Analysis of Interaction of Cry1C protein from *Bacillus thuringiensis* with Aminopeptidase N receptor of *Spodoptera litura*

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In
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by

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Certificate

This is to certify that the research work embodied in this thesis entitled "Analysis of Interaction of Cry1C protein from *Bacillus thuringiensis* with Aminopeptidase N receptor of *Spodoptera litura*" has been carried out in Insect Resistance, International Centre for Genetic Engineering and Biotechnology, New Delhi, India. This work is original and no part of this thesis has been submitted for the award of any other degree or diploma to any other university.

Date: 11 April 2008

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Declaration

I hereby declare that the research work embodied in this thesis entitled "Analysis of Interaction of Cry1C protein from *Bacillus thuringiensis* with Aminopeptidase N receptor of *Spodoptera litura*" has been carried out by me under the supervision of Dr. Raj K Bhatnagar in Insect Resistance Group, International Centre for Genetic Engineering and Biotechnology, New Delhi. This work is original and has not been submitted so far, for the award of any degree or diploma to any other university, to the best of my knowledge and belief.

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Dedicated to my Husband and Our Families

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ABBREVIATIONS

AP	Alkaline phosphatase
APN	Aminopeptidase N
BBMV	Brush Border Membrane Vesicle
BCIP	5-bromo-4-chloro-3-indolyl phosphate toluidine
β-ME	β-mercaptoethanol
BSA	Bovine Serum Albumin
B.t.	*Bacillus thuringiensis*
CBB	Coomassie Brilliant blue
cmc	Critical micellar concentration
Cry	Crystal
DNA	Deoxy ribonucleic acid
DTT	1, 4-Dithio-DL-threitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay (ELISA)
GPI	Glycosyl phosphatidyl inositol
HRP	Horse Radish Peroxidase
ICP	Insecticidal Crystal Protein
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	Luria Broth
LpNA	L-leucine-p-nitroanilide
MCS	Multiple Cloning Site

NBT	p-nitroblue tetrazolium chloride
NC	Nitro cellulose
OD	Optical density
PAGE	Polyacrylamide Gel electrophoresis
PCR	Polymerase Chain Reaction
PMSF	Phenylmethanesulfonyl fluoride
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
Sf	*Spodoptera frugiperda*
TBS	Tris Buffered Saline (10 mM Tris-HCl, pH 7.5, 150 mM NaCl)
TE	Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)
T_m	Melting Temperature
X-Gal	5-Bromo-4-chloro-3-indolyl β-D-galactoside

Preface

The proposed thesis is aimed to identify the binding epitopes of the Cry1C toxin and the recombinant aminopeptidase N receptor expressed in *Sf*21 insect cell line. Since this required analyzing two different proteins with respect to their binding and toxic behavior, the performed work has been divided into different sections for the ease of understanding and flow of information. The first section **"Mapping the Cry1C binding epitope(s) of Aminopeptidase N receptor of *Spodoptera litura*"** deals with attempts to map the toxin binding epitope(s) of the recombinantly expressed full length APN receptor (SlAPN). Briefly, this section involves expression, purification and characterization of 60kDa active Cry1C toxin and 108 kDa SlAPN receptor. This section also demonstrates the identification of a 7 amino acid region (APN-CRY: ^{128}HLHFHLP^{134}) of SlAPN as the putative toxin binding region of the receptor. Identification of APN-CRY region led to cloning, expression, purification and characterization of recombinant BR-APN protein (deletion derivative of *SlAPN* expressing the APN-CRY region). Involvement of APN-CRY region in toxin binding and susceptibility to target insects is further reinforced by competition binding assays and in vivo toxicity assays. The second section **"Mapping the SlAPN Binding Epitope of Cry1C"** deals with attempts to map the receptor binding region of the Cry1C toxin. Competition binding assays and immunoprecipitation experiments suggest that domain II has higher binding affinity for SlAPN than domain III. Further, phage display and competition binding assays reveal that loops 2&3 of domain II and loop a of domain III are putative receptor binding epitopes of Cry1C toxin. The BR-APN protein which was shown to bind to Cry1C toxin in the previous section is studied in more detail in this section. Competition binding experiments suggest that loops 2 and 3 interact with APN-CRY region of the receptor. The third section **"Homology based Modeling of Cry1C Toxin and SlAPN Receptor AND In-silico Docking of the derived Structures"** aims to identify the cognate toxin and receptor epitopes by in-silico means. This section deals with homology based modeling of the toxin and receptor molecules using MODELLER. Docking of the derived structures was done by GRAMM v1.03. The docked complex M1.8 reveals interaction between loops 2&3 of Cry1C toxin and APN-CRY region aminopeptidase receptor thus validating the results obtained in the previous sections. The individual residues of the binding epitopes of the toxin and the receptor are analysed further in the last section **"Elucidating the Role of Critical Residues of Toxin and Receptor Epitopes in Receptor recognition and Toxicity"**. This section deals with various mutations introduced in loop regions of the toxin and APN-CRY region BR-

APN protein. The effect of these mutations on ligand binding and toxicity is described in this section. Each of the four sections is further divided into Introduction, Materials and Methods, Results and Discussion categories. To avoid repetition, clones and techniques already discussed in earlier section are not described in detail in later sections. The last section is followed by a common Summary and Conclusions chapter.

INTRODUCTION

Bacillus thuringiensis (B.t.)[1] is an entomopathogenic, aerobic, Gram positive bacterium that synthesizes crystalline inclusions during sporulation process. These heterologous inclusions (δ-endotoxins) consist of one or more insecticidal crystal proteins (Cry) proteins, that are classified according to their sequence similarity into 22 classes namely Cry1 to Cry22 (Crickmore et al, 1998). Upon ingestion, the insecticidal proteins are activated and acquire activity against Lepidopteran, Dipteran, Coleopteran or Hymenopteran insect larvae (Shcnepf et al, 1998; and de Maagd et al, 2001).

Elucidation of 3-D structure of Cry toxins (Cry1Aa, Cry 2Aa, Cry3Aa, Cry3Bb, Cry 4Aa and Cry 4Ba) by X-ray crystallography has been an important advance in the understanding of the mode of action of these toxins (Grochulski et al, 1995; Morse et al, 2001; Li et al, 1991; Galitsky et al, 2001; and Boonserm et al, 2005 & 2006). The structural analysis has revealed existence of three distinct domains; domain I, domain II and domain III. The N-terminal domain (domain I) is a bundle of 6 amphipathic helices surrounding a central hydrophobic helix-α 5. Most of the helices are longer than 30A° and would thus be capable of spanning a hydrophobic membrane. Various site-directed mutagenesis (SDM) studies and studies on isolated helical hairpin α 4-α 5 suggest that it is equipped for membrane insertion and pore formation (Chen et al, 1995; and Puntheeranurak et al, 2004). The variable domain II is a β-prism consisting of 3 antiparallel sheets, each terminating in a loop, packed around a triangular hydrophobic core (Morse et al, 2001). Mutagenesis studies have suggested that this domain participates in receptor recognition and hence insect specificity (Jurat- Fuentes et al, 2001). Lastly, domain III, a 'jelly roll' of 2 antiparallel β-sheets is believed to play a role in structural integrity of toxin molecule (Morse et al, 2001). However, in domain swapping experiments and SDM studies have shown domain III correlation with receptor binding and insect-specificity (Lee et al, 1995; and de Maagd et al, 1996) and ion-channel formation (Schwartz et al, 1997).

Most of the B.t. Cry toxins are synthesized as inactive protoxins which upon ingestion by a susceptible lepidopteran larvae, dissolve in the alkaline and reducing environment of the larval midgut, and release bioactive soluble proteins. The soluble protoxin is processed proteolytically by midgut enzymes, yielding 60-70 kDa protease-resistant toxic fragment. The activated toxin then binds specific receptor molecules located at the midgut epithelial cell brush border

membranes of the host insect (Rajamohan et al, 1998). The specific binding involves two-steps, a reversible step followed by an irreversible step leading to insertion of the toxin oligomer into the cell membrane (Rajamohan et al, 1998; and Knowles et al, 1994). The insertion of oligomer disrupts the transepithelial potential difference leading to the formation of pores in the membrane resulting in a net influx of ions and an accompanying influx of water, leading to the swelling of cells and eventual lysis (Lorence et al, 1995; and Carroll et al, 1993). However, the exact involvement of each toxin domain in the overall toxic process remains to be understood.

The receptors of the Cry toxins have been classified into four major groups: a cadherin like protein (CADR), a glycosyl phosphatidyl-inositol (GPI)-anchored aminopeptidase-N (APN), a GPI-anchored alkaline phosphatase (ALP), a 270 kDa glycoconjugate. Additionally certain glycolipids have also been recognized as toxin receptor molecules. Since 1994, more than 60 different APNs from different Lepidopterans have been sequenced and registered in databases. These APNs display high diversity in primary sequence and have been grouped into isoforms. These exopeptidases are believed to be anchored in lipid rafts and serve as interacting ligand molecule for Cry1A, Cry1C, Cry1F and Cry1J proteins in diverse Lepidopteran species (Jurat-Feuntes et al, 2001; Luo et al, 1996; and Agrawal et al, 2002). The aminopeptidase receptors have been implicated in toxin insertion (Bravo et al, 2004), shown to enhance pore formation when incorporated into lipid bilayers (Schwartz et al, 1997) and if inactivated by any means, render the insect populations resistant to B.t. toxins (Herrero et al, 2005; and Rajagopal et al, 2002). However, the molecular details of APN-toxin interaction are controversial and require extensive research.

Employing highly purified insecticidal protein and receptor, Liang et al (1995) analysed kinetics of interaction by BIACORE. The saturation kinetic analysis of Cry1A-type toxin binding to *Lymantia dispar* BBMVs was described according to the two-step interaction scheme. Briefly, the scheme proposes that at an early stage of binding there is a large saturable accumulation of reversible receptor-toxin complex followed by the formation of an irreversibly and tightly associated complex with the receptor. This led to the proposition of direct correlation between the rate of irreversible association and toxicity. Thus, toxin-receptor interaction is a complex process wherein both domain II and domain III are believed to be involved in the receptor recognition.

The domain III of Cry toxins is involved in the APN-Cry interaction, as shown by the interchange of domain III between Cry1Ac and Cry1Ab toxins (de Maagd et al, 1999). They suggested a sequential-binding mechanism was proposed in the interaction of Cry1Ac with APN. In this model, Cry1Ac -domain III initiates docking through recognition of an N-acetylgalactoseamine moiety on APN facilitating the subsequent interaction of domain II loop regions with a higher affinity region in this receptor (Jenkins et al, 2001). Although some APN binding epitopes in the toxin have been characterized, little is known about the APN domains involved in Cry toxin binding. Thus a significant effort is being put towards elucidation of the specifics of receptor-toxin interaction, interest in this input is further guaranteed by the perceived threat of emergence of resistance in insects towards insecticidal protein. Several possible mechanisms for the generation of resistance towards B.t.-protein have been proposed, among them, the most frequent mechanism has been the selection of insects affected on receptor-toxin interaction. Therefore, it is of paramount importance to determine the molecular basis of Cry toxin-receptor interactions. Insights gained from the fascination receptor-B.t. protein interaction could be useful in designing new toxins that could overcome the threat of generation of resistant insect populations under natural cultivation conditions of transgenic crops.

Previous work in our lab demonstrated that a 110 kDa recombinant aminopeptidase N is a receptor for Cry1C toxin in *Spodoptera litura* midgut and is responsible for Cry1C toxin sensitivity in the larvae (Agrawal et al, 2002). In the present study the identification and mapping of domains of interacting moieties has been carried out to identify critical residues in the interaction of APN and Cry1C

The following objectives were undertaken for this study –

- Mapping APN binding epitopes of Cry1C
- Mapping Cry1C binding epitopes of APN
- In-silico modeling of Cry1C and APN
- Mutational Studies on binding epitopes of Cry1C and SlAPN to investigate the role of critical individual amino acids

The present investigation was pursued to map and identify interacting domains of Cry1C and aminopeptidase N through phage display, deletion mutant analysis and site directed mutagenesis.

