STUDIES OF THE MANDUCA SEXTA CADHERIN-LIKE RECEPTOR BINDING EPITOPES OF BACILLUS THURINGIENSIS CRY1Aa TOXIN AND PROTEIN ENGINEERING OF MOSQUITOCIDAL ACTIVITY

DISSERTATION

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

BY

Xinyan Liu, B.S.
The Ohio State University
2005

Dissertation Committee:
Dr. Donald H. Dean
Dr. Ross Dalbey
Dr. Russ Hille
Dr. Z. Justin Wu

Approved By

Dr. Russ Hille
Adviser
Ohio State Biochemistry Program
ABSTRACT

*Bacillus thuringiensis*, an aerobic, gram-positive spore-forming bacterium commonly found in soil, produces parasporal crystal (Cry) proteins with insecticidal activity against a wide range of pests. Cry toxin binding to receptors on the brush border membrane in insect midgut is required, among other factors, to exert the toxic effect. The binding epitopes on Cry1Aa toxin to the cadherin-like receptor in *Manduca sexta* (tobacco hornworm) was mapped by a combined approach of molecular modeling, site-directed mutagenesis, bioassay on insect larvae, and kinetic analysis.

CAD-D, a truncated fragment (CR11 and 12) of Bt-R1a (63), the cadherin-like receptor from *Manduca sexta* for *Bacillus thuringiensis* Cry1A toxins, was expressed and purified as a soluble MBP (maltose binding protein) fusion protein. Binding affinity of Cry1Aa to CAD-D measured by real time SPR was at the 10nM level. Some of the CAD-D binding epitopes on Cry1Aa toxin were mapped by alanine scanning mutagenesis. Designing of the area targeted for mutagenesis was based on structural information derived from topology prediction and computational docking of the toxin with the receptor. Loop 2 residues in domain II and three clusters of surface residues in domains II
and III were demonstrated to be involved in binding to CAD-D. The interaction surface was defined by the loss of binding for mutants on the predicted face of the toxin and no effects on another set of substitutions located on the opposite face of domain III.

The *Bacillus thuringiensis* crystal protein Cry1Aa is naturally selectively active to caterpillar larvae. In further manipulation of receptor binding epitopes through rational design, toxicity (μg/ml) to the mosquito *Culex pipiens* was introduced by selected deletions and substitutions of the loop residues of domain II. Toxicity to its natural target *Manduca sexta* was concomitantly abolished. The successful grafting of the alternate mosquito toxicity onto the original lepidopteran Cry1Aa toxin by exchanging the specificity-determining loop regions demonstrates the possibility of designing and engineering a desired toxicity into any toxin of a common scaffold by reshaping the receptor binding region with desired specificities.

Taken together, these studies provided promising evidence that epitope-mapping and protein-engineering under the guidance of molecular modeling can serve as a rational and useful tool in understanding the mode of action of Cry toxins, and ultimately in producing better toxins.
DEDICATION

Dedicated to my family
ACKNOWLEDGEMENTS

I am truly grateful to my adviser, Dr. Donald H. Dean. His intellectual guidance and unwavering support at all times made my graduate study possible. He has steadfast confidence in me even when I doubt myself. I shall treasure my graduate study always.

A warm thank you goes to my past and present lab members, with whom I shared challenging ideas, exciting discussions and warm friendship. They are Dr. Jeremy Jenkins, Dr. Taek H. You, Dr. Mi K. Lee, Dr. Mongkon Audtho, Dr. Amir Abudulla, Tara Grove, April Curtiss; Dr. Sasha Popova-Butler, Dr. Jung-yul Roh, Manoj Nair, Betina McNeil, Pei-yu Chiang, Steven Lin, Yoshio Ikeda and Sean McClory, Dr. Dan Zeigler, Carol Zeigler and Mary Beth Dunn.

I am much obliged to my graduate study committee, Dr. Ross Dalbey, Dr. Venkat Gopalan and Dr. Russ Hille. I appreciate Dr. Zhengrong Justin Wu to serve on my dissertation committee on a very short notice. I would like to extend my special thanks to Drs. Charles Brooks and Scott Walsh, who gave me insightful advices on BIAcore techniques.

I am forever indebted to my mother, Prof. Meimin Yu, for continuously giving me faith and inspiration to pursue life and science. I am also thankful to my elder brother Si-ze Liu, who is also my best friend. Most especially, I wish to express my appreciation to my husband, Junwen Yu, for his unconditional support, pure honesty and true love.
VITA

May 25, 1974 .......................... Born - Beijing, P.R. of China

1992 to 1997 ........................ B.S., Applied Chemistry
             Beijing University, P.R of China

1997 to 1998 ........................ Engineer
             Metronix® Medical Co., LTD.
             Beijing, P. R. of China

1999 to present  ...................... Graduate Teaching and Research Associate
             Ohio State Biochemistry Program (OSBP)
             The Ohio State University
             Columbus, OH

FIELD OF STUDY

Major Field: Ohio State Biochemistry Program, Protein-protein interaction, protein engineering
# TABLE OF CONTENTS

ABSTRACT ........................................................................................................................ ii
DEDICATION ................................................................................................................... iv
ACKNOWLEDGEMENTS ................................................................................................. v
VITA ............................................................................................................................. vi
LIST OF TABLES ............................................................................................................. ix
LIST OF FIGURES ............................................................................................................ x

CHAPTER 1 LITERATURE REVIEW ........................................................................1
1.1. *Bacillus thuringiensis* δ-endotoxin as a biopesticide: a century of progress .......... 1
1.2. General mode of action .............................................................................................. 5
1.3. Risks and Risk Management in *Bt* crops ................................................................. 12
1.4. Resisting Resistance to *Bt* crops ............................................................................. 15
1.5. Protein Engineering .................................................................................................... 19

CHAPTER 2 SUBCLONING AND OVER-EXPRESSION OF *Manduca sexta* CADHERIN-LIKE RECEPTOR BT-R1a FRAGMENT ......................................................... 22
2.1 Introduction .................................................................................................................. 22
2.2 Material And Methods ............................................................................................... 27
   Subcloning of cadherin-like receptor fragment ......................................................... 27
   Over-expression and purification of the receptor ..................................................... 28
   Bt toxin purification ................................................................................................... 29
   Surface Plasmon Resonance analysis of toxin affinity ............................................... 30
2.3 Results......................................................................................................................... 32
   Over-expression and purification of MBP-CAD-D fusion protein ............................ 32
   Bt toxin purification ................................................................................................... 32
   Kinetic analysis of sensorgrams .............................................................................. 35

CHAPTER 3 STUDIES OF THE *Manduca sexta* CADHERIN-LIKE RECEPTOR BINDING EPITOPES ON CRY1Aa TOXIN ................................................................. 39
3.1. Introduction................................................................................................................... 39
3.2. Material and methods ............................................................................................... 40
   Homology modeling .................................................................................................. 40
   Simulated docking ..................................................................................................... 41
   Molecular Visualization .............................................................................................. 42
   Construction of Mutants ........................................................................................... 42
   Toxin structural analysis ........................................................................................... 47
   Toxicity bioassay ........................................................................................................ 47
   Slot-blotting analysis of binding affinity .................................................................... 47
3.3. Results......................................................................................................................... 49
   Homology modeling and simulated docking ............................................................ 49
   Construction and purification of mutants ................................................................. 51
CHAPTER 4 RATIONAL DESIGN AND PROTEIN ENGINEERING TO CREATE MOSQUITOCIDAL ACTIVITY IN CRY1Aa TOXIN ............75

4.1. Introduction..................................................................................................................75
4.2. Material and methods..................................................................................................76
   Sequence and Structural alignments ............................................................................76
   Construction of mutants ...............................................................................................77
   Toxin structure analysis ...............................................................................................80
   Toxicity bioassay to Manduca sexta ...........................................................................80
   Toxicity bioassay to Culex pipiens .............................................................................81
4.3. Results.........................................................................................................................82
   Sequence and Structural alignments ............................................................................82
   Construction of mutants and Bt toxin purification ......................................................85
   Toxin structure analysis ...............................................................................................85
   Toxicity bioassay to Manduca sexta and Culex pipiens ...............................................85
4.4. Discussion....................................................................................................................88

REFERENCES ..................................................................................................................91
# LIST OF TABLES

Table 3.1 List of primer design for loop2 residues in Domain II ................................. 44
Table 3.2 List of primers for Domains II and III surface residues................................. 45
Table 3.3 List of primers for two clusters of Domains II and III................................ 46
Table 3.4 List of all mutants constructed and expressed. ............................................. 54
Table 3.5 Bioassay of *Manduca sexta* larvae with mutant toxins................................. 61
Table 3.6 Binding properties of mutant toxins to receptor. .......................................... 68
Table 3.7 Properties of Cry1Aa mutant toxins binding and toxicity. ............................. 73
Table 4.1 Sequences of primers used in site-directed mutagenesis. .............................. 79
Table 4.2 Sequence Alignment of Cry1Aa and Cry4Ba............................................... 83
Table 4.3 Toxicity of Cry4Ba and Cry1Aa toxins......................................................... 87
Table 4.4 Ranges of Reported Toxicity (ng/ml) of Mosquitocidal Proteins..................... 90
LIST OF FIGURES

Figure 1.1 The three-domain structure of Cry1Aa ........................................................ 7
Figure 2.1 BT-R₁ structure model ................................................................................. 24
Figure 2.2 Purification of MBP-CAD-D fusion protein .............................................. 33
Figure 2.3 Purification of wt Cry1Aa toxin ............................................................ 34
Figure 2.4 BIACore analysis representation ............................................................ 37
Figure 2.5 Processed binding curves ....................................................................... 38
Figure 3.1 Docking Model of Cry1Aa and CAD-D by GRAMM ......................... 53
Figure 3.2 Molecular visualization of mutation positions ........................................ 55
Figure 3.3 SDS-PAGE of wt, loop2, and charged mutant toxins ............................ 56
Figure 3.4 CD spectra of loop2 and charged mutant toxins ..................................... 58
Figure 3.5 CD spectra of mutant toxins .................................................................. 59
Figure 3.6 Bioassay of mutant toxins against 1st instar Manduca sexta larvae ....... 60
Figure 3.7 Slot-blot of toxin and receptor binding .................................................. 66
Figure 3.8 Slot-blot of toxin and receptor binding .................................................. 67
Figure 3.9 Distribution of mutants affecting binding ............................................... 74
Figure 4.1 Loop regions of Cry1Aa and Cry4Ba ..................................................... 84
Figure 4.2 CD spectra of mutant toxins ................................................................. 86
1.1. **Bacillus thuringiensis** δ-endotoxin as a biopesticide: a century of progress

Insect pests are a major cause of damage to the world's commercially important agricultural crops. Due to pests of various nature, worldwide crop losses are estimated to be about 30% of pre-harvest and 10% of post-harvest yields (83). Pesticides clearly play a significant role in meeting the demand of foodstuff resulted from the steady increase in world population and very limited addition of arable land. As the application of synthetic pesticides more than a century resulted in environmental damage and pest resistance, *Bacillus thuringiensis* emerges as a valuable alternative in pest control.

*Bacillus thuringiensis* (*Bt*), an aerobic gram-positive endospore-forming bacterium, was first isolated from *Bombyx mori* larvae (silkworms) killed by sotto disease (sudden collapse disease) in Japan by Shingetane Ishiwata, who named it *Bacillus sotto* in 1901. Ten years later, Ernst Berliner, a Germany entomologist, isolated another variety of this bacterium from dead *Anagasta kuehniella* (Mediterranean flour moth) found in stored grain in Thuringia. In 1915, Berliner recorded the first scientific description of the bacterium, and was credited for naming it *Bacillus thuringiensis* after the town (9). Berliner reported the existence of a crystal within *Bt*, but the activity of this crystal was not discovered until much later.
Farmers started to spray $Bt$ spores as a pesticide as early as in 1920. The first commercial formulation of $Bt$ spores (Sporeine) was available to control flour moths in France in 1938. In the US, $Bt$ was used commercially starting in 1958. By 1961, $Bt$ was registered as a pesticide to the EPA. Field trials yielded inconsistent results in the 1960’s until the discovery of $Bt$ kurstaki isolate, HD-1, which proved 2 to 200 times more toxic against key agricultural pests (32). For many years, $Bt$ remained only a minor component of pest management, because highly efficient synthetic pesticides were readily available, while $Bt$ spray had limitations such as short range of field stability, lack of capacity to reach cryptic pests, and narrow spectrum of activity.

Basic research aimed at understanding the mode of action of Bacillus thuringiensis lagged far behind its commercial development. In late 1950, Angus, Fitz-James and Hannay found that the insecticidal activity of $Bt$ was mainly due to protein crystals formed during sporulation (4, 38, 58). In early 1980, Gonzalez et al. discovered that genes encoding for the crystal (Cry) proteins reside on transmissible plasmids(13, 50). A $Bt$ kurstaki gene encoding a δ-endotoxin was first cloned into $E. coli$ in 1981 (118).

Up until 1977, only thirteen $Bt$ strains had been described, all of which were toxic only to certain species of Lepidoptera (butterflies and moths) larvae. In 1977, the first subspecies toxic to Diptera (flies and mosquitoes) was found, and the first discovery of strains toxic to species of Coleoptera (beetles and weevils) followed in 1983. To date, thousands of different $Bt$ strains have been isolated, producing well over 200 crystal proteins that are active against several orders of insects (> 150 species) and some other invertebrates and recently to leukemic cells. The current nomenclature for the $Bt$ pesticidal crystal proteins was based on the amino acid identity. (20), (21). Two major
classes of crystal proteins or δ-endotoxins are the cytolytic toxins (Cyt) and the other crystal proteins (Cry). The former are produced by Bt subspecies active on Diptera. The latter, also the focus of this study, are more prevalent and active on at least three orders of insects.

In the 1980s use of Bt increased when insects became increasingly resistant to the synthetic insecticides, and scientists and environmentalists became aware that the chemicals were harming the environment. Bt-based insecticides are considered safe for mammals and birds, and safer for non-target insects than conventional insecticides. They affect specific insects but do not persist in the environment. These factors ensure a place for Bt as a commercially viable product. Insecticides containing Bt and its toxins have been sold under trademarks such as Dipel, Thuricide, and Vectobac. Bt spore spray was also applied to control forest defoliators such as Choristoneura fumierana (spruce budworm) and Lymantria dispar (gypsy moth). Governments and private industries started to fund research on Bt.

With the advancement in molecular biology, introduction of genes encoding the toxic crystals (cry genes) into a plant became feasible. Prior to 1990, Bt was rather ineffective against certain pests, because the spore spray could not reach the cryptic insects inside the stalk or near the root. In 1995, introducing the cry gene into corn plants resulted in Bt-corn, the first genetically engineered plant registered with the EPA. The GM (genetically modified) crops make the cry proteins in each and every cell of the plant. The "in-plant" protection reduces or eliminates the traditional spraying of Bt in the field and outperforms spraying with Bt.
Influenced by an increased confidence in the benefits of \textit{Bt} crops and the availability of new \textit{Bt} varieties that confer protection against additional pests, adoption of \textit{Bt} crops is rising rapidly. Global acreage of \textit{Bt} crops increased 24\% between 2002 and 2003. In 2003, \textit{Bt} varieties account for 9\% and 17\% of the world’s corn and cotton acreage, respectively. Eight years after the first \textit{Bt} crop was cultivated, \textit{Bt} corn and cotton have been grown on a cumulative area of more than 80 million hectares in 17 countries worldwide (8). In addition, other \textit{Bt} crops, including potato, rice, canola, soybean, tobacco, tomato, apple, peanuts and broccoli, are in various stages of development (8, 123).
1.2. GENERAL MODE OF ACTION

The general mode of action of *Bt* Cry toxins has been studied extensively through several decades, but is not yet completely understood. The insecticidal property of *Bt* Cry toxin is attributed to the formation of pores in epithelial cells lining the insect midgut (5, 14, 34, 45, 62, 114). During the sporulation phase of the bacterium, *Bt* produces parasporal inclusions containing one or more crystal proteins (δ-endotoxins). Upon completion of sporulation, the parent bacterium lyses to release the spores and the inclusions. When ingested by susceptible larvae, *Bt* crystals in the inclusions are solubilized under the alkaline and reducing condition in insect midgut, and an inactive protoxin is released (65). Protoxin is further processed to active Cry toxin by proteases present in the midgut (16, 17).

