65 kDa toxin by gut protease (Tojo & Aizawa, 1983). The activated toxin binds to the receptor on the microvillar membrane of the susceptible insect with high binding affinity (Hofmann *et al.*, 1988a). Subsequent to binding, the toxin molecules are
believed to insert into the membrane and either create non-specific pores or ion channels (Knowles & Ellar, 1987; Schwartz et al., 1993). This leads to a loss of homeostasis across the brush border membrane, leading to gross structural changes in the midgut and eventually results in the death of the larvae (Bauer & Pankratz, 1992).

The binding of toxin to the receptors was considered to be related directly to the toxicity (Hofmann et al., 1988b; Van Rie et al., 1990). Wolfersberger (1990) has made a paradoxical observation that there is an inverse relationship between binding affinities and toxicities for Cry1Aa and Cry1Ac against gypsy moth larvae. A two-step process, involving initial reversible binding and irreversible binding, is considered to be important for the insecticidal activity of the δ-endotoxins (Chen et al., 1995; Liang et al., 1995).

Recent elucidation of the crystal structures of Cry1Aa (Grochulski et al., 1995) and earlier reported Cry3A structure (Li et al., 1991) have proven that all the δ-endotoxins, having five highly conserved amino acid blocks, adopt a similar three-domain tertiary structure. Domain I is a bundle of α-helices encircling a relatively more hydrophobic α5. Domain II has three β-sheets in Greek-key topology. Domain III has anti-parallel β-ribbon in jelly-roll sandwich (Li et al., 1991; Grochulski et al., 1995).
Genetic and electrophysiological studies have been carried out to determine the function of various domains. Domain II is mainly involved in receptor binding (Ge et al., 1989; Lee et al., 1992; Lu et al., 1994; Rajamohan et al., 1996). Domain III has a role in structural integrity (Li et al., 1991), and also ion conductance and receptor binding (Chen et al., 1993; Lee et al., 1995; Wolfersberger et al., 1996). Domain I is considered to be involved in membrane insertion and ion channel formation as shown in planar lipid bilayer (Walter et al., 1993; Cummings et al., 1994). Ahmad and Ellar (1990) have shown that point mutations in the hydrophobic amino acid residues of α5 can reduce the toxicity of Cry1Aa toxin, but does not affect the binding to the lepidopteran membrane. Recently, mutations in the α-helices connecting loops of domain I have shown that certain residues of Cry1Ab and Cry1Ac, such as A92, R93, and Y153, play an important role in toxicity and irreversible binding (Wu & Aronson 1992; Chen et al., 1995), suggesting that domain I (or certain α-helices) are inserted into the membrane. This notion is further demonstrated by the studies on the proteolytically derived N-terminal fragment corresponding to domain I of Cry1Ac which has been shown to form ion channel similar to the intact toxin (Walter et al., 1993). Studies with synthetic peptides corresponding to α5 and α7 helices of Cry3A and Cry1Ac, have also supported the role of domain I in insertion and ion conductance (Cummings et al., 1994; Gazit & Shahi 1995).
The central α5 helix appears to be particularly important because of the mutations with altered toxicity for three insects (Wu & Aronson, 1992). A central highly conserved residue in the helix, H168, is of particular interest in the Cry1A δ-endotoxins. Substituting Histidine168 with Glutamine, Asparagine or Arginine had different effects on toxicity. In particular, there was a increase in toxicity for Manduca sexta by the H168R toxin. It was suggested that since H168 is in the middle of the hydrophobic face of α5 helix it may be important for sub-unit interactions within the membrane (Wu & Aronson, 1992).

In this study, we have utilized biophysical and electrophysiological approaches, along with toxicity assays and receptor binding, to determine the possible effect of mutating H168 in Cry1Ac. We found that the increase in toxicity with H168R is due to better ion-conductance and is correlated to greater thermostability. These results indicate that substituting His168 for Arg will increase the integrity of α5 segment which can serve as a structural element in the pore formation.
EXPERIMENTAL PROCEDURES

Mutagenesis and Expression of Mutant Proteins

Cry1Ac mutants, H168Q and H168R, were prepared as described earlier (Wu & Aronson, 1992). Briefly the cry1Ac template was constructed by inserting 2.09 kb HincII-BclI fragment into M13mp19 vector. Site-directed mutagenesis was carried out according to the method of Kunkel (1985). In this method M13mp19 vector carrying the cry1Ac template was transformed into dut, ung Escherichia coli strain CJ236. Isolated phagemids and DNA was extracted. Annealed the mutagenic oligonucleotide and using the mutagenic oligomer as primer synthesized complimentary strand with T4 DNA polymerase and dNTPs (deoxy nucleoside triphosphate) Transformed wild-type E. coli strain MV1190 which has active uracil-N-glycosylase thus inactivates parental uracil-containing strand. Only mutant strand replicates. Cells are grown on LB ampicillin plates. Mutant colonies are screened by isolating DNA and sequencing by dideoxy method (Sanger et al., 1977). For the expression of mutant genes, 1.3 kb NsiI-SacI fragment was subcloned into the vector pOS4201. The construction of pOS4201 and expression in E. coli have been described (Ge et al., 1989). This vector carried the wild type cry1Ac gene which was restricted with NsiI-SacI. The mutants, H168Q and H168R, were overexpressed in the E. coli host strain MV1190.
Purification and Protease Digestion of Protoxin

After being expressed in E. coli, Cry1Ac and its mutants were purified from MV1190 by the method described by Ge, et al. (1991). Briefly cells were grown in 400 ml of Luria Broth (LB) with 50mg/ml of ampicillin for 48 hrs at 37°C and 250 rpm. Cells were harvested (7,000 g, 10 min) and pellet was resuspended in 50 ml lysis buffer (50mM Tris-HCl, 50mM EDTA, 15% sucrose, pH 7.5, supplemented with 1mg/ml of lysozyme) and incubated for six hours at 37°C with gentle shaking. The cell suspension was sonicated on ice (2 x 1.5 min, large tip, Fischer sonic disemembrator model 300) After sonication the cells were centrifuged and the pellet washed three times with ice cold Crystal wash I (0.5 M NaCl, 2% Triton X-100) and then two times each with Crystal wash II and double distilled water. After solubilizing the crystal proteins in 50 mM sodium bicarbonate buffer (pH 9.6) supplemented with 10 mM dithiothreitol (DTT), the protoxin concentration was determined by Coomassie proteins assay reagent (Pierce). The solubilized protoxin was then activated with trypsin/protoxin ratio of 1:30 (w/w) at 37°C for 2h. Protoxins and toxins were analyzed by SDS-polyacrylamide gel (12.5%) electrophoresis (PAGE) as described by Laemmli (1970). The structural stability of wild type and mutant toxins were determined by treating with Manduca sexta gut juice as described earlier (Lu, et al., 1994).
Toxicity Assay

Bioassays on *M. sexta* were performed by the surface contamination method (Chen *et al.*, 1995). Five toxin concentrations were used to calculate the medium lethal concentration (LC$_{50}$) values with 12 neonate larvae per dilution. Mortality was recorded after four days. PROBIT.SAS program (Raymond, 1985) was used to determine the LC$_{50}$ value and 95% fiducial limits.

Preparation of Brush Border Membrane Vesicles (BBMV)

The midguts were isolated from fifth instar *M. sexta* larvae. BBMV were prepared from these isolated midguts by differential magnesium precipitation method of Wolfersberger *et al.* (1987). The midguts are placed in a variable speed homogenizer (Model S63C, Tri-R instrument, Inc) with nine times their weight of buffer A (300mM mannitol, 5mM EDTA, 17mM Tris-HCl, pH 7.5). An equal volume of 24mM mgCl$_2$ was added to the homogenate and incubated on ice for 15 min. After incubation the suspension was centrifuged for 15 min at 4,500 rpm at 4°C. Supernatant was collected in another tube and centrifuged for 30 min. at 16,000 rpm using JA-20 rotor and Beckman J2-21 centrifuge. The pellet was resuspended in half the original volume of buffer A and equal volume of 24 mM MgCl$_2$. The suspension was again centrifuged first at 4,500 rpm and the supernatant was recentrifuged at 16,000 rpm. The final BBMV pellet was then suspended in binding buffer (8mM NaH$_2$PO$_4$, 2mM KH$_2$PO$_4$, 150 mM
NaCl, pH 7.4) Protein concentration in the BBMV preparation was measured by Coomassie Protein Assay reagent (Pierce). The enrichment of alkaline phosphatase (a brush border marker) was also determined to check the purity of BBMV. The BBMV was either used immediately or stored in liquid nitrogen until further use.

**Iodination of Toxin**

The activated toxins were iodinated using IODO-BEADS (Pierce) following manufacturer’s protocol. 1 mCi of Na$^{125}$I (Amersham) was used to label 25 μg of each toxin. After the labeling reaction, the $^{125}$I-labeled toxin was separated on an Excellulose column (Pierce). The purity of labeled toxins were analyzed on SDS-(12.5%)PAGE. Specific activity was determined by cutting the gel fragment and counting on a gamma-counter (Beckman Instruments). The specific activity for Cry1Ac and mutant toxins ranged from 0.46 to 0.51 μCi/μg.

**Competition Binding Assay**

Heterologous competition assays were performed according to Lee, et al. (1992) using 200 μg/ml of *M. sexta* BBMV and 1 nM of $^{125}$I-Cry1Ac. BBMV were incubated with 1nM of $^{125}$I-labeled toxins (wild-type) and increasing concentration of unlabeled mutant toxins in 100μl of binding buffer containing 0.1% bovine serum albumin (BSA). After 1h of incubation at 25°C the sample was centrifuged
for 10 min at 15,000 rpm and the BBMV pellet washed three times with binding buffer to remove the unbound 125I-labeled toxin. The radioactivity on the washed pellet was determined by counting in a gamma counter (Beckman). Binding affinity ($K_{\text{com}}$) and binding site concentration ($B_{\text{max}}$) of the bound toxin was analyzed by LIGAND program (Munson & Rodbard, 1980). The term $K_{\text{com}}$ was introduced recently by Wu & Dean (1996) to describe the binding constant derived specifically from competition studies of Cry toxins with BBMV that involve both reversible (initial) and irreversible binding.

**Dissociation assay and the rate of irreversible binding**

Dissociation assays were performed according to the method of Chen *et al.* (1995) using 1 nM of $^{125}$I-toxin and 200 μg/ml of *M. sexta* BBMV. The $^{125}$I-toxin-BBMV mixture was incubated for 1 h at 25°C and then competed off at different time intervals (0-60 min) with 400-fold excess of unlabeled toxin. The BBMV were then centrifuged, washed, and counted on a gamma counter (Beckman Instruments).

The irreversible binding rate was determined according to the method of Liang *et al.* (1995). Briefly in this pulse-chase experiment, $^{125}$I-toxin is incubated with *M. sexta* BBMV for different time intervals (0-80 min) and then chased off with 400-fold excess of unlabeled toxin for 1 h. BBMV is then pelleted, washed, and the radioactivity was counted on a gamma counter.
Voltage Clamp Analysis

Inhibition of short circuit current ($I_{sc}$) by wild type and mutant toxins, across the *M. sexta* midgut membrane was determined by voltage clamp as described earlier (Chen *et al.*, 1993). The anterior midgut of a freshly dissected larva is mounted on an orifice (0.25 cm diameter) and inserted into the voltage clamping chamber. The equipment for voltage clamp consists of D.C. 1000 voltage/current clamp, and an A-310 Accupulser (World Precision Instruments) and a strip chart recorder (Kipp and Zonen) linked to a Macintosh computer with a MacLab data acquisation system. Voltage was measured with Calomel electrodes. KCl-4% agar bridges, made from polyethylene tubing PE-190 with i.d 1.19 mm (Clay Adams Becton, Dickinson & Co.), connected the electrodes to the chamber. The voltage clamp chamber, holder and disc were constructed at The Ohio-State University medical workshop. Each side of the chamber holds 3.5 ml of the liquid. The bathing solution used was as described by Chamberlin (1990): 2mM Hepes, 5mM MgCl$_2$, 1mM CaCl$_2$, 5.8mM KOH, 9mM NaOH, 3mM Na-methylsulfate, 7.7 mM K-citrate, 2.8 mM Na-succinate, 5.6 mM malic acid, 2mM glucose, 27mM trihalose dihydrate, 9.4 mM glutamine, 8.9mM serine, 7.4mM proline, 12.8 mM glycine, 4.6 mM threonine, 3.6mM alanine, 23.1mM N-methyl-D-glucamine, 140 mM PEG-400 pH 6.7. The chamber buffer was continuously bubbled with oxygen. After stabilizing the current, 200 ng/ml of either wild type or mutant, toxin was added on the luminal side of the chamber and the change in
short circuit current (Isc) over time was recorded. The recorded data was plotted as the percent inhibition of Isc against time.