Review Of Literature

2.1 Biological Control of Insect Pests

Insect pests have played important role in human life either as vectors for various disease causing parasites or in the destruction of plants as well as stored plant products. Every year farmers loose a considerable amount of their crops due to the infestation by plant pests. Pest damage is the major yield-destabilizing factor in agricultural system. It is estimated that 20-40% of the crop yields are annually lost due to the insect pests. In India, annual crop loss due to insect pests is estimated to be Rs 20,000 millions. Chemical insecticides lack specificity as a result beneficial insects are also killed along with the target pest. A number of these chemicals are xenobiotic and show serious side effects on man and other animals as they biomagnify along the food chain and pollute the environment irreversibly. The extensive use of chemical insecticides is showing serious deleterious consequences on the environment. The harmful effects of overwhelmed usage of chemical insecticides led to a search of alternative, environment friendly biological control agents, among which bacterial insect pathogens are the most extensively studied. Slowly, *Bacillus thuringiensis* (B.t.), with its narrow insect specificity, environmental favorable nature and the slow rate of resistance development by insect populations against it became the most successful commercially proved control agent for agronomically important insect pests. The principal reasons for the success of B.t. include the existence of a diversity of proteins that are effective against a range of important pests, its relative safety to nontarget insect predators and parasites, mammals and birds, its ease of mass production at a relatively low cost, its adaptability to conventional formulation and application technology, and its biodegradability. Various preparations of spores and crystals have been used to control insects in the orders Lepidoptera, Diptera and Coleoptera. Such biopesticides are a useful alternative in forest pest management, agriculture and vector borne disease control (Fedirici, 2005; and Schnepf et al, 1998). B.t. accounts for 90-95% of the insect biocontrol market. Recently, introduction of *cry* genes into plants have led to the development of "B.t. crops" (Shelton et al, 2002; and Van Rie, 102000) and in 2004 B.t. maize and B.t. cotton were grown on 22.4 million hectares worldwide (James, 2004).

2.1.1 Bacillus thuringiensis: Knowing the leading Biorational Pesticidal Bacterium

Bacillus thuringiensis comprise a group of ubiquitous aerobic, Gram-positive, spore-forming bacteria closely related to *Bacillus cereus* (Carlson et al, 1994). It was first isolated in Japan by Ishiwata (1901), from diseased larvae of the silkworm, *Bombyx mori*, and was considered an insect pathogen. In 1915, Berliner isolated another strain of B.t. from diseased larvae of Mediterranean flour moth, *Anagasta kuehniella* which is a stored grain pest, in Thuringia, Germany; hence this strain was named *Bacillus thuringiensis*. Strains have been isolated from many habitats, including soil, insects (Carozzi et al, 1991), stored-product dust (Burges and Hurst, 1977; and Meadows, et al, 1992), and deciduous and coniferous leaves (Smith and Couche, 1991). *B.thuringiensis* seems to have been more readily isolated from the latter sources than the former. The bacterium is a rod-shaped spore-former. When nutrients are abundant in the environment, it grows vegetatively but when the nutrients are exhausted, it starts sporulating and makes a dormant spore. During sporulation, the bacterium produces large amounts of insecticidal proteins which accumulate in the spore mother cell compartment to form a crystal inclusion that can account for 20 to 30% of the dry weight of the sporulated cells (Bechtel and Bulla, 1976). Some of the B.t. strains contain multiple protoxin genes. Related protoxins in each class are mostly packaged into intracellular crystals of 0.6-1.0 micron. Parasporal crystals of different shapes, bipyramidal, irregular and flat rectangular have been identified in different B.t. strains (Hofte and Whiteley, 1989). The bacterium produces many virulent compounds in these crystals that allow its development in dead or weakened insect larvae. These include phospholipases (Zhang et al, 1993), proteases (Lovgren et al, 1990), chitinases, secreted vegetative insecticidal proteins (VIPs) (Estruch et al, 1997) and β-exotoxins (Levinson et al, 1990).

2.1.2 Expression and Regulation of cry genes

The Cry toxins (δ-endotoxins) are the most prominent of the larvicidal compounds formed by B.t. and to date, over 140 cry genes have been cloned and sequenced from various *B.thuringiensis* strains. These strains have a genome size of 2.4 to 5.7 million base pairs with several circular and linear extrachromosomal elements (Carlson et al, 1994). The *cry* genes are usually encoded by large plasmids (40-150 mDa) although exceptions are known in which

the gene is located on the chromosome. Many transposable elements, including insertion sequences and transposons have been found to be associated with the cry genes. The *cry1A* gene is flanked by two sets of inverted repeat sequences (IS 231 and IS 232) while the *cry4A* is flanked by two repeated sequences in opposite directions (IS 240). Insertion sequences have also been found upstream of the *cry1Ca* gene and downstream of a cryptic *cry2Ab* gene. Tn4430 has been found in the vicinity of the genes of the *cry1A* type in various Lepidopteran-active strains. Tn5401 is structurally associated with cry3Aa gene. It has been hypothesized that these transposable elements are involved in the amplification of the *cry* genes in the bacterial cell and horizontal dissemination of the genetic material (by a conduction process involving the formation of cointegrate structures between self conjugative plasmids and chromosomal DNA or nonconjugative plasmids) within the species (Schnepf et al, 1998).

The products of *cry* gene can account for 30-35% of the dry weight of the sporulated culture. The high level of crystal protein synthesis and its coordination with the stationary phase are controlled by a variety of transcriptional, posttranscriptional, translational and posttranslational mechanisms. Transcription of sporulation-dependent *cry1Aa, cry1Ba, cry2Aa, cry4Aa, cry4Ba, cry11Aa, cry15Aa* and many others is controlled by the activation of sporulation specific sigma factors (σ^E or σ^K) (Agaisse et al, 1995; and Baum et al, 1995) while the *cry3Aa* expression is activated by a non-sporulation dependent mechanism during the transition from the exponential growth to the stationary phase. The *cry3Aa* promoter resembles promoters recognized by the primary sigma factor of vegetative cells, σ^A. The disappearance of σ^A in the mother cell during sporulation turns off *cry3Aa* expression making it sporulation-independent (Salamitou et al, 1996). Several investigations have analysed reasons for high amount of synthesis of B.t. insecticidal proteins. In the case of *cry1Aa* and various other genes putative transcriptional terminators (a stem-loop structure) have been hypothesized to act as a positive retroregulator which increases the cry mRNA stability by protecting it from exonucleolytic degradation from the 3'end. At the same time stability of the transcript is achieved from interaction between the 3'end of 16s rRNA and Shine-Dalgarno sequence (GAAAGGAGG) mapped at position between -125 and –117 in the case of *cry3Aa* gene (Agaisse et al, 1996). Such potential sequences are also present in similar positions upstream of the *cry3Ba, cry3Bb and cry3Ca* genes. In addition the storage of proteins in the form of crystals decreases the susceptibility of the toxins to premature proteolytic degradation. Crystallization of Cry2A requires the presence of accessory proteins that may act

at a posttranslational level to stabilize the nascent protoxin molecule and to facilitate crystallization. Stability of crystalline inclusions is believed to be a consequence of structural features of C-terminal end of these proteins. It is generally assumed that the cysteine-rich C-terminal half of the Cry1, Cry4, Cry5 and Cry7 protoxins contribute to crystal structure through the formation of disulfide bonds (Bietlot et al, 1990) while in the cysteine-rich lacking Cry3A toxin intermolecular salt bridges participate in the formation of crystal inclusion (Li et al, 1991). Taken together, these elements are believed to be responsible for accumulation of B.t. insecticidal proteins.

2.1.3 Diversity of Cry Proteins

The extraordinary diversity of Cry toxins is believed to be due to high degree of genetic plasticity. The large number of Cry proteins thus generated has permitted comparative sequence analysis and identification of elements important for both toxin function and insect specificity. The first detailed analysis of Cry protein sequences was carried out by Hofte and Whiteley (1989) who classified the Cry proteins into four major classes based on their host range and primary structure.

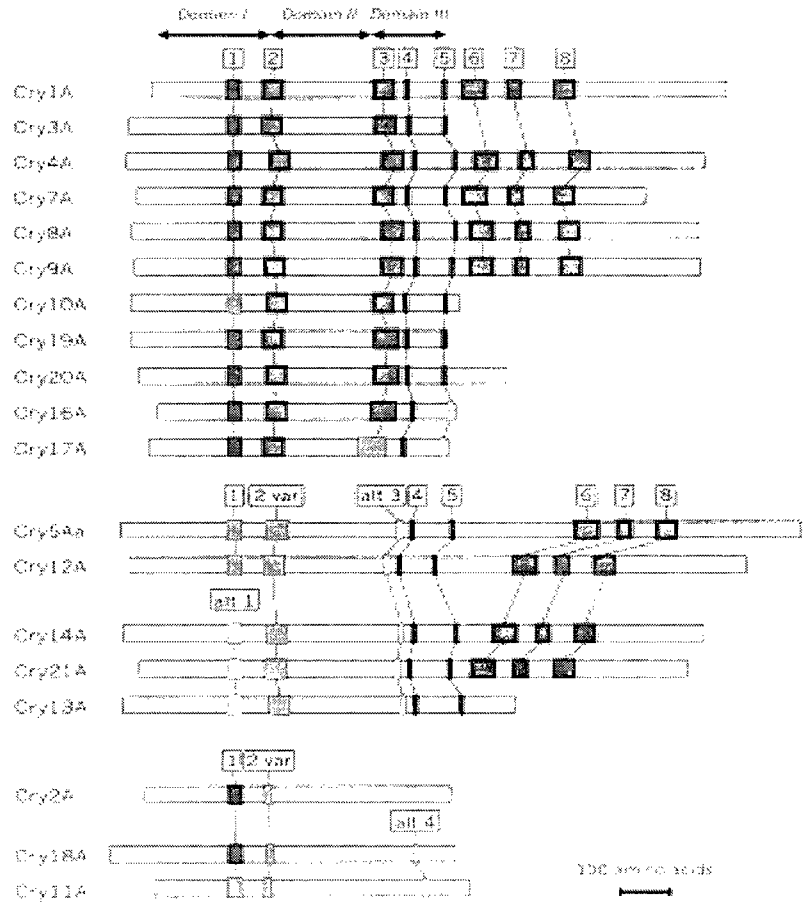
Table 1. Hofte and Whiteley classification

Class	Protein size	Host specificity
CryI	60-65 kDa	Lepidoptera
CryII	68-71 kDa	Lepidoptera and Diptera
CryIII	73-kDa	Coleoptera
CryIV	72-135 kDa	Diptera

However, inconsistencies existed in the original scheme due to attempts to accommodate genes that were highly homologous to known genes but did not encode a toxin with a similar insecticidal spectrum. For example, the cryIIB gene received a place in the lepidopteran class with *cryIIA*, even though toxicity against dipterans could not be demonstrated for the toxin designated CryIIB; the protein named CryIC was reported to be toxic to both dipterans and lepidopteran and many other anomalies were found out later. The discovery of new Cry proteins prompted further analyses: first by Bravo in 1997 (Bravo, 1997) and then by de

Maggd in 2001 (de Maggd et al, 2001). Now more than 300 Cry toxins, distributed among 49 classes, have been identified and classified based on sequence homology (www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/). In the proposed revision, Roman numerals have been exchanged for Arabic numerals in the primary rank (e.g. Cry1Aa) to better accommodate the large number of expected new proteins. The definition of a Cry protein is set as: "*a parasporal inclusion (crystal) protein from Bacillus thuringiensis that exhibits some experimentally verifiable toxic effect to a target organism or any protein that has obvious sequence similarity to a known Cry protein*". Similarly, Cyt denotes a "*parasporal inclusion (crystal) protein from B. thuringiensis that exhibits hemolytic activity or any protein that has obvious sequence similarity to a known Cyt protein*".

In 1989, Hofte and Whiteley drew attention to the five blocks of amino acids conserved among most of the Cry toxins active core then known. Comparison of the carboxyl-terminal halves of sequences with more than 1000 residues suggests the presence of three additional blocks lying outside the active toxic core.



Figure, 1. Positions of conserved blocks among Cry proteins. The cartoon shows the sequence arrangement for each holotype toxin (e.g., Cry1Aa1) having at least one of the conserved blocks. Sequence blocks are shown as dark gray, light gray, or white to indicate high, moderate, or low degrees of homology, respectively, to the consensus sequence for each conserved block. Variant sequences (var) conform to the consensus sequence of the highly conserved group at 50 to 75% of the positions. Alternate blocks (alt) are derived from groups of proteins having a consensus sequence over that sequence block that differs from the corresponding highly conserved sequence at more than half of its positions. The lengths of each protein and the conserved blocks within them are drawn to scale. (Reference: Schnepf et al, 1998)

Block 1 encompasses helix 5 of domain I. This helix has been implicated in pore formation, a role that might explain its highly conserved nature. The central location of helix 5 within domain I also suggests an essential role in maintaining the structural integrity of the helical bundle.