The three-dimensional structure of activated Cry1Aa toxin has been determined by X-ray crystallography (Figure 1.1) (86). The toxin has three distinct domains. The N-terminal domain I is a bundle of α-helices with the central hydrophobic helix surrounded by six amphipathic helices. The middle (II) and C-terminal (III) domains are made of mostly β-sheets. Domain II consists of three anti-parallel β-sheets connected by loops in a “Greek key” conformation, while domain III comprises a sandwich of anti-parallel β-sheets with lectin-like, jellyroll topology.

Active toxin passes through the peritrophic membrane lining the insect midgut, which prevents traverse of aggregate toxins (151). The initial reversible binding to specific receptors, located on the brush border membrane of columnar epithelial cells, is largely dependent upon domains II and III (24). Upon binding to receptors, toxin possibly undergoes conformational changes and oligomerization / aggregation that are essential
for subsequent steps to take places. Irreversible insertion of toxins into the membrane follows, leading to formation of cation-conductive pores (144). As demonstrated by many studies, the transmembrane pores are comprised of certain amphipathic helices in domain I, while domain III was suggested to contribute to ion-channel formation as well (15, 120, 143).

Although it is accepted in the field that pore formation in the membrane is lethal to the insect, the exact mechanism of mortality is still under debate. A relatively general hypothesis was proposed that influx of water resulted in swelling and bursting of columnar cells lining the midgut (5, 75). Eventually insects stop feeding and die of starvation. Alternatively, it has been postulated that lethality is due to depolarization of the membrane potential by formation of pores, giving rise to an elevated cytoplasmic pH (59, 142). While most animal cells utilize various combinations of Na\(^+\), K\(^+\), and Cl\(^-\) channels along with the Na\(^+\)/K\(^+\)- and H\(^+\)/K\(^+\)-ATPases to maintain a constant resting potential across the plasma membrane, in the case of the lepidopteran midgut, the apical plasma membrane of columnar epithelial cells has been demonstrated to regulate K\(^+\) efflux and influx only indirectly through reliance upon neighboring goblet cell H\(^+\)/K\(^+\)-ATPases. This unique situation has been proposed to make the epithelial lining of the lepidopteran midgut susceptible to the toxins of _Bt_. Any major influx of K\(^+\) across the apical membrane of the columnar cell will result in depolarization of the membrane with subsequent efflux of H\(^+\) down the large pH gradient normally maintained in the highly alkaline midgut.
Figure 1.1  The three-domain structure of Cry1Aa

Domain I (blue) is a bundle of eight α-helices. Domain II (cyan and green, bottom) consists of three anti-parallel β-sheets connected by loops, while domain III (yellow and green, top) comprises a sandwich of anti-parallel β-sheets with lectin-like, jellyroll topology.
Overall, events occurring after toxin binding, such as the cellular mechanisms leading to changes in midgut cell membranes and their contributions to the death of larvae, are still poorly understood. Smouse *et al.* (126) reported that Cry toxins *Bt* subsp. *israelensis* (Bti), besides capable of forming pores in cell membranes (77, 138), induces apoptosis, or programmed cell death, in the larval midgut cells of the mosquito, *Culex pipiens*. The relationship between pore formation and apoptosis is unknown, but may be part of the insecticidal mechanism of the toxin.

Among steps in the general mode of action, Cry toxin binding to receptors on midgut epithelial membrane has been proven essential for its toxic activity. A technique for studying binding at molecular level was developed using insect brush border membrane vesicles (BBMV) and $^{125}$I-labeled wild-type or mutant toxins (140). Saturation and competition binding data provide valuable insight into toxin-receptor interaction. BBMV binding study represents complex affinities, since two consecutive steps are involved: an initial reversible receptor binding and a subsequent irreversible membrane insertion. Although irreversible insertion depends on reversible binding, a direct correlation to toxicity has only been observed for the irreversible insertion (87). Further analysis of the initial binding using purified receptor will provide helpful information to dissect the mode of action.

To date, two types of membrane proteins have been identified as primary Cry toxin receptors: the enzyme aminopeptidase and a cadherin-like glycoprotein. The 120kDa metalloprotease aminopeptidase-N (APN), a ubiquitous gut enzyme, was first identified as the Cry1Aa binding protein by ligand blot analysis using *Manduca sexta* BBMV (43, 76). Further characterization indicated that APN was glycosyl-
phosphatidylinositol (GPI) anchored to the membrane at its C-terminus (43, 74, 76). Since the discovery of this APN (MsAPN-1), two other *M. sexta* APNs have been reported (29, 93), as well as APN’s from several other Lepidoptera including *Lymantria dispar* (44, 84, 135), *Plutella xylostella* (29, 93), *Heliothis virescens* (94), *Bombyx mori* (149), and *Trichoplusia ni* (89), with the sizes ranging from 105 to 170kDa. Although affinity of Cry1A to purified APN is in the 100-200nM level, approximately 100-fold lower than that to BBMV (81, 116), it has been shown that APN acts as a functional receptor in its enhancement of channel forming capacity when reconstituted in liposomes (119) and reduction in inhibition of short circuit current when released from the GPI anchor (84).

APN from *Lymantria dispar* has been cloned and expressed transgenically using a baculovirus vector system in *Spodoptera frugiperda* Sf9 insect cells (44), but failed to produce Cry1Ac binding receptor. One possible explanation originated from the observation that Cry1Ac binding to APN was inhibited by GalNAc, inferring that proper glycosylation of APN is essential in binding. On the other hand, Cry1Aa binding to APN does not require carbohydrate moieties (94, 97), may be the reason why recombinant *Bombyx mori* APN from *E. coli* was able to bind to Cry1Aa toxin (150).

The focus of this study is on the second type of receptor for Cry toxin, the cadherin-like protein. The first Cry toxin-binding cadherin-like protein, BT-R₁, was purified and cloned from *Manduca sexta* (132, 133). BT-R₁ shares extensive sequence homology and identity to other members of the cadherin superfamily. Binding of BT-R₁ and Cry1A toxins is at the nM (10⁻⁹) level (73), comparable to the affinity of toxin to BBMV. The apparent molecular weight (210kDa) of BT-R₁ is higher than the molecular
mass calculated from its DNA sequence (172kDa), as a result of post-translational glycosylation. However, treatment of the receptor with de-glycosylating enzymes does not reduce its binding, suggesting that its sugar moieties are not essential in binding.

Cadherin-like proteins of various sizes which have been purified from other insect species, e.g. 175 kDa BtR175 from *Bombyx mori* (103), 270 kDa BTR-CAD from *Lymantria dispar* (136), 180 kDa HvCAD from *Heliothis virescens* (147), and 220 kDa OnBt-R1 from *Ostrinia nubilalis* (39), also show high affinity to Cry1A toxins.

When expressed on the surface of *Drosophila* S2 cells, *Spodoptera frugiperda* Sf9 cells, or mammalian COS-7 cells, cadherin-like proteins from several species all induce Cry1A cytotoxicity (63, 64, 133). Insect resistance to Bt Cry toxins in *Heliothis virescens* (42), *Helicoverpa armigera* (148), *Ostrinia nubilalis* (18), *Pectinophora gossypiella* (101), *Plutella xylostella* (117) have also been linked to elimination or alteration of cadherin-like proteins from the midgut. This further confirms the cadherin-like proteins act as functional receptors for Cry toxins.

Recently, a third category of Bt receptors was reported to be invertebrate glycolipid (54). Studies have shown the capacity for Bt toxins to bind directly and specifically to insect glycolipids (28, 54). Glycolipid levels have also been found to be substantially reduced in a field-evolved Cry1Ac-resistant *Plutella xylostella* strain (79) and *Caenorhabditis elegans* strain.

Besides the three types of receptors for Cry toxins reviewed above, there are a few other toxin-binding proteins identified by various methods. English and Readdy (35) first identified a 72 kDa alkaline phosphatase as a Cry1Ac binding protein in *H. virescens* through ligand blot analysis with traditional one-dimensional electrophoresis (1DE), the
same method used when both APN and the cadherin-like protein were initially identified. Using a combination of two-dimensional electrophoresis (2DE), mass spectroscopy and western blotting, Adang et al. (99) has identified actin and alkaline phosphatase from Manduca sexta as novel Cry1Ac-binding proteins in addition to the previously reported APN. However, the detailed binding characteristics and potential mediation of toxicity will be necessary to establish for these proteins.

Enhanced knowledge of the binding of insecticidal toxins to receptors will provide the basis of improvements in toxicity and reduction of toxicity toward beneficial and non-target insects.
1.3. RISKS AND RISK MANAGEMENT IN BT CROPS

Insect pests are a major cause of damage to the world's commercially important agricultural crops. By estimation, the European corn borer causes well over $1 billion of damage yearly to corn in the US. Other insects like corn rootworm, cotton bollworm, tobacco budworm, etc. cause combined damages of over $7 billion yearly in the US. After decades of application of synthetic pesticides which resulted in severe insect resistance and adverse effects on the environment, Bt has emerged as a valuable alternative in pest control. US farmers alone used 450,000 kg less synthetic pesticides on Bt cotton than they would have used on conventional varieties in 1998 (36). Yields and profits also improved notably in Bt crop fields. The estimated total net savings to the grower using Bt cotton in the US was approximately $92 million in 1998 (10). The extremely rapid adoption of these Bt-protected crops demonstrates the outstanding grower’s satisfaction of the performance and value of these products.

As Bt has clearly become the mainstay of non-chemical control of pests, either as sprays or through incorporation of Bt toxins into transgenic crops, studies on the environmental fate of transgenic crops and their genetic constructs have begun to accumulate in the last decade. Basic ecological knowledge has been needed to assess four types of potential negative environmental consequences: 1) Increased weediness of the crops (131); 2) invasiveness of Bt genes in natural plant population and cross-contamination of genes; 3) The disruption of non-target herbivores; and 4) The development of resistant pests (52).
There is a myth about new biotech plant varieties, especially those with genes from unrelated organisms, entailing greater risks than their traditionally bred counterparts. This line of thinking lacks an appreciation of the fact that genes from an organism are often times not unique to that organism and as likely to be found in a variety of unrelated species. High conservation of genes between species has become increasingly apparent in the current era of genome research. The potential of GM crops to transfer foreign genes through pollen to related plant species has been cited as an environmental concern as well. Molecular approaches with potential for controlling gene flow among crops and weeds include maternal inheritance, male sterility, and seed sterility. Several other containment strategies may also prove useful in restricting gene flow, including apomixis (vegetative propagation and asexual seed formation), cleistogamy (self-fertilization without opening of the flower), genome incompatibility, chemical induction/deletion of transgenes, fruit-specific excision of transgenes, and transgenic mitigation (transgenes that compromise fitness in the hybrid). As yet, however, no strategy has proved broadly applicable to all crop species, and a combination of approaches may prove most effective for engineering the next generation of GM crops.

Another argument of emotional importunity, spurred largely by a study published in Nature (90) a few years ago that pollen from Novartis® Bt corn harmed Monarch butterflies. By design, the study was flawed and thus reviewed by peers skeptically. As put by one critic, “There probably was not an entomologist in the world who was not aware that corn pollen containing the Bt gene could harm butterflies – if butterflies ate corn pollen, which they don’t.” (40) In response, the US Department of Agriculture
(USDA) in conjunction with the several universities studied the impact of *Bt* corn pollen on non-target insect species, represented by Monarch butterfly larvae. The results (60, 106, 109, 121, 122, 127) showed that there was a distinct temporal and spatial overlap between monarch larvae and *Bt* corn pollen. The migration pattern of Monarch butterflies does not even bring the larvae in contact with corn during its short period of shedding pollen (125). Furthermore, the overlay artificially environment of the experiments (90) did not resemble the natural conditions. *Bt* pollen is not dispersed widely. The pollen concentrations in the original experiment were far higher than what was found in a field setting. At its naturally low concentration, a single rain shower can remove more than half of the pollen from leaves on the upper portion of milkweed plants, where monarch larvae tend to feed. Taken together, the impact of *Bt* corn pollen from current commercial hybrids on monarch butterfly populations is negligible. The fact that there may be non-target impacts of *Bt* corn is not in itself surprising. There are non-target impacts of any pest management approach. It should be recognized that any potential harm to non target species should be weighed relative to other pest control techniques, as for chemical insecticides are much more likely to harm other species, and contaminate water supplies, and food chains than the narrow-spectrum *Bt* toxins.

However, even with this new body of evidence, it may still be hard to overcome the generally negative image of GM foods. Although the talk of 'Franken-foods' is often exaggerated and misleading, government and the biotech industry has the responsibility to respond and address many of the concerns of consumer groups and environmentalists in a scientific fashion. Myths surrounding GM food, including *Bt* crops, may yield to an
understanding of its benefits only through persistent public education and advocate for scientific awareness that

The use of biotechnology in GM foods has also raised concerns about the potential risks to human, which mainly lies in the uncertainty of their safety for consumption, and argument that relies heavily on perceived risks. The feeling is that growers and producers characterize GM foods as safe largely because they have not been proven harmful. This sentiment led to the regulatory “precautionary principle” in Japan and European countries, calling for government restrictions to be made even in the absence of scientific evidence (125).

1.4. RESISTING RESISTANCE TO BT CROPS

By killing pest insects, Bt crops create selection pressure on pests to evolve resistance. In the face of the enormous selective pressure generated by widespread use of Cry proteins in crops and organic farming, development of Cry toxin resistance among target populations is considered a serious threat to long term potency of Bt toxins (54). Bt represents a natural pesticide, and as with any kind of pesticide the problem of resistance is almost unavoidable. Given the wide use of Bt technology, it is quite striking that not many cases of resistance have emerged. When Bt spores is applied as spray, two pest species, Trichoplusia ni (cabbage looper) (66) in the greenhouse and Plutella xylostella (diamondback moth) outside the laboratory, have developed significant resistance. The only field-evolved resistance that has been documented is Helicoverpa armigera (cotton
bollworm) (57) to Bt transgenic crops. These evidences suggest that insect resistance management (IRM) strategies have been effective thus far. However, current strategies to delay resistance remain far from ideal. Eight years without resistance provides a timely opportunity for researchers, regulators and industry to reassess the risk of resistance and the most effective strategies to preserve Bt and other novel insect-resistant crops in development (8).

Three different biochemical mechanisms of resistance to Bt have been proposed so far: i) proteolytic processing of protoxins (12), ii) improved repair of damaged midgut cells, and iii) modification of Cry protein–binding. However, only for the binding reduction mechanism has a relatively direct link been observed between the biochemical modification and decreased susceptibility (resistance) (37). Mutations affecting the Cry1A-binding cadherin-like protein have shown to be tightly linked with laboratory-selected resistance in Heliothis virescens (42), Helicoverpa armigera (148), Ostrinia nubilalis (18), Pectinophora gossypiella (101), Plutella xylostella (117). Cadherin genes have been considered the prime target for DNA-based screening for resistance. However, recent work has reported that field-evolved resistance in Plutella xylostella were caused by glycolipid alteration, suggesting that a single molecular test will not be suitable for detecting resistance-allele frequency in all insect species (8).

Using two-dimensional electrophoresis (2DE), Candas et al. (12) compared the differences between B. thuringiensis-susceptible and -resistant Indianmeal moth, Plodia interpunctella. In resistant insects, increases in levels of several specific proteins, such as glutathione transferase, cytochrome c oxidase subunit I, and NADH dehydrogenase
subunit 5, were found. Additionally, there was a shift in both charge and size of an ortholog of mitochondria F\textsubscript{1}F\textsubscript{0}-ATPase subunit δ between susceptible and resistant *P. interpunctella*, implicating that there were alterations in the gene expression of key proteins in oxidative metabolism in the midgut epithelial cells. These findings suggest that the mode of action of Cry toxins is complex and resistance to Cry toxins is multifaceted.