Circular Dichroism Spectroscopy

The wild type and mutant toxins were purified by size exclusion HPLC using Sec 30-XL column (Bio Rad). Sodium bicarbonate (50 mM) at pH 9.6 was used as an elution buffer. The purified proteins were dialyzed against 10 mM sodium phosphate buffer pH 9.5.

CD spectra for Cry1Ac and its mutants, H168Q and H168R, were recorded on a Jasco J-500A spectropolarimeter equipped with a computer interface software. Ellipticity was calibrated with 26.69 mM D-10-camphorosulfonic acid solution. Temperature was controlled with NESLAB operating temperature system connected to a NESLAB water bath RTE-100 and a NESLAB bath/computer interface M-RS-232. HELLMMA cuvettes of 1.0 cm path length were employed containing 100 µg of either wild type or mutant toxin in 10 mM sodium phosphate buffer (pH 9.5). The wave length scanned ranged from 190-265 nm at room temperature. The data were transformed into molar ellipticity by using the equation (Woody, 1995).

\[
\theta = 100 \frac{\theta}{lc}
\]

Where \([\theta]\) is Molar Ellipticity [Deg. cm\(^2\). dmol\(^{-1}\)]. \(\theta\): Ellipticity (mdeg). \(l\): cell path (cm). \(c\): molar concentrations.
Thermal denaturation profile of the mutant toxins as compared to the wild type toxin was followed at 222 nm with temperature ranging from 40° to 80°C in 2°C increments.

RESULTS

Stability and toxicity of Mutant Proteins

Protoxin inclusion of Cry1Ac and the mutants, H168Q and H168R, produced stable toxins upon trypsin digestion. These activated toxins, when treated with M. sexta gut juice, showed that both the mutants have similar stability to wild type Cry1Ac toxin. (data not shown). Bioassays were performed with Cry1Ac, H168Q and H168R toxins, against M. sexta (Table 1.1). The LC\textsubscript{50} value indicates that mutant toxin, H168R is 2.5 fold more toxic than Cry1Ac. On the other hand, H168Q mutant toxin was about 3 fold less toxic than Cry1Ac for M. sexta (Table 1.1).

Competition Binding Studies

Heterologous competition assays were performed in order to determine if the mutant toxins affected the initial binding to the M. sexta membrane receptors.
$^{125}$I-Cry1Ac toxin was competed against unlabeled wild-type and mutant toxins for *M. sexta* BBMV (Figure 1.1). It was observed that although H168Q toxin has reduced toxicity for *M. sexta* larvae, it did not affect its initial binding ($K_{\text{on}}$ 3.65 nM) significantly as compared to the wild-type toxin (2.4 nM). The two-fold better toxic mutant H168R, also has binding affinity (1.96 nM) similar to the wild-type toxin. Binding site concentration ($B_{\text{max}}$) was also similar for Cry1Ac, H168Q, and H168R, indicating that the increase in toxicity of H168R for *M. sexta* is not due to the differences in either binding affinity or the number of binding sites. (Table 1.1).
Table 1.1: The effect of mutations of the H168 residue of Cry1Ac on larval toxicity, binding, to midgut membranes and short circuit current.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>$a\text{LC}_{50}$ (ng/cm²)</th>
<th>$bK_{\text{com}}$ (nM)</th>
<th>$cB_{\text{max}}$ (pmol/mg)</th>
<th>$dK_{\text{obs}}$ (min⁻¹)</th>
<th>$I_{\text{sc}}$slope µA/min/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry1Ac</td>
<td>5.25 (3.1-7.9)</td>
<td>$b2.45 \pm 0.8$</td>
<td>$62.2 \pm 1.4$</td>
<td>$3.4 \times 10^2$</td>
<td>$-15.2 \pm 2.3$</td>
</tr>
<tr>
<td>H168Q</td>
<td>15.5 (11.1-20.2)</td>
<td>$3.65 \pm 0.6$</td>
<td>$60.4 \pm 1.7$</td>
<td>$^c\text{ND}$</td>
<td>$-5.42 \pm 1.5$</td>
</tr>
<tr>
<td>H168R</td>
<td>2.04 (1.1-3.2)</td>
<td>$1.95 \pm 0.7$</td>
<td>$59.6 \pm 1.6$</td>
<td>$2.9 \times 10^1$</td>
<td>$-32.0 \pm 4.2$</td>
</tr>
</tbody>
</table>

$a$50% lethal concentration (95% fiducial limit). $b$Heterologous competition with labeled H168R and unlabeled Cry1Ac. $c$Binding site concentration on BBMV. $d$Rate of irreversible binding. $^e$Not determined. The values are expressed as means of three determinations.
Figure 1.1. Competition binding assay of $^{125}$I-labeled Cry1Ac. Binding is expressed as the percentage of labeled toxin bound to *M. sexta* BBMV after competing with increasing amount of mutant toxins.
**Dissociation Assay and the Rate of Irreversible Binding**

Dissociation binding experiment were performed to determine the percentage of toxin remaining irreversibly bound to the BBMV after prolonged incubation with labeled toxin. In the presence of excess amounts of unlabeled toxin, Cry1Ac and H168R showed less dissociation with more than 92% and 85%, respectively, and remained bound to the BBMV after 60 min. of chase with 400 fold excess of corresponding unlabeled toxin. On the other hand, less toxic mutant H168Q showed greater dissociation with only 68% remaining bound at 60 min (Figure 1.2).

The rate of irreversible binding ($K_{obs}$) was measured to determine if greater toxicity of H168R toxin, as compared to Cry1Ac toxin, is due to faster insertion into *M. sexta* BBMV (Table 1). It was surprising to note that H168R toxin has a slower insertion rate ($2.9 \times 10^{-1} \text{ min}^{-1}$) as compared to Cry1Ac ($3.4 \times 10^{-2} \text{ min}^{-1}$).

**CD Spectra of Wild Type and Mutant Toxin**

To determine the changes in the secondary structure of the mutant toxins, H168Q and H168R, as compared to Cry1Ac toxin, CD spectra was measured from 192 to 265 nm (Figure 1.3). The global structure of the mutant toxins was not affected, however, there were some differences in CD spectrum between Cry1Ac and the two mutant toxins in the range from 205 to 223 nm. (Figure 1.3).
Figure 1.2. Dissociation assay of Cry1Ac mutants. Binding is expressed as the percentage of toxin remaining bound to the BBMV after adding 400 fold excess of corresponding unlabeled toxin. Non-specific binding is subtracted from the total binding.
Figure 1.3. Circular dichroism spectra in the far UV regions of the wild-type and mutant toxins. Measurements were made in 10 mM sodium phosphate buffer (pH 9.5). Protein concentration was 0.1 mg/ml.
Effect on the inhibition of $I_{sc}$

To determine the effect of mutations on ion-conductance voltage clamp analysis was carried with isolated midgut of $M$. sexta larvae. It was observed that, at a toxin concentration of 200 ng/ml, H168R showed a greater inhibition of the slope of $I_{sc}$ (slope -32.0 $\mu$A/min/cm$^2$), than the wild-type toxin (slope -15.2 $\mu$A/min/cm$^2$). On the other hand under the same conditions H168Q caused a slower decline in $I_{sc}$ (Table 1.1; Figure 1.4).

Thermal CD Analysis

In order to determine the structural stability, all three toxins were heated from 40 to 80°C. Cry1Ac toxin and H168Q showed similar melting temperature ($T_m$) with 50% unfolded at 56°C and 57.4°C respectively. However, H168R toxin showed a shift in $T_m$ with 2.8° and 1.7°C higher melting temperature than Cry1Ac and H168Q toxins respectively (Figure 1.5).
Figure 1.4. Inhibition of short circuit current ($I_{sc}$) across *M. sexta* midgut. Wild-type and mutant toxins were applied on the luminal side of the voltage clamp chamber. The final concentration for each toxin was 200 ng/ml. A control experiment without any toxin was also carried out. Time zero is the time for the addition of toxin.
Figure 1.5. Thermal CD spectra of wild-type and mutant toxins. The data recorded at 223 nm with temperature ranging from 40° to 80°C. Molar ellipticity is converted to percent unfolded.
DISCUSSION

Since the α5 helix has a critical role in toxicity, the effects of specific mutations on a highly conserved residue, H168, were extensively analyzed. It is likely that this amphipathic α5 helix serves as a transmembrane pore-lining segment and H168, being in the center of the helix, may be involved in sub-unit interaction (Gazit & Shahi 1995; Wu & Aronson 1992).

Both mutant H168Q and H168R protoxins were converted to stable trypsin-resistant toxins. The CD spectra of the mutants showed relatively higher absorbance in the peptide region suggesting that the mutated residues may have reoriented the alpha helices in order to adopt a more compact globular conformation (Figure 1.3). Incubation of Cry1Ac, H168Q and H168R toxins with *M. sexta* gut juice indicated that the mutants are as stable as the wild-type toxin (data not shown). Therefore, gross structural alterations are not a likely reason for more than two fold higher toxicity of H168R for *M. sexta* (Table 1.1).
The binding affinities of the mutant toxins were determined by heterologous competition binding analyses. None of the toxins have a significant effect on initial binding affinities, $K_{\text{com}}$, indicating the fact that the mutants bind to the same receptor with similar binding affinity as the wild-type toxin (Table 1.1; Figure 1.1.). Studies with other Cry1A toxins have shown that mutations in domain I may have a drastic effect on toxicity but the initial receptor binding remains unaffected (Chen et al., 1995). This indicates that domain I is not involved in initial binding to the membrane.

The binding of toxin to the BBMV has now been suggested to include initial reversible binding and an irreversible binding stage (Liang et al., 1995). Several studies suggest that irreversible binding indirectly indicates toxin insertion into the membrane (Wolfersberger et al., 1987; Van Rie et al., 1989; Ihara et al., 1993; Chen et al., 1995). Higher irreversible association of Cry1Ac (92%) and H168R (85%) indicates that the toxic proteins can associate properly with the *M. sexta* membrane. On the contrary the less toxic mutant, H168Q, also shows greater dissociation from the *M. sexta* BBMV indicating a defective insertion into the membrane or lesser tendency to associate with the membrane receptor and may account for the lower toxicity (Table 1.1; Figure 1.2).

Rate of irreversible binding is another criteria to determine if the toxin is inserting efficiently into the membrane. Since H168R has two fold greater
toxicity than the wild-type toxin we speculated that it will also show a faster rate of insertion. However, our experiment indicated a converse relationship (Table 1.1).

Dissociation assays and the rate of irreversible binding does not explain the reason for 2.5 fold higher toxicity with H168R toxin as compared to the wild-type toxin. Voltage clamp method was, therefore, used to determine the channel forming efficiency of wild-type and mutant toxins. In contrast to reversible binding and irreversible binding rate data, sharp differences in the inhibition of Isc are observed. These differences are correlated with the toxicity of the toxins for *M. sexta* with H168R showing a two-fold steeper drop in Isc (-32.0 μA/min/cm²), than the wild-type toxin (-15.2 μA/min/cm²). Similarly three fold less toxic mutant H168Q, caused a shallower drop in Isc (Table 1.1; Figure 1.4). This relationship suggests that H168R can form better ion-channels than Cry1Ac.