Block 2 includes helix 7 of domain I and the first β-strand of domain II. These two structures comprise the region of contact between the two domains. There are three structurally

equivalent salt bridges present between domain I and domain II in Cry1Aa and Cry3A; the residues involved in the salt bridges lie within block 2.

Blocks 3, 4, and 5 each lie on one of the three buried strands within domain III. Block 3 contains the last β-strand of domain II, a structure involved in interactions between domains I and III. The central two arginines of block 4 may be involved in intermolecular salt bridges affecting crystal or oligomeric aggregation. Grochulski et al (1995) have noted, however, that the first and last arginines are solvent exposed. These residues have been implicated in channel function.

The group consisting of Cry1, Cry3, Cry4, Cry7 to Cry10, Cry16, Cry17, Cry19, and Cry20 contains all five of the core blocks. A second group consisting of Cry5, Cry12 to Cry14, and Cry21 contains recognizable homologs of blocks 1, 2, 4, and 5 (Figure 1). The members of the second group show more variability in block 1, possess a block 2 variant but lack block 3 altogether. An unrelated sequence, highly conserved within the second subgroup but absent from the first, lies between blocks 2 and 4 (Figure 1). For both groups, when a protein possesses the C-terminal extension, blocks 6, 7, and 8 are invariably present. Members of a third sequence similarity group, composed of Cry2, Cry11, and Cry18, possess block 1 and a truncated variant of the block 2 core (Figure 1) but lack convincing homologs of the other conserved blocks. An alternating arginine tract not otherwise homologous to block 4 is found near the C terminus of Cry11 and Cry18.

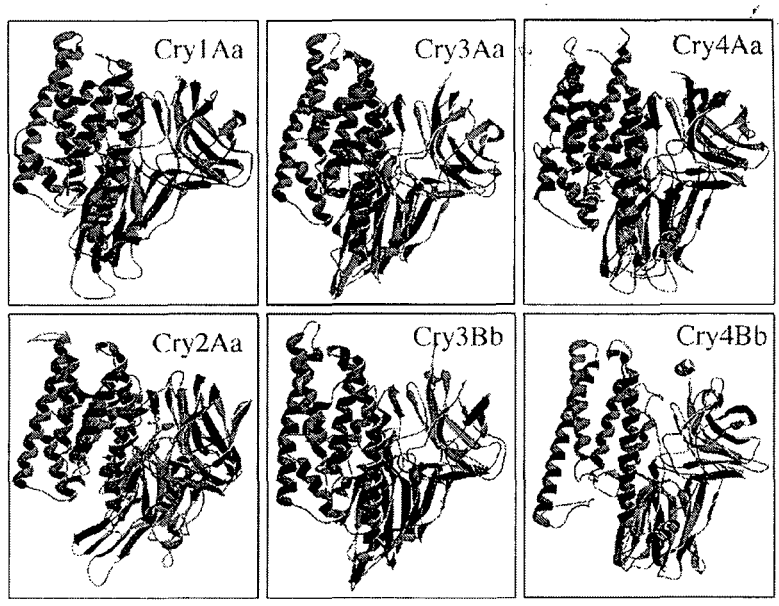
The conservation of blocks 1 through 5 is at least consistent with the notion that the proteins within the first subgroup, which includes Cry1 and Cry3, might adopt a similar three-domain tertiary structure. It is possible, too, that the second subgroup-Cry5, Cry12 to Cry14, and Cry21-could possess a variation of the same structural theme. The degree of sequence similarity found in the Cry2, Cry11, and Cry18 group of proteins suggests that a fold similar to that in domain I of Cry3A may be present. Somewhat more surprisingly, the 2.2A°crystal structure of the Cry2Aa revealed that this toxin also possesses second and third domains strikingly similar to those of Cry3A, despite the apparent absence of primary sequence homology between the two proteins over this region.

2.2 Toxin Structure and Function

One particular feature of the members of Cry family is the presence of protoxins. The C-terminal extension found in the long protoxins is dispensable for toxicity and is believed to play a role in the formation of the crystal inclusion bodies within the bacterium (de Maagd et al, 2001). Since most of the cysteine residues are located in this fraction of the protoxin, it has been suggested that the alkaline and reducing conditions required for the solubility of these proteins are related to disulfide bridge formation within the protoxin fragment (Choma and Kaplan, 1992). However, this fragment is not found in some toxins (Cry3A, Cry3Ba, Cry3Bb, Cry3Ca, Cry2Aa Cry2Ab, Cry2Ac, and Cry11Aa) or is very small in other protoxins, like Cry1Ia and Cry1Ib and Cry13A (Bravo, 1997).

2.2.1 Three-Domain Topology of the Active Toxic Core

The members of the three-domain family, the larger group of Cry proteins, are globular molecules containing three structural domains connected by single linkers. To date, the tertiary structures of seven different three-domain Cry proteins, Cry1Ac Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, Cry4Aa and Cry4Ba have been determined by X-ray crystallography (Li et al, 2001 & 1991; Grochulski et al, 1995; Morse et al, 2001; Galitsky et al, 2001; and Boonserm et al, 2005 & 2006). These toxins show considerable differences in their amino acid sequences and insect specificity but, remarkably, they all display a high degree of similarity with a three-domain organization, suggesting a similar mode of action of the Cry three-domain protein family (Figure 2).



Figure, 2. Three-dimensional structures of insecticidal toxins produced by Bacillus thuringiensis Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, Cry4Aa and Cry4Bb. (Reference: Bravo et al, 2007)

Domain I

The N-terminal domain (domain I) shares structural similarities with other Pore Forming Toxins like colicin Ia and N and diphtheria toxin, supporting the role of this domain in pore-formation. This domain is the most conserved part of the active core among various groups. It is a bundle of seven α-helices in which the central helix-α5 is hydrophobic and is encircled by six other amphipathic helices. The six amphipathic helices are long enough to span the 30-A^0- thick hydrophobic region of a membrane bilayer. The structure of this domain together with the results of site-directed mutagenesis (SDM) studies and studies on isolated helical hairpin α 4-α 5 suggest that it is equipped for membrane insertion and pore formation (Chen et al, 1995). Fluorometric studies with synthetic α 5 peptide indicated that this peptide is involved in the toxic mechanism of delta-endotoxins (Gazit and Shai, 1993). Later on, membrane permeation studies together with spectrofluorometric studies provided evidence for the role of α 4-loop-α 5 as the membrane-inserted pore-forming hairpin in which α 4 and α 5 line the lumen of the channel and α 5 also participates in the oligomerization of the toxin (Gerber et al, 2000).

Domain II

In the case of domain II, structural similarities with several carbohydrate-binding proteins like vitelline, lectin jacalin, and lectin Mpa have been reported (de Maagd et al, 2003). The domain II is a β-prism consisting of 3 antiparallel β-sheets, each terminating in a loop, packed around a triangular hydrophobic core. Thus, the β-sheets are joined in a "Greek key" topology, arranged in a β-prism fold. Genetic studies involving segment swapping and mutagenesis studies have suggested that this domain participates in receptor recognition and hence insect specificity. This domain represents the most divergent part in structure of the toxic core. The three surface-exposed loops of domain II which connect the three β-pleated sheets have been implicated in receptor binding (Li and Ellar, 1991; and Grochulski et al, 1995). These loops are of different lengths and are located between β1 and β2 (loop 1), β6 and β7 (loop 2) and β10 and β11 (loop 3). There is another exposed loop (α 8 loop) located between α-helix 8 and β1 sheet in Cry3A (Li and Ellar, 1991) or between α8a and α8b in Cry1Aa (Grochulski et al, 1995). Extensive mutagenesis has been carried out in the residues constituting the loops of domain II specifically in Cry3A, Cry1A and Cry1C toxins to evaluate their role in binding and toxicity (Schnepf et al, 1998). Loops 1, 2 and 3of domain II correspond to the regions that showed the largest structural differences between Cry1Aa and Cry3A toxins. This domain has been described as the specificity-determining domain (Schnepf et al, 1998).

Domain III

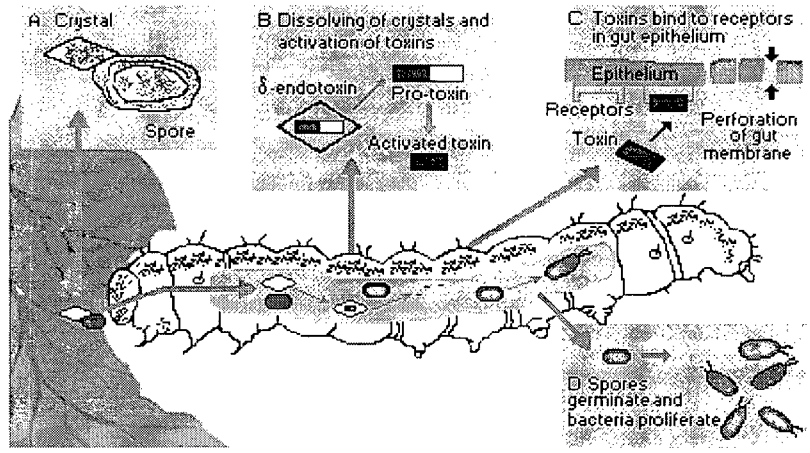
Lastly, domain III is a sandwich of two antiparallel β-sheets in a 'jelly roll' configuration. Domain III, shares structural similarity with other carbohydrate-binding proteins such as the cellulose binding domain of 1,4-bglucanase C, galactose oxidase, sialidase, b-glucoronidase, the carbohydrate-binding domain of xylanase U and b-galactosidase (de Maagd et al, 2003). These similarities suggest that carbohydrate moieties could have an important role in the mode of action of three-domain Cry toxins. The function of domain III remains obscure even after the elucidation of its structure. It may be involved in ion channel formation as a voltage sensor, since conservative mutations in R521K and R527K reduced toxicity without reducing binding (Chen et al, 1993). The salt bridges and hydrogen bonds formed between arginine residues present in the β-17 sheet and other residues in the vicinity play an important role in

stabilizing the toxin structure (Grochulski et al, 1995). Both β-sheets (β-17 and β-23) are important determinants for the proper folding of the toxin (Grochulski et al, 1995). Domain III has also been implicated in receptor binding and hence insect specificity. Detailed experimental evidences of domain II and domain III involvement in receptor binding is explained in the following sections.

2.3 Domain Swapping between domain II and domain III

An analysis of the phylogenetic relationships of the isolated domains of members of the three domain Cry family revealed interesting features regarding the creation of diversity in this protein family (Bravo, 1997; and de Maagd et al, 2001). Domains I and II have coevolved. The analysis of domain III sequences, revealed a different topology due to the fact that several examples of domain III swapping among toxins occurred (Bravo, 1997; and de Maagd et al, 2001). Some toxins with dual specificity (coleopteran, lepidopteran) are clear examples of domain III swapping among coleopteran and lepidopteran specific toxins. This suggests that domain III swapping could create novel specificities. In this regard, in vitro domain III swapping of certain Cry1 toxins resulted in changes in insect specificity (Bosch et al, 1994; and de Maagd et al, 2000). The independent evolution of the three structural domains and domain III swapping among different toxins generated proteins with similar mode of action but with very different specificities (Bravo, 1997; and de Maagd et al, 2001).

Though the detailed mechanism of action of the δ-endotoxin is not completely understood, a significant amount of information has been obtained in recent years. Most of the information was obtained from studies of lepidopteran insects because of their large size and easy maintenance under laboratory conditions. It is widely accepted that the primary action of Cry toxins is to lyse midgut epithelial cells (hence the name-gut poisons) in the target insect by forming pores in the apical microvilli membrane of the cells (Aronson and Shai, 2001; de Maagd et al, 2001; and Bravo et al, 2005). Nevertheless, it has been recently suggested that toxicity could be related to G-protein mediated apoptosis following receptor binding (Zhang et al, 2006).