To combat or at least delay resistance development, insect resistance management (IRM) plans are being implemented to reduce the risk of to enable the prolonged effectiveness of *Bt* products. For example, farmers that plant *Bt* corn are required to follow resistance management requirements set by the EPA. Conventional IRM includes moderate dosage to ensure survival of fraction of susceptible population, high dosage to kill insects heterozygous for resistance, combination/stacking/pyramid of toxins, temporal or tissue-specific expression of toxin, and provision of non-toxin plants. Refuge planting, an area planted to a non-*Bt* variety that is physically close to a field planted with a *Bt* variety, is a big part of resistance management in transgenic farming. Alternating *Bt* applications with synthetic insecticides also helps that any resistance to any one class of insecticide does not develop. Crop rotation is another method used to combat resistance. Since different crops are attacked by different insect pests, different types of *Bt* will be used. By rotating crops, the pressure of one specific strain of *Bt* on an insect is minimized. The pest insect has less time to develop resistance before a new strain is used for a different insect.
Clearly, it is important to maintain a “diverse toolbox” for pest management. Careful use of these tools can help to preserve their utility as well as to maintain environmental quality. On the other hand, further and detailed understanding of the mode of action of \textit{Bt} toxins will allow tailoring of safer and better \textit{Bt} toxins in application, facilitate monitoring of insect resistance, and in the long term enable safeguarding of the value of \textit{Bt} for insect control.
1.5. PROTEIN ENGINEERING

Protein-protein interactions are involved in essentially all cellular processes. A detailed understanding of these interactions is one of the fundamental goals of modern biology. In the face of rapid adoption of Bt and Bt crops world wide, clear understanding in receptor binding and specificity will aid in the construction of safer and better biopesticide. For example, application of lepidopteran-specific Cry1A toxins is restricted in sericultural countries, due to its harm on Bombyx mori (silkworm). Judicious modification of the B. mori specificity region without altering the structure and activity against other pests would ease concerns over the adverse effect. On the other hand, identification of residues involved in specificity will help in construction of toxins targeting a wider range of insects. Engineering mutant toxins with new or broadened insecticidal properties, among other approaches, is useful to forestall resistance development.

Site-directed mutagenesis is a powerful tool for probing protein structure and function. Analysis of the functional importance of side-chains by mutational study has the caveat that the mutant protein may exaggerate the effect by imposing a structural disturbance or an unusual steric, electrostatic or hydrophobic interaction. Alanine-scanning, by systematic replacement of side-chains with alanine, is least disruptive to the structure, thus has been particularly successful in mapping functional binding epitopes (i.e., residues in direct contact with a ligand). Because substitution with alanine removes all side chain atoms past the β-carbon, the effects of individual alanine mutations can be used to infer the roles of individual side chains. Alanine-scanning mutagenesis provides a detailed map of a protein-binding interface, but the method is laborious. Many mutant
proteins must be produced and purified, and the structural integrity and binding constant of each mutant must be assessed separately.

Additional structural analysis, such as CD spectrum, can be performed to confirm the structural integrity of the proteins that contain the alanine substitutions. Residues important for binding cluster in a small region near the center of the structural epitope can be evaluated. The functionally “null” contact residues tend to be near the periphery. On average the energetics for electrostatic interactions are considerably weaker than estimated from mutagenesis of enzyme-substrate complexes (53).

In recent years, considerable effort has gone into mapping of binding epitopes (protein-protein interfaces). The key residues involved in binding are termed “hotspots.” Hotspots are defined as residues that, when mutated to alanine, gives rise to a distinct drop in the binding constant (typically 10-fold or higher K_D). The result of this effort has been disappointing in that general predictive rules for a priori identification of likely hotspots have not arisen. The surface topology of enzymes allow the prediction that the deepest clefts contain the active sites, but this is not so for hotspots in binding epitopes. Protein binding sites have neither the largest total surface area nor the most extensive non-polar buried surface area. They are not distinguished by their electrostatic characteristics or the number of hydrogen bonds. Although new combinatorial methods are now in the embryonic stage there is no current replacement for the brute force approach of alanine scanning mutagenesis. In general hotspot residues may result in the loss of (a) optimal van der Waals contacts; (b) the loss of essential electrostatic pairings (which may or may not be net-stabilizing with respect to the solvent); (c) loss of buried (or exposed) non-polar surface area; (d) local conformational change; (e) local unfolding;
(f) increased entropy of unbound states; (g) aggregation or (h) global unfolding. In the present proposal we are not interested in the results of mutagenesis that would result in global conformational changes (g) and (h) of this list, but would focus on those mutational products that provide the mapping of distinct areas. Global conformational changes can be detected by alterations in circular dichroism spectra, differences in thermal denaturation from the wild type protein and greater sensitivity to proteases (especially useful for Cry proteins, given their trypsin-resistant native state). The take home lesson from a review of the literature is that studies on protein-protein interaction are not only a very active, topical field, but there is still much to learn about the nature of binding epitopes. Cry toxin-receptor interactions hold the potential for revealing new information on protein-protein interaction. In particular, the binding of Cry1Aa to the cadherin-like receptor, Bt-R1, has very high affinity binding (~1 nM), and promises to yield important information on the nature of hotspot residues.
CHAPTER 2

SUBCLONING AND OVER-EXPRESSION OF Manduca sexta CADHERIN-LIKE RECEPTOR BT-R1a FRAGMENT

2.1 INTRODUCTION

To fully realize the potential of Bt toxins as biopesticides and in transgenic crops, sufficient understanding of the mechanism of toxicity must be gained in order to engineer toxins with maximum activity and wider range of pest toxicity. Determining toxin-receptor interactions will facilitate understanding of how toxin receptors are involved in eliciting toxicity and assist in the design of insecticidal proteins with higher affinity for and greater specificity toward targeted insects.

One of the Cry toxin receptors is a cadherin-like protein. Cadherin is a large family of calcium-dependent, transmembrane glycoproteins that are responsible for maintaining the integrity of cell-cell contacts in multicellular organisms. Cadherins mediate Ca\(^{2+}\) dependent cell-cell adhesion, provide extracellular information to the cell, and participate in tissue morphogenesis and maintenance of the epithelium (104).

A cadherin-like protein (denoted as BT-R1) with high binding affinity to Cry1A toxins was first isolated from Manduca sexta (tobacco hornworm) (133). Isolation and identification of cadherin-like proteins from other species followed. The 175 kDa BtR175
23

from *Bombyx mori* (103), 270 kDa BTR-CAD from *Lymantria dispar* (136), 180 kDa HvCAD from *Heliothis virescens* (147), and 220 kDa OnBt-R₁ from *Ostrinia nubilalis* (39, 103) also showed tight binding to Cry1A toxins.

When expressed on the surface of *Drosophila* S2 cells, *Spodoptera frugiperda* Sf9 cells, or mammalian COS-7 cells, cadherin-like glycoproteins from several species induce Cry1A cytotoxicity (63, 64, 133). Recombinant and synthetic peptides containing both amino acid sequences inhibited Cry1Ab toxicity *in vivo* when fed to *M. sexta* (31, 49) and *H. virescens* (147) larvae, demonstrating their involvement in toxicity.

Gahan *et al.* (42) identified that a cadherin-like gene is disrupted in the Bt-Resistant line YHD2 of *H. virescens*. Loss of expression of the correspondent cadherin-like protein (HevCaLP) accounts for the major portion of resistance to Cry1Ac observed in YHD2 larvae. Correlation between alteration of cadherin genes and insect resistance has been reported in other species, such as *Ostrinia nubilalis* (18), *Pectinophora gossypiella* (101), *Helicoverpa armigera* (148), and *Plutella xylostella* (117).

Bt-R₁ from *M. sexta* is a classical cadherin protein, sharing 30 to 60% homology and 20 to 40% identity to other members of the cadherin superfamily. It is composed of four domains (Domain representation shown in Figure 2.1). Downstream of a putative membrane signal sequence, the ectodomain (EC) consists of tandem repeats of homologous domains, called cadherin repeats (CR), which are numbered from the outermost 1 to the innermost 12. The membrane-proximal extracellular domain (MPED) is joined to a membrane-spanning domain (TM), and a short cytoplasmic domain (CYTO) (41) in the C terminus.
Figure 2.1  BT-R₁ structure model.

Proposed structure of the cadherin receptor BT-R₁ is based on the domain organization of the cadherin superfamily of proteins. (from Reference (31)

BT-R₁ is composed of four domains: (i) EC, (ii) MPED, (iii) TM, (iv) CYTO.

Downstream of a putative membrane signal sequence, the ectodomain consists of 12 cadherin repeats that are structured in EC modules (EC1 through EC12).

The EC harbors a pair of cell-adhesion sequences, HAV (His–Ala–Val), which is characteristic to all cadherins and two integrin-binding sequences, RGD (Arg–Gly–Asp) and LDV (Leu–Asp–Val).

The Cry1A toxin-binding region folds into the EC11, which is close to MPED.
Mapping of the toxin binding regions (TBRs) have been carried out on the cadherin-like proteins from different species by different methods. Three toxin-binding regions (TBRs) have been reported. Using the phage display technique, Gomez et al. identified that the toxin binding region 1 (TBR1), a stretch of eight amino acid residues ($^{869}$HITDTNNK$^{876}$) in CR7, interact with loop 2 of Cry1Aa and Cry1Ab toxins (48). Dorsch et al. mapped a second Cry1Ab binding region (TBR2) within CR11 (aa 1296-1362) in protein blotting, which was later narrowed down to 12 amino acid residues ($^{1331}$IPLPASILTVTV$^{1342}$) (31) and was shown by Gomez et al. to bind loop α8 (47). This TBR2 overlaps with a 219 residue region (aa 1245-1464) of the B. mori cadherin, BtR175, which is responsible for Cry1Aa binding (103). A third region, aa 1363-1464 of M. sexta BT-R$_{1a}$ CR12, was established to be involved in toxin binding and cytotoxicity (63), and subsequently confirmed in a homologous region ($^{1423}$TGVLTLNFQPTASMHGMFE$^{1439}$) in HvCAD from H. virescens (147).

The Adang lab cloned a full length BT-R$_{1a}$ from M. sexta, which was virtually identical (5 amino acid differences) to the Bt-R$_1$ originally cloned in Bulla lab. Truncated fragments of cadherin repeats of different lengths were then sub-cloned into a pET (His-tag) vector system. Over-expression of His-tagged CAD-D (CR 11 and 12) in E. coli resulted in inclusion bodies. Fusing proteins of interest with MBP (maltose-binding protein) has been shown to elevate the solubility of the target proteins (55, 96). A pMAL vector system was utilized in this study to produce a soluble form of the CAD-D fragment.

The binding kinetics of several Bt toxins with midgut receptors has been observed using optical biosensors, such as the BIAcore (BIAcore AB, Uppsala, Sweden) (69).
BIAcore measures the affinity of a flowing molecule for another molecule immobilized on a surface as they form a real-time complex. As the molecule in solution is adsorbed by the ligand, changes in mass on the surface are monitored using surface plasmon resonance (SPR). To determine the affinity of the partial *M. sexta* cadherin protein to Cry1A toxins, SPR was performed to obtain kinetic parameters.
2.2 MATERIAL AND METHODS

Subcloning of cadherin-like receptor fragment

Full length *Manduca sexta* cadherin-like receptor gene was cloned into pMECA vector at the Adang lab. The 790 bp CAD-D fragment was amplified by PCR, double digested with restriction enzymes (EcoRI and PstI), and inserted into pMAL-c2G vector (New England Biolabs®) at the compatible positions. The *cad-D* gene was positioned downstream of the *malE* gene, which encoded for a maltose-binding protein (MBP).

The primer pair for PCR was:

Fw CAD-D-5 (underlined is a built-in EcoRI site)

```
GCA ACA GCG AGG GTG AAT TCG GCG TGG ATC CTG TTC GC
```

Re CAD-3 (underlined is a built-in PstI site)

```
GGT CAA CAA GTC CTG CAG CAC CAG CGA TCG CGC
```

The recipe of PCR reaction was:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template (CAD in pMECA)</td>
<td>100ng 1μl</td>
</tr>
<tr>
<td>Forward primer (25pmol/μl)</td>
<td>10pmol 0.4μl</td>
</tr>
<tr>
<td>Reverse primer (25pmol/μl)</td>
<td>10pmol 0.4μl</td>
</tr>
<tr>
<td>dNTP’s</td>
<td>10mM each 0.5μl</td>
</tr>
<tr>
<td>5X Tgo buffer</td>
<td>1X 5μl</td>
</tr>
<tr>
<td>Tgo polymerase</td>
<td>1μl</td>
</tr>
<tr>
<td>Water</td>
<td>to 25μl 16.7μl</td>
</tr>
</tbody>
</table>
The program for PCR Reaction is:

1. 94°C, 2 min
2. 94°C, 30 sec
3. 55°C, 1 min
4. 72°C, 1 min
5. go to step 2, 29 more cycles
6. 72°C, 7 min
7. END

Over-expression and purification of the receptor

pMAL-CAD-D plasmid was transformed into E. coli DH5α cells. Procedures of over-expression and purification of the MBP-CAD-D fusion protein was based on a protocol from pMAL system with minor modifications. 500ml of rich media (1L: 10g tryptone, 5g yeast extract, 5g NaCl, 2g glucose, 100 μg/ml Ampicillin) was inoculated with 1% of overnight culture. Glucose was included in the medium to suppress amylase expression, which interfere with a subsequent affinity chromatography step. Cell growth was closely monitored by OD₆₀₀ measurement. When A₆₀₀ reached ~ 0.5 (~2x10⁸ cells/ml), IPTG was added at a final concentration of 0.3mM. The culture was grown for another 2 hours.

Cells were harvested by centrifugation at 4,000 xg for 20 min, and resuspended in 50 ml of column buffer (10 mM HEPES, 200mM NaCl, pH 7.4). Note: EDTA was
eliminated from the buffer system to prevent degradation of CAD receptor (11) Cell resuspension was frozen at -20°C overnight. Cell suspension was thawed in cold water, and sonicated in an ice-water bath twice, each for 2 minutes at a 10 sec on / 10 sec off pace, with a 10-minute interval on ice. Cell debris (pellet) and crude extract (supernatant) was separated by centrifugation at 9,000xg for 30 min. Batches 0.5 ml of crude extract were loaded onto amylose resin at equal volume in microfuge tubes. MBP fusion protein was allowed to bind to the amylose resin during an overnight incubation at 4°C with shaking. The resin and crude extract mixture was centrifuged at 5,000xg for 2 minutes. Supernatant containing unbound portion was discarded, while the pelleted resin with bound fusion protein was washed twice in equal volume of column buffer, and finally eluted with 0.5 ml of elution buffer (column buffer + 50mM maltose).

MBP-CAD-D Protein concentration was determined by Coomassie Reagent (Pierce®) based on Bradford method. Purity and quality of eluted protein was examined on 8% SDS-PAGE.

**Bt toxin purification**

Wild-type and mutant Cry1Aa toxins were expressed in *Escherichia coli* DH5α strain, purified and trypsin-activated as described previously. 500ml of TB media with 100μg/ml of Ampicillin was inoculated with 3ml of an over-night seed culture. Cells were grown for 72 hours at 37°C. Cells were harvested by centrifugation at 9,000 X g for 10 minutes. The pellet was resuspended in 100ml of lysis buffer (50mM Tris-HCl, 50mM EDTA, 15% sucrose, pH 8.0, 10mg lysozyme). After overnight lysis, cells were
spun down, and resuspended in 50ml of Crystal Wash I (2% Triton X-100, 0.5 M NaCl). The cell suspension was sonicated in an ice-bath, twice for 2 minutes with a 5 minutes interval. After three washes in Wash I and three washes in Wash II (0.5 M NaCl), the crystal protein was finally solubilized to protoxin in high salt sodium carbonate-bicarbonate buffer (0.5M NaCl, 30mM Na$_2$CO$_3$, 20mM NaHCO$_3$, pH 9.5) at 37°C with shaking for 2 hours. Solubilization of protoxin was examined on 8% SDS-PAGE, and protoxin concentration was determined by Bradford method using Coomassie protein assay reagent (Pierce). Solubilized protoxin was activated by bovine trypsin (Sigma) at a trypsin: protoxin ratio of 1:20 (W/W) in 37°C water-bath for 2 hours. Solubilized toxin was loaded onto a Superdex-200 size exclusion column (Amersham Pharmacia Biotech, Uppsala, Sweden). Elution was at 1ml/min in 0.5M NaCl, 50mM sodium carbonate-bicarbonate buffer, pH 9.5. Monomeric toxin was separated from protoxin/oligomeric toxin and peptides.