In order to understand the reason for better ion-conductance activity of H168R we performed thermal CD analysis. H168R was found to be more thermostable with 2.8°C higher Tm than Cry1Ac and 1.7°C higher Tm than H168Q toxin (Figure 1.5).

The molecular modeling approach was adopted to determine the reason for increased thermostability. One of the δ-endotoxin, Cry3A although shows
only 37% sequence homology to Cry1Aa and Cry1Ac. The crystal structure of Cry3A and Cry1Aa show high overall similarity with domain I of Cry1Aa superimposed on Cry3A (Grochulski et al., 1995). Cry 1Ac is more than 89% homologous to Cry1Aa, therefore, it is considered to have a similar 3-D structure. The coordinates for the crystal structure of Cry1Aa are not available at present, however, based on the overall crystal homology between Cry1Aa and Cry1Ac we used program Quanta (Byosys. Inc.) to determine the effect on electrostatic interaction, between residues of domain I, by substituting Arg and Gln in Cry3A at position equivalent to His168. It was found that in wild-type toxin H168 only forms hydrogen bonds with two alanine residues, A164 and A165, within the α5 helix (Fig. 1.6A). Mutant toxin H168Q showed higher hydrogen bonding interaction than the wild-type toxin with an additional H-bond with a highly conserved Y203 in α6 helix Fig. 1.6B). Mutant H168R, besides forming H-bonds with A164, A165 and Y203, is also likely to form a salt bridge with E136 in α4 helix (Fig. 1.6C). As α5 is a central helix, therefore, forming a salt bridge with α4 helix and H-bond with α6 helix may cause domain I to have greater stabilization energy which may account for the higher thermostability of mutant H168R.
Figure 1.6. Cross-section of domain 1 α-helical bundles viewed from the bulky end of the Cry3A molecule. Electrostatic interactions (H-bonds and salt bridge) around conserved His residue in Cry3A, at position equivalent of Cry1A, H168, are shown from side-chain atoms by using program Quanta (Biosys. Inc). A, B and C are wild-type, H168Q and H168R, respectively.
There are several possibilities to explain why an additional salt bridge in H168R may make the toxin a better ion-conductor. In Cry1Aa toxin, similar to Cry3A toxin, α4 helix form a helical hairpin with α5 helix (Grochulski et al., 1995). It has been also observed that one of the Cry1 type δ-endotoxin, Cry1Ac tend to oligomerize at alkaline pH (personal observation, M. K. Lee and D. H. Dean). Based on this information it is possible that formation of a salt bridge in H168R, increases the stability and reduces the mobility of α-4 with respect to α5 helix giving the whole molecule a greater ability to oligomerize and disrupt the membrane.

CONCLUSION

Substitution of highly conserved H168 for Q or R, although produced subtle changes in toxicity, has helped us to find some of the residues which are critical for the ion channel function of Cry1Ac δ-endotoxin. Our studies showed that in α5 helix mutating H168 to R increases the toxicity, ion conductance and thermostability. On the other hand although H168Q increases in thermostability there is a lower inhibition of short-circuit current and toxicity towards M. sexta. We conclude that stability of domain I alone does not fully explain the reason for increased toxicity. Orientation of the toxin molecule with respect to the membrane of the susceptible insect or direct participation of 168 residue in ion channel function may also be important factors in determining toxicity.
CHAPTER 2

Positively charged residues on the proximal side of domain I are important in irreversible binding of Bacillus thuringiensis Cry1A δ-endotoxins

INTRODUCTION

The entomopathogenic crystalline inclusions produced by Bacillus thuringiensis have been widely used in the control of agricultural pests because of their great specificity and lack of harmful side effects. These parasporal crystals are comprised of δ-endotoxins, or Cry proteins, which are expressed as protoxins. Upon ingestion by susceptible insect larvae, the protoxins are solublized in the alkaline midgut and are converted to active toxins by midgut proteases (Tojo & Aizawa, 1983). The 65 kDa activated toxins pass through the peritrophic membrane and bind to receptors located on the surface of midgut epithelial cells of the larvae (Hofmann et al., 1988) and then
insert into the membrane. The inserted toxins disrupt the ionic balance but the
decision is not clear, being described as either forming small non-specific
pores (Knowles & Ellar 1987) or ion channels (Schawrtz et al., 1993). This
leads to a gradual elongation and swelling of the midgut epithelial cells (Lüthy

Most of the Cry proteins contain five conserved blocks of amino acids
(Höfte & Whiteley 1989). Based on the crystal structures of the Cry3A and the
recently elucidated Cry1Ac toxins (Li et al., 1991; Grochulski et al., 1995) it is
likely that all the Cry proteins have a similar tertiary structure comprised of three
domains. Domain I is a bundle of seven amphipathic α-helices with α5 in the
center. Domain II is comprised of three β-sheets with loops at the apex of the
β-hairpin extensions. Beta-sheet three has an axis of pseudo-symmetry nearly
parallel to the α5 helical axis. Domain III is a sandwich of two anti-parallel β-
sheets (Grochulski et al., 1995). It has been postulated that domain I (or
certain α-helices) inserts into the membrane and either interacts with a pre-
existing channel or forms an ion channel (Gill et al., 1992; Hodgman & Ellar

Studies with synthetic peptides corresponding to the α-5 and α-6
helices have supported the role of domain I in insertion and ion-channel
formation (Gazit & Shahi, 1995). In addition, the effects of mutations in various
regions of domain I are consistent with this model. In particular, it was observed that introduction of a negatively charged residue at position Y153 in the loop between helices $\alpha$-4 and $\alpha$-5 and at the proximal side of helix $\alpha$-3 at positions A92 and A93, drastically reduced toxicity for target insects (Wu & Aronson, 1992; Chen et al., 1995). This reduction in toxicity was reported to be directly correlated with a reduction in irreversible binding and channel formation (Chen et al., 1995).

It is interesting that these particular domain I mutations were on a surface of the toxin which is the same as certain loops in domain II which have been implicated in the initial reversible binding to the receptor. In this communication we have further analyzed the importance of amino acid residues of the Cry1Ab and Cry1Ac toxins on both the proximal and distal sides of the $\alpha$-helices of domain I relative to these loops in domain II, for toxicity and irreversible binding. Only mutations on the proximal side resulted in reduced irreversible binding and toxicity.

**MATERIALS AND METHODS**

*Construction, Expression and Stability of mutant proteins.*

Site-directed mutagenesis was carried out according to the method of Kunkel et al. (1985) using a uracil-containing pSB033 vector and *E. coli*
MV1190 as a host (Chen et al., 1995). This system was used both for mutations and the expression of proteins. Mutations in the cry1Ac gene were prepared as previously described (Wu & Aronson, 1992). Mutant colonies were screened by the dideoxy sequencing method using Sequenase Version 2.0 kit (US Biochemicals). The solubilized protoxins were activated to 65 kDa toxins with 5% (w/w) trypsin (Chen et al., 1993).

Toxicity assay.

Bioassays were performed with Manduca sexta neonate larvae by surface contamination method (Chen et al., 1995). Artificial diet was was prepared following manufactures instruction (BioServ Inc. Frenchtown New Jersey). Two ml. of the molten diet was poured in Falcon tissue culture dishes with a diameter of 2cm² and allowed to congeal. Various dilutions of toxin solution (50µl) was poured on the diet surface and allowed to dry. Two day old M. sexta larvae was placed on the the dried surface and the mortality was monitored after five days. The effective dose estimate (LC50) was calculated using the Probit program (Raymond, 1985).

Preparation of Brush border membrane vesicles (BBMV)

Briefly M.sexta fifth instar larvae were chilled on ice until dead. The chilled lavae were dissected behind the second thoracic appendage pairs. The peritropic membrane was removed and the midgut was rinsed in ice cold
buffer A (300 mM mannitol, 5mM EGTA, 17 mM Tris-HCl, pH 7.5). The midguts were kept in liquid nitrogen before making BBMV.

*M. sexta* brush border membrane vesicles (BBMV) were prepared from fifth instar larvae by the differential magnesium precipitation method of Wolfersberger *et al.* (1987). The final BBMV pellet was resuspended in binding buffer (150mM NaCl, 8mMNa_{2}HPO4, 2mM KH_{2}PO4, pH 7.4) and stored in liquid nitrogen until further use.

**Ligand binding assays**

Iodination of trypsin-activated toxins and heterologous competition assays were performed as described by Lee *et al.* (1992). For the competition binding assay 20 μg of *M. sexta* BBMV was incubated for 1h with 0.5 nM ¹²⁵I-labeled toxin in 100μl of binding buffer in the presence of varying concentrations of unlabeled toxin. Data was analysed using the LIGAND computer program (Munson and Rodbard, 1980).

**Dissociation assays**

Dissociation assays were performed according to the procedure of Chen *et al.* (1995). Briefly, 1 nM of ¹²⁵I-labeled wild type or mutant toxin was first incubated with 20μg of BBMV for 1h at 27°C. At various time intervals thereafter (0-60 min), a 500 fold excess of unlabeled toxin was added to chase
off reversibly bound toxin. The pellet was washed twice with binding buffer and counted in a gamma counter (Beckman Instruments). Non-specific binding was determined by adding labeled toxin and a 500 fold excess of unlabeled toxin together to the BBMV and was subtracted from the total counts per minute (cpm).

RESULTS AND DISCUSSION

Effect of mutations on the stability of protoxin and toxin

Mutations in domain I of the Cry1Ab and Cry1Ac toxins are listed in Fig. 2.1 A&B. The double mutant A2 (147LF148--AA), in which leucine and phenylalanine were replaced by alanines, did not produce stable toxin upon trypsin digestion. Another double mutant, A3 (180QR181--AA), failed to yield any detectable protoxin in E. coli. Other Cry1Ab mutants, R115A, A119D, A119E, A119G, and A1(117WE118--AA) produced stable toxin upon trypsin digestion. Similarly, the selected Cry1Ac mutants, A92D, A92C, and R39F, all formed toxins as stable as the wild type toxin when incubated with M. sexta midgut juice (data not shown).
Figure 2.1. Mutations in domain I of Cry1Ab and Cry1Ac. A: Protoxin and stable toxin mutants of Cry1Ab. Amino acid residues between 122-144 and 151-178 are not shown. B: Mutants of Cry1Ac which give stable toxins. (+) stable expression of protoxin or toxin, (−) unstable protein, (±) unstable upon storage. Classification of δ-endotoxins is based on the revised nomenclature (Cickmore et al., 1995).
### [A]

<table>
<thead>
<tr>
<th>Allele</th>
<th>Amino Acid Sequence</th>
<th>Protoxin</th>
<th>Toxin</th>
</tr>
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<tr>
<td>Cry1Ab</td>
<td>SFREWEADP&lt;sup&gt;121&lt;/sup&gt;.. IPLFAV&lt;sup&gt;150&lt;/sup&gt;.. GQRW&lt;sup&gt;182&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R115A</td>
<td>SFAEWEADP</td>
<td>IPLFAV</td>
<td>GQRW</td>
</tr>
<tr>
<td>A119D</td>
<td>SFREWEDDP</td>
<td>IPLFAV</td>
<td>GQRW</td>
</tr>
<tr>
<td>A119E</td>
<td>SFREWEDDP</td>
<td>IPLFAV</td>
<td>GQRW</td>
</tr>
<tr>
<td>A119G</td>
<td>SFREWEGDP</td>
<td>IPLFAV</td>
<td>GQRW</td>
</tr>
<tr>
<td>A1&lt;sub&gt;(117WE&lt;sub&gt;118&lt;/sub&gt;−AA)&lt;/sub&gt;</td>
<td>SFREAADP</td>
<td>IPLFAV</td>
<td>GQRW</td>
</tr>
<tr>
<td>A2&lt;sub&gt;(147LF&lt;sub&gt;148&lt;/sub&gt;−AA)&lt;/sub&gt;</td>
<td>SFREWEADP</td>
<td>IPAAAV</td>
<td>GQRW</td>
</tr>
<tr>
<td>A3&lt;sub&gt;(180QR&lt;sub&gt;181&lt;/sub&gt;−AA)&lt;/sub&gt;</td>
<td>SFREWEADP</td>
<td>IPLFAV</td>
<td>GAAW</td>
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</tbody>
</table>

### [B]

<table>
<thead>
<tr>
<th>Allele</th>
<th>Amino Acid sequence</th>
<th>Toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry1Ac</td>
<td>EEFARNQ&lt;sup&gt;65&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>A92C</td>
<td>EEFCRNQ</td>
<td>+</td>
</tr>
<tr>
<td>A92D</td>
<td>EEFDRNQ</td>
<td>+</td>
</tr>
<tr>
<td>R93F</td>
<td>EEFAFNQ</td>
<td>+</td>
</tr>
</tbody>
</table>

[Figure 2.1]
Location of mutant proteins.