Figure, 3. Mechanism of toxicity of B.t. toxin

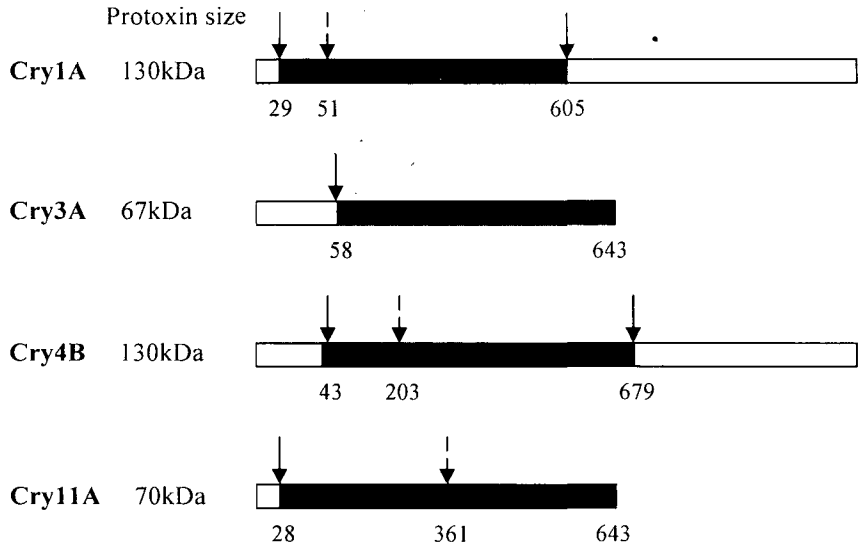
2.4 Mode of Action of Cry Toxins in Lepidopteran Insects

Despite its importance as a systemic pesticide in genetically modified plants, the mechanism of action of B.t. δ-endotoxins remains unclear. The mode of action of Cry toxins has been characterized principally in lepidopteran insects. In general, the mechanism of action of the *B. thuringiensis* Cry proteins involve solubilisation and activation of the ingested inclusion bodies to generate soluble active toxins that interact with the receptors on the brush border membranes of the insect gut leading to the insertion of the activated toxin oligomers. This disrupts the transepithelial potential difference leading to the formation of pores in the membrane resulting in a net influx of ions and an accompanying influx of water, so that the cells swell and lyse. This results in paralysis of the midgut and subsequent larval death. Nevertheless, it has been recently suggested that toxicity could be related to G-protein mediated apoptosis following receptor binding. The exact mechanism of toxicity and role of individual domains in the overall process of receptor binding and toxicity has been reviewed many times (Pigott and Ellar, 2007; Bravo et al, 2007; Soberon et al, 2007; and Schnepf et al, 1998) but still remains unclear.

2.4.1 Solubilization and Activation

To exert their toxic effect, a transition from crystal inclusion protoxins to membrane-inserted activated toxins is required for formation of pores in the apical membrane of the insect midgut epithelial cells. The mixture of protoxins within an inclusion determines the solubility of these protoxins and thus the effectiveness for a particular insect (Aronson et al, 1991). The crystal inclusions ingested by the susceptible larvae dissolve in the alkaline environment of the larval midgut, thereby releasing soluble inactive protoxins. The inclusions composed of CryI, CryII and CryIV protoxins are solubilized at high pH (9-10) and under reducing conditions, found in the midgut of lepidopteran and dipteran larvae. Inclusions containing the CryIIIA protoxin are soluble at pH 7-8, close to that found in susceptible coleopteran larval guts. Differences in the extent of solubilization can sometimes account for diversity in toxicity of Cry proteins among target insects. In some insects an alteration in conditions affecting Cry solubility is correlated with development of resistance to Cry toxins.

Following solubilization, inactive protoxins are cleaved by midgut proteases yielding 60-70 kDa protease resistant proteins. Toxin activation involves the proteolytic removal of an N terminal peptide (25-30 amino acids for Cryl toxins, 58 residues for Cry3A and 49 for Cry2Aa) and approximately half of the remaining protein from the C-terminus in the case of the long Cry protoxins. Figure 4 shows a schematic representation of the Cry protoxin structure and their protease cleavage sites.



Figure, 4 Relative lengths of Cry protoxins and position of protease digestion. White boxes represent the protoxin and solid boxes represent the activated toxin. Solid arrows show the amino- and carboxy- terminal cleavage sites of the activated toxins. Doted arrows show the intra-molecular cleavages. Cleavage of Cry1A at residue 51 resulted in loss of helix a-1 and pre-pore formation. Cleavage of Cry4B resulted in two fragments of 18 and 46 kDa, while Cry11A resulted in two fragments of 34 and 32 kDa. (Bravo et al, 2007)

In general for Cry1 protoxins, only limited cleavage of 28 amino acids occurs at the N-terminal while approximately 500 amino acids are removed from the C-terminal. In contrast, the Cry2, Cry3, and Cry4D proteins do not undergo protease-mediated C-terminal cleavage because these proteins appear to be naturally truncated (Carroll and Ellar, 1989; Widner and Whiteley, 1989). These naturally truncated proteins may undergo protease cleavage in the insect midgut (Chilcott and Ellar, 1988; Carroll and Ellar, 1989). The midgut environment probably plays a crucial role in establishing the specificity of insecticidal protein. The specificity of Cry1Ab toxin, from *B. thuringiensis* subsp. *aizawai* strain IC1 depends on the midgut protease processing of the toxin. When activated with lepidopteran *Pieris brassicae* midgut extract, the toxin kills both *P. brassicae* and dipteran *Aedes aegypti* larvae; however, when activated by *A. aegypti* midgut extract, the isolate is toxic only to these mosquito larvae (Haider and Ellar, 1989a).

One interesting feature of Cry toxin activation is the processing of the N-terminal end of the toxins. The three-dimensional structure of Cry2Aa protoxin showed that two a-helices of the N-terminal region occlude a region of the toxin involved in the interaction with the receptor (Morse et al, 2001). Also, it was found that a Cry1Ac mutant that retained the N-terminus end

after trypsin treatment binds nonspecifically to *Manduca sexta* membranes and was unable to form pores on *M. sexta* brush border membrane vesicles (BBMV) (Bravo et al, 2002). Therefore, processing of the N-terminal end of Cry protoxins may unmask a domain II hydrophobic patch involved in toxin-receptor or toxin-membrane interaction.

Candas et al (2003) used differential-in-gel electrophoretic (DIGE) analysis to compare B.t. susceptible and resistant larvae of *Plodia interpunctella*. They observed that reduced levels of alkaline chymotrypsin in the resistant *P. interpunctella* larvae were associated with reduced capacity for protoxin activation.

2.4.2 Receptor Binding

Activated Cry toxins have two known functions, receptor binding and ion channel activity. The receptor binding properties of B.t. toxins have been extensively examined in recent years to understand the toxicity, specificity and in some cases, insect resistance mechanisms. The activated toxin readily binds to specific toxin binding proteins exposed in the brush border membrane of the midgut epithelium columnar cells (de Maagd et al, 2001; and Bravo et al, 2005). Such proteins are usually called receptors, although this name is somewhat incorrect because the real function of these proteins in the organism is not related with their interaction with the toxin.

Receptor binding is the key determinant of specificity and efficiency of toxic effect. Binding of the Cry toxins to the microvillate apical membrane of epithelial cells has been demonstrated by *in vivo* immunocytochemical studies (Fast et al, 1988; Bravo et al, 1992). The interaction with high-affinity binding sites in the midgut epithelium of susceptible insect species is a major determinant for the selectivity of the ICPs or delta-endotoxins of *B. thuringiensis*. In view of the important contribution of these high-affinity binding sites in the mode of action of *B. thuringiensis* ICPs, much effort is put on the cloning and characterization of ICP-binding proteins. An *in vitro* system using insect midgut BBMVs and radiolabeled (^{125}I) toxins was developed to study binding at the molecular level (Wolfersberger et al, 1987). This binding assay system became a tool to compare binding affinities of native and mutant toxins, to correlate toxin binding with insecticidal activity (Hofmann et al, 1988b; Van Rie et al, 1990a) and to probe the mechanism of resistance development in insects. The toxin-

binding proteins (receptors) from several insects have been identified by ligand blotting studies (Garczynski et al, 1991; Oddou et al, 1991; Oddou et al, 1993; Vadlamudi et al, 1993; and Cowles et al, 1995) using BBMVs and iodinated or biotinylated toxins. Recently, however, new techniques of receptor identification have emerged. Proteomic approaches based on two-dimensional (2D) gel electrophoresis and mass spectrometry have been used to discover novel B.t. toxin binding proteins and elucidate changes in midgut proteins associated with B.t. resistance. McNall and Adang (2003) identified a membrane-bound form of alkaline phosphatase (mALP) and actin as novel Cry1Ac toxin-binding proteins in the brush border midgut membrane proteome of *Manduca sexta* larvae. Candas et al (2003) used differential-in-gel electrophoretic (DIGE) analysis to compare B.t. susceptible and resistant larvae of *Plodia interpunctella*. Additional Cry1Ac binding proteins in *H. virescens* BBMV have also been identified by Krishnamoorthy et al (2007) using 2D gel electrophoresis and peptide mass fingerprinting (PMF). Using PIPLC treated BBMVs, V-ATP synthase subunit A and actin were identified as novel Cry1Ac binding proteins in *H. virescens*. Phage display has also been speculated to provide means for receptor identification, since it is possible to expect that peptide-phages that bind the toxin and mimic the receptor may share some primary sequence similarity with the receptor (Fernandez et al, 2005; Kasman et al, 1998; and Marzari et al, 1997).

2.4.2.a General Receptor binding and Kinetic Considerations

Early work by Hofmann et al (1988), Van Rie et al (1990), and others employed competition binding studies, using purified BBMVs and ^{125}I-labelled toxins, to demonstrate the correlation between toxin binding affinity and insecticidal activity. In many cases there is a direct correlation between binding affinity, binding site concentration, and toxicity, i.e., the highly active toxins constitute more binding sites and bind with higher affinity to the BBMVs than do the less active toxins. However, in a paradoxical finding, Wolfersberger (1990) observed an inverse relationship between binding affinity and toxicity in the gypsy moth (*Lymantia dispar*). A highly active toxin, Cry1Ab exhibited weaker binding affinity compared to a less active toxin, Cry1Ac. Garczynski et al (1991) investigated the interactions of Cry1Ac toxin with BBMVs from sensitive (*M. sexta* and *H. virescens*) and naturally resistant insects (*S. frugiperda*). Homologous competition binding assays showed that all the species tested have high-affinity binding sites for Cry1Ac toxin. The presence of Cry1Ac toxin binding sites in

susceptible and resistant insects support a hypothesis in which toxin receptors are necessary, but insufficient for insecticidal activity. Ihara et al (1993) observed that Cry1Aa and Cry1Ab toxins bind to *B. mori* BBMVs with similar affinity but Cry1Aa was found to be 100-fold more toxic than Cry1Ab. They demonstrated that the differences in the rate of irreversible association with BBMVs could account for variations in toxicity. Other examples of this phenomenon-a lack of correlation between receptor binding affinity and insecticidal activity-are now known (Van Rie et al, 1989; Garczynski et al, 1991; Sanchis and Ellar, 1993; and Cowles et al, 1995). Mutational studies in domain II, loop 2 residues of Cry1Ab extended the knowledge of Cry toxin membrane insertion beyond the analyses of initial binding. It is now widely accepted that receptor-binding process of Cry 1 toxins in several lepidopteran insects involves two steps. Liang et al (1995) described the saturation kinetic analysis of Cry1A-type toxin binding to *L.dispar* BBMVs according to the two-step interaction scheme shown by equation 1

$$T + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} T{\equiv}R \xrightarrow{k_2} {}^{*}T \text{ (or } {}^{*}TR) \qquad \text{.....Equation (1)}$$

Where, T is a Cry toxin, R is a receptor for this toxin, $T{\equiv}R$ is a toxin that is reversibly bound to the receptor, k_1 is the on rate constant, k_{-1} is the off rate constant and k_2 is the irreversible rate constant. *T is an irreversibly bound toxin, presumably inserted into the membrane but not associated with a receptor; and *TR is an irreversibly bound toxin which is still associated with a receptor.

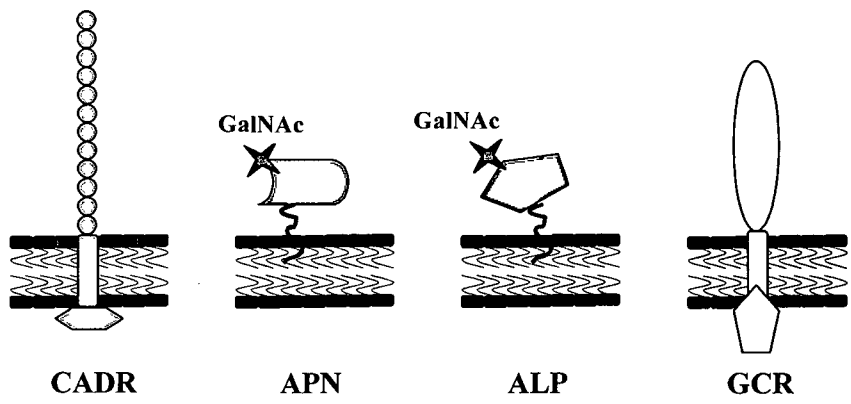
In summary, this scheme shows that that at an early stage of binding there is a large saturable accumulation of reversible receptor (R) and toxin (T) complex ($T{\equiv}R$) and then the formation of an irreversibly or tightly associated complex either with the receptor *TR or membrane *T at a rate constant of k_2. This led to the proposition of direct correlation between the rate of irreversible association (k_2) and toxicity, i.e. the higher the k_2 value the greater the toxicity to the insect.