Surface Plasmon Resonance analysis of toxin affinity

Surface plasmon resonance experiments on BIAcore 3000 were performed for kinetic analysis. Sensor Chip CM5, BIAcore’s versatile and high-binding capacity sensor chip with a carboxymethylated dextran matrix, was used. The analysis temperature was set to 25°C.

Around 15,000 RU of anti-MBP IgG was immobilized on the surface of flow cell 2 on the CM5 sensor chip using an EDC/NHS-mediated amine coupling procedure. A freshly prepared solution of 50mM NHS (N-hydroxysuccinimide) and 0.2 M EDC [1-
ethyl-3-(3-dimethylaminopropyl) carbodiimide] was injected for 7 minutes to activate the flow cell. Anti-MBP IgG was reconstituted in 10mM NaAc, pH 5.0, and injected at a flow rate of 10 µl/min. Excess activated ester groups on the surface were deactivated using a 7-minute injection of 1 M ethanolamine-HCl, pH 8.5. Flow cell 1 was activated with 50mM NHS and 0.2 M EDC, and blocked with M ethanolamine-HCl, pH 8.5, without immobilization of anti-MBP IgG, serving as the reference surface.

MBP (maltose binding protein)-receptor fusion protein, MBP-CAD-D, at 50 µg/ml was injected at a flow rate of 5µl/min to interact with IgG on the chip. Over a period of time around 3 min, approximately 100 RU of MBP-receptor was captured. The (anti-MBP IgG)-(MBP-CAD-D) surface was allowed to stabilized for 1min, before wild type toxin at various concentrations were injected. Buffer only was included as a blank. The flow rate for toxin injections was at 30 µl/min. The association phase was 3 min and the dissociation phase was 10 min. Regeneration was achieved by two 30-second injections of 10mM glycine, pH 1.8 at 100µl/min. The control flow cell that was activated and blocked without immobilization of the antibody had both MBP-receptor fusion protein and toxin flowing through in each cycle. Running buffer in all experiments was HBS-P buffer (0.01M HEPES, pH 7.4, 0.15M NaCl, 0.005% surfactant). MBP-CAD-D fusion protein was purified in the same buffer, while toxin was prepared in sodium carbonate buffer and filter-dialyzed into the HBS-P buffer.
2.3 RESULTS

Over-expression and purification of MBP-CAD-D fusion protein

CAD-D fragment of around 30kDa contains CR11 and 12 of Manduca sexta cadherin BT-R1a. When cloned into a pET vector system and expressed as a His-tagged fusion protein, it became insoluble and resulted in the inclusion body. By fusing it to a maltose-binding protein (MBP), the solubility problem was solved. The insertion of a MBP tag creates a stable fusion product that does not interfere with the bioactivity of the protein or with the bio-distribution of the MBP-tagged product. The expression of polypeptides in-frame with maltose binding protein also allows for their easy, single-step purification from bacterial extracts under mild conditions using amylose resin (96). MBP-CAD-D fusion protein remained in the soluble fraction of the crude extract. The crude extract was loaded on amylose affinity column, washed and eluted in maltose-containing buffer. SDS-PAGE of these purification steps are shown in Figure 2.2. The yield of MBP-CAD-D fusion protein was around 20 μg / ml of culture. Further binding studies was made possible by the successful over-expression and purification of the MBP-CAD-D receptor fusion protein

Bt toxin purification

65kDa wild type Cry1Aa toxin was prepared and purified as described in the Material and Method section. SDS PAGE and chromatography profile of toxin purification on a size-exclusion column are shown in Figure 2.3.
Figure 2.2  Purification of MBP-CAD-D fusion protein

Lanes: 1) MW marker, 2) crude extract, 3) unbound protoxin from the resin, 4) wash I, 5) wash II, 6) proteins bound on resin, 7) elution (75kDa), 8) MW marker.
Figure 2.3  Purification of wt Cry1Aa toxin.

A. Lanes: 1) MW marker (Roche High Range), 2) solubilized protoxin (130kDa), 3) MW marker (Roche High Range), 4) column-purified toxin (65kDa)

B. Chromatography of active toxin on size-exclusion column. The second peak (fraction number: 9-11) is the monomeric form of toxin.
Surface Plasmon Resonance analysis of binding affinity

Sensor chip CM5 is designed for molecule interaction analysis in BIAcore® system. The sensor chip is fixed to a polystyrene support frame located in a protective sheath. Inside, the chip surface has a carboxymethylated dextran matrix covalently attached to a gold film. A “sandwich” method was used in this study. A capturing molecule, the monoclonal antibody against MBP (anti-MBP IgG), was amine-coupled onto the chip using well-established chemistry. The ligand, MBP-CAD-D fusion protein, was then affinity-captured. Interaction analysis was performed as toxin analytes in solution were injected in a concentration series over the surface of sensor chip. Regeneration condition of the immobilized capturing molecule was chosen to achieve complete dissociation of both the analyte (toxin molecule) and the ligand (MBP-CAD-D fusion protein) without affecting the binding characteristic of the capturing molecule (anti-MBP IgG).

Kinetic analysis of sensorgrams

Raw data (shown in Figure 2.4) from SPR was processed before evaluation. This included setting the baseline to zero, cropping the data so that the staggered start times could be analyzed globally, referencing responses across flow cells by subtracting data generated across an unmodified flow cell (Fc1) and double referencing within flow cells by subtracting an average buffer (blank) response from data collected within the same flow cell (Fc2). The processed data is shown in Figure 2.5.

The binding responses were analyzed globally by fitting data to various interaction models using BIAevaluation 4.1 software. A short period after injection start
and stop was excluded from the fitting to avoid sample dispersion effects. The best fitting is derived from “1:1 Langmuir binding with drift baseline” model. The “closeness-of-fit” was indicated by $\chi^2 < 3$. All other models, including “two binding sites” model and “sequential binding” model, had $\chi^2 > 10$, indicating higher non-random deviation from the fitted curve. The association rate constant (ka) and dissociation rate constant (kd) obtained from the best fitting is $ka = 1.01 \times 10^5 \text{M}^{-1}\text{S}^{-1}$, $kd = 1.08 \times 10^{-3} \text{S}^{-1}$. The equilibrium dissociation constant ($K_D$) was calculated from $kd/ka$ ratio, yielding 10.6 nM.

Previously, a $K_D$ value of 1 nM for Cry1Ab toxin binding to native BT-R$_1$ was established in competition binding assays using $M. sexta$ midgut BBMV preparations (132). Another study using SPR technique reported the affinity at 2.6nM between Cry1Aa and tissue-purified $B. mori$ cadherin (68). Full-length BT-R$_1$a expressed on the surface of S2 cells binds to Cry1Aa and Cry1Ab with similar affinities, 1.7 and 3.2nM, respectively (63). Recombinant fragments of BT-R$_1$ from $E. coli$ cells (CR11 + 12 to Cry1Aa/b/c at $K_D = 39$ nM) (31), BT-R$_1$a from S2 cells (CR11 + 12 to Cry1Ab at $K_D = 10$ nM) (63) and Hv CAD from $E. coli$ cells (CR12 to Cry1Ac at $K_D = 96$ nM) (147) all resulted in lower affinity to Cry1A toxins, but still well within the acceptable range for tight binding. In this study, $K_D$ of 10.6nM for Cry1Aa wt toxin and MBP-CAD-D binding is in line with these reported affinities. Thus, MBP-CAD-D will serve as the binding partner in subsequent Cry1Aa epitope-mapping experiments.
A. Representation of binding components on a BIACore chip.

B. Raw sensogram of binding steps: Binding of MBP-CAD-D with immobilized anti-MBP IgG, binding of toxin to CAD-D, regeneration of the chip (Both toxin and MBP-CAD-D will be released.).
Figure 2.5. Processed binding curves.

Concentration of Analyte (toxin): 7.8nM (red), 15.6nM (pink), 62.5nM (green), 125nM (blue).
CHAPTER 3
STUDIES OF THE Manduca sexta CADHERIN-LIKE RECEPTOR BINDING EPITOPES ON CRY1Aa TOXIN

3.1. INTRODUCTION

Cry1Aa is the only toxin in the Cry1A group to have its 3-D structure determined by crystallography. Although Cry1Aa is recognized as binding to the APN and Bt-R₁ (cadherin-like) receptors of M. sexta (132), there are few studies that identify the binding epitopes on Cry1Aa. Early work by Lu et al. (91) used short deletions and block alanine-scanning mutagenesis to associate residues of loop 2 in domain II with a loss of competition binding to B. mori BBMV. These defined a binding epitope of residues RRIILGSG (367-374). Likewise a short deletion of 4 hydrophobic residues in loop 3 of domain II (AAGA, 440-443) was shown to disrupt competition binding to both M. sexta and B. mori BBMV (113). The effect of individual residue substitutions has not yet been conducted. Domain swapping experiments among Cry1Aa, 1Ab and 1Ac indicated that residues in domain III direct Cry1Aa to a 210-kDa receptor (presumably Bt-R₁) (85). But the more specific region has yet to be defined. Overall, the receptor binding epitopes of Cry1Aa toxin are incompletely mapped, especially to the important receptor, Bt-R₁.

Cry1Aa binding epitopes for Bt-R₁ will be mapped by alanine scanning, which is an effective method in hotspots scanning (95) (25). The strategy for mutation design is
based on topological contact between Cry1Aa and an abstract surface (the receptor). Starting with domain II loop regions 371-374 and 440-443 mentioned above, and projecting a straight line onto domain III, the line touches residues 285, 286 and 289 in domain II and residues 556-559 in domain III. Alternatively, a line may be projected to a surface exposed group of residues, 591-593, to the right. There is a concave area of the toxin that will be not in contact with the straight line (rigid receptor or flat surface). If the straight line were allowed to bend into the concave area (as a flexible receptor or convex surface) residue 489 would be contacted because it is the most exposed residue in this area. These residues were constructed as alanine blocks and as individual alanine substitution mutants. Alanine scanning mutagenesis allows evaluation of the effect of individual residues on binding.

The *M. sexta* BT-R1 shares extensive tracts of amino acids with a large number of other cadherins, including several that have determined know three-dimensional structures. The known structure of a closely related cadherin can serve as a template to build the model of a portion of the BT-R1 cadherin-like protein. To theoretically predict the interaction of Cry1Aa with the CR 11-12 region of BT-R1, indicated by Dorsch *et al.* as the specific binding site of *Bt* toxins, the robust protein-protein docking hydropathy complementarity analysis program, GRAMM was used.

3.2. MATERIAL AND METHODS

*Homology modeling*

Three main programs were used to model the structure of cadherin-like receptor: i) An internet-based CLUSTAL W version available at (http://npsa-pbil.ibcp.fr/cgi-
bin/npsa_automat.pl?page=npsa_clustalw.html); ii) SWISS-MODEL available at (http://www.expasy.org/swissmod/SWISS-MODEL.html); iii) Swiss-Pdb Viewer Version 3.7b2. CLUSTAL W was used to align the protein sequence of the target protein with the template of known tertiary structure. Models were constructed using the “Optimize (project) mode” in SWISS-MODEL, in conjunction with Swiss-Pdb Viewer. The sequence of the target protein was aligned with the template sequence in Swiss-Pdb Viewer according to the alignment produced by CLUSTAL W earlier. Unaligned residues at the N and C terminal of the target protein were removed prior to submitting the project to the SWISS-MODEL site.

Simulated docking

A program for protein docking called GRAMM was used to simulate docking between Cry1Aa and Cads 11-12 of BT-R1. The protein docking used the high-resolution generic setting for hydrophobic docking. The hydrophobic docking was reported to yield markedly higher signal-to-noise ratio so that the correct match is discriminated better from false positive fits. Docking was performed using the model structures of cadherin-like receptor and Cry1Aa. Ten highest scoring complex based on the lowest-energy matches were scrutinized based on the close association of domain II loop 2 of Cry1Aa and cadherin-like receptor. One complex that matched the criteria also showed association of clusters in domain III loop region with cadherin-like receptor. These sites were analyzed further because it suggested that the loop region could be a potential site for binding to receptor.
Molecular Visualization

Cry1Aa three-dimensional structure was visualized in SWISS-pdbViewer v.3.1 with Q3D rendering (Glaxo Wellcome) and RasMol (RasWin Molecular Graphics Windows, Version 2.6).

Construction of Mutants

*Bacillus thuringiensis* δ-endotoxin gene, cry1Aa, an NdeI-NdeI fragment (nucleotide 445 to 4210) was blunt-ended with Klenow enzyme, and subcloned into pBluescript KS (-) phagemid vector at blunt end EcoRV site under pLac promoter.

Mutagenic primers were purchased from Integrated DNA Technology Inc. (listed in Table 3.2, Table 3.1 and Table 3.3). Site-directed mutagenesis was performed using ssDNA Kunkel method, modified from Muta-Gene phagemid *in vitro* mutagenesis kit (Bio-Rad). The pBluescript- cry1Aa construct (AmpR) was first transformed into *E. coli* strain CJ 236 (ChloropenicolR, duR, ungR). A 2X YT culture inoculated with 1% overnight seed culture was incubated to OD600 around 0.4, when helper phage M13KO7 (KanR) was added at MOI of 20. Kanamycin was added one hour after the infection and the culture was incubated for additional 6 hours. *E. coli* cells were separated by two rounds of high speed (17,000xg) centrifugation. Supernatant containing phage particles was treated with RNAse A. And phage particles were finally harvested by high
concentration of PEG-NH₄Ac. Multiple rounds of phenol-chloroform extraction released and purified the uracil-containing ssDNA from phage particle coats.

Mutagenic primers were phosphorylated by polynucleotide kinase (Roche®) and annealed to ssDNA in water-bath at decreasing temperature (70°C to 30°C over 40min). In vitro synthesis of the complementary strand was achieved using T7 DNA polymerase (USB®) in the presence of dNTPs, ATP and proper buffer. The resulting ds-DNA was transformed into regular *E. coli* strain DH5α (*dut⁺, ung⁺*), so that the wild-type uracil-containing strand would be cleaved, resulting in lower wt background during later screening procedures.