The mutated residues 92-93 (A92D & R93F) and 147-148 (A2) are present at the base of helices α-3 and α-4, respectively. Based on the structural alignment with the Cry1Aa toxin, this region is on the same side as potential receptor binding loops in domain II (Grochulski, et al., 1995). Mutations of amino acid residues 115-119 (R115A, A1, A119D,E or G) and 180-181 (A3), however, are located in loops between helices α-3-α-4 and α-5-α-6, respectively. This region is on the side opposite to the loops of domain II. The face of domain I aligned with the loops in domain II is designated the proximal side, whereas the opposing face is considered the distal side or face.

Effect on toxicity

There was no significant variation in the LC50 values for the wild type Cry1Ab and distal side mutants R115A, A119D, A119E, A119G, and A1 (Table 2.1). On the other hand, mutation A92D in Cry1Ac did result in the loss of toxicity (Wu & Aronson, 1992). All other changes at Ala92, such as A92C, were as toxic for M. sexta as the wild type toxin. In the case of Arg93, any mutation that removed the positive charge, such as R93F, resulted in reduced toxicity (Wu & Aronson, 1992). Similar results were obtained with A92E and Y153D in Cry1Ab (Chen et al., 1995). These bioassay results showed that mutations which removed a positive charge on the presumptive distal side of the toxin did not affect toxicity.
Table 2.1. Toxicity of Cry1Ab and its mutants, located on the distal surface of domain I, against *M. sexta* larvae

<table>
<thead>
<tr>
<th>Toxin</th>
<th>*LC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>FL&lt;sub&gt;95&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry1Ab</td>
<td>12.5</td>
<td>8.7-18.5</td>
</tr>
<tr>
<td>R115A</td>
<td>17.2</td>
<td>9.5-24.6</td>
</tr>
<tr>
<td>A119D</td>
<td>10.7</td>
<td>7.5-17.9</td>
</tr>
<tr>
<td>A119E</td>
<td>11.6</td>
<td>8.7-17.2</td>
</tr>
<tr>
<td>A119G</td>
<td>12.2</td>
<td>8.5-18.2</td>
</tr>
<tr>
<td>A1</td>
<td>18.2</td>
<td>9.5-25.2</td>
</tr>
</tbody>
</table>

* 50% lethal concentration (LC<sub>50</sub>) and 95% fiducial limits (FL<sub>95</sub>) are expressed in ng/cm<sup>2</sup> of the diet surface area. 12 neonate larvae were used for each dilution. LC<sub>50</sub> value is the average of three assays.
Effect of mutations on initial binding

In order to determine whether the low toxicity of mutants A92D and R93F was due to reduced initial binding to the membrane receptor, heterologous competition assays were done. ¹²⁵I-labeled Cry1Ab or Cry1Ac toxin was competed with unlabeled mutant toxins for binding to *M. sexta* BBMV (Fig. 2.2 A&B). It was observed that none of the mutations had any significant effect on the initial binding as reflected by their binding affinities [K<sub>com</sub>] (Table 2.2). Studies with a BIA Core surface plasma resonance biosensor (Pharmacia) have also shown that the A92D toxin does not affect the rate of initial binding (L. Masson, personal communication). Similar observations have been made with the Cry1Ab toxin when Ala92 was mutated to Glu (A92E) (Chen *et al.*, 1995). Earlier studies had also shown that mutations F50N, V51N, A165P, and L167M in the N-terminal hydrophobic region of the Cry1Aa toxin did not affect binding to tissue culture cells, but there was lower cytotoxicity (Ahmad & Ellar, 1990).

Effect of mutation on irreversible association

In order to determine if the post-initial binding events had been altered in mutants with no toxicity for *M. sexta*, irreversible binding experiments were performed. In the presence of an excess amount of unlabeled toxin, ¹²⁵I-labeled A92D and R93F showed greater dissociation than wild type Cry1A or mutant A92C toxins (Fig. 2.3A). More than 94% of the Cry1Ac toxin remained...
Figure 2.2. Competition binding assay of $^{125}$I-labeled Cry1Ab (A) and Cry1Ac (B). Binding is expressed as the percentage of labeled toxin bound to the *M. sexta* BBMV (200μg/ml) after competition with increasing amounts of mutant toxins.
[Figure 2.2]
[Figure 2.2 cont.]

![Graph showing the percentage of bound toxin with different competitors vs. competitor concentration.]

**COMPETITOR (nM)**

B
Table 2.2 Effect of Cry1Ab and Cry1Ac mutations on the initial binding to the BBMV of *M. sexta*.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>$K_{\text{com}}$</th>
<th>$B_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cry1Ab</em>(wt)</td>
<td>8.1 ± 0.53</td>
<td>58.6 ± 1.3</td>
</tr>
<tr>
<td>R115A</td>
<td>4.7 ± 1.25</td>
<td>38.7 ± 2.5</td>
</tr>
<tr>
<td>A119D</td>
<td>5.2 ± 1.45</td>
<td>42.5 ± 1.7</td>
</tr>
<tr>
<td>A119E</td>
<td>6.0 ± 0.75</td>
<td>60.2 ± 1.2</td>
</tr>
<tr>
<td>A119G</td>
<td>6.9 ± 0.83</td>
<td>60.1 ± 1.2</td>
</tr>
<tr>
<td>A1</td>
<td>6.9 ± 1.24</td>
<td>55.3 ± 2.1</td>
</tr>
<tr>
<td><em>Cry1Ac</em> (wt)</td>
<td>2.75 ± 0.53</td>
<td>55.2 ± 1.8</td>
</tr>
<tr>
<td>A92C</td>
<td>2.92 ± 0.08</td>
<td>42.1 ± 2.7</td>
</tr>
<tr>
<td>A92D</td>
<td>2.95 ± 0.13</td>
<td>47.5 ± 1.7</td>
</tr>
<tr>
<td>R93F</td>
<td>1.85 ± 0.11</td>
<td>60.1 ± 1.6</td>
</tr>
</tbody>
</table>

* Dissociation constants ($K_{\text{com}}$) are expressed in nM. Binding site concentrations ($B_{\text{max}}$) are expressed in pmol/mg of BBMV. $K_{\text{com}}$ (binding affinity) has earlier been used by Wu & Dean (1996) to define the binding constant derived from the competition assay for Cry toxins. 
(i) data obtained by competing labeled A119G and unlabeled Cry1Ab. (ii) heterologous competition with labeled A92C and unlabeled Cry1Ac. The values are the mean of four determinations.
bound to the BBMV after 80 min of chase. On the other hand, the non-toxic mutants, A92D and R93F, continued to dissociate with only 65% and 76%, respectively, remaining irreversibly bound after 80 min. These observations are in agreement with earlier reports demonstrating that in the Cry1Ab domain I mutants, A92E and Y153D (with reduced toxicity), there was less irreversible binding although the initial binding was not affected (Chen et al., 1995). All of the toxic mutants have irreversible binding comparable to the wild type toxin.

In order to confirm these findings, we performed dissociation assays with the fully toxic Cry1Ab mutant toxins, A119D and A119G, and compared the results with those obtained with the non-toxic mutant, A92E. As noted previously, the percentage of active mutant toxin bound irreversibly to BBMV was similar to the wild type toxin, while the A92E toxin had a sharp decline in the percentage that remained bound (Fig. 2.3B). It was observed, however, that the Cry1Ab mutant toxin, A92E, was displaced more than the Cry1Ac mutant toxin, A92D. This may be due to the greater propensity of Cry1Ac to aggregate and thus greater difficulty in removing surface bound toxin from BBMV.

Irreversible binding is indicative of toxin insertion into the membrane (Wolfersberger et al., 1987; Hofmann et al., 1988a; Ihara et al., 1993). Reduced irreversible binding implies that the membrane insertion process has been disturbed and thus results in a reduction in ion-conductance and toxicity (Chen et al., 1995).
Figure 2.3 Dissociation assay of Cry1Ac mutants (A) and Cry1Ab mutants (B). Binding is expressed as the percentage of toxin remaining bound to the BBMV after adding 1000 fold excess of corresponding unlabeled toxin. Non-specific binding has been subtracted from the total binding.
[Figure 2.3]
B
A defective insertion process as observed with mutants A92E, A92D and R93F (Fig. 2.3 A&B) can be explained on the basis of the recently determined crystal structure of the Cry1Aa toxin (greater than 95% sequence homology with the Cry1Ab and Cry1Ac toxins). Arg93 is involved in salt bridges with Glu81 and Asp74 (Grochulski et al., 1995), so a mutation of Arg93 to Phe (R93F) would be destabilizing to this charge interaction. Similarly, Ala92 is not solvent exposed but rather faces the apolar α4-α5 loop. Introduction of a negatively charged residue may have a repulsive effect when the toxin comes in contact with the negatively charged environment of the insect membrane.

On the other hand, when a negatively charged residue was introduced at position Ala119 (A119D and A119E), which lies in the loop between α3-α4 on the distal side of domain I, no effect on toxicity or irreversible binding was found (Table 2.1, Fig. 2.3B). Arg115 is reported to form a salt bridge with Asp112 (Grochulski et al., 1995) but mutating Arg115 to Ala (R115A) did not result in a reduction of the toxicity or irreversible binding of the mutant toxin for M. sexta (Table 2.1, Fig 2.3A.). This indicates that, unlike the positively charged residue on the proximal site (R93), a positive charge (R115) on the distal surface does not appear to have as critical a role in toxin insertion.
We have noted that the proximal surface of domain I is the same as the loops in domain II which are involved in the initial reversible binding of the toxin to the receptor. This correlation implies that this orientation of the toxin is critical for the first two steps of binding to the receptor and toxin insertion into the membrane. This latter step may appear to involve ionic interactions with the membrane of these loops in domain I, an interaction which is essential for the subsequent insertion into the membrane of the amphipathic, pore-forming helices.
CHAPTER 3

Substitution of *Bacillus thuringiensis* Cry1Ab δ-endotoxin Domain II Loops with Cry1Aa sequences enhances toxicity of the hybrid for *Bombyx mori*

**INTRODUCTION**

Recent elucidation of the X-ray crystal structure of, *Bacillus thuringiensis*, Cry3A and Cry1Aa, δ-endotoxins have shown that the three domains of the δ-endotoxins are built on five conserved blocks of amino acids with high structural similarity (Li *et al.*, 1991; Grochulski *et al.*, 1995). Several studies have attempted to analyze the important regions of toxins for receptor binding and toxicity through site-directed mutagenesis or homolog-scanning approaches. These studies have shown that domain I mutations affect toxicity and ion-conductance (Ahmad & Ellar, 1990; Wu & Aronson, 1992; Chen *et al.*, 1995). The functional role of domain III has not yet been clearly dissected. Li *et al.*
(1991) consider this jelly-roll sandwich of β-sheets to be involved in structural stability. However, mounting evidence suggests that domain III also plays a role in ion-conductance (Chen et al., 1993; Wolfersberger et al., 1996), specificity (Bosch et al., 1994) and receptor binding (Lee et al., 1995).