Prior to the work of Liang et al (1995), kinetic analysis of Cry toxin-receptor binding had used equations based on the Hill (Hofman et al, 1988) or Scatchard equations (Van Rie et al, 1990), which assume a strictly reversible binding. In reality, the toxin becomes irreversibly associated with the apical membrane by insertion. Given the irreversible rate component of k_2,

the whole equation cannot reach equilibrium, because as the toxin-receptor complex is formed its concentration will be depleted by insertion. Therefore competition or binding experiments under conditions whereby insertion can take place do not yield true K_d values. Alternate designations such as IC_{50} or K_{com} have been used to represent binding affinity under these conditions. These studies do, however, suggest that the biological activity of the toxin is directly correlated to the sum of toxin irreversibly associated with the membrane rather than strictly to initial binding. In order to calculate individual binding constants (k_1, k_{-1}, and k_2) precisely solubilized BBMV proteins or purified receptors attached to a solid support, on which membrane insertion does not occur have been used (Sangadala et al, 1994; Masson et al, 1995; Lee et al, 1996a and 1996b).

2.4.2.b Identification and Validation of Receptors

At least four different binding proteins have been described in different lepidopteran insects; a cadherin-like protein (CADR), a glycosyl phosphatidyl-inositol (GPI)-anchored aminopeptidase-N (APN), a GPI-anchored alkaline phosphatase (ALP) and a 270 kDa glycoconjugate (Vadlamudi et al, 1995; Knight et al, 1994; Jurat- Fuentes and Adang, 2004; and Valaitis et al, 2001). In addition, it has been proposed that glycolipids are important Cry-receptor molecules in insects and nematodes (Griffits et al, 2005). Figure 5 shows a representation of the four types of putative Cry1A-receptor molecules characterized so far.



Figure, 5. Receptor molecules of Cry1A proteins. CADR, cadherin receptor; APN, aminopeptidase-N, ALP, alkaline phosphatase, GCR, 270 kDa glyco-conjugate receptor.

2.4.2.b.i AMINOPEPTIDASE

Classification And Nomenclature

Aminopeptidase catalyze the cleavage of amino acids from the amino terminus of many proteins. They were some of the earliest proteases discovered. Many aminopeptidase activities have been described, and they appear to be widely distributed in the plant and animal kingdoms. They are essential for protein maturation (Moerschell et al, 1990), degradation of nonhormonal (Botbol and Scornik, 1991) and hormonal peptides, and possibly determination of protein stability (Bachmair et al, 1986), etc. By virtue of their ability to remove NH2-terminal residues at differential rates, it has also recently been proposed that aminopeptidases are involved in regulation of rates of hydrolysis of proteins that are degraded by the ubiquitin-dependent pathway. Taken together, this indicates that aminopeptidases perform regulatory as well as "housekeeping" functions. Many disease states are associated with impaired proteolytic function (Taylor et al, 1992).

Aminopeptidases have been classified in numerous ways: with respect to their substrate, their location in cell, according to metal ion content, pH of maximal activity etc. Given the labyrinth of classifications, it is not surprising that in recent years several enzymes previously thought to be distinct were shown to be identical. For example, hkLAP and hog intestinal prolyl aminopeptidase are indistinguishable (Matsushima et al, 1991). Perhaps it would be advantageous to assay an aminopeptidase with many peptide substrates before choosing a name.

Enzymatic activity and diversity of aminopeptidase

Aminopeptidase N (APN; EC 3.4.11.2) is an extensively studied Cry receptor identified and isolated from several lepidopteran insects. It is a zinc-dependent metallopeptidase having a well-conserved HEXXHXW motif, the marker for zinc binding site (Taylor, 1993; Hooper, 1994) that catalyzes the removal of N-terminal, preferentially neutral residues from peptides. In the epithelial cells of mammalian kidney and intestine, aminopeptidase N is a type II membrane protein, anchored by an uncleavable N-terminal signal peptide sequence of approximately 60 amino acids and with a C-terminal extracellular domain (Kenny et al,

1987). In insects, however, APN is a type I membrane protein, anchored in the epithelium by a glycosyl-phosphatidyl-inositol (GPI) moiety located at the C terminus (Takesue et al, 1989 and 1992; and Garczynski and Adang, 1995). The differences in the mode of attachment and thus the orientation of the membrane anchored aminopeptidases in insect and mammals might account for the inability of B.t. toxins to interact with mammalian aminopeptidases. Mammalian and insect APNs are glycosylated proteins possessing N-glycosylation sites and O-glycosylation sites. APN serves as a receptor for B.t. δ-endotoxins and coronaviruses (Delmas et al, 1992; Yeager et al, 1992; and Tresman et al, 1996). Viruses and Cry toxins bind at a site distinct from the catalytic site as APN enzyme activity was not affected by their binding (Luo et al, 1996). In the course of this study, we also investigated the toxin binding site and the substrate binding site of the *Spodoptera litura* APN and observed that the functions of the two sites are not coupled or related in any way. We show that the binding of Cry1C toxin to the APN does not inhibit the binding of the substrate or the active site inhibitor to the enzyme-toxin complex (Kaur et al, 2007).

Multiple aminopeptidases have been isolated from different insects. For insects, the presence of multiple related aminopeptidases is important for hydrolysis of amino acids from peptides and their subsequent transport by amino acid transporter across the brush border membrane. The existence of multiple aminopeptidases has been explained by gene duplication mechanism (Chang et al, 1999; and Garner et al, 1999). Oltean et al (1999) carried out a dendrogram analysis of the previously described insect aminopeptidases and divided them into four homology groups. The differences in the susceptibilities of different insects toward the Cry toxins can partly be explained by the aminopeptidase diversity.

APN as a Cry-binding protein

The first lepidopteran aminopeptidase N to be discovered was a 120 kDa Cry1Ac receptor, observed on ligand blots of *M. sexta* BBMVs (Garczynski et al, 1991; and Knowles et al, 1991). It was demonstrated that Cry1Ac binding to this band was abolished by N-acetylgalactosamine (GalNAc) (Knowles et al, 1991), suggesting the presence of competing GalNAc moieties on the receptor itself (Sangadala et al, 1994). The protein was identified as the metalloprotease aminopeptidase N (Knight et al, 1994). Since the discovery of the *M. sexta* APN receptor (MsAPN-1), two other *M. sexta* APNs have been reported (Luo et al,

1996; Denolf et al, 1997). Isoforms of APN ranging in size from 105-170 kDa have been isolated from several lepidopteran insects including *L. dispar*, *P. xylostella*, *H. virescens*, *B. mori*, and *P. interpunctella*. Different APN isoforms in the midgut can interact differentially with various subtypes of the Cry toxins (Rajagopal et al, 2003). APNs have also been discovered in other insect organs like fat bodies (Budatha et al, 2007) in addition to midgut.

APNs have generally been purified from nonionic detergent, CHAPS-solubilized BBMV proteins by using a combination of toxin affinity chromatography and ion-exchange chromatography. The activity of purified APN protein has been shown to be inhibited by inhibitors such as amastatin and bestatin (competitive active site inhibitor) and metal chelators like EDTA (Luo et al, 1996 & 1999). Unlike mammals, insect APN has been demonstrated to be anchored to the membrane by a GPI anchor located at the C terminus. In 1996, Luo et al purified and characterized a Cry1C-binding 106 kDa protein from *M. sexta* BBMVs that was found to be distinct from a previously described 115 kDa Cry1Ac-binding APN (Knight et al, 1994; Sangadala et al, 1994; and Masson et al, 1995). Their results have shown that Cry1Ac and Cry1C toxins recognize functionally related, but structurally distinct isoforms of APN in *M. sexta* midgut. Hua et al (1998) cloned a cDNA encoding 110 kDa APN isozyme anchored on the brush border membrane of *B. mori* midgut. The 170 kDa aminopeptidase N (receptor A) purified from midgut of *H. virescens* bound to Cry1Aa, Cry1Ab and Cry1Ab toxins. Kinetic binding analyses using surface plasmon resonance showed that the purified APN contained two Cry1A toxin-binding sites; a higher-affinity site (K_d = 41 to 95 nM) and a lower-affinity site (K_d = 325 to 632 nM). Later, Oltean et al (1999) characterized two Cry1Ac toxin binding 130 kDa and 170 kDa APNs isolated from *H. virescens* BBMVs. The two APNs had the same N-terminal sequence but showed different molecular masses due to the difference in their extent of glycosylation. They cloned the cDNA corresponding to the 170 kDa APN. They also showed the inhibition of Cry1Ac toxin binding to the 170 kDa protein in the presence of GalNAc. These results are in agreement with results that show that GalNAc competes with Cry1Ac binding (Gill et al, 1995) but not with Cry1Aa and Cry1Ab binding in experiments performed with ligand blots, in binding assays and in SPR analyses (Luo et al, 1997; and Lee et al, 1996c) evaluated the binding of Cry toxins to APN purified from *L. dispar* BBMV. By ligand blotting experiment, they demonstrated that Cry1Ac recognizes 120 kDa APN while Cry1Aa and Cry1Ab recognize 210 kDa molecule in the *L. dispar* BBMV.

Expression of the cloned *apn* genes has been attempted in both *E. coli* and insect cell culture. Yaoi et al (1999) successfully expressed a 110 kDa *B. mori* aminopeptidase in *E. coli*. Though the protein was expressed in inclusion bodies, they were able to demonstrate its binding to Cry1Aa toxin. Thus raising concerns about the requirement of carbohydrate moiety for toxin binding to the receptor. There are reports in which aminopeptidase-encoding genes have been cloned and expressed in heterologous insect cell cultures. Denolf et al (1997) expressed a *P. xylostella* 105 kDa APN in *Sf*9 cells which was glycosylated and anchored in the membrane of the cells by a GPI anchor but it did not show binding to Cry toxins. Luo et al, (1999) successfully expressed the 120 kDa APN cDNA (APN1a) from *M. sexta* midgut in *Sf*21 cells. But the expressed APN did not exhibit binding towards the Cry1Ac toxin, inspite of being glycosylated. Simpson and Newcomb (2000) could demonstrate the binding of the surface expressed *Epiphyas postvittana* APN to Cry toxins by ligand blot assays but failed to show toxin binding to the intact cells. Agrawal et al (2002) successfully cloned and expressed a 108-kDa APN from *Spodoptera litura* in *Sf*21 cells using baculovirus expression system. Intact *Sf* cells expressing the recombinant APN protein were shown to bind to the bioactive Cry1C toxin. The high expression levels of APN obtained in *Sf*21 cells provided with a source of protein for structural and functional characterization and facilitated further analysis of B.t. toxin-APN interactions.

The SPR study of Cry toxins with purified receptors showed that Cry1Aa, Cry1Ab and Cry1Ac could bind to *M. sexta* APN (Masson et al, 1995). Stoichiometric analysis indicated that Cry1Ac bound to two sites on the APN, while Cry1Aa and Cry1Ab bound only one APN site thus Cry1Aa/b binding fit to a one-site model and Cry1Ac binding fit to a two-site model.

L. dispar APN is unique from *M. sexta* and *H. virescens* APNs because it binds Cry1Ac, but not Cry1Aa or Cry1Ab toxins. With SPR, the binding stoichiometry of Cry1Ac and *L. dispar* APN has been approximated to be 1:1 (Valaitis et al, 1997). A sequential-binding mechanism has been proposed in the interaction of Cry1Ac with APN (Jenkins et al, 2000) which suggests that Cry1Ac domain III initiates docking through recognition of an N-acetylgalactoseamine moiety on APN facilitating the subsequent interaction of domain II loop regions with a higher affinity region in this receptor.

APN as a mediator of Cry toxin susceptibility

Multiple toxin-binding proteins have been identified in BBMVs of several insects, but it is important to identify the ones that serve as functional receptors in which binding leads to pore formation. Aminopeptidase N genes have been isolated from the midgut of a variety of insects and several in vitro as well as in vivo studies have authenticated the role of APN in B.t. pathogenesis. Voltage clamping assays using reconstituted mixtures of purified APN and Cry toxin in membrane vesicles have shown decrease in the amount of toxin required to induce $^{86}Rb^{+}$ efflux (Sangadala et al, 1994; and Luo et al, 1997) in the presence of the APN receptors. A laboratory-selected *Spodoptera exigua* colony resistant to Cry1C did not express APN1, suggesting that the lack of APN production correlated with resistance to Cry1C toxin (Herrero et al, 2005). Also, inhibition of APN1 production in *S. litura larvae* by dsRNA interference showed that insects with low APN levels became less susceptible to Cry1C toxin (Rajagopal et al, 2002). Finally, heterologous expression of *M. sexta* APN1 in midguts and mesodermal tissues of transgenic *Drosophila melanogaster* caused sensitivity to Cry1Ac toxin (Gill and Ellar, 2002). All these studies imply that APN serves not only as a binding molecule for the Cry toxins but also influences their toxicity.