Plasmids from transformants were extracted using Qiagen® kit. Automated DNA sequencing was performed at the Plant Microbe Genomics Facility at OSU.
**Wild type sequence of Domain II loop 2:**

\[
LGIFRTLSSP^{365}LY^{367}RR^{369}IIL^{372}G^{373}SGPNNQELFV
\]

<table>
<thead>
<tr>
<th>Primer Design</th>
<th>Length</th>
<th>Sequence Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D 3</strong> (25MER)</td>
<td>(^{365})LYRRI I L (^{371}) to (\Delta)</td>
<td>CA TTA TCT TCA CCT GGT TCA GGC CC1</td>
</tr>
<tr>
<td><strong>A 3</strong> (46MER)</td>
<td>(^{365})LYRRI I L (^{371}) to AAAAAAA</td>
<td>CA TTA TCT TCA CCT GCA GCT GCA GCA GCT GCA GCT GGT TCA GGC CC</td>
</tr>
<tr>
<td><strong>RR367AA</strong> (28MER)</td>
<td></td>
<td>CA CCT TTA TAT GCA GCA ATT ATA CTT GG</td>
</tr>
<tr>
<td><strong>I I L369AAA</strong> (32MER)</td>
<td></td>
<td>CT TTA TAT AGA AGA GCT GCA CTT GGT TCA GGC</td>
</tr>
<tr>
<td><strong>I369A</strong> (28MER)</td>
<td></td>
<td>CT TTA TAT AGA AGA GCT ATA CTT GGT TC</td>
</tr>
<tr>
<td><strong>I370A</strong> (19MER)</td>
<td></td>
<td>GA AGA ATT GCA CTT GGT TC</td>
</tr>
<tr>
<td><strong>L371A</strong> (23MER)</td>
<td></td>
<td>GA AGA ATT ATA GCT GGT TCA GGC</td>
</tr>
<tr>
<td><strong>I370C</strong> (22MER)</td>
<td></td>
<td>GA AGA ATT TGC CTT GGT TCA GG</td>
</tr>
<tr>
<td><strong>S373A</strong> (22MER)</td>
<td></td>
<td>GA ATT ATA CTT GGT GCA GGC CC</td>
</tr>
</tbody>
</table>

**Table 3.1** List of primer design for loop2 residues in Domain II
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp, Tyr, Phe</td>
<td></td>
</tr>
<tr>
<td><strong>F461C (25MER)</strong></td>
<td>GT GCT GAA TgT AAT AAT ATA ATT CC</td>
</tr>
<tr>
<td><strong>Lys, Arg</strong></td>
<td></td>
</tr>
<tr>
<td><strong>K489C (19MER)</strong></td>
<td>CT GTC GTT tgt GGA CCA GG</td>
</tr>
<tr>
<td><strong>R521C (26MER)</strong></td>
<td>CCA TTA TCA CAA tGa TAT CGG GTA AG</td>
</tr>
<tr>
<td><strong>R523C (24MER)</strong></td>
<td>CAA AGA TAT tGe GTA AGA ATT CGC</td>
</tr>
<tr>
<td><strong>R525C (20MER)</strong></td>
<td>CGG GTA tGe ATT CGC TAC GC</td>
</tr>
<tr>
<td><strong>R527C (21MER)</strong></td>
<td>G GTA AGA ATT tGC TAC GCT TC</td>
</tr>
<tr>
<td><strong>R457C (18MER)</strong></td>
<td>GG CAG CAT tGC AGT GCT G</td>
</tr>
<tr>
<td><strong>R500C (22MER)</strong></td>
<td>GAT ATT CTT tGe AGA ACT TCA C</td>
</tr>
<tr>
<td><strong>R501C (17MER)</strong></td>
<td>CTT CGA tGe ACT TCA CC</td>
</tr>
<tr>
<td><strong>R511C (28MER)</strong></td>
<td>CA ACC TTA tGe GTA AAT ATT ACT GCA CC</td>
</tr>
<tr>
<td><strong>R543C (26MER)</strong></td>
<td>CA ATT GAC GGA tGe CCT ATT AAT CAG GG</td>
</tr>
<tr>
<td><strong>R566C (20MER)</strong></td>
<td>GGA AGC TTT tGe ACT GTA GG</td>
</tr>
<tr>
<td><strong>Asp, Glu</strong></td>
<td></td>
</tr>
<tr>
<td><strong>D541C (19MER)</strong></td>
<td>CA TCA ATT tGC GGA AGA CC</td>
</tr>
<tr>
<td><strong>E460C (29MER)</strong></td>
<td>CGC AGT GCT tgc TTT AAT AAT ATA ATT CC</td>
</tr>
<tr>
<td><strong>E595C (24MER)</strong></td>
<td>CA GGC AAT tGe GTT TAT ATA GAT CG</td>
</tr>
<tr>
<td><strong>E607C (24MER)</strong></td>
<td>GTT CCG GCA tge GTA ACC TTT GAG</td>
</tr>
</tbody>
</table>

Table 3.2 List of primers for Domains II and III surface residues.
<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Q285A</td>
<td>(22mer)</td>
<td>GGA ATG GCT gcG AGA ATA GAA C</td>
</tr>
<tr>
<td>R286A</td>
<td>(24mer)</td>
<td>GGA ATG GCT CAG gcA ATA GAA CAG</td>
</tr>
<tr>
<td>Q289A</td>
<td>(23mer)</td>
<td>G AGA ATA GAA gcG AAT ATT AGG C</td>
</tr>
<tr>
<td>QR285AA</td>
<td>(24mer)</td>
<td>GGA ATG GCT gcG gcA ATA GAA CAG</td>
</tr>
<tr>
<td>QRQAAA</td>
<td>(34mer)</td>
<td>GGA ATG GCT gcG gcA ATA GAA gcG AATATT AGG C</td>
</tr>
<tr>
<td>S556A</td>
<td>(26mer)</td>
<td>CT ATG AGT gcT GGG AGT AAT TTA CAG</td>
</tr>
<tr>
<td>G557A</td>
<td>(22mer)</td>
<td>G AGT AGT GcG AGT AAT TTA CAG</td>
</tr>
<tr>
<td>S558A</td>
<td>(24mer)</td>
<td>GT AGT GGG gcT AAT TTA CAG TCC G</td>
</tr>
<tr>
<td>N559A</td>
<td>(22mer)</td>
<td>GT GGG AGT gcT TTA CAG TCC GG</td>
</tr>
<tr>
<td>SGSN556AAAA</td>
<td>(35mer)</td>
<td>GCA ACT ATG AGT gcT GeG gcT gcT TTA CAG TCC GG</td>
</tr>
</tbody>
</table>

Table 3.3   List of primers for two clusters of Domains II and III.
Toxin structural analysis

To detect changes in secondary structure, CD spectra of wild-type and mutant toxins were measured with an Aviv Circular Dichroism spectrometer-model 62A DS (Lakewood, NJ) in a 32-Q-10 quartz cuvette at 25 °C. The control software was Star Stationary 3.0. Toxins were at 2 μM in the high salt sodium carbonate-bicarbonate buffer. Readings were taken from 200nm to 300nm with 100 sampling numbers and averaged from five replicate measurements.

Toxicity bioassay

*Manduca sexta* eggs (Carolina Biological Supply Company) were hatched at room temperature for 3 to 4 days. Larvae were reared on artificial diet (BioServ, Inc.) to 1st instar. Activity of toxins was determined by the surface contamination method. Briefly, artificial diet was poured into 24-well tissue culture plates (Corning Costar, Corning, NY). Each well had a surface area of 2cm². Toxins were serially diluted in high salt sodium carbonate-bicarbonate buffer, pH 9.5, and 50μl of toxin dilution was applied to one well, with four wells for each concentration. After toxins were absorbed into the diet surface and dried, two larvae were placed in each well. Mortality was recorded after 5 days. LC₅₀ (50% lethal concentration) values was calculated by Probit analysis in SoftTOX 1.1 program (WindowChem, Fairfield, CA).

Slot-blotting analysis of binding affinity
Bio-Dot SF apparatus (Bia-Rad®) was assembled according to manufacture’s instruction. Nylon membrane and three layers of filter paper were pre-wetted in TBS buffer (20 mM Tris, pH 7.5, 500 mM NaCl) prior to placing in. Screws were tightened in a diagonal fashion under vacuum to insure that there would not be any cross-well contamination. The membrane was rehydrated with 100 µl TBS per well to insure uniform binding of the antigen. The chamber was adjusted to open to atmosphere, the appropriate wells was filled with 400 µl of toxins per well in the center, and the valve re-adjusted to allow samples to filter through the membrane by gentle vacuum. Sample wells were washed with 200µl of fresh TBS buffer. Screws were loosened with the vacuum on, so that the membrane would not move when the top sample template was removed. The vacuum was turned off and the membrane transferred to 100 ml of blocking solution (3% dry milk in TBS). The membrane was blocked with gentle agitation on a shaker platform for 30 minutes, and transferred into 50ml of TTBS (TBS buffer with 0.05% Tween 20) containing 100µg/ml of MBP-CAD-D protein. Binding between toxins on the membrane and receptor in solution was at room temperature with shaking for 1 hour. The membrane was then challenged by primary (Rabbit anti-MBP) and secondary (anti-rabbit-AP conjugate) antibodies with thorough washes in TTBS between steps. Alkaline phosphatase substrates were used in the color development reaction.
3.3. RESULTS

*Homology modeling and simulated docking*

The *M. sexta* Bt-R₁ shares extensive tracts of amino acids with a large number of other cadherins, including several that have determined known three-dimensional structures. We have used this information to build a model of a portion of the Bt-R₁ cadherin-like protein. Using 3D-PSSM, and a closely related cadherin with known structure, the mouse E-cadherin, as a template, a model is obtained with a 95% structural confidence. (Structure is shown in red in Figure 3.1). Breaks in the model structure were repaired with Swiss-Pdv Viewer. To predict the interaction of Cry1Aa with the cad 11-12 region of Bt-R₁, indicated by Dorsch *et al.* (31) as the specific binding site of *Bt* toxins, the robust protein-protein docking hydropathy complementarity analysis program, GRAMM was used.

There are several methods available for the study of protein docking. Because molecular recognition consists of geometrical and chemical aspects, the computational algorithms for molecular recognition can be separated into geometry-based docking procedures that attempt to find the best steric fit between two molecules (GRAMM, FTDOCK), and approaches based on the minimization of the energy of interaction (AUTODOCK). The former perform exhaustive six-dimensional search of all possible conformations, the latter carry out statistical sampling of the conformational space. Some programs (DOT) attempt to combine both approaches. All these algorithms have good predicting ability, as a root mean standard distance of the obtained complexes was within 2.5Å from known crystal structures (102).
To predict the docking structure of a complex, GRAMM requires only the atomic coordinates of the two molecules (no information about the binding sites is needed). The program performs an exhaustive six-dimensional search through the relative translations and rotations of the molecules (72). It estimates surface complementarity between two proteins treated as rigid bodies. The atomic coordinates of the two proteins obtained from PDB files are projected onto a three-dimensional grid, yielding a digital representation. Small positive numbers are assigned to the surface of the bigger molecule (receptor) and large negative numbers are assigned to its interior to penalize for penetration in the core of the protein. Next, the smaller molecule (ligand) is translated and rotated around the receptor searching through all the conformational space in six dimensions. At each rotational step the correlation function using Fourier transformation is calculated. The correlation function evaluates the degree of the geometric match between two molecules. Thus, the best geometric fit yields the highest score, and low scores represent the poor matches, as a result of penetration in the interior.

Based on the fact that protein-protein interfaces are more hydrophobic than the rest of the protein surface (71), the simplified approach, called hydrophobic docking, was proposed by Vakser and Aflalo (134). GRAMM method considers both geometrical fit and hydrophobicity. Geometric algorithms used in GRAMM, which deal mostly with van der Waals interactions, were more effective in prediction than those concerned with energy considerations. This can probably be explained by the hydrophobic nature of the complex of interest.

Among the 10 strongest predicted binding models, all predict binding to loops of domain II and one predicts tight binding (-29 kcal/mole) to both the loops of domain II
and domain III (Figure 3.1). The Cry1Aa residues indicated in the topological approach correspond to the predicted contacts in this docking model, with the exception that Cry1Aa alternate domain III residues, 591-593, are not contacted by this docking model. The interactions between the two molecules confirmed the mutagenesis design based on topological contact.

Construction and purification of mutants

Cry1Aa has the advantage of a known 3D structure and models may be made to predict the location of potential binding epitopes. 65kDa toxin proteins were prepared and purified according to protocol described in the Materials and Methods section. The list of all mutants constructed and expressed is shown in Table 3.4.

Surface charged / bulky mutations, E460C, F461C, K489C, R527C, D541C, R543C and E595C gave rise to normal yield of crystal protein, protoxin and active toxin (SDS-PAGE of these mutants shown in Figure 3.3 A). Several others turned out to have significant structural alterations, as evidenced by their low level of expression, insolubility from inclusion bodies or unusual sensitivity to trypsin. R457C, R566C, and E607C had normal level of expression but low level of solubilization. Arg to Cys mutants at other positions, R500C, R501C, R511C, R521C, R523C and R525C, resulted in very unstable proteins that were susceptible to trypsin degradation.

Not surprisingly, mutations in loop 2 of domain II (Figure 3.3 B) had much better production of proteins, because the flexible loop region can accommodate changes without causing major disruption to the overall structure. Most point mutations, with the exception of an Ala block substitution (A3) and a half-loop deletion (D3) resulted in
normal production of toxins. The only two problematic substitutions are G374A and P375A, resulting in poor expression. These two residues locate on the very end of loop 2, presumably important in maintaining the overall integrity of the loop 2 and the next β sheet.

Among the Q285/R286/Q289 cluster, the Q285A mutant resulted in loss of expression. R286A, Q289, QR285/286AA and QRQ mutants produced active toxin. In the S556/G557/S558/N559 cluster, alanine substitutions of the first three residues expressed stable proteins, while N559A and the SGSN to AAAA mutation didn’t express soluble protoxin (SDS-PAGE in Figure 3.3 C). V444A, located between loop 2 and QRQ cluster gave rise to insoluble protoxin as well.
Figure 3.1   Docking Model of Cry1Aa and CAD-D by GRAMM.

Cry1Aa (in blue) is oriented with domain II at the bottom, while domain III is at the upper right corner. CAD-D (in red) has it CR11 interacting with domain II loops and CR12 in contact with domain III loops.
<table>
<thead>
<tr>
<th>Domain II</th>
<th>Domain III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q285A*</td>
<td>E460C</td>
</tr>
<tr>
<td>R286A</td>
<td>F461C</td>
</tr>
<tr>
<td>285QR286 to AA</td>
<td>K489C</td>
</tr>
<tr>
<td>Q289A</td>
<td>R500C***</td>
</tr>
<tr>
<td>Q285R286Q289 to AAA</td>
<td>R501C***</td>
</tr>
<tr>
<td>D 3</td>
<td>R511C***</td>
</tr>
<tr>
<td>A 3</td>
<td>R521C***</td>
</tr>
<tr>
<td>RR367/368AA</td>
<td>R523C***</td>
</tr>
<tr>
<td>I I L369/370/371 AAA</td>
<td>R525C***</td>
</tr>
<tr>
<td>I 369A</td>
<td>R527C</td>
</tr>
<tr>
<td>I 370A</td>
<td>D541C</td>
</tr>
<tr>
<td>L 371A</td>
<td>R543C</td>
</tr>
<tr>
<td>G372A</td>
<td>S556A</td>
</tr>
<tr>
<td>S373A</td>
<td>G557A</td>
</tr>
<tr>
<td>G374A*</td>
<td>S558A</td>
</tr>
<tr>
<td>P375A*</td>
<td>N559A**</td>
</tr>
<tr>
<td>V444A**</td>
<td>SGSNAAAAA**</td>
</tr>
<tr>
<td>R457C**</td>
<td>R566C**</td>
</tr>
<tr>
<td></td>
<td>E595C</td>
</tr>
<tr>
<td></td>
<td>E607C**</td>
</tr>
</tbody>
</table>

**Table 3.4** List of all mutants constructed and expressed.

* *. Low level of expression.

** *. Low level of solubilization of protoxin from inclusion body.

*** *. Unusual sensitivity to trypsin.
Figure 3.2  Molecular visualization of mutation positions.

A and B. Surface residues in domain III. 180° mirror images.
C. Loop 2 residues in domain II. The orientation of 3D structure is the same as in A.
Figure 3.3  SDS-PAGE of wt, loop2, and charged mutant toxins.

A. Lanes: 1) wt, 2) D3, 3) IIL, 4) RR, 5) I369A, 6) I370A, 7) L371A, 8) G372A, 9) S373A


C. Lanes: 1) IIL, 2) MW marker (Invitrogen Benchmark), 3) R286A, 4) Q289A, 5) QR, 6) QRQ, 7) S556A, 8) G557A, 9) S558A, 10) MW marker (Roche High Range)
**Structural Analysis**

Mutant toxins were subjected to CD analysis to ensure the integrity of their overall secondary structure. The far UV regions revealed some changes in secondary structure caused by mutations. The near UV region reflecting the micro-environment of aromatic residues showed no obvious disturbance. CD spectra of all available mutations were shown in Figure 3.4 and Figure 3.5. All surface residue substitutions are similar to wild type. Loop2 deletion showed higher extent of distortion, agreeing with loss of most part of a flexible loop. In the Q285/R286/Q289 cluster, even the triple mutation did not cause significant change in the secondary structure. Individual substitutions in S556/G557/S558/N559 cluster resulted in minor “dips” in CD spectra, hinting a more “compacted” secondary structure overall. CD spectrum of R501C, a trypsin-sensitive mutant, was included to show the obvious disturbance in its secondary structure.