Domain-swapping between homologous portions of the toxins has successfully identified the specificity region of Cry1A-type and CryII toxins for lepidopteran and dipteran, respectively (Ge et al., 1989, 1991; Liang et al., 1994). These experiments have marked the residues from 332-450 within the hypervariable region of Cry1Aa to be critical for Bombyx mori toxicity and receptor binding (Ge et al., 1989; Lee et al., 1992). This region has now shown to be in the domain II of Cry1Aa (Grochulski et al., 1995).

Alanine block and deletion of the residues (366LYRRIIL370) in loop 2 and contiguous β-sheet 6, of domain II in Cry1Aa caused considerably reduced binding affinity and essentially no toxicity for B. mori (Lu et al., 1994). Recent studies involving Cry1Ab residues, 368RRP370, have also been shown to be important for initial binding affinity (Rajamohan et al., 1995), whereas residues F371 and G374 affect irreversible binding to Manduca sexta BBMV (Rajamohan et al., 1996). Further studies on loop 3 of domain II in Cry1Aa and Cry1Ab have
shown that hydrophobicity of this loop is also important for initial receptor binding (Rajamohan et al., 1996).

These studies have identified that at least loop 2 and loop 3 of domain II play a critical role in insect specificity and toxicity. In the present study, we have substituted a region of loops 2 and 3 of Cry1Aa into Cry1Ab in order to alter its specificity for *B. mori*. Bioassays, receptor binding, and voltage clamp analysis showed that double substitution (loop 2 and loop 3) resulted in a hybrid Cry1Ab protein with increased toxicity and ion conductance for *B. mori*.

**MATERIALS AND METHODS**

**Construction of Cry1Aa Mutants**

In order to make mutations in Cry1Aa, a 0.35 kb *EcoR1-Sac1* fragment of *cry1Aa* gene was sub-cloned into pBluescript KS' (Stratagene). Alanine-block substitution (HL3) and a deletion mutant (DL2) were created according to the method of Kunkel (1985). The mutant gene was then re-cloned in pOS4102 carrying a *cry1Aa* gene. The construction and expression of pOS4102 has been described earlier (Ge et al., 1989).
Cry1Ab Loop Substitution Mutants

The cry1Ab 7 gene (pSB033b) was obtained from Dr. T. Yamamoto (Sandoz Agro, Inc., Palo Alto, CA). A uracil-containing template of pSB033b was mutated by site-directed mutagenesis using oligomers corresponding to cry1Aa loop 2 and loop 3, according to the manufacturer's protocol (Muta-Gene M13 in vitro mutagenesis kit, BioRad). Mutants were expressed in Escherichia coli MV1190.

Thermal Stability of Purified Mutant Toxins

Cry1Aa and Cry1Ab mutant proteins were solubilized and activated according to Lee et al. (1992). Wild-type Cry1Ab and loop substitution mutants were digested with thermolysin, according to a modified procedure of Almond and Dean (1993). Toxin in 15 μg aliquots were incubated for 15 min. at 55°C and 65°C with 4% (w/w) of thermolysin. The reaction was terminated by adding 20 mM EDTA (final conc.) and 5 μl of Laemmeli SDS-loading buffer. Samples were analyzed by loading equal volumes on SDS-12% PAGE and Coomassie Brilliant Blue staining.

Preparation of brush border membrane vesicles (BBMV)

BBMV, from the fifth instar B. mori and M. sexta larval midguts, were prepared by the differential precipitation method of Wolfersberger et al. (1987).
Iodination and Competition Assays

Purified Cry1Aa toxin (25 μg) was labeled with 1 mCi of $^{125}$I-Na (Amersham) using IODO-BEADS (Pierce), as described earlier (Lee et al., 1992). Competition assays were performed with 0.5 nM $^{125}$I-labeled toxin, 100μg/ml of BBMV and increasing concentrations of the unlabeled toxin, as described earlier (Lu et al., 1994).

Toxicity Assays

Toxicity for B. mori larvae was determined by surface contamination of Mulberry leaf discs with various toxins. Neonate larvae were put on the discs and mortality recorded after 24 h. Bioassays against M. sexta neonate larvae was carried out as described earlier (Chen et al., 1995).

Voltage Clamp

B. mori larvae were dissected and mounted on the orifice (0.25 cm$^2$) according to the method of Harvey et al. (1990). The equipment for voltage clamp consists of D.C. 1000 voltage/current clamp, and an A-310 Accupulser (World Precision Instruments). The Isc was traced with a Kipp and Zöner recorder. The inhibition of short circuit current ($I_{sc}$) across the midgut was recorded after stabilization of the midgut membrane and addition of 1000 ng/ml
of toxin on the lumen side. Inhibition of short-circuit current (%) was measured and compared with other toxins.

RESULTS

Generation and Analysis of the Mutants

Fig. 3.1 shows the alignment of Cry1Ab domain II loops 2 and 3 sequences in comparison with the known loops of Cry1Aa (Grochulski et al., 1995). Cry1Aa alanine block mutant HL3 (368RIL370->AAA) and deletion mutant DL2 (Δ368ILGS373) produced stable toxin upon trypsin digestion (Fig. 3.1A). Loop substitution mutants, LS1 and LS2 in loop 2 of Cry1Ab, are shown in Fig. 3.1B. Similarly, Fig. 3.1C shows two loop substitution mutants, LS3 and LS4, in loop 3 of Cry1Ab. LS3 mutant made loop 3 smaller by two residues and did not produce a stable toxin upon trypsinization, however, trypsin treatment of LS4 protoxin produced active toxin. A double-loop hybrid mutant (DLS), with loop 2 (LS1) and loop 3 (LS4), sub-cloned in cry1Ab, also produced stable toxin fragments when treated with trypsin (Fig. 3.1C). The stability of these proteins were analyzed on SDS-12% PAGE.
**LOOP 2:**

<table>
<thead>
<tr>
<th>Cry1Aa</th>
<th>362SSpLYRRiilgsppNNQeL$_{380}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry1Ab</td>
<td>362SStLYRR---pfnigiNNQqL$_{379}$</td>
</tr>
</tbody>
</table>

**[A] Cry1Aa Mutants:**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL3</td>
<td>(368-370)</td>
</tr>
<tr>
<td>DL2</td>
<td>(369-373)</td>
</tr>
</tbody>
</table>

**[B] Cry1Ab Mutants:**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS1</td>
<td>(369-375)</td>
</tr>
<tr>
<td>LS2</td>
<td>(364/369-375)</td>
</tr>
</tbody>
</table>

**LOOP 3:**

<table>
<thead>
<tr>
<th>Cry1Aa</th>
<th>433HVtM-lsqagavtyt-RAP$_{450}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry1Ab</td>
<td>432HVsMfrsgfsnssvsiiRAP$_{451}$</td>
</tr>
</tbody>
</table>

**[C] Cry1Ab Mutants:**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS3</td>
<td>(438-446)</td>
</tr>
<tr>
<td>LS4</td>
<td>(440-443)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DLS</th>
<th>RRiilgsppNN........MfrsgaagaatsvsiiRAP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LS1......................................</td>
</tr>
<tr>
<td></td>
<td>LS4......................................</td>
</tr>
</tbody>
</table>

Figure 3.1. Alignment of domain II loop 2 and 3 of Cry1Ab toxin with respect to Cry1Aa. A: mutants in loop 2 of Cry1Aa. B: loop 2 substitution mutants of Cry1Ab. C: loop 3 substitution mutants and a double substitution mutant (DLS) of Cry1Ab.
**Structural stability of the mutants**

Cry1Aa and Cry1Ab mutant protoxins (except LS3) produced stable 65 kDa toxins upon trypsin digestion. Comparison of thermal stability of wild-type Cry1Ab and loop-substitution mutants (LS1, LS2, LS4 and DLS) shows that LS1 and LS4 has similar stability as wild-type Cry1Ab. However, mutant LS2 and DLS shows greater degradation at 65°C (Fig. 3.2).

**In vivo toxicity assays**

Purified Cry1Aa, Cry1Ab and the mutant toxins were assayed for toxicity for *B. mori* and *M. sexta* larvae. It was observed that Cry1Aa mutants, HL3 and DL2 have greatly reduced toxicity towards both *B. mori* and *M. sexta* (Table 3.1A). Comparison of wild-type Cry1Ab and loop-substituted mutants (LS1, LS2, LS4 and DLS) shows that at Cry1Ab cause only 15% mortality in *B. mori* neonate larvae where as, mutant LS1 showed no toxicity at 2.5 ug/cm² concentration however, LS4 was relatively more toxic than the wild-type Cry1Ab (Table 3.1B). The most significant increase in toxicity was observed with the double mutant (DLS) with nearly 70% greater toxicity than wild-type Cry1Ab against *B. mori* (Table 3.1B). However, LC₅₀ values showed only 10 fold less toxicity of DLS toxin than Cry1Aa against *B. mori* (Table 3.2).

In contrast, toxicity of mutant toxins, LS4 and DLS against *M. sexta* was comparable to the wild-type Cry1Ab. LS1and LS2 showed showed slightly lower toxicity towards *M. sexta* (Table 3.1B).
Figure 3.2. Thermal stability of Cry1Ab mutants. Lanes 1 and 11 molecular weight marker. Lanes 2, 5, 8, 12 and 15 are controls of Cry1Ab, LS1, LS2, LS4 and DLS, respectively, kept at room temperature without adding thermolysin. Lanes 3, 6, 9, 13 and 16 are with thermolysin at 55°C for the corresponding toxins. Lanes 4, 7, 10, 14 and 17 are at 65°C with thermolysin.
Table 3.1. Sensitivity and binding affinity of Cry1Aa and Cry1Ab mutants to *B. mori* and *M. sexta*

### [A]

<table>
<thead>
<tr>
<th>Toxin</th>
<th><em>B. mori</em></th>
<th><em>M. sexta</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a% Mort</td>
<td>b*K&lt;sub&gt;com&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>(150ng/cm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>(nM)</td>
</tr>
<tr>
<td>Cry1Aa</td>
<td>100</td>
<td>1.87 ± 0.8</td>
</tr>
<tr>
<td>HL3</td>
<td>40</td>
<td>9.56 ± 1.3</td>
</tr>
<tr>
<td>DL2</td>
<td>30</td>
<td>6.95 ± 1.7</td>
</tr>
</tbody>
</table>

### [B]

<table>
<thead>
<tr>
<th>Toxin</th>
<th><em>B. mori</em></th>
<th><em>M. sexta</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%Mort</td>
<td>d*I&lt;sub&gt;sc&lt;/sub&gt; slope</td>
</tr>
<tr>
<td></td>
<td>(2.5μg/cm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>(μA/min/cm&lt;sup&gt;2&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Cry1Ab7</td>
<td>15</td>
<td>-2.6 ± 0.4</td>
</tr>
<tr>
<td>LS1</td>
<td>00</td>
<td>5</td>
</tr>
<tr>
<td>LS2</td>
<td>50</td>
<td>ND</td>
</tr>
<tr>
<td>LS4</td>
<td>35</td>
<td>-4.2 ± 0.7</td>
</tr>
<tr>
<td>DLS</td>
<td>80</td>
<td>-6.9 ± 0.8</td>
</tr>
</tbody>
</table>

* a Mortality (%) is the mean of three assays
* b Binding affinity (K<sub>com</sub>) is derived specifically from competition studies of Cry toxin with BBMV (Wu and Dean, 1996).
* c Insufficient competition to calculate K<sub>com</sub>
* d Short-circuit current (I<sub>sc</sub>)
* e Insufficient inhibition of I<sub>sc</sub> to calculate slope
Table 3.2. Lethal concentration (LC₅₀) of Cry1Aa and loop-substitution mutant for *B. mori*

<table>
<thead>
<tr>
<th>Toxin</th>
<th>LC₅₀ (ng/cm²)</th>
<th>CL₉₅ (ng/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry1Aa</td>
<td>68.45</td>
<td>(44.5-90.4)</td>
</tr>
<tr>
<td>Cry1Ab7</td>
<td>&gt;2500</td>
<td>-</td>
</tr>
<tr>
<td>DLS</td>
<td>730.2</td>
<td>(607.5-890.4)</td>
</tr>
</tbody>
</table>
Binding assays

Heterologous competition between Cry1Aa and unlabeled toxins showed that HL3 and DL2 have reduced ability to displace, B. mori BBMV, bound Cry1Aa with a $K_{\text{com}}$ of 9.6 and 6.9, respectively as compared to high affinity binding ($K_{\text{com}}$ 1.87) for Cry1Aa. Similarly two to three fold lower binding was observed when Cry1Aa was competed with HL3 and DL2 on M. sexta (Table 3.1; Fig. 3.3A & B).