The possibility of existence of alternative receptors in the gut of susceptible larvae was put forth by Carroll et al (1997) who demonstrated that APN in *M. sexta* midgut is predominantly located in the posterior midgut while binding of Cry toxins can take place throughout the midgut. This indicates that receptors other than APN are functional in binding Cry toxins in the anterior region of the gut.

2.4.2.b.ii CADHERIN

Cadherin proteins represent a large family of glycoproteins that are responsible for inter-cellular contacts. They are transmembrane proteins with a cytoplasmic domain and an extracellular ectodomain with several cadherin repeats, 12 in the case of Bt-R1 (Vadlamudi et al, 1995). The ectodomain contains calcium-binding sites, integrin interaction sequences and cadherin binding sequences. SPR experiments have shown that the binding affinity of monomeric Cry1A toxins to the *M. sexta* Bt-R1 is in the range of 1 nM (Vadlamudi et al, 1995), while that of APN is in the range of 100 nM (Jenkins and Dean, 2000).

Like APN, several cadherin molecules have been identified and demonstrated to bind to the active toxin molecule in different target insects. With the use of Cry1Aa-Cry1Ac hybrids, it has been demonstrated that domain III substitutions direct the binding of these toxins to different *L.dispar* midgut receptors. Hybrid toxins which had the residues 451 to 623, comprising essentially domain III, from CryIAc toxin exhibited strong binding to purified aminopeptidase-N and 120 kDa brush border membrane protein. In contrast, the hybrid toxins which had the residues 451 to 623 from CryIAa toxin failed to bind to aminopeptidase-N, but did bind to another receptor, a 210 kDa protein (Lee et al, 1995b). Ligand blotting experiments have demonstrated that CryIAc recognized a 120-kDa peptide (APN), while CryIAa and CryIAb recognized a 210-kDa molecule in *L. dispar* BBMV. In contrast, CryIAa and CryIAb bound to both the 120- and 210-kDa molecules in *M. sexta* BBMV, while CryIAc recognized only the 120-kDa peptide (Lee et al, 1996c). A 180 kDa protein with sequence similarity to Bt-R_1 has been purified from *B. mori* and shown to have high affinity for Cry1Aa on ligand blots (Nagamatsu et al, 1997 & 1998).

The role of toxin-receptor interaction has been particularly well described in *M. sexta*. In this insect, at least two Cry1A-binding proteins, a CADR protein (Bt-R1) and a GPI-anchored APN have been described as receptors (Vadlamudi et al, 1995; and Knight et al, 1994). Bt-R_1, a 210 kDa protein showing 30-60% similarity to proteins in the cadherin superfamily has been purified and cloned from *M.sexta* (Vadlamudi et al, 1995). The receptor bound to Cry1Aa, Cry1Ab and Cry1Ac (Francis and Bulla, 1997). The Bt-R_1 cDNA has been expressed in both insect and mammalian cell cultures and shown to display high-affinity binding for Cry1A toxins (Keeton and Bulla, 1997). The interaction of Cry1A toxins with the Bt-R1 receptor is rather complex involving at least three binding epitopes in the two molecules. Using a synthetic phage-antibody library, Gomez et al (2001, 2002a) characterized a scFv antibody (scFv73) that bound to domain II loop 2 (β6- β 7 loop) of Cry1A toxins. This antibody inhibited binding of Cry1A toxins to Bt-R1 but not to APN. Sequence analysis of the CDR3H region of scFv73 led to the identification of an eight amino acid epitope in Bt-R1 CADR repeat 7 (869HITDTNNK876) involved in binding to domain II loop 2 of Cry1A toxins (Gomez et al, 2001 & 2002a). Additionally, another binding epitope in Bt-R1 CADR repeat 11 (1331IPLPASILTVTV 1342) that interacts with domain II loop α8 (α8a-α8b loop) and loop 2 of Cry1Ab toxin has been identified (Gomez et al, 2003). Finally, a third region in CADR repeat 12 of Bt-R1 (residues 1363-1464) involved in Cry1Ab interaction and toxicity was reported by Hua et al (2004). In the case of *Heliothis virescens*, site-directed mutagenesis

identified residues 1422-1440 of CADR as the cognate binding epitope of Cry1Ac domain II loop 3 (β10-β11 loop) (Xie et al, 2005). Similar to other lepidopteran cadherins that bind B.t. toxins, BtR from *Pectinophora gossypiella* has at least two Cry1Ac-binding domains in cadherin-repeat regions 10 and 11, which are immediately adjacent to the membrane proximal region. However, unlike cadherins from *Manduca sexta* and *Bombyx mori*, toxin binding has not been seen in regions more distal from the membrane proximal region. The finding that both the protoxin and activated toxin forms of Cry1Ac bound to recombinant BtR fragments, suggests that Cry1Ac activation may occur either before or after receptor binding (Fabrick et al, 2006).

It has been observed that the most frequent mechanism of resistance to Cry toxins involves a change in receptor binding (Ferre and van Rie, 2002). In the case of the laboratory selected *H. virescens* Cry1Ac-resistant line YHD2, it was shown that a single mutation was responsible for 40-80% of Cry1Ac resistance levels and that this was linked to a retrotransposon insertion in the CADR gene (Gahan et al, 2001). Also, characterization of CADR alleles in field derived and laboratory selected strains of *Pectinophora gossypiella* and *Helicoverpa armigera* has revealed different mutated CADR alleles that were associated with Cry1Ac resistance (Morin et al, 2003; and Xu et al, 2005).

2.4.2.b.iii ALKALINEPHOSPHATASE

Membrane-bound alkalinephosphatase (ALP) from *B. mori* and *M. Sexta* are attached to the brush border cell membrane by a GPI anchor (McNall and Adang, 2003; Takeseue et al, 1989; and Ikezawa et al, 1976). Specific interactions between Cry1Ac and ALPs under native conditions have resulted in inhibition of phosphatase activity in *M. sexta* (Sangadala et al, 1994) and *H. virescens* (English and Readdy, 1989). However, the potential role for alkaline phosphatases in Cry1Ac intoxication has not been addressed directly. In the case of *H. virescens* resistant line YHD2, part of the Cry1Ac-resistant phenotype is due to mutations that lower the production of the GPI-anchored ALP receptor (Jurat-Fuentes and Adang, 2004). In *M. sexta*, proteomic analysis of BBMV Cry1Ac binding proteins has also revealed ALP as a putative receptor molecule (McNall and Adang, 2003). As APN and ALP are both GPI-anchored proteins, these proteins have been proposed to be selectively included in lipid rafts

that are conceived as spatially differentiated liquid-ordered microdomains in cell membranes. Lipid rafts are detergent-resistant membranes (DRM) enriched in glycosphingolipids, cholesterol and in GPI-anchored proteins and are proposed to be involved in signal transduction, sorting and trafficking of plasma membrane proteins (Munro, 2003). They also function as pathogen portals for different viruses, bacteria and toxins (Rosenberger et al, 2000; and Cabiaux et al, 1997). Disruption of lipid rafts membrane microdomains has been shown to abolish pore formation of Cry1A toxins (Zhuang et al, 2002). The interaction of pore-forming toxins with lipid rafts could result in additional cellular events, including toxin internalization, signal transduction and cellular response.

2.4.2.b.iv GLYCOLIPIDS

Glycolipids are another important class of putative Cry toxin receptor. While an interaction between glycosphingolipids and Cry toxins was first reported in 1986 (Dennis et al, 1986) the importance of this receptor class was demonstrated only recently using the nematode *Caenorhabditis elegans* (Griffitts et al, 2005). Chemically mutagenized *C. elegans* strains were selected for their resistance to Cry5Ba (Griffitts et al, 2003; and Marroquin et al, 2000) and using forward genetics, four genes were identified that could restore toxin susceptibility. These genes were named the *bre* genes for *B. thuringiensis* toxin *re*sistant (Griffitts et al, 2003 & 2001). The first gene to be characterized, *bre*-5, was found to be a member of the β1, 3-galactosyltransferase family and encoded a protein most similar to the *Drosophila* protein BRAINIAC (Griffitts et al, 2001). Subsequently, *bre-2*, *bre-3*, and *bre-4* were characterized and found to encode glycosyltransferases that showed no similarity to putative toxin receptors previously identified in insects (Griffitts et al, 2003).

Experiments showing that fluorescently labeled Cry5Ba was readily endocytosed by wild-type animals but not by *bre* mutants suggested that the glycosyltransferases synthesized a component necessary for the toxin to interact with intestinal cells (Griffitts et al, 2003 & 2001). Additionally, the finding that *bre* mutants failed to produce certain ceramide-based glycolipids that specifically bound to Cry5Ba demonstrated that the *bre* genes function to produce a glycosphingolipid toxin receptor (Griffitts et al, 2005). Glycolipids extracted from the midguts of *M. sexta* have also been shown to bind to Cry1Aa, Cry1Ab, and Cry1Ac. However, the importance of this interaction in mediating toxin susceptibility has not been reported yet and remains to be explored.

2.4.2.b.v OTHER RECEPTORS

Some reports have suggested that Cry toxins may bind to two additional types of receptors. The first, a 270-kDa glycoconjugate has been isolated from *L. dispar* and is called BTR-270. It has been shown to bind strongly to Cry1Aa, Cry1Ab, and Cry1Ba, weakly to Cry1Ac, and not at all to Cry1Ca, Cry2Aa, Cry2Ba, and Cry3Aa (Valaitis et al, 2006). The receptor has thus far been difficult to characterize; however, it appears to be a highly glycosylated anionic protein that may be a component of the brush border membrane glycocalyx. The second receptor, named P252, has been isolated from a Triton X-100-soluble fraction derived from *B. mori* BBMV (Hossain et al, 2004). The protein has a molecular mass of 252 kDa; however, a 985-kDa homo-oligomer has also been detected by gel filtration chromatography. The purified receptor has been shown to bind Cry1Aa, Cry1Ab, and Cry1Ac under nondenaturing conditions but GalNAc did not inhibit toxin binding. Two internal peptides from this protein have been found to have significant matches (12/15 and 15/15 identical residues) (Pigott and Ellar, 2007) with a recently described protein called chlorophyllid A-binding protein (ChBP) (Mauchamp et al, 2006). ChBP protein has been identified in the midgut of *B. mori* and characterized by its ability to bind to a derivative of chlorophyll. It is an unusual member of the lipocalin family (Flower, 1996; and Flower et al, 2000) and contains 15 prototypic lipocalin domains but whether ChBP and P252 are the same protein remains to be confirmed.

2.4.2.c Determinants of APN-Toxin Binding

The major concern regarding the use of B.t.-crops is the threat of generation of insect populations resistant to Cry toxins. Different mechanisms of insect resistance using laboratory selected insect populations have been characterized (Oppert et al, 1994; Gahan et al, 2001; and Darbaux et al, 2002). Among them, the most frequent mechanism has been the selection of insects affected on receptor-toxin interaction. Therefore, it is crucial to determine the molecular basis of Cry toxin-receptor interactions since this knowledge could be useful in designing new toxins that could overcome the threat of resistant insect populations under natural conditions.

2.4.2.c.i Toxin Determinants

Domain II

The prediction that domain II is involved in receptor binding (Ge et al, 1989; and Li et al, 1991) has led to extensive substitution of loop residues in this domain in Cry3A, Cry1A and Cry1C by mutagenesis. Studies on the effects of mutations in sequences encoding domain II loop regions of selected Cry toxins have indicated that mutations may have either a negative or positive effect on binding and toxicity and that mutation in different loop regions, sometimes involving the same type of amino acid residue, can have a different effect on binding. Furthermore, either binding affinity (as measured by competition binding) or irreversible binding may affect toxicity, and for a few mutant proteins one of these parameters may be positive (increased affinity) while the other may be negative (increased dissociation), with an overall negative effect on toxicity. It is apparent that the same mutation in a toxin can have quite different results on different insects.