**Toxicity Bioassay**

Active toxins were tested for their toxicity against 1\textsuperscript{st} instar *M. sexta* larvae (Figure 3.6). LC\textsubscript{50} (lethal concentration of 50% mortality) in ng/cm\textsuperscript{2} is listed in Table 3.5. As expected, deletion or alanine substitution of the first half of loop 2 in domain II caused a loss of toxicity. Individual alanine substitutions did not cause great effect in toxicity with the exception of I370A, which had a 5 fold increase in toxicity. Among the surface charged / bulky residues, toxicity fluctuated to a minor extent, with no prominent effect. In the Q285/R286/Q289 cluster, double mutation QR285/286AA lost 2.5 fold of toxicity. G557A among individual substitutions in S556/G557/S558/N559 cluster led to 2.8 fold of loss in toxicity.
Figure 3.4   CD spectra of loop2 and charged mutant toxins.

Toxins were diluted to 2μM in sodium carbonate buffer at pH 9.5. CD signals were collected from 200 to 300 nm and were base-line corrected.
Figure 3.5  CD spectra of mutant toxins.

Toxins were diluted to 2μM in sodium carbonate buffer at pH 9.5. Spectra were acquired from 200 to 300 nm and were base-line corrected.
Figure 3.6  Bioassay of mutant toxins against 1st instar *Manduca sexta* larvae
<table>
<thead>
<tr>
<th>Toxin</th>
<th>LC(ng/cm²)</th>
<th>confidence limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3.37</td>
<td>(1.92-7.67)</td>
</tr>
<tr>
<td><strong>Domain II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R286A</td>
<td>2.09</td>
<td>(0.20-3.55)</td>
</tr>
<tr>
<td>Q289A</td>
<td>1.56</td>
<td>(1.40-1.76)</td>
</tr>
<tr>
<td>QR285/286AA</td>
<td>8.73</td>
<td>(4.58-17.69)</td>
</tr>
<tr>
<td>QRQ</td>
<td>2.34</td>
<td>(0.41-3.91)</td>
</tr>
<tr>
<td><strong>Loop 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>12.95</td>
<td>(7.89-26.07)</td>
</tr>
<tr>
<td>D3</td>
<td>10.67</td>
<td>(6.39-43.30)</td>
</tr>
<tr>
<td>RR</td>
<td>2.67</td>
<td>(1.16-3.95)</td>
</tr>
<tr>
<td>IIL</td>
<td>3.68</td>
<td>(0.01-8.44)</td>
</tr>
<tr>
<td>I369A</td>
<td>1.5</td>
<td>(1.37-1.66)</td>
</tr>
<tr>
<td>I370A</td>
<td>0.66</td>
<td>(0.00-1.39)</td>
</tr>
<tr>
<td>L371A</td>
<td>1.5</td>
<td>(1.37-1.66)</td>
</tr>
<tr>
<td>G372A</td>
<td>3.08</td>
<td>(2.00-4.62)</td>
</tr>
<tr>
<td>S373A</td>
<td>1.55</td>
<td>(1.40-1.73)</td>
</tr>
<tr>
<td><strong>Domain III</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E460C</td>
<td>1.54</td>
<td>(0.65-2.39)</td>
</tr>
<tr>
<td>F461C</td>
<td>2.79</td>
<td>(1.67-4.00)</td>
</tr>
<tr>
<td>K489C</td>
<td>1.51</td>
<td>(0-3.10)</td>
</tr>
<tr>
<td>R527C</td>
<td>1.55</td>
<td>(1.40-1.73)</td>
</tr>
<tr>
<td>D541C</td>
<td>3.22</td>
<td>(1.63-4.86)</td>
</tr>
<tr>
<td>R543C</td>
<td>2.31</td>
<td>(0.89-3.30)</td>
</tr>
<tr>
<td>S556A</td>
<td>2.80</td>
<td>(0.90-5.56)</td>
</tr>
<tr>
<td>G557A</td>
<td>9.58</td>
<td>(5.78-29.38)</td>
</tr>
<tr>
<td>S558A</td>
<td>2.64</td>
<td>(1.73-3.90)</td>
</tr>
</tbody>
</table>

**Table 3.5**  Bioassay of *Manduca sexta* larvae with mutant toxins.

Surface Contamination on 1st Instar *Manduca sexta* larvae. Major numbers are LC<sub>50</sub> in ng/cm<sup>2</sup> calculated by SOFTTOX software. Numbers in brackets are lower and upper 95% confidence limit.
Slot-blot and Surface Plasmon Resonance analysis of binding affinity

Wild type and mutant toxins were blotted on Nylon membrane, and bound to MBP-CAD-D in solution. Binding of toxin and MBP-CAD-D was detected by the presence of MBP components on membrane. Results from slot blot (shown in Figure 3.7 and Figure 3.8) qualitatively demonstrated the effect of Ala substitutions on binding affinity. Additionally the slot blot results also guided the design of proper concentration series for SPR experiments.

In the Surface Plasmon Resonance analysis of binding affinity, a capturing molecule, the monoclonal antibody against MBP (anti-MBP IgG), was amine-coupled onto the chip using well-established chemistry. The ligand, MBP-CAD-D fusion protein, was then affinity-captured. Interaction analysis was performed as toxin analytes in solution were injected in a concentration series, including a buffer only sample served as the blank. Regeneration conditions were chosen to achieve complete dissociation of both the analyte (toxin molecules) and the ligand (MBP-CAD-D fusion protein) without affecting the binding characteristic of the capturing molecule (anti-MBP IgG).

Kinetic analysis of sensorgrams

Raw data from BIACore was processed before evaluation. This included setting the baseline to zero, cropping the data so that the staggered start times could be analyzed globally, referencing responses across flow cells by subtracting data generated across an unmodified flow cell (Fc1) and double referencing within flow cells by subtracting an average buffer (blank) response from data collected within the same flow cell (Fc2). A short period after injection start and stop was excluded from the fitting to avoid sample
dispersion effects. The binding responses were analyzed globally by fitting data to “1:1 binding with drift baseline” interaction model using BIAevaluation 4.1 software. The association rate constant (ka) and dissociation rate constant (kd) were calculated for each data set and the equilibrium dissociation constant (K_D) was derived from their ratio, kd/ka (data shown in Table 3.6).

Binding affinity of alanine or cysteine substitutions was compared to that of wild type Cry1Aa and CAD-D at K_D of 10.6nM. Most alanine substitutions in loop 2 of domain 2, including RR367/368AA, I370A, L371A, G372A and S373A, affected binding negatively at various levels. Two exceptions are I369A and IIL369/370/371 to triple alanines. I369A, a mutation located near the bottom of domain II loop 2 on the opposite surface from the rest of the loop, showed increases in both binding and toxicity, which resembles the observation of a homologous residue (I375A) in Cry1Ac (67). Affinity of I369/I370/L371 to AAA triple substitution is comparable to that of wild type toxin, roughly the sum of effect on binding from the three individual mutations.

Among substitutions of charged and bulky residues on domain III surface, K489C and R527C disrupt binding affinity, while E460C, F461C, D541C and R543C result in tighter binding. Among substitutions of two clusters of residues on the same face of the main part of loop2, S556/G557/G558 cluster shows clear loss in affinity. However, effects of binding from the Q285/R286/Q289 cluster yield rather conflicting results when mutated one by one or in groups. Q285A does not express normally, R286A has enhanced affinity, and Q289A maintains similar affinity like wild type. QR285/286AA results in lower affinity, but Q285/R286/Q289 to triple alanine substitution does not disturb affinity. Although all the well-expressed mutants are structurally stable, the role
of Q285 in maintaining the right structure may be critical in determining the affinity of this region.

Taken together, an interaction surface between Cry1Aa and CAD-D fit the hypothesis. Starting from the bottom of the domain II loop2 region, the receptor touches Q285A/R286A/Q289A cluster, and reaches the G556/S557/N558 cluster in domain III. The fact that K489A in the concave portion of the structure reduces binding 10 fold indicates that the receptor is not a flat surface, but is either convex, matching the contour of the toxin or has the flexibility to bend and interact with the toxin surface. Two residues in the receptor binding epitope, I369 and R286, when mutated to alanine, display tighter binding to CAD-D. The increased affinity from alanine scanning mutants is not unprecedented. Alanine substitutions in alpha helix 8 and loop3 (113) has shown by Rajamohan et al. to increase the initial binding for 30 fold.

Charged or bulky residues that do not disturb binding affinity are all located outside the area defined above. E460C and F461C are on the opposite side of the toxin, while D541C and R543C are about 90 degree away. None of the substitutions on the distal side affect binding negatively, further confirming the orientation of the proposed interacting surface. Interestingly, Phe 461, a bulky hydrophobic residue located on the opposite side of the interacting surface, when substituted by a cysteine, had higher affinity to the CAD-D receptor. In the case of cytochrome c, the asymmetric distribution of charges on the surface leads to a dipole moment and orients the molecule with respect to its binding partner in a specific fashion (78). It is possible that the substitution of Phe to Ala on the surface of the flip side of Cry1Aa translates into changes in hydrophobicity
of the other side, which is more directly involved in receptor-binding, and causes an increase in affinity.

As previously shown by Jenkins (67), in the hotspots for Cry1Ac-APN binding, Cry1Aa-CAD-D binding epitopes are located in two separate domains. For Cry1Ac the binding-epitopes include the depression (GalNAc binding-pocket) in domain III, and loops (3) of domain II. Cry1Aa lacks the GalNAc binding-pocket in domain III and is a much weaker binding partner to APN. Instead, Cry1Aa interacts with BT-R₁ receptor at a much higher affinity. The CAD-D interaction surface in Cry1Aa domain III characterized in this study is in line with the involved loop regions of domain II, but away from the homologous region for APN interaction. The distribution of several clusters of hotspots may be one of the reasons why Cry1A toxins bind to BT-R₁ much tighter than to APN, which involves two separate regions on the opposite sides of the toxin (69).
Figure 3.7  Slot-blot of toxin and receptor binding.

Each Toxin was tested with three concentrations (25, 50 and 100μg)).

Positive binding is in bold.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>WT+</td>
<td>WT+</td>
<td>WT+</td>
<td>BLANK</td>
<td>BLANK</td>
<td>BLANK</td>
</tr>
<tr>
<td>2</td>
<td>IIL+</td>
<td>IIL+</td>
<td>IIL+</td>
<td>I369A+</td>
<td>I369A+</td>
<td>I369A+</td>
</tr>
<tr>
<td>3</td>
<td>I370A</td>
<td>I370A</td>
<td>I370A</td>
<td>L371A</td>
<td>L371A</td>
<td>L371A</td>
</tr>
<tr>
<td>4</td>
<td>S373A</td>
<td>S373A</td>
<td>S373A</td>
<td>K489C</td>
<td>K489C</td>
<td>K489C</td>
</tr>
<tr>
<td>5</td>
<td>E460C+</td>
<td>E460C+</td>
<td>E460C+</td>
<td>F461C+</td>
<td>F461C+</td>
<td>F461C+</td>
</tr>
<tr>
<td>6</td>
<td>D541C+</td>
<td>D541C+</td>
<td>D541C+</td>
<td>E595C+</td>
<td>E595C+</td>
<td>E595C+</td>
</tr>
<tr>
<td>7</td>
<td>CAD-D+</td>
<td>CAD-D+</td>
<td>CAD-G+</td>
<td>CAD-G+</td>
<td>BSA</td>
<td>BSA</td>
</tr>
</tbody>
</table>
Figure 3.8  Slot-blot of toxin and receptor binding.

Each Toxin was tested with two concentrations (25μg and 50μg).

Positive binding is in bold.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th></th>
<th>B</th>
<th></th>
<th>C</th>
<th></th>
<th>D</th>
<th></th>
<th>E</th>
<th></th>
<th>F</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><strong>R286A</strong>+</td>
<td>R286A+</td>
<td><strong>Q289A</strong>+</td>
<td>Q289A+</td>
<td><strong>QR</strong>+</td>
<td>QR+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><strong>QRQ</strong>+</td>
<td>QRQ+</td>
<td><strong>I369A</strong>+</td>
<td>I369A+</td>
<td>I370A</td>
<td>I370A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>L371A</td>
<td>L371A</td>
<td>S373A</td>
<td>S373A</td>
<td>RR</td>
<td>RR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td><strong>IIL</strong>+</td>
<td>IIL+</td>
<td>D3</td>
<td>D3</td>
<td>A3</td>
<td>A3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td><strong>L123</strong>+</td>
<td>L123+</td>
<td>G372A</td>
<td>G372A</td>
<td>R511C</td>
<td>R511C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>R527C</td>
<td>R527C</td>
<td><strong>D541C</strong>+</td>
<td>D541C+</td>
<td><strong>R543C</strong>+</td>
<td>R543C+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>S556A</td>
<td>S556A</td>
<td>G557A</td>
<td>G557A</td>
<td>S558A</td>
<td>S558A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>mark</td>
<td>wt</td>
<td>BSA</td>
<td>mark</td>
<td>CAD-D</td>
<td>CAD-G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 3.6 Binding properties of mutant toxins to receptor.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>( k_a \text{ (M}^{-1}\text{S}^{-1}) )</th>
<th>( k_d \text{ (S}^{-1})</th>
<th>( K_D \text{ (M)} )</th>
<th>Slot Blot</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.01E+05</td>
<td>1.08E-03</td>
<td>1.06E-08</td>
<td>+</td>
</tr>
<tr>
<td><strong>Domain II</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R286A</td>
<td>2.45E+04</td>
<td>6.63E-05</td>
<td>2.71E-09</td>
<td>+</td>
</tr>
<tr>
<td>Q289A</td>
<td>9.85E+04</td>
<td>1.07E-03</td>
<td>1.09E-08</td>
<td>+</td>
</tr>
<tr>
<td>QR</td>
<td>1.19E+05</td>
<td>2.52E-03</td>
<td>2.12E-08</td>
<td>-</td>
</tr>
<tr>
<td>QRQ</td>
<td>1.82E+04</td>
<td>1.98E-04</td>
<td>1.09E-08</td>
<td>+</td>
</tr>
<tr>
<td><strong>Loop 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>1.47E+05</td>
<td>3.33E-03</td>
<td>2.26E-08</td>
<td>-</td>
</tr>
<tr>
<td>D3</td>
<td>2.66E+04</td>
<td>2.77E-03</td>
<td>1.04E-07</td>
<td>-</td>
</tr>
<tr>
<td>RR</td>
<td>5.78E+04</td>
<td>1.24E-03</td>
<td>2.14E-08</td>
<td>-</td>
</tr>
<tr>
<td>IIL</td>
<td>1.90E+04</td>
<td>2.32E-04</td>
<td>1.22E-08</td>
<td>+</td>
</tr>
<tr>
<td>I369A</td>
<td>1.84E+04</td>
<td>1.62E-04</td>
<td>8.83E-09</td>
<td>+</td>
</tr>
<tr>
<td>I370A</td>
<td>2.64E+04</td>
<td>2.02E-03</td>
<td>7.63E-08</td>
<td>-</td>
</tr>
<tr>
<td>L371A</td>
<td>1.87E+04</td>
<td>1.16E-03</td>
<td>6.18E-08</td>
<td>-</td>
</tr>
<tr>
<td>G372A</td>
<td>6.71E+04</td>
<td>1.13E-03</td>
<td>1.68E-08</td>
<td>-</td>
</tr>
<tr>
<td>S373A</td>
<td>1.50E+04</td>
<td>2.36E-03</td>
<td>1.58E-07</td>
<td>-</td>
</tr>
</tbody>
</table>

**Domain III**

| E460C  | 4.94E+04                               | 3.69E-04       | 7.46E-09         | +         |
| F461C  | 2.06E+04                               | 3.36E-05       | 1.63E-09         | +         |
| K489C  | 1.38E+04                               | 2.54E-03       | 1.85E-07         | -         |
| R527C  | 2.26E+04                               | 2.37E-03       | 1.05E-07         | -         |
| D541C  | 1.37E+05                               | 1.05E-03       | 7.62E-09         | +         |
| R543C  | 5.50E+04                               | 2.80E-04       | 5.09E-09         | +         |

| S556A  | 1.25E+05                               | 1.62E-03       | 1.29E-08         | -         |
| G557A  | 2.45E+04                               | 8.78E-04       | 3.58E-08         | -         |
| S558A  | 3.88E+04                               | 1.85E-03       | 4.77E-08         | -         |
3.4. DISCUSSION

Using real-time SPR measurement of toxin-receptor binding, an interaction surface between Cry1Aa and CAD-D is established as starting from the bottom of the domain II, loop 2, touching Q285A/R286A/Q289A cluster, and reaching the domain III G556/S557/N558 cluster. Relative changes in binding rates (ka and kd), overall affinity (K_D), and toxicity of mutant toxins are combined in Table 3.7.