Competition binding studies with labeled Cry1Aa and unlabeled wild-type Cry1Ab and Loop-substitution toxins showed a weak competition on B. mori BBMV. Mutant toxin, LS1 was only able to compete off 10% of the bound Cry1Aa. Other mutants showed relatively better ability to compete for the Cry1Aa receptor binding site with a maximum of 45% labeled toxin being competed off with DLS (Fig. 3.4). Due to weak competition with the loop-substitution mutant, binding affinity ($K_{\text{com}}$) was not determined.

On the contrary wild-type Cry1Ab and all the loop-substitution mutant toxins (LS1, LS2, LS4, DLS) showed much greater ability to compete with the wild-type Cry1Aa for the receptor sites on M. sexta BBMV (Table 3.1B; Fig 3.5).
Figure 3.3. Binding of $^{125}$I-labeled Cry1Aa. [A] B. mori BBMV and [B] M. sexta BBMV were incubated with 0.5 nM of labeled toxin and competed off with an excess of Cry1Aa loop 2 mutants. Data is expressed as the percentage of $^{125}$I-Cry1Aa remained bound.
[Figure 3.3]

A

B

[Figure 3.3]
Figure 3.4. Heterologous competition with $^{125}$I-Cry1Aa on *B. mori* BBMV. Increasing concentration of unlabeled Cry1Ab and loop-substitution mutants are used to compete off 0.5 nM of $^{125}$I-Cry1Aa.
Figure 3.5. Competition binding assay of $^{125}$I-Cry1Aa with Cry1Ab mutants.

Binding is expressed as the percentage of labeled toxin bound to *M. sexta* BBMV after competing with increasing amount of unlabeled toxin.
Inhibition of short-circuit current

Voltage clamp analysis was performed to determine the possible effect on the ability of Cry1Ab and loop-substitution mutant toxins to disrupt ion-flux by forming an adventitious ion-channel. It was observed that the percentage of $I_{sc}$ inhibition of LS4 and DLS toxins was better than Cry1Ab. On the other hand LS1 did not show any significant drop in current (Fig. 3.6).

DISCUSSION

Although Cry1Aa and Cry1Ab are more than 90% homologous it is reported that Cry1Ab shows at least 20 fold lower toxicity towards B. mori (Ihara et al., 1993). We have observed that the natural allelic Cry1Ab7 toxin has even lower toxicity, for B. mori, than the Cry1Ab from B.t. sub-spp. aizawai (Unpublished observation). This difference in toxicity may be due to the variation in amino acid residues (G281A and S282L) in the loop between $\alpha$8 helix and $\beta$-sheet 1 of Cry1Ab7 which is shown to have reduced toxicity and binding affinity to Lymantria dispar (Lee et al., 1996). Earlier studies have shown that residues $^{365}$LYRRIIL$^{371}$ of Cry1Aa are involved in initial binding to the receptor on B. mori BBMV (Lu et al., 1994). 3-D structure of Cry1Aa shows that two of these residues $^{365}$LY$^{366}$ lies within the $\beta$-sheet 6 contiguous to loop 2, however the rest
Figure 3.6. Inhibition of short-circuit current ($I_{sc}$) across *B. mori* midgut.

Toxins (1000 ng/ml) were applied on the luminal side of the voltage clamp chamber. Cry1Aa toxin is used as a positive control. Vertical arrow indicates the time for the addition of toxins. Data is converted to percentage drop in $I_{sc}$. An average of four experiments were used to determine the $I_{sc}$ (%).
of the residues are part of loop2 (Grochulski et al., 1995). In the present studies we have done fine-point mapping of the residues which play a critical role in receptor binding and toxicity to B. mori. Cry1Aa, alanine block mutant HL3 and deletion mutant DL2 cause reduction in toxicity and receptor binding to both B. mori and M. sexta (Table 3.1A; Fig. 3.3A & B). Analogous studies in Cry1Ab loop 2 residues 368RRP370 have shown that the two conserved Arginine residues are involved in initial binding to the M. sexta 210-kDa receptor, whereas F371 and G374 of the same loop are involved in irreversible association to the M. sexta BBMV (Rajamohan et al., 1995, 1996a).

After establishing that loop 2 is important in receptor binding we attempted to alter the specificity region of Cry1Ab toxin by loop-substitution mutations. Loop 2 substitution mutant LS1 showed binding and toxicity even lower than the wild-type Cry1Ab toxin (Table 3.1B; Fig. 3.4). Although there is not a significant difference in toxicity between Cry1Ab (15%) and LS1 (0%) repeated assays have shown a similar effect on toxicity. However, thermal denaturation studies show that the mutant toxin, LS1, is equally stable as wild-type Cry1Ab, therefore gross structural changes are not likely to be the cause of this effect (Fig. 3.2).

The LS2 mutant substituting Pro364 of Cry1Aa, for the more conserved Thr, was speculated to make the Cry1Ab hybrid loop similar to Cry1Aa and thus improve the binding and toxicity to B. mori. However, this mutant was only able
to partially improve the receptor binding with no significant effect on toxicity (Table 3.1; Fig. 3.4). Furthermore reduced thermal stability at 65°C indicates that some structural destabilization has occurred, which may account for the lack of toxicity for *B. mori*.

Studies with domain II, loop 3 mutations in Cry1Aa and Cry1Ab have shown reduced toxicity and binding to the receptors on both *B. mori* and *M. sexta* (Rajamohan *et al.*, 1996b). Therefore, we substituted loop3 residues (440AAGA443) of Cry1Aa in Cry1Ab to construct the mutant LS4. This mutant toxin was able to increase the toxicity for *B. mori* by 20% as compared to the wild-type Cry1Ab (Table 3.1). However, initial binding to *B. mori* BBMV was not greatly improved (Fig.3.4). Similarly, the double loop-substitution mutant (DLS) also shows weak initial binding however, this mutant has significantly higher toxicity towards *B. mori* (Table3.1C, Table 3.2). These observations suggest that initial binding is not always the limiting factor for determining toxicity. Recent studies have also shown that substituting alanine block in loop3 of Cry3A results in increased activity against *Tenebrio molitor*, although the initial binding was reduced (Wu & Dean, 1996).

It has been further proposed that alanine substitution increases the hydrophobicity of the loop which may facilitate the irreversible binding of toxin to the receptor or membrane (Wu & Dean, 1996). Indirect studies suggest that
irreversible binding corresponds to the toxin insertion into the membrane (Wolfersberger, et al., 1987; Gill et al., 1992; Ihara et al., 1993). Since insertion is synonymous to ion-channel function, voltage clamp analysis was performed on B. mori midgut to determine the channel forming efficiency of the mutants (LS1, LS4 and DLS), as compared to the wild-type Cry1Ab. The non-toxic mutant, LS1, did not show any significant drop in current. However, in contrast to the initial receptor binding, LS4 showed a shift in the inhibition of I_{sc} (slope -4.21 μA/min^{-1}/cm^{2}), compared to the wild-type Cry1Ab (-2.65 μA/min^{-1}/cm^{2}). Similarly, in the case of the double mutant (DLS) the curve shifted further to the right with a nearly three fold steeper drop in I_{sc} (-6.81 μA/min^{-1}/cm^{2}) as compared to Cry1Ab. This suggests better overall membrane insertion and ion channel formation which is also reflected in relatively greater toxicity (Table 3.2; Fig 3.6). Thus our voltage clamp analysis is in good agreement with the bioassay data.

Since loop-substitution mutants have not completely gained the initial receptor binding and toxicity as Cry1Aa for B. mori, it can be assumed that other residues within the hypervariable segment may be important. One candidate region which has not yet been explored is loop 1 of domain II. In Cry1Aa this loop is predominantly hydrophobic (310GFNY313), whereas the presence of Glu in Cry1Ab (310GEYY313) makes this region hydrophilic. It can hypothesized that the
Phe\textsubscript{311} in loop 1 of Cry1Aa might be important for irreversible association. The raison d' être for this assumption arises from recent studies involving residue Phe\textsubscript{371} in loop2 of Cry1Ab showing that substituting Phe\textsubscript{371} with non-aromatic, hydrophilic residues reduces the toxicity and irreversible binding (Rajamohan et al., 1996a). Other possibility for the defective binding of loop-substituted mutants may be that since loop 2 is highly mobile in the X-ray structure of Cry1Aa (Grochukski et al., 1995), substituting this region in Cry1ab may have affected the intrinsic fluidity of the loop.

Both Cry1Aa and Cry1Ab toxins are highly toxic to \textit{M. sexta} larvae (Wu & Aronson, 1992). Studies have shown that like Cry1Aa, loop2 and 3 of Cry1Ab toxin play a critical role in binding to \textit{M. sexta} receptors (Rajamohan et al., 1995; 1996 submitted). Therefore the second part of our studies was to observe the effect on toxicity and receptor binding with loop-substitution mutants of Cry1Ab. Earlier it was suggested that highly conserved 366RR\textsubscript{369} in Cry1Ab are important for initial contact with \textit{M. sexta} receptor (Rajamohan et al., 1996a). This is in agreement with our observations, since all the loop-substituted mutants have conserved Arg residues and therefore initial binding to \textit{M. sexta} is not affected (Table 3.1B; Fig. 3.5). Furthermore, the relatively similar toxicity of Cry1Ab and loop-substituted mutants for \textit{M. sexta} suggest that different regions of the same receptor or two different receptors on \textit{M. sexta} recognize the loops of Cry1Aa and Cry1Ab.
In conclusion these results show that loops 2 and 3 within domain II of Cry 1Aa are important in dictating the insecticidal specificity and toxicity for *B. mori*. However when isolated from the rest of the Cry1Aa molecule these loops cannot exert full potential, suggesting that these loops may be interacting with other residues in a manner that cause the molecule to bind with high affinity to *B. mori* receptor.
CHAPTER 4

L371K: A *Bacillus thuringiensis* Cry1Aa δ-endotoxin split mutant toxin giving insight into binding and irreversible association with *Manduca sexta* membrane

INTRODUCTION

The mode of action of *Bacillus thuringiensis* δ-endotoxin consists of solubilization of protoxin, activation of protoxin into toxin molecule, and binding of the toxin to the midgut epithelial cell receptor (Tojo *et al*., 1983; Hofmann *et al*., 1988). The binding of toxin changes the membrane permeability allowing the influx of cations through the formation of either small non-specific pores or ion channels (Knowles & Ellar, 1987; English & Slatin, 1992; Schwartz *et al*., 1993). There is a gradual elongation and swelling of midgut epithelial cells.
Finally the cells rupture at the apical surface causing the leakage of cytoplasmic material into the gut lumen (Bauer & Pankratz, 1992).