A triple substitution (R345A, Y350A and Y351A) in loop 1 of Cry3A reduced binding but not toxicity of this toxin to its susceptible insect. In contrast, single-substitution mutants, N353A and D354A in loop 1, completely lost binding capacity and toxicity in *Tenebrio molitor* (Wu and Dean, 1996). Mutations located in loop 1 of Cry1Aa and Cry1Ab toxins demonstrated that the residues in this region were essential for binding to the midgut brush border membranes of *B. mori* & *M. sexta* and *H. virescens* respectively. Smith and Ellar (1994) have shown that mutations in loops 1 and 2 of Cry1C toxin could modulate toxicity and specificity.

In *B. mori*, deletion of a major portion of loop 2 (365 LYRRIL371) caused a substantial loss in binding affinity and toxicity of Cry1Aa (Lu et al, 1994). However, alanine substitution of only three residues (368RRP370) in loop 2 of Cry1Ab toxin led to a substantial reduction in binding affinity for BBMVs and toxicity in *M. Sexta* and *H. virescens* (Rajamohan et al, 1996). In addition to this, loop 2 has also been implicated in irreversible binding of the toxin to the receptor molecule. Substitution mutations have shown that F371 and G374 residues affect the irreversible binding of Cry1Ab toxin to midgut membrane vesicles of *M. sexta* (Rajamohan et al, 1995). Based on these findings, it has been proposed that the two arginine residues at the first position of the loop affect the earliest toxin-receptor interactions (initial binding) whereas the remaining hydrophobic residues affect irreversible binding. These

studies have proposed that positive charges might be required to orient the toxin to the receptor, and that binding is strengthened by a hydrophobic aromatic side-chain after initial recognition of the receptor.

Substitution mutations in loop α-8 in Cry1Ac (R281A and R289A) resulted in loss of toxicity which correlated with reduction in binding affinity to APN receptor suggesting the involvement of this loop in initial receptor recognition (Lee et al, 2001). While it has been shown that binding of loop 2 of Cry1Ab with Bt-R1 (region 865-876) was determined by hydropathic complementarily (Gomez et al, 2002) the nature of binding between loop α-8 and another Bt-R1 region (1331-1342) is not clear.

Deletion of hydrophobic residues of loop 3 of Cry1Ab and Cry1Aa toxins reduced their toxicity to the susceptible hosts *M.sexta* & *H.virescens*, and *B.mori* & *M.sexta* respectively (Rajamohan et al, 1996). Substitution studies have suggested that the loop 3 residues of these toxins establish hydrophobic interactions with the receptor molecule, and mutations at these hydrophobic residues affect initial binding. However, loop 3 residues of Cry3A have been shown to affect irreversible binding of the toxin to membrane vesicles of *T. molitor* (Wu and Dean, 1996).

Domain III

One of the several speculated roles of domain III is its involvement in receptor binding. Several groups (Ge et al, 1989; and Schnepf et al, 1998) have suggested a role for domain III of Cry1Ac in *H. virescens* specificity. Aronson et al (1995) have mutated a hypervariable region of domain III (residues 500-509) of Cry1Ac and shown that mutations S503A and S504A resulted in lower toxicity to *M. sexta*, with a corresponding decrease in binding to BBMV proteins on ligand blots. Lee et al (1995) analyzed homolog scanning mutants that exchanged domain III between Cry1Aa and Cry1Ac and demonstrated that hybrid proteins containing the Cry1Aa domain III bound a 210-kDa receptor while hybrid proteins containing the Cry1Ac domain III bound a 120-kDa receptor in *L. dispar*. Subsequently, it was shown that GalNAc could completely disrupt this interaction (Jenkins et al, 2000).

Domain switching experiments have also suggested a role for Cry1Ab domain III in binding to *S. exigua* (de Maagd et al, 1996). Burton et al (1999) mutated several residues on the outer

sheet of domain III (within and around β-strand 16) and observed that the greatest decrease in receptor binding was with the triple N506D Q509E Y513A mutant. The interaction between Cry1Ac and *M. sexta* APN at this site was found to be GalNAc dependent. Similar results were obtained by Jenkins et al (1999) who showed that mutants with alanine substitutions at Q509, R511, Y513, or 509QNR511 failed to bind to purified *M. sexta* APN and bound to purified *L. dispar* APN with lower affinity (Jenkins et al, 2000). It is hypothesis that ligand binding at domain III may increase the mobility of the pore-forming domain, possibly triggering a conformational change leading to membrane insertion (Li et al, 2001). Consistent with this hypothesis, Pardo-Lo´pez et al (2006) have recently reported that GalNAc binding to an oligomeric form of Cry1Ac enhanced toxin membrane insertion.

Domain II/III interface

A third region that has been proposed to act as an APN binding site was first characterized for Cry1Aa (Atsumi et al, 2005). This region has been identified at the interface between domain II and III and shown to interact with the 64-amino-acid toxin-binding fragment of class 1 *B. mori* APN (Yaoi et al, 1999). The putative binding site was identified by mapping the epitopes of two monoclonal antibodies that bound to Cry1Aa and blocked its interaction with APN. Both antibodies were shown to bind to the outer sheet of domain III at distinct but overlapping sites that included residues 508STLRVN513 and 582VFTLSAHV589. Additional studies showed that the APN binding site was close to, but did not include, this region. The interface between domain II and domain III was proposed as a candidate APN binding site based on its proximity to the antibody binding sites and on the presence of conserved residues in this region that are common to Cry toxins with specificity for class 1 *B. mori* APN. Recently, Gomez et al (2006) have shown that a similar region in Cry1Ab may interact with APN from *M. sexta*.

2.4.2.c.i ***APN Determinants***

While many reports have identified APNs as Cry toxin-binding proteins, little is known about how the molecules interact. As discussed earlier, GalNAc appears to be an important determinant of the Cry1Ac-APN interaction; however, the nature and position of the glycan structure involved in binding have not yet been determined. Recently, Stephens et al (2004)

carried out an extensive characterization of the N-linked oligosaccharides by mass spectrometry. Three of the four glycosylation sites (N295, N623, N752) were found to be linked to highly fucosylated glycans with unusual difucosylated cores. The fourth site (N609) was glycosylated with the paucimannosidic glycan common to insect glycoproteins (Tomiya et al, 2004). However, GalNAc was not identified as a component of any of the glycans supporting the theory that Cry1Ac interacts with APN at its threonine-rich C-terminal stalk predicted to be O glycosylated with mucintype (GalNAc-α-O-Ser/Thr) glycans (Knight et al, 2004 & 1994).

Attempts have also been made to locate putative toxin binding sites in APNs believed to interact with toxin in a glycan independent manner. In regard to this, a region of 63 residues (I135-P198) involved in Cry1Aa binding has been identified in *B. mori* APN1 (Nakanishi et al, 2002). Domain swapping studies between Cry1Ac and Cry1Ab toxins have suggested that domain III of Cry toxins is involved in the APN-Cry interactions (de Maagd et al, 1999). In another study, (Yaoi et al, 1999) binding was assessed using fragments of APN expressed as GST fusion proteins in *E. coli* and the smallest fragment that retained toxin binding affinity corresponded to APN amino acids residues Ile135 to Pro198. More recently, using monoclonal antibodies that competed binding of Cry1Aa with *Bombyx mori* APN, domain III residues (508STRVN513 and 582VFTLSAHV589) of Cry1Aa were shown to be involved in binding to this I135-P198 APN fragment (Atsumi et al, 2005).

2.4.3 Pre-Pore Formation

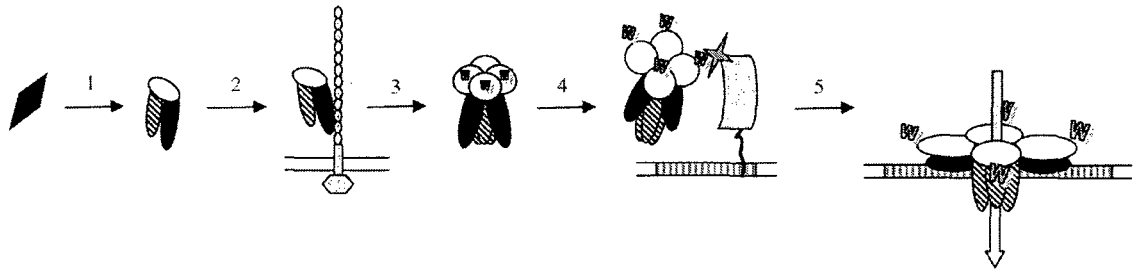
Several pore forming toxins have been proposed to form a soluble oligomeric structure before membrane insertion. In the case of Cry1Ab toxin, binding of this toxin to *M. sexta* Bt-R1 has been shown to promote an additional proteolytic cleavage in the N-terminal end of the toxin (helix α-1) facilitating the formation of a pre-pore oligomeric structure that is believed to be important for insertion into the membrane and for toxicity (Gomez et al, 2002b; and Rausell et al, 2004a). Incubation of Cry1Ab protoxin with the single chain antibody scFv73 that mimics the CADR receptor or with the toxin-binding peptides of Bt-R1 (CADR repeats 7 and 11), and treatment with *M. sexta* midgut juice, has resulted in toxin preparations with the formation of a 250 kDa oligomer that lacked the helix α-1 of domain I (Gomez et al, 2002b and 2003). It has been reported that oligomeric structures of Cry1Ab and Cry1Ac increase

their binding affinity to the APN receptor to 100-200 fold (Gomez et al, 2003; and Pardo-Lopez et al, 2006). Intrinsic fluorescence of tryptophan residues and analysis of membrane permeability using black lipid bilayers have shown the oligomer, in contrast to the 60 kDa monomer, to be membrane insertion competent (Rausell et al, 2004a). Kinetic characteristics of oligomeric structures of Cry1Ab have been observed to be different from the monomeric Cry1Ab toxin. Firstly, pore formation by pure oligomer preparations has been observed at much lower toxin concentrations than the monomeric toxin and secondly, oligomeric Cry1Ab showed stable channels that had a high open probability in contrast to the monomeric toxin that showed an unstable opening pattern (Rausell et al, 2004a). The formation of Cry oligomeric structures has been demonstrated for Cry1Aa, Cry1Ab, Cry1Ca, Cry1Da, Cry1Ea, Cry1Fa and Cry3 toxins (Gomez et al, 2002b; Rausell et al, 2004a & 2004c; and Munoz-Garay et al, 2006). In all cases, the Cry toxin samples containing oligomeric structures have been correlated with high pore activity, in contrast to monomeric samples that showed marginal pore-formation activity, supporting the hypothesis that oligomer formation is a necessary step in the mechanism of action of Cry toxins.

2.4.4 Membrane Insertion

After exposure of BBMV to Cry1A toxins, these toxins have been found associated with lipid rafts microdomains and it has been reported that the integrity of these microdomains is essential for in vitro Cry1Ab pore-forming activity (Zhuang et al, 2002). Lipid rafts are detergent-insoluble lipid microdomains enriched in cholesterol and sphingolipids, and GPI-anchored proteins (Simons and Toomre, 2000). The Bt-R1 receptor is located in soluble membrane in contrast with APN and ALP receptors, which are attached to the membrane by GPI anchors, and are preferentially partitioned into lipid rafts (Zhuang et al, 2002). Lipid rafts have been implicated in membrane and protein sorting, and in signal transduction (Simons and Toomre, 2000). Interaction of different bacterial PFT with receptors located in lipid rafts has been shown to be a crucial step in the oligomerization and insertion of PFT into the membrane (Cabiaux et al, 1997). In the case of Cry1A toxins, the APN receptor has been implicated in Cry1A toxin insertion, since cleavage of APN by phosphatidylinositol phospholipase C treatment, which cleaves out the GPI-anchored proteins, substantially decreased the levels of Cry1Ab oligomer in insoluble membranes and reduced drastically the pore-formation activity of the toxin (Bravo et al, 2004). In addition, APN incorporation into

lipid bilayers has been shown to enhance Cry1Aa pore-formation activity (Schwartz et al, 1997). Using tryptophan fluorescence analysis, structural changes after binding of the oligomeric Cry1Ac toxin to the APN receptor have been studied by analyzing the binding of GalNAc to the Cry1Ac toxin (Pardo-Lopez et al, 2006). The in vitro interaction of GalNAc with oligomeric Cry1Ac has been demonstrated to induce a conformational change in the toxin which enhanced its insertion into lipid membranes indicating that the interaction of the pre-pore oligomer of Cry1A toxins with APN is important for facilitating membrane insertion. It has been proposed that proteins must partially unfold to facilitate membrane insertion and channel formation. In the case of PFT active against mammalian cells, unfolding is triggered by acidic pH (Parker and Feil, 2005). Interestingly, lepidopteran and dipteran insects have a basic pH (up to pH 11) in their midgut lumen (Dow, 1986). Unfolding analysis of pure Cry1Ab structures at different pHs has demonstrated that the molten globe state of the prepore complex was induced by alkaline pH (Rausell et al, 2004b). These analyses also showed that the pre-pore and membrane inserted oligomer, had a more flexible conformation than the monomeric toxin (Rausell et al, 2004b). Although not proven, it's may be possible that the conformational change observed after interaction of the pre-pore oligomer with APN could be related to molten globule state since, this interaction facilitated membrane insertion (Pardo-Lopez et al, 2006). Additionally, in the membrane-inserted pore, only domain I has been shown to be protected from heat denaturation, suggesting that it may be inserted into the membrane in contrast to domains II and III (Rausell et al, 2004b). Finally, the alkaline pH has been found to induce a looser conformation of the membrane inserted domain I that is important for an active channel formation (Rausell et al, 2004b). Based on the data described above, a model involving sequential interaction of Cry1A toxins with Bt-R1 and APN receptor molecules has been proposed. It is believed that the interaction of monomeric Cry1A toxins with Bt-R1 facilitates the formation of a pre-pore oligomeric structure that gains binding-affinity to APN, the pre-pore toxin binds APN, a conformational change occurs and a molten globule state of the toxin is induced, the pre-pore is inserted into lipid rafts inducing pore formation and cell swelling (Bravo et al, 2004; Figure 6).