Clearly, functionality of any given receptor requires that Cry toxins utilized the receptor for binding en route to membrane insertion and pore formation. At face value it would seem receptor affinity and receptor concentration may serve as accurate predictors of toxin activity. In fact, it is not necessarily true that loss of binding to a functional receptor in vivo will significantly reduce toxicity, as shown in Figure 3.9, binding affinity and toxicity do not correlate directly in all cases. Three very interesting mutants, I370A L371A, and S373A in loop 2, show more 5 to 14 fold decreases in binding affinity, but more than 2 fold increases in toxicity. Similarly, K489C, located in the concave region in the middle of the interaction surface, also show the inverse correlation between affinity and toxicity.

It should be noted that the binding affinity measured in this study is the initial binding of toxin and cadherin-like receptor. Multiple receptor specificity in vivo works in concert to elicit toxicity. The possible oligomerization step, the proposed secondary receptor-hopping step, and irreversible membrane insertion step are not represented by K_D value, but are equally critical contributing factors to the total toxicity. There are prior precedents of miscorrelations between insect pathogenicity and binding affinity. For instance, Wolfersberger et al. found that the toxicity of two Bacillus thuringiensis δ-
endotoxins (HD1-9 and HD-7) to gypsy moth larvae is inversely related to their affinity of binding sites on midgut brush border membranes (141). Similarly, Garczynski et al. (43) when studying the binding of $^{125}$I-Cry1Ac to BBMVs from four insects which differed greatly in their sensitivity to the Cry1Ac toxin, found high-affinity, saturable binding sites in all four of the insects tested, including the highly resistant *S. frugiperda* larvae. In similar tests with strains of three other moths resistant to the three Cry1A toxins, binding of Cry1Ac was not reduced (61, 82, 98). Binding of Cry1Ab but not Cry1Ac was reduced in the PHI strain of *Plutella xylostella* (129) and the Dplr strain of *Plodia interpunctella* (51), whereas binding of Cry1Aa but not Cry1Ab or Cry1Ac was reduced in the YHD2 strain of *H. virescens* (82). Masson et al. (98) compared the kinetics of toxin binding to BBMVs from susceptible and resistant *Plutella xylostella* larvae. The binding kinetics for Cry1Ac did not differ significantly between susceptible and resistant larvae, and it was concluded that factors other than binding were altered in the resistant insect (128).

In a study comparing binding property and toxicity of several Cry toxins to a group of insects at various stages, binding of Cry1Ac to *M. sexta* APN in neonates was lower than that in third instar larvae, which inversely correlates with toxicity, while Cry1Ca and *M. brassicae*, which exhibited a significant increase in binding site concentration during larval development despite the resistance seen in the third-instar larvae (46). Ballester et al. (7) demonstrated that the loss of a low-affinity binding site for Cry1Ab in *P. xylostella* resulted in resistance in this organism even though a high-affinity binding site was still available. Liu et al. (88) showed there were no significant differences in Cry1C binding to resistant and susceptible strains of *P. xylostella*. Van Rie
et al. (137) observed that Cry1E had two binding sites in BBMV prepared from *M. sexta* and *Spodoptera littoralis*. Although these two insects had similar susceptibilities to Cry1E and similar affinities for the low-affinity binding sites (correlating with toxicity), the high-affinity binding site for *M. sexta* had a $K_{\text{com}}$ at least 10 times lower than the $K_{\text{com}}$ for *S. littoralis* and was therefore not positively correlated with potency.

The conflicting results could be due in part to interpretation of the binding data. Most bioassays have been carried out with neonate larvae, whereas binding assays are usually done with midguts dissected from fifth-instar larvae. However, as clearly shown in a study by Gilliland *et al.* (46), using binding data for a larval stage different from the stage used for bioassays could be problematic and some cases lead to misinterpretation of data.

The irreversible associations, as well as the reversible associations, of the toxins with BBMV; further complicates interpretation of binding data. Perhaps the more subtle differences in potency may go undetected unless these two components are measured separately. Liang *et al.* (87) showed the importance of obtaining such measurements by demonstrating that irreversible binding was directly correlated with toxicity but not with $B_{\text{max}}$ and $K_D$. Wu *et al.* (145) found that there was a direct correlation between potency and irreversible binding of the loop III block mutant but no correlation with reversible binding. Thus, even distinguishing between irreversible binding and reversible binding can make data difficult to interpret, as concluded by Luo *et al.* (92), who found that Cry1Ac, Cry1Ca, and Cry1Bb bound irreversibly to *S. exigua* BBMV, even though the toxicity of the molecules were different.
Binding characteristics also did not correlate positively with potency in every case in various studies of mutations of binding epitopes. Cry1Ac Q509, R511, Y513 alanine substitution greatly affected binding to *L. dispar* BBMV with only minor reductions in toxicity (83). The difficulties in correlating APN activity and binding parameters with potency underscore the complexity of the mode of action of these toxins, and further study of the toxins is warranted (46). The fact that loss in binding did not correlate with loss in toxicity implies that other steps such as partitioning of the toxin into the membrane, plays equally important role in toxicity (70).

From previous studies on Cry1Ab, there are two possibilities for the role of loop2 of domain II: Loop 2 may irreversibly bind to receptor(s) or it may insert into the midgut in an irreversible manner. Mutations in loop 2 did not affect the initial binding property of Cry1Ab toxin to *M. sexta* BBMV (112). Instead, toxicity directly correlated with irreversible binding to BBMV. Investigation of these Cry1Ab loop 2 mutants on *H. virescens* showed decrease in the initial binding (112), indicating that residues in this loop play quite different roles on different insects.

In the present study, residues in Cry1Aa loop 2 have been demonstrated to be involved in the initial binding, but only affect the overall toxicity to *M. sexta* when deleted or substituted in a group. With other residues in the same interaction surface, a line of surface clusters are characterized to play significant roles in toxin-receptor recognition. Their contribution in other aspects that leads to mortality, such as irreversible partitioning into membrane, needs to be studied further.
Table 3.7  Properties of Cry1Aa mutant toxins binding and toxicity.

Shown are relative changes in binding rates (ka and kd), overall affinity (K_D), and toxicity. For K_D, bold font represents loss in binding, while gray font represents increase in binding.
Figure 3.9  Distribution of mutants affecting binding.

Red:  Negative effect on binding and toxicity. (-/-)
Orange:  Negative effect on binding, with no effect on toxicity. (-/=)
Yellow:  Negative effect on binding, but increase on toxicity. (-/+)

A3(-/-), D3(-/-), G372A(-/-), S373A(-/-), K489C(-/-), S556A(-/-), G557A(-/-), S558A(-/-), QR285/286AA(-/-), I370A(-/-), L371A(-/-)
CHAPTER 4

RATIONAL DESIGN AND PROTEIN ENGINEERING TO CREATE MOSQUITOCIDAL ACTIVITY IN CRY1Aa TOxin

4.1. INTRODUCTION

Due to the enormous selective pressure imposed by widespread use of Bacillus thuringiensis Cry proteins in agriculture worldwide, the development of better Cry toxins is of ever increasing importance. The ultimate goal of protein engineering of the insecticidal crystal proteins from Bacillus thuringiensis is to be able to design any Cry toxin to possess toxic activity against any insect. A more immediate goal is to introduce a specific activity into a toxin that does not possess it.

Several examples of protein engineering of Bacillus thuringiensis Cry toxins have demonstrated enhancements of activity in toxins that already expressed some level of activity. In vivo domain substitutions of Cry1Ab resulted in a 4-fold enhancement of activity against Spodoptera (23). Site-directed mutations of individual residues in domain II loop regions of Cry3Aa led to a 10-fold increase of activity against Tenebrio molitor (146) and mutations in domain II loop regions of Cry1Ab resulted in a 34-fold increase in activity against the gypsy moth, Lymantria dispar (111). More extensive deletions and substitutions of domain II loop regions of a mosquitocidal toxin, Cry4Ba, that has activity...
against *Anopheles* and *Aedes*, mosquitoes but no measurable activity against *Culex* mosquitoes, resulted in robust activity against *Culex* species (1).

To date no manipulation of Cry proteins has completely changed the specificity of a toxin to a different order of insect. This project was a test of the ability of rational design, based on current knowledge of receptor binding epitopes, to synthesize a completely new activity into a Cry protein.

Previous successful protein engineering on Cry toxins, especially on the loop regions of domain II of Cry4Ba (1) to enhance *Culex* toxicity, suggest that a rational design to improvement of these toxins by protein engineering has great potential.

In the present study, homology alignment and sequence comparison served as the basis for protein engineering design. Here we show that by rational design, a mutant of Cry1Aa, after several rounds of deletions and substitutions made in loops of domain II, displays enhanced toxicity against *C. pipiens*. These results further suggest that the introduction of short variable sequences of the loop regions from one Cry toxin into another might provide a general rational design approach to enhancing toxicity of *B. thuringiensis* Cry toxins.

4.2. MATERIAL AND METHODS

*Sequence and Structural alignments*

Three programs were used to model the structure of Cry4Ba:

i) a free online software Clustal W (http://www.ebi.ac.uk/clustalw/);

ii) SWISS-MODEL (http://www.expasy.org/swissmod/SWISS-MODEL.html);

iii) Swiss-Pdb Viewer Version 3.7b2 (56, 108)
Sequence alignment of Cry1Aa and Cry4Ba was done by Clustal W, a general purpose multiple sequence alignment program for DNA or proteins. It produces biologically meaningful multiple sequence alignments of divergent sequences. It calculates the best match for the selected sequences, and aligns them to reveal the identities, similarities and differences.

Model structure of Cry4Ba was constructed using the “Optimize (project) mode” in SWISS-MODEL, in conjunction with Swiss-Pdb Viewer. The sequence of the target protein (Cry4Ba) was aligned with the template sequences (Cry4Aa modeled based on Cry1Aa and 3Aa) in Swiss-Pdb Viewer according to the alignment produced by CLUSTAL W (Figure 2). Unaligned residues at the N and C terminal of the target protein were removed prior to submitting the project to the SWISS-MODEL program.

Construction of mutants

cry1Aa gene cloned in pBluescript vector was expressed under the control of the lac^uv5 promoter in DH5α E. coli cells for DNA isolation and protein expression. Several rounds of site-directed mutagenesis (primer sequences are listed in Table 1.) were performed using the modified QuickChange (Stratagene) method. DNA templates were purified using Qiagen Miniprep Kit. Purified templates (3 μg) were methylated using 8 U of dam methylase (New England Biolabs) for 15 min at 37°C. The reactions were quenched by plunging the tubes on ice. For polymerase chain reaction (PCR), 100 ng of methylated DNA was mixed with 15 pmol of forward and reverse mutagenic primer, 300 μM (final concentration) of each deoxynucleotide triphosphate (dNTP mix, Roche), 0.5
U of Expand Long Template Polymerase (Roche), 1X Buffer I (Roche) in a total volume of 25 μl. The sequences of the primers are listed in Table 4.1.

The programmed steps for the PCR reaction were as follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Reaction</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Initial Denaturation</td>
<td>94°C</td>
<td>2 min</td>
</tr>
<tr>
<td>2.</td>
<td>Denaturation</td>
<td>94°C</td>
<td>10 s</td>
</tr>
<tr>
<td>3.</td>
<td>Annealing</td>
<td>48°C</td>
<td>30 s</td>
</tr>
<tr>
<td>4.</td>
<td>Elongation</td>
<td>68°C</td>
<td>4 min</td>
</tr>
<tr>
<td>5.</td>
<td>Repeat steps 2-4</td>
<td>94°C</td>
<td>15 s</td>
</tr>
<tr>
<td>6.</td>
<td>Denaturation</td>
<td>94°C</td>
<td>30 s</td>
</tr>
<tr>
<td>7.</td>
<td>Annealing</td>
<td>68°C</td>
<td>4 min + 20 s every successive cycle</td>
</tr>
<tr>
<td>8.</td>
<td>Elongation</td>
<td>68°C</td>
<td>4 min + 20 s</td>
</tr>
<tr>
<td>9.</td>
<td>Repeat steps 6-8</td>
<td>68°C</td>
<td>4 min + 20 s</td>
</tr>
<tr>
<td>10.</td>
<td>Final elongation</td>
<td>68°C</td>
<td>7 min</td>
</tr>
<tr>
<td>11.</td>
<td>Cooling</td>
<td>4°C</td>
<td>on hold</td>
</tr>
<tr>
<td>12.</td>
<td>End</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

After the PCR was completed (on a MiniCycler, MJ Research Inc.), the reaction product was digested with DpnI (Roche) to remove the methylated wt template DNA.

The digested PCR product was transformed into E. coli DH5α competent cells. Plasmid DNA from different clones was extracted using Qiagen Miniprep Kit. Mutations were confirmed by automated DNA sequencing (Plant-Microbe Genomics Facility, The Ohio State University).
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→ 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fw1Aa4BL1</td>
<td>TAT ACT GAT GTG CAT TAC CAG GAT ATC TTT AAT TAT TGG TCA GGG</td>
</tr>
<tr>
<td>Re1Aa4BL1</td>
<td>ACC AAT AAT TAA AGA TAT CCT GGT AAT GCA CAT CAG TAT AAA TGG</td>
</tr>
<tr>
<td>D3</td>
<td>CA TTA TCT TCA CCT GGT TCA GGC CC</td>
</tr>
<tr>
<td>Fw1AaD2</td>
<td>GGT TCA GGC CCA GGA CTG TTT GTC CC</td>
</tr>
<tr>
<td>Re1AaD2</td>
<td>GGG ACA AAC AGT CCT GGG CCT GAA CC</td>
</tr>
</tbody>
</table>

Table 4.1 Sequences of primers used in site-directed mutagenesis.
Bt toxin purification

The method used was essentially as described in previous chapters. Toxins were expressed in *E. coli* cells. Crystal inclusion protein was obtained. For mosquito toxicity analysis, 100 μl of inclusion body resuspended in Crystal Wash II was spun down, and solubilized in 100μl of high salt sodium carbonate buffer (30mM Na₂CO₃, 20mM NaHCO₃, pH 9.5, 0.5M NaCl) at 37°C for 1 hour. Protein concentration was measured using the Coomassie protein assay reagent (Pierce) with bovine serum albumin as standard. The amount of protoxin that could be solubilized from 100μl of inclusion body was calculated. For other assays, wild-type and mutant Cry1Aa 130kDa protoxins were treated with trypsin to produce the 65kDa toxin.

Toxin structure analysis

To detect changes in secondary structure, CD spectra of wild-type and mutant toxins were measured with an Aviv Circular Dichroism spectrometer-model 62A DS (Lakewood, NJ) in a 32-Q-10 quartz cuvette at 25 °C. The control software was Star Stationary 3.0. Toxins were at 2 μM in the high salt sodium carbonate-bicarbonate buffer. Readings were taken from 200nm to 300nm with 100 sampling numbers for five times.