The crystal structure of Cry3A and Cry1Aa has already been determined. The three domains of these toxin molecules are built on five conserved blocks of amino acid residues (Li et al., 1991; Grochulski et al., 1995). Most of the δ-endotoxins are proposed to have a similar three-domain structure. These domains are responsible for the receptor recognition and pore formation. Domain I, consisting of α-helices, is believed to be capable of transversing the membrane (Li et al., 1991; Grochulski et al., 1995). Mutations in domain I of Cry1Ac and Cry1Ab have shown that certain residues play an important role in toxicity and irreversible binding to the BBMV (Wu & Aronson, 1992; Chen et al., 1995). Domain II consists of three beta-barrel sheets, each terminating with a hypervariable loop. These loops are considered to be involved in receptor recognition and initial binding (Li et al., 1991; Lee et al., 1992; Lu et al., 1994). Recent studies have shown that mutation in the loop regions, in domain II, can also affect the irreversible association of δ-endotoxin with the midgut epithelial cells (Rajamohan et al., 1995, 1996; Wu & Dean, 1996). The function of domain III was proposed to be involved only in the structural integrity of the toxin (Li et al., 1991). However, recent report involving mutation in the conserved block 4 at the C-terminal have shown that domain III also plays a role in ion-conductance (Chen et al., 1993). Switch mutation studies between Cry1Aa and
Cry 1Ac have also shown that domain III may also be involved in receptor binding (Lee et al., 1995).

Several theoretical assumptions have been made in recent years regarding the insertion of toxin into the membrane and pore formation. In general, these models suggest that the hypervariable loop region of domain II first binds to midgut epithelial receptor of the susceptible insect. This leads to conformational changes in the toxin molecule causing the α-helices of domain I to insert into the membrane (Hodgman & Ellar, 1990; Knowles & Dow, 1993; Yamamoto & Powell, 1993). Most of these models have ignored the possibility that other domains might also play a role in receptor binding or membrane insertion. Gill et al. (1992), however, alluded to the possibility that, besides hydrophobic α-helices, β-sheets may also form a membrane-spanning channel. Taken together, both experimental evidence and hypothesis suggest that insertion of toxin into the membrane most probably involves several regions of δ-endotoxin. These domains associate with the brush border membrane of the target insect, in a complex manner, in order to allow the insertion of the toxin into the membrane.

We have constructed a trypsin-sensitive mutant of Cry1Aa in the loop 2 region at leucine 371, which gives two fragments of the toxin. Functional
studies such as toxicity, receptor binding and insertion with wild type and the mutant protein, have led us to propose a topology of Cry1Aa toxin in the BBMV of target insect.

EXPERIMENTAL PROCEDURES

Mutant construction and screening

The construction of pOS4102 (cry1Aa), by cloning cry1Aa gene from Bacillus thuringiensis HD-1 into pKK233-3 and overexpression in Escherichia coli, has been described previously (Ge et al., 1989). To target the putative loop region in domain II, we sub-cloned the 354 bp EcoRI-Sacl fragment from pOS4102 into pBluescript KS, to obtain pBS350. Site-directed mutagenesis was performed using a Bio-Rad Muta-Gene M13 In vitro mutagenesis kit following manufacturers protocol. Mutagenic oligonucleotide, to convert Leu371 to Lys (L371K), was obtained from Sandoz Agro. Inc.

Potential mutant colonies were screened by the dideoxy sequencing method of Sanger et al. (1977), using sequenase kit (U.S Biochemical). After mutagenesis and selection, the 354 bp EcoRI-Sacl fragment was sub-cloned into the expression vector pOS4102. The resulting construct was named as L371K.
Purification and solubilization of inclusion bodies.

The mutant L371K gene was overexpressed in host strain MV1190. δ-endotoxin was purified from *E.coli* by the method described by Ge *et al.* (1991). The purified crystal proteins were solubilized in sodium carbonate buffer (50 mM Na₂CO₃; 10 mM dithiothreitol, pH 9.5). The concentration of the solubilized protein was determined by Coomassie blue protein assay reagent (Pierce).

Trypsin digestion of protoxin

The wild type (Cry1Aa) and the mutant (L371K) protoxins were digested with 3% (w/w) of trypsin at 37°C for 4hrs. The digestion of 130 kDa protoxin was analyzed by SDS-12% PAGE according to the method of Laemmli, (1970).

Determination of structural alterations

Thermolysin digestion was carried out with the trypsin resistant core of Cry1Aa and L371K. The procedure employed was the modification of Almond & Dean (1993). Toxins in 30 μg aliquots were incubated for 20 minutes at 50°C, and 58°C with 4% (w/w) of thermolysin in 50 mM Tris-HCl (pH 9.5) buffer containing 10mM CaCl₂. The reaction was terminated by adding 20 mM EDTA (final concentration). Samples were analyzed by SDS-12% PAGE and Coomassie brilliant blue staining. The relative intensities of the toxin
fragments at various temperatures were determined by scanning with a densitometer (Hoefer Scientific Instruments) and recorded as the percentage of peptide remaining versus temperature.

Toxicity assay

*Manduca sexta* eggs were obtained from Dr. D. L. Dahlman from the Dept. of Entomology at the University of Kentucky, Lexington, Kentucky. Bioassay were performed by surface contamination of artificial diet obtained from Bio-Serve Inc. The diet, containing molten agar, was first poured into tissue culture plates of 2cm² surface area and allowed to congeal. Fifty microliters of various toxin dilutions were then added on to the surface and allowed to air dry. Six toxin concentrations were used to calculate the median lethal concentration (LC₅₀) value with 12 *M. sexta* neonate larvae for each concentration. Effective dose estimate (LC₅₀) and 95% fiducial limits were calculated with the Probit program (Raymond, 1985) by using an average of three bioassays.

Preperation of BBMV

The midguts from fifth instar *M.sexta* larvae were isolated according to the method described by Chen *et al.* (1995). BBMV was prepared from these isolated midguts by the differential magnesium precipitation method of Wolfersberger *et al.* (1987). The BBMV pellet was then resuspended in
binding buffer (8 mM Na₂HPO₄, 2 mM KH₂PO₄, 150 mM NaCl, pH 7.4) and stored in liquid nitrogen until further use.

**Competition assay**

Iodination of toxins was carried out according to the method of Lee *et al.* (1993). The Homologous competition assay was performed by competing 1nM of ¹²⁵I-labeled toxin with an increasing concentration of the same unlabeled toxin as described earlier (Rajamohan *et al.*, 1995). Binding data were analyzed by the LIGAND computer program (Munson & Rodbard, 1980).

**Dissociation assay**

For the dissociation experiments, 50 µg of *M. sexta* BBMV was incubated with 2 nM of either ¹²⁵I-labeled Cry1Aa or ¹²⁵I-labeled L371K in 100 µl of binding buffer at room temperature. After 1hr of incubation, 500 fold excess of unlabeled toxin was added to the ¹²⁵I-labeled toxin-BBMV suspension. The reaction was terminated by spinning down the mixture at various time intervals (0 to 60 mins). The pellets were washed twice with 300 µl of binding buffer in order to remove any unbound toxin. The final pellets were counted in a gamma counter (Beckman instruments). Non-specific binding was determined by adding together labeled toxin and 500 fold excess of the corresponding
unlabeled toxin to the BBMV. Non-specific binding was subtracted in the final data analysis.

**Toxin insertion into the membrane and gut juice digestion**

*M. sexta* BBMV (45 μg) was incubated with 0.120 μg of $^{125}$I-Cry1Aa or $^{125}$I-L371K. The reaction volume was adjusted to 60 μl with binding buffer supplemented with 0.1% BSA. After 150 mins of incubation at 25°C, the suspension was centrifuged and the pellets were washed with binding buffer to remove any unbound toxin. The pellets were resuspended in 30 μl of binding buffer (no BSA) and *M. sexta* gut juice (10 μl) was added. The other vials containing no gut juice were used as a control and the volume was adjusted with binding buffer. The suspension was incubated at 25°C for 1 hr. At the end of the incubation period 5 μl of 50X protease inhibitor cocktail (Boehringer Mannheim Biochemicals) was added to stop the reaction. The BBMV was then solubilized by adding denaturing gel loading buffer (Laemmli, 1970), and boiled for 5 mins. Toxin fragments were separated from BBMV on SDS-12% PAGE and analyzed from the autoradiograph. As a negative control mouse BBMV was incubated with either $^{125}$I-Cry1Aa or $^{125}$I-L371K and subjected to same treatment as *M. sexta* BBMV. Cry1Aa and L371K toxins were also treated with *M. sexta* gut juice (10 μl) without adding any BBMV however, 60 μg
of BSA was added to compensate for the BBMV proteins. Relative intensities of gut juice treated and untreated fragments were determined by densitometer (Hoefer Scientific Instruments). The data was recorded as the percentage of peptide remaining after protease digestion as compared to undigested toxin.

RESULTS

Tryptic digestion of mutant protein

The protoxins were digested with trypsin and analyzed on SDS-PAGE. Both Cry1Aa and its mutant L371K gave a 130 kDa protoxin band. The mutant protein was cleaved into two peptides of approx. 35 kDa (fragment A) and 30 kDa (fragment B) upon trypsin digestion in contrast to a single 65 kDa trypsin-activated toxin band for Cry1Aa (Fig. 4.1). Since the trypsin-sensitive site in L371K was created in the loop2 of domain II, we consider the 35 kDa peptide as domain I and a portion of domain II. Domain III and the remainder of domain II corresponds to the 30 kDa fragment. Non-denaturing PAGE shows that L371K toxin behaves as the wild type toxin (Fig. 4.2).

Structural stability of Cry1Aa and L371K.

In order to determine the structural alteration in the trypsin-resistant core of L371K, it was treated with thermolysin at various temperatures and
Figure 4.1. SDS-PAGE pattern of Cry1Aa and L371K. 130 kDa protoxin bands of Cry1Aa and L371K, lanes 1 and 3, respectively. Trypsin activated Cry1Aa toxin (lane 2). Trypsin digested fragments of L371K (lane 4). Fragment A is 35 kDa and fragment B is 30 kDa.
Figure 4.2. Non-denaturing PAGE of Cry1Aa and L371K toxins. Cry1Aa toxin (lane 1). L371K toxin (lane 2). Three bands in each lane are the isomeric forms of the toxin as determined by Chen & Dean (unpublished observations). Conditions for non-denaturing (7%) PAGE are as described by Lu & Adang (1996).
Table 4.1. Thermal stability of Cry1Aa and L371K in the presence of thermolysin.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Cry1Aa (%)</th>
<th>L371K (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>°C</td>
<td>((^A))</td>
</tr>
<tr>
<td>Control*</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>80</td>
<td>38</td>
</tr>
<tr>
<td>58</td>
<td>15</td>
<td>10</td>
</tr>
</tbody>
</table>

Stability is expressed as the percentage of toxin remaining at a given temperature as compared to the control. (i) L371K fragment A. (ii) L371K fragment B.

*: Toxin kept at room temperature without adding thermolysin.
compared with the wild type toxin under the same conditions. Our data shows that the fragment B of L371K was more susceptible to thermolysin than fragment A (Table 4.1).

Bioassay

Trypsin-activated L371K and Cry1Aa were analyzed for toxicity against *M. sexta* neonate larvae. The mutant protein showed 6 to 7 fold less toxicity against the insect as compared to wild type (Table 4.2).

Competition binding studies

In order to study the binding ability of L371K toxin toward the membrane receptor of *M. sexta* midgut, a competition assay was performed. Homologous competition with either $^{125}$I-Cry1Aa to unlabeled Cry1Aa or $^{125}$I-L371K to unlabeled L371K indicated that the mutant has three-fold less initial (reversible) binding affinity ($K_{com}$) than Cry1Aa toxin (Table 4.2, Fig.4.3).

Irreversible association.