Toxicity bioassay to Manduca sexta

*Manduca sexta* eggs (Carolina Biological Supply Company) were hatched at room temperature for 3 to 4 days. Larvae were reared on artificial diet (BioServ, Inc.) to 1st instar. Activity of toxins was determined by surface contamination method. Briefly, artificial diet was poured into 24-well tissue culture plates (Corning Costar, Corning, NY).
Each well had a surface area of 2cm². Toxins were serially diluted in high salt sodium carbonate-bicarbonate buffer, pH 9.5, and 50μl of toxin dilution was applied to one well, with four wells for each concentration. After toxins were blow-dried, two larvae were placed in each well. Mortality was recorded after 5 days. LC₅₀ (50% lethal concentration) values was calculated by Probit analysis in SoftTOX 1.1 program (WindowChem, Fairfield, CA).

Toxicity bioassay to Culex pipiens

Colonies of the mosquitoes were reared in an environment-controlled room at 28°C and 85% humidity, with a photoperiod of 14h light/10h dark. Culex pipiens egg rafts were purchased from Carolina Biological Supply Company. Adult mosquitoes were maintained on heparinated cow blood, sugar cane cubes (Domino Dots) and dechlorinated tap water. Larvae were maintained on fish food pellets (Koi Floating Blend, Aquaricare™), as suggested by Mark Q. Benedict (Centers for Disease Control and Prevention). Bioassays were performed as described previously (1). C. pipiens larvae were used 2 days after hatching. A total of four larvae per 2.5 ml of water with one replicate in a 24-well Costar cell culture plate (Corning) were fed a serial dilution of Cry toxins (as inclusions), and the number of mortalities was counted after a 48-h incubation at 28°C. The bioassay was repeated to obtain a concentration range on Cry toxin inclusions yielding 10 to 90% mortality. The 50% lethal concentration was calculated by a Probit method using SoftTOX version 1.1 (WindowChem).
4.3. RESULTS

Sequence and Structural alignments

Sequence alignment analysis by Clustal W analysis is shown in Table 4.2. RMSD of Cry4Ba structure by molecular modeling is at 0.53Å.

Loop regions are excellent targets for genetic re-designing of novel toxins with diverse specificity by exchanging residues or chain lengths of the active sites without major disruption of the overall integrity of the toxin. Based on sequence alignment and structural analysis, we found significant differences in the first two of the three loops in domain II. Loop 1 (residues 311RG312) in Cry1Aa was replaced by YQDL, the loop 1 sequence in Cry4Ba, to extend its length. Using structural modelling, LY367RRILGSGPNQ378 of Cry1Aa loop2 was designed to be altered in two separate steps. LYRRIIL was first deleted in mutant D3. D2 was a substitution of NNQ by G, to maintain the turn between two β-sheets and to mimic the shorter second loop in Cry4Ba. Interestingly, 4BL3PAT (1), a mutant derivative of Cry4Ba toxin (model structure shown in Figure 2; D454 was replaced with P and AT was inserted after position 454) that contains mutations in a putative loop 3 in domain II, has a 200-fold enhanced toxicity toward mosquito Culex. However it was also noted that further modification of loop three where PAT was replace with GAV, to match loop 3 of Cry1Aa was even more toxic to Culex (1). For this reason the third loop of Cry1Aa was left unchanged in this study.
| Cry1Aa | 33 | YTPIDISLSL TQFLLLSEFVP GAGFVLGLVD I---IWGIFG PSQW-----D |
| Cry4Ba | 57 | KFVNPP AGTVLTVLSA VLPILWP--T NTPTPERVWN |

```
Cry1Aa
Cry4Ba

33   YTPIDISLSL TQFLLLSEFVP GAGFVLGLVD I---IWGIFG PSQW-----D
57   KFVNPP AGTVLTVLSA VLPILWP--T NTPTPERVWN

* ..  .  *   .   .*      .       .
```

Table 4.2 Sequence Alignment of Cry1Aa and Cry4Ba

| Cry1Aa | 75 | AFLVQIEQILI QFNDMNSALT TAIPPLLAVQN YQVPPLLSSYV QAANLHLSVL |
| Cry4Ba | 91 | DFMTNTGNLI DQTVTAYVRT DANAKMTVVK YDLOQYTTKF NTWKRPPMNQ |

```
Cry1Aa
Cry4Ba

75   AFLVQIEQILI QFNDMNSALT TAIPPLLAVQN YQVPPLLSSYV QAANLHLSVL
91   DFMTNTGNLI DQTVTAYVRT DANAKMTVVK YDLOQYTTKF NTWKRPPMNQ
```

---

Table 4.2 Sequence Alignment of Cry1Aa and Cry4Ba

“*” stands for identical residues, “:” for conserved substitutions, and “.” for semi-conserved substitutions. Three loop regions are in red.
Figure 4.1  Loop regions of Cry1Aa and Cry4Ba.

Loops in domain II of Cry1Aa structure and the corresponding putative loops in model structure of Cry4Ba are as labelled.
Construction of mutants and Bt toxin purification

Cry1Aa wt and mutant toxins were constructed and expressed. Yield of mutant toxins were at wild type level, agreeing with the flexibility of loop region alterations.

Toxin structure analysis

The near UV spectral region (Shown in Figure 4.2) of wt and mutant Cry1Aa showed no significant variation, indicating that the defined tertiary structure was not disturbed. The gradual differences in far UV region agree with the changing ratio of loop components.

Toxicity bioassay to Manduca sexta and Culex pipiens

Bioassay results shown in Table 4.3 indicated that Cry1Aa wild type and intermediate mutants have no apparent toxicity to C. pipiens, while the final construct with changes in both loops 1 and 2 has enhanced C. pipiens activity in μg/ml level (45.73 μg/ml). This engineered protein was named Cry1AaMosq. Concomitant with the gain in mosquito toxicity, toxicity toward Manduca sexta was abolished during several rounds of changes in loop residues, confirming the importance of the domain II loops in specificity and activity.
Figure 4.2  CD spectra of mutant toxins.
Toxins & \( \text{LC}_{50} \) & \text{Manduca sexta}^a (ng/cm^2) & \text{C. pipiens}^b \\
4BRA & ND\(^d\) & >20,000 ng/ml \\
4BL3PAT & ND\(^d\) & 95 ng/ml (69-130) \\
4BL3GAV & ND\(^d\) & 70 ng/ml (34-129) \\
1Aa & 3.37 (1.92-7.67) & no mortality\(^c\) \\
1Aa L1 & 6.29 (4.48-7.98) & no mortality\(^c\) \\
1Aa D3 & 10.67 (6.39-43.30) & no mortality\(^c\) \\
1Aa L1D3 & 1664 (1302.15-2175.93) & no mortality\(^c\) \\
1Aa L1D3D2 & no mortality & 45.73 \(\mu\)g/ml (32.18-59.76) \\

**Table 4.3** Toxicity of Cry4Ba and Cry1Aa toxins.

a. 2-day old larvae of *Manduca sexta* were used for bioassays. Mortality was recorded after 5 days exposure to a serial dilution of the toxins. The 95% confidence limit is indicated in parentheses.

b. 2-day old larvae of *Culex pipiens* were used for bioassays. Mortality was recorded after 24 hours exposure to a serial dilution of the toxins.

c. No mortality at 100\(\mu\)g/ml.

d. ND: not determined.
4.4. DISCUSSION

Cry1Aa and Cry4Ba are presumed to share a similar mode of action, but target distinct insect species. With its known tertiary structure and relatively well characterized receptor binding regions, Cry1Aa is an ideal candidate for the design of alternate specificity. Cry1Aa is a lepidopteran toxin with no natural activity toward mosquito. In this study we have introduced alterations in domain II loops of Cry1Aa to introduce mosquito toxicity.

Loop regions are excellent targets for genetic re-designing of novel toxins with diverse specificity by exchanging residues or chain lengths of the active sited without affecting the structural framework of the toxin. The idea of using a protein of known three-dimensional structure to present motifs of various functions or specificity has been a goal since the beginnings of the protein engineering discipline (33). The idea of using a protein of known three-dimensional structure to present motifs of various functions or specificity has been a primary goal of the protein engineering (33). The use of so-called protein scaffolds for generation of novel binding proteins via combinatorial engineering has emerged as a powerful alternative to natural or recombinant antibodies (105).

The results of this study present an example of enhancing Cry toxicity through an approach that integrates sequence comparison, computational prediction and rational design of mutagenesis. Table 3 shows the toxicity of known mosquitocidal toxins from *B. thuringiensis* and *B. sphaericus*. Toxicity of engineered Cry1AaMosq is greater than several natural toxins (Cry1Ca, Cry2Aa, Cry4Ba, and Cry20Aa). The successful grafting of the alternate mosquito toxicity onto the original lepidopteran Cry1Aa toxin
demonstrates the possibility to design and engineer desired toxicity into any toxin of a common scaffold by reshaping the receptor binding region with desired specificities. By varying the specificity elements in loop regions on a general scaffold, a customized toxin can be selectively tuned to target different insect species.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Ae. aegypti</th>
<th>An. quadrimaculatus</th>
<th>An. stephansi</th>
<th>An. gambiae</th>
<th>Cx. quinquefasciatus</th>
<th>Cx. pipiens</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry1AaMosq</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>42,000</td>
<td>Present work</td>
</tr>
<tr>
<td>Cry1C</td>
<td>141,000</td>
<td></td>
<td></td>
<td>283,000</td>
<td>126,000</td>
<td></td>
<td>(124)</td>
</tr>
<tr>
<td>Cry2Aa</td>
<td>500-1000</td>
<td>38</td>
<td>--</td>
<td>--</td>
<td>1630</td>
<td>&gt;200,000</td>
<td>(6, 22, 100, 139)</td>
</tr>
<tr>
<td>Cry4Aa</td>
<td>563-1600</td>
<td>7400</td>
<td>1117</td>
<td>251-980</td>
<td>400</td>
<td></td>
<td>(3, 26)</td>
</tr>
<tr>
<td>Cry4Ba</td>
<td>61</td>
<td>25</td>
<td>&gt;80,000</td>
<td>&gt;20,000</td>
<td></td>
<td></td>
<td>(2, 26)</td>
</tr>
<tr>
<td>4BRAL3\text{PAAT}</td>
<td>53</td>
<td>44</td>
<td>3</td>
<td>65</td>
<td>95</td>
<td></td>
<td>(2)</td>
</tr>
<tr>
<td>4BRAL3\text{GAV}</td>
<td>44</td>
<td>52</td>
<td>114</td>
<td>37</td>
<td></td>
<td></td>
<td>(2)</td>
</tr>
<tr>
<td>Cry10Aa</td>
<td>low toxicity</td>
<td>not active</td>
<td></td>
<td></td>
<td>not active</td>
<td></td>
<td>(27, 130)</td>
</tr>
<tr>
<td>Cry11Aa</td>
<td>20-287</td>
<td>455</td>
<td>39.7-64</td>
<td>268-372</td>
<td></td>
<td></td>
<td>(19, 27)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(30, 107, 110)</td>
</tr>
<tr>
<td>Cry11Ba</td>
<td>18-30</td>
<td>42.7</td>
<td>6.5</td>
<td>10</td>
<td></td>
<td></td>
<td>(27, 107)</td>
</tr>
<tr>
<td>Cry11Bb</td>
<td>17.9</td>
<td>166.3 (An. albimanus)</td>
<td>34.1</td>
<td></td>
<td></td>
<td></td>
<td>(107)</td>
</tr>
<tr>
<td>Cry19Aa</td>
<td>1,400,000</td>
<td>3</td>
<td>1039</td>
<td>35</td>
<td>6-187</td>
<td></td>
<td>(2)</td>
</tr>
<tr>
<td>Cry19Ba</td>
<td>nd</td>
<td>not active</td>
<td></td>
<td></td>
<td>7520</td>
<td></td>
<td>(115)</td>
</tr>
<tr>
<td>Cry20Aa</td>
<td>648,000</td>
<td></td>
<td></td>
<td>700,000</td>
<td></td>
<td></td>
<td>(80)</td>
</tr>
</tbody>
</table>

**Non-Cry Proteins**

<table>
<thead>
<tr>
<th>Protein</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BinA-B</td>
<td>not active</td>
<td>not active</td>
<td></td>
<td></td>
<td></td>
<td>15.4-487</td>
</tr>
</tbody>
</table>

**Table 4.4**  Ranges of Reported Toxicity (ng/ml) of Mosquitocidal Proteins
REFERENCES


22. Dankocsik, C., W. P. Donovan, and C. S. Jany. 1990. Activation of a cryptic crystal protein gene of Bacillus thuringiensis subspecies kurstaki by gene fusion and
determination of the crystal protein insecticidal specificity. Mol. Microbiol. 4:2087-2094.


44. **Garner, K. J., S. Hiremath, K. Lehtoma, and A. P. Valaitis.** 1999. Cloning and complete sequence characterization of two gypsy moth aminopeptidase-N cDNAs, including the receptor for *Bacillus thuringiensis* Cry1Ac toxin. Insect Biochem. Mol. Biol. 29:527-535.


Crops Expressing *Bacillus thuringiensis* Cry1Ac Toxin. Applied and environmental microbiology 71:2558–2563.


67. **Jenkins, J. L.** 2000. Investigation of the *Bacillus thuringiensis* Insecticidal Cry1A Toxin Binding Mechanism to Lepidopteran Midgut Receptors. Thesis. Ohio State University, Columbus.


70. **Jenkins, J. L., M. K. Lee, S. Sangadala, M. J. Adang, and D. H. Dean.** 1999. Binding of *Bacillus thuringiensis* Cry1Ac toxin to *Manduca Sexta* aminopeptidase-N receptor is not directly related to toxicity. FEBS Lett. **462:**373-376.


73. **Keeton, T. P., and L. A. Bulla, Jr.** 1997. Ligand specificity and affinity of BT-R1, the *Bacillus thuringiensis* Cry1A toxin receptor from *Manduca sexta*, expressed in mammalian and insect cell cultures. Appl. Environ. Microbiol. **63:**3419-3425.

74. **Knight, P. J. K., B. H. Knowles, and D. J. Ellar.** 1995. Molecular cloning of an insect aminopeptidase N that serves as a receptor for *Bacillus thuringiensis* Cry1A(c) toxin. J. Biol. Chem. **270:**17765-17770.


76. **Knowles, B. H., P. J. K. Knight, and D. J. Ellar.** 1991. N-acetyl galactosamine is part of the receptor in insect gut epithelia that recognizes an insecticidal protein from *Bacillus thuringiensis*. Proc. R. Soc. Lond. B **245:**31-35.


78. **Koppeno, W. H., and E. Margoliash.** 1982. The asymmetric Distribution of Charges on the Surface of Horse Cytochrome C. JBC **257:**4426-4437.

79. **Kumaraswami, N. S., T. Maruyama, S. Kurabe, T. Kishimoto, T. Mitsui, and i. H. Hor.** 2001. Lipids of brush border membrane vesicles (BBMV) from *Plutella xylostella* resistant and susceptible to Cry1Ac delta-endotoxin of *Bacillus thuringiensis*. Comp Biochem Physiol B Biochem Mol Biol. **129:**173-83.


92. Luo, K., D. Banks, and M. J. Adang. 1999. Toxicity, binding, and permeability analyses of four Bacillus thuringiensis Cry1 delta-endotoxins using brush border
membrane vesicles of *spodoptera exigua* and *Spodoptera frugiperda*. Appl Environ Microbiol *65*:457-64.


100. **Moar, W. J., J. T. Trumble, R. H. Hice, and P. A. Backman.** 1994. Insecticidal activity of the CryIIA protein from the NRD-12 isolate of *Bacillus thuringiensis* subsp. *kurstaki* expressed in *Escherichia coli* and *Bacillus thuringiensis* and in a leaf-colonizing strain of *Bacillus cereus*. Appl. Environ. Microbiol. *60*:896-902.


135. **Valaitis, A. P., M. K. Lee, F. Rajamohan, and D. H. Dean.** 1995. Brush border membrane aminopeptidase-N in the midgut of the gypsy moth serves as the receptor for the Cry1A(c) delta-endotoxin of *Bacillus thuringiensis*. Insect Biochem Mol Biol **25:**1143-1151.


140. Wolfersberger, M. G. 1990. The toxicity of two Bacillus thuringiensis δ-endotoxins to gypsy moth larvae is inversely related to the affinity of binding sites on midgut brush border membranes for the toxins. Experientia 46:475-477.