The effect of mutation on irreversible binding to the BBMV of *M. sexta* BBMV was studied. $^{125}$I-labeled wild type and mutant toxins were first allowed to bind to the BBMV for 1h. In the presence of excess amounts of unlabeled
Table 4.2. Effect of mutation on binding and toxicity for *M. sexta*

<table>
<thead>
<tr>
<th>Toxin</th>
<th>$^{a}\text{LC}_{50}$ (ng/cm²)</th>
<th>$^{b}\text{K}_{\text{com}}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry1Aa</td>
<td>2.5 (1.7-3.6)</td>
<td>4.37 ±0.72</td>
</tr>
<tr>
<td>L371K</td>
<td>16.8 (12.5-20.8)</td>
<td>14.25 ±0.51</td>
</tr>
</tbody>
</table>

$^{a}$ 50% lethal concentration (95% confidence limits)

$^{b}$ Data were obtained from homologous competition assay expressed as a mean of four readings. $^{*}\text{K}_{\text{com}}$ (binding affinity) has earlier been used by Wu & Dean (1996) to define the binding constant derived from the competition assay for Cry toxins.
Figure 4.3. Homologous competition assay of 125I-Cry1Aa and 125I-L371K to *M. sexta* BBMV. Binding is expressed as the percentage of labeled toxin bound to the BBMV after competition with increasing amount of the corresponding unlabeled toxin.
toxin, 45% of the mutant toxin was chased off in the first 20 minutes and only 50% of the toxin remained bound to BBMV at 60 min. In contrast to L371K, the wild type toxin showed more irreversible association with only 17% being chased off after 60 min. (Fig. 4.4).

**Evidence for toxin insertion into the BBMV**

We also investigated the possibility of identifying the domain(s) or region(s) of 8-endotoxin which may be involved in insertion into the membrane. It was observed that in the absence of *M. sexta* BBMV Cry1Aa toxin has a very faint band with an excess of *M. sexta* gut juice with more than 95% of the toxin being digested after incubation for 1h at 25°C (Table 4.3). The toxin remained after digestion was however, of the same size as the untreated toxin (Fig. 4.5A lane 1&2). On the other hand only 40% of the *M. sexta* BBMV-bound toxin was degraded with gut juice (Table 4.3, Fig. 4.5A). An interesting observation was made that, on the SDS-PAGE, the digested fragment size was slightly smaller than the undigested toxin (Fig 4.5A, lanes 3&4). At present we have not determined if the degradation is at the N-terminal or C-terminal.
Figure 4.4. Dissociation of the wild type and mutant toxins from *M. sexta* BBMV. Binding is expressed as the percentage of labeled toxin remain associated with BBMV after adding 400 fold excess of corresponding unlabeled toxin at various time intervals. Non-specific binding is subtracted from the total binding.
In the case of L371K toxin, the effect of *M. sexta* gut juice on fragments A (35 kDa) and B (30 kDa) were studied. Both the fragments showed low band intensities, as compared to the untreated toxin, in solution, in the absence of *M. sexta* BBMV. There was however, a relatively greater degradation of fragment B as compared to fragment A (Table 4.3, Fig 4.5B, lane 1 & 2). In sharp contrast to solution conditions, L371K toxin behaved differently after incubation with *M. sexta* BBMV with lesser degradation of fragment B than fragment A (Fig. 4.5B, lanes 3 & 4).

In order to eliminate the possibility of non-specific interaction of Cry1Aa or L371K, to any lipid-bilayer and thus protection from proteases, mouse BBMV was used. It was observed that there was only a weak binding of either toxin to these vesicles. This may be due to the non-specific binding of toxin to the mouse BBMV, however both Cry1Aa toxin and L371K toxin were completely degraded by gut juice proteases (Fig. 4.5A&B lanes 5&6).
Figure. 4.5. Autoradiogram of $^{125}$I-labeled Cry1Aa and L371K toxins incubated with *M. sexta* gut juice with or without BBMV. Panel A: $^{125}$I-Cry1Aa toxin as a control (lane 1). Cry1Aa toxin treated with gut juice as described in experimental procedures (lane 2). *M. sexta* BBMV pellet incubated with Cry1Aa (lane 3). *M. sexta* BBMV with Cry1Aa after gut juice treatment (lane 4). Mouse BBMV incubated with $^{125}$I-Cry1Aa (lane 5). *M. sexta* gut juice treated mouse BBMV with Cry1Aa (lane 6). Panel B: $^{125}$I-L371K as a control (lane 1). L371K (fragments A&B) after *M. sexta* gut juice digestion (lane 2). *M. sexta* BBMV incubated with L371K (lane 3). Incubation mixture of *M. sexta* BBMV and L371K after gut juice digestion (lane 4). Mouse BBMV incubated with $^{125}$I-L371K (lane 5). Mouse BBMV with L371K after gut juice digestion (lane 6). Fragment A corresponds to 35 kDa band and Fragment B gives a 30 kDa band on SDS-12% PAGE.
[Figure 4.5]
[Figure 4.5 cont.]

[B]
Table 4.3: Protection of Cry1Aa and L371K against *M. sexta* gut juice digestion.

<table>
<thead>
<tr>
<th>TOXIN</th>
<th>WITHOUT</th>
<th>BBMV(%)*</th>
<th>WITH BBMV(%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry1Aa</td>
<td>5.21</td>
<td></td>
<td>69.4</td>
</tr>
<tr>
<td>L371K:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fragment A</td>
<td>3.75</td>
<td></td>
<td>6.3</td>
</tr>
<tr>
<td>Fragment B</td>
<td>0.74</td>
<td></td>
<td>25.7</td>
</tr>
</tbody>
</table>

Relative intensities of both wild type and mutant were compared to the untreated Cry1Aa and L371K (fragment A&B) respectively using scanning densitometer.

*% of toxin remaining after *M. sexta* gut juice digestion.
DISCUSSION

The mechanism of action of δ-endotoxins involves binding to the microvillar membrane of the midgut. A major portion of this interaction becomes irreversible (Hofmann et al., 1988; Van Rie et al., 1990). Liang et al. (1995) found a direct correlation between toxicity and the rate of irreversible binding. Several studies indirectly suggest that irreversible binding indicates toxin insertion into the membrane (Wolfersberger et al., 1987, Gill et al., 1992, Ihara et al., 1993). More specifically, membrane integration and channel formation has been attributed to domain I (Li et al., 1991).

In this report we have attempted to test this hypothesis by performing some functional studies with Cry1Aa and its mutant toxin, L371K. We constructed this trypsin-cleavable mutant based on the biochemical studies by Convent et al. (1991), showing that Cry1Ab consists of two domains linked by a protease susceptible loop region which can be cleaved by chymotrypsin at amino acid residue 371. Our work has shown that treating L371K protoxin with trypsin separates it into a 35 kDa [fragment A] and a 30 kDa [fragment B] (Fig 4.1). In agreement with the N-terminal sequence analysis of the fragments, performed by Convent et al. (1991), we believe that fragment A corresponds to domain I and a portion of domain II (residues 1 to 370), whereas fragment B contains domain III and the remainder of domain II (residues 372 to 623).
Under non-denaturing PAGE these fragments are not separated from each other and give a similar electrophoresis pattern as wild type Cry1Aa (Fig. 4.2). This indicates that the non-covalent interactions are strong enough to hold the fragments together under native conditions.

Thermal denaturation profile of L371K shows a greater degradation of fragment B as compared to fragment A in the presence of thermolysin (Table 4.1). This may be due to the fact that fragment A mainly has domain I, which consists of amphipathic α-helices. These may be close-packed in order to keep the hydrophobic residues away from the solvent (Li et al., 1991; Grochulski et al., 1995).

It has been observed earlier that in Cry1Aa toxin, block deletion of residues 369-372 affects both initial and irreversible association to M. sexta BBMV (Hussain et al., 1995). The importance of this region in Cry1A family of δ-endotoxins is further strengthened by the recent studies showing that substituting aliphatic, hydrophilic and small side-chain residues for Phe371 can reduce the irreversible association and toxicity of Cry1Ab to M. sexta (Rajamohan et al., 1995, 1996). Based on similar analogy it can be assumed that the seven fold reduction in toxicity for M. sexta larvae is mainly due to
defective irreversible association (insertion) of L371K toxin (Fig. 4.4), however, reduced binding affinity is also a factor (Fig. 4.3).

Protection against externally added proteases has been used to determine the topology of diphtheria toxin in lipid vesicles (Cabiaux et al., 1994). Using a similar technique, protection of $^{125}$I-Cry1Aa and $^{125}$I-L371K toxins against M. sexta gut juice proteases was studied. It was observed that, after incubation with M. sexta BBMV, almost 65% of the Cry1Aa fragment was resistant to digestion by externally added gut juice, while 95% of Cry1Aa is degraded in the absence of M. sexta BBMV (Table 4.3; Fig. 4.5A). This protection may be due to the toxin insertion into the membrane, because of this transmembrane configuration the toxin is no longer susceptible to proteolytic cleavage. Another possibility is that the toxin may be very closely associated with the charged phospholipid head groups of the membrane thus can resist proteolytic attack.

We do not eliminate the possibility of conformational alterations as suggested in various models put forward to explain the insertion/pore formation of toxin in the membrane (Li & Ellar, 1991; Yamamoto & Powell, 1993; Knowles, 1994). We can however, rule out the possibility of protection by only being closely applied to the membrane surface because in that case we
would have seen protection in the presence of mouse BBMV which is not susceptible to δ-endotoxins (Fig. 4.5 A&B).

Proteolytic digestion studies with mutant L371K showed differential resistance of fragment A (domains I & II) and fragment B (domains III & II) in the presence and absence of *M. sexta* BBMV. Although both the fragments were degraded in solution form, fragment A showed more resistance than fragment B in the absence of *M. sexta* BBMV (Fig. 4.5B). This is in agreement with the thermal stability study showing that amphipathic helices are packed more tightly in aqueous environment (Table 4.1). In the presence of BBMV however, fragment B (30 kDa) showed more protection against protease than 35 kDa fragment A (Fig. 4.5B). To account for this discrepancy we propose that at least some of the β-sheets of domain II and/or domain III may be in the transmembrane orientation. Our hypothesis is based on the recent studies involving the C-terminus of δ-endotoxins. It has been shown, by voltage clamp and light scattering studies, that the mutation of arginine residues in the conserved block 4, of domain III, causes a reduction in ion-channel functions of Cry1Aa toxin (Chen *et al.*, 1993; Wolfersberger *et al.*, 1996). Furthermore mutations in domain II of Cry1Ab and Cry3A has been shown to affect the irreversible association with the membrane (Rajamohan *et al.*, 1995 & 1996; Wu & Dean, 1996). Our interpretation is further supported by the earlier work
indicating that the whole toxin associated with BBMV is neither attacked by proteases nor bound by monoclonal antibodies (Wolfersberger et al., 1987). The possibility of anti-parallel β-sheets being inserted into the bilipid layer is demonstrated by the protease protection and analytical studies on diphtheria toxin, and aerolysin (Cabiaux et al., 1994, Parker et al., 1994). Fourier-transform infrared (FTIR) spectroscopy of nicotinic acetylcholine receptor also shows a β-structure immersed in the membrane. The β-sheets have been suggested to serve as scaffold, so that α-helices can acquire most a favourable position to serve as the ion-channel (Hücho et al., 1994).

Relative higher degradation of fragment A in the presence of M. sexta BBMV is an enigma. It is possible that since L371K is cleaved at loop 2 in domain II, after binding of toxin to the receptor, α-helices of domain I tend to separate from domain II and III. This may result in the partial insertion of domain I thus accounting for the lower toxicity and irreversible association (Table 4.2; Fig. 4.4).

The current observations indicate that both α-helices and β-sheets of δ-endotoxin are protected from protease. This suggests that the organization in the membrane is quite complex and involves all three domains. Thus we propose that the pre-insertion events involve binding of toxin to the membrane.
receptor by regions of domain II and possibly domain III. This leads to a conformational change in the molecule causing the hydrophobic helices of domain I, to insert into the membrane. The protection of domains II and III may either be due to the possibility that β-sheets are inserted into the membrane. Other possibilities are that some folding conformation on the surface of the membrane make these domains inaccessible to the protease, or β-sheets may form oligomer, after binding tightly to the receptor, thus protecting the whole toxin from degradation. However, in order to differentiate between true insertion or surface association of domain II and domain III we need to perform further experiments such as using membrane specific probe to determine membrane insertion.


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