Figure, 6. Sequential interaction of Cry toxins with different receptor molecules in lepidopteran larvae. (1) Solubilization and activation of the toxin; (2) binding of monomeric Cry toxin to the first receptor (CADR or GCR), conformational change is induced in the toxin and α-helix 1 is cleaved; (3) oligomer formation; (4) binding of oligomeric toxin to second receptor (GPI-APN or GPI-ALP), a conformational change occurs and a molten globule state of the toxin is induced; (5) insertion of the oligomeric toxin into lipid rafts and pore formation. (Bravo et al, 2007)

2.5 Ion Channel Activity

The ion channel activity of Cry toxins has been explored by a wide variety of techniques like voltage clamping using insect midgets, BBMV selling assays, insect tissue culture cells, phospholipid vesicles, and lipid bilayers. Knowles and Ellar (1987) used CF-1 cells and proposed a "colloidal osmotic lysis" theory as a model for the cytolytic activity of Cry toxins. According to their proposal, the Cry toxins make small holes in the midgut membrane, leading to the influx of water and ions, resulting in cell swelling and eventually lysis. The toxin has been studied with complete proteins, with domain I in isolation, with synthetic peptides mimicking particular a-helices, and with mutants that disrupt ion channel function (Gazit & Shai, 1993; and English et al, 1995). The ion channels of Cry toxins have been shown to be affected by several factors. For Cry1C the channel activity has been shown to be pH dependent (Schwartz et al, 1993); at low pH it formed anion-selective channels and at high pH it formed cation-selective channels. The physical basis of pH dependent selectivity might be related to the observation that α-helical content changes radically with pH (Choma & Kaplan, 1990; and Feng & Becktel, 1994). It is speculated that pH can alter the pitch or arrangement of α-helices of domain I and change the nature of the ion channel. The channel activity of Cry toxins have also been shown to be affected by insect midgut membrane receptor proteins. Studies with synthetic peptides corresponding to α-helix 5 and α-helix 7 of Cry3A and Cry1Aa have concluded that α-helix 5 was involved in formation of the ion

channel while α-helix 7 acted as a scaffolding helix. Another study involving membrane permeation studies with Cry1Ac revealed that the α4-loop-α5 hairpin was extremely active compared with the isolated helices or their mixtures, indicating the complementary role of the two helices and the need for the loop for efficient insertion into membranes (Gerber & Shai, 2000). Spectrofluorometric studies have revealed that α4-loop-α5 hairpin inserts in the membrane to form pores in which α4 and α5 helices line the lumen of the channel and α5 helix also participates in the oligomerization of the toxin. However, channels formed by the α5 helix and domain I are different from those formed by whole toxins. Additionally, although domain I contains ample information to generate ion channels, such channels are significantly different than those formed by native toxin. Thus, other domains may also play an indirect role in channel formation either by assisting in structural conformation, in oligomerization, or in regulation of channel activity. Domain III has also been reported to play a role in ion channel activity. On analyzing the alternating arginine region in β-sheet 17 (conserved block 4), Chen et al (1995) have observed that mutations in central arginines caused structural instability in Cry1Aa whereas conservative substitutions of the outermost arginine caused reduction of toxicity due to alteration in the ion channel activity. It has been suggested that toxin induced leakage of K^+ ions into the cell might result in an immediate loss of H^+ and a change in cytoplasmic pH from 7.1 to 9.5 (Wolfersberger, 1992). The resulting reduction of the apical membrane potential from 180 to 30mV could cause collapse of the transepithelial membrane potential and disruption of cell physiology. The contradictory results concerning the selectivity and size of the pore have reflected the adaptability of the toxin to different physiological conditions which exists in its functional environment. In the alkaline midgut, the toxin may function as a cation channel (Schwartz et al, 1993), taking advantage of the large K^+ gradient that exists in some insect midgut environments. As the pH falls due to cell lysis or leakage, the toxin may function as an anion channel, further wounding the epithelial cells. In large amounts the Cry protein may form very large leakage pores resulting in cell lysis and disruption of the midgut epithelium.

2.6 Pros and Cons of Current Pore Formation Models

Numerous proposals have been made regarding the mode of action of the Cry toxins, and most have included models. The current mechanism of insertion of the toxin into the membrane is conjectural. The hydrophobic surfaces of the six outer amphipathic helices

surrounding a central α-helix 5 face inwards in the toxin structure. Since the helices have an opposite arrangement, a conformational change in the toxin is required for the insertion of the domain I into the membrane and pore formation. Li et al (1991) have proposed that α-helices 4 and 5 penetrate the membrane while the remainder of α-helices remains on the surface (the umbrella model). Hodgman and Ellar (1990) have proposed that α-helices 5 and 6 flip out of domain I and penetrate the membrane as a "penknife" ("the penknife model"). Both the well known models assume that binding of the toxin to its receptor triggers the conformational change needed for insertion but that the receptor does not form part of the pore (Knowles and Dow, 1993; Knowles, 1994). Additionally, the role of domain III has been largely ignored in the above proposed models.

Site-directed mutagenesis studies have indicated that the residues of the loop between α-helices 4 and 5 affect penetration (Chen et al, 1995) but that the residues in the loop between α-helices 5 and 6 do not (Hussain et al, 1996). These results argue against the penknife model. Furthermore, the insensitivity of the inserted toxin to a variety of proteases and monoclonal antibodies argues against both of these models. Dean et al (1996) have presented an alternative model that suggests that the whole of domain I might penetrate the membrane. Though there are many theories, the ion channel is believed to involve at least residues from α-helices 5 and 7 from domain I, and β-sheet 17 from domain III. The determination of the structural changes involved in membrane insertion is likely to contribute to the understanding of the mode of action of the insecticidal Cry toxins and of the final structure of the membrane-inserted channel.

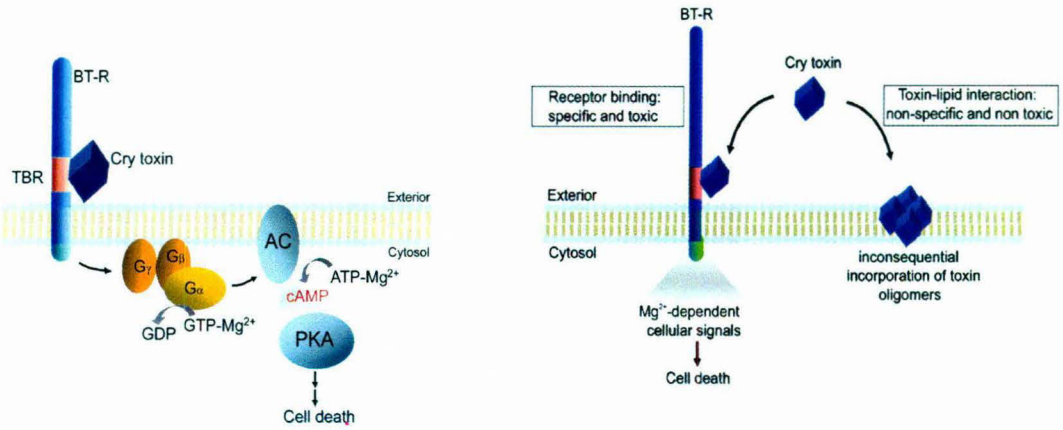
2.7 The Zhang Model of Cry induced toxicity

Particular ligand-receptor complexes are known to generate specific cellular signals essential to differentiation, proliferation, pathogen recognition, immune response, and cell death (Gilman, 1987 and Juliano, 2002). Interestingly, many pathogenic organisms and their toxins target host cell receptors, the consequence of which is altered signaling events that lead to aberrant activity or cell death (Finlay & Cossart, 1997; Fiorentinin et al, 2003; and Fivaz et al, 2001).

Cytotoxicity and cell death have long been considered a direct result of univalent binding of a Cry toxin monomer to its respective cadherin receptor (Zhang et al, 2005). Impeding the toxin-receptor interaction by receptor modification has been linked to the development of resistance to Cry toxins (Gahan et al, 2001). However, recent studies have revealed that neither resistance nor cytotoxicity can be explained solely by toxin binding. For example, both the number of Cry toxin receptors and the affinity of toxin to receptor have been shown to be similar in the brush border membrane vesicles isolated from resistant and susceptible European corn borer larvae. Also, removing Mg^{2+} by EDTA completely blocked Cry1Ab toxin induced cell death of cabbage looper cells but did not interfere with toxin-receptor binding (Zhang et al, 2005). Evidently, the interaction of toxin with the receptor is pre-requisite, but not sufficient to induce cell death.

Until very recently, Cry proteins were believed to be pore forming toxins that kill cell by osmotic lysis. Changes in membrane permeability were shown to co-relate with the incorporation of Cry toxin oligomers into lipid bilayers rafts and brush border membrane vesicles (de Maggd et al, 2003; and Schnepf et al, 1998). However, studies of mutated Cry toxin proteins have shown that neither the toxin oligomer complex nor commensurate changes in membrane vesicle permeability correlated directly with toxicity (Kumar et al, 1999; Luo et al, 1999; and Vachon et al, 2004). Interestingly, Cry toxin oligomers also were incorporated into the cell membrane of non susceptible cabbage looper cell and were carried by the cells for several generations with no adverse effect. Apparently, toxin action is much more complicated than simply osmotic lyses. Recently, Zhang et al (2005) used a cell based system to reveal that Cry1Ab toxin formed oligomeric complexes in the membranes of cell expressing BT-R_1 as well as in those of cells devoid of receptor. Toxin oligomers integrated into cell membrane did not produce lytic pores and did not kill the cells whereas monomeric Cry 1AB toxin specifically bound to BT-R1, activating a Mg^{2+} dependent cellular signaling pathway that was believed to lead to necrotic cell death. Such binding was demonstrated to provoke cell death in insect cells by activating signaling pathway involving simulation of the stimulatory G protein A-subunit ($G_{\alpha s}$) and adenylyl cyclase (AC), increased cyclic adenosine monophosphate (cAMP) levels, and activation of protein kinase A (PKA). Activation of the (AC/PKA) signaling pathway is thought to initiate a series of cytological events that include membrane blebbing , appearance of nuclear ghosts, and cell swelling followed by cell lysis (Zhang et al, 2006). Based on above findings, a new model of Cry toxin action has recently been proposed (Figure, 7). That Cry toxins of B.t. aggravate critical intracellular signaling

pathways through receptor-coupled interactions might have implications in insecticide and drug discovery (Fishman et al, 2005).



Figure, 7. Zhang model for Cry toxin action. According to the model, there are two kinds of interaction between toxin and cell. The first is a nonspecific toxin-lipid interaction, mediating assembly of Cry toxin molecules as oligomers and their insertion into membrane. The membrane-incorporated oligomer complex does not form lytic pores in the membrane and has no toxic effect on cells. The second kind is specific interaction between Cry toxin and the cadherin receptor BT-R, which mediates cytotoxicity. Binding of toxin to receptor brings about cell death through activation of a Mg^{2+}-dependent signaling pathway downstream of the toxin-receptor interaction. Cry toxin binds to BT-R and stimulates G protein and AC, which promotes production of intracellular cAMP. In turn, PKA activation destabilizes the cytoskeleton and ion channels, leading to cell death. (Zhang et al, 2005 & 2006)

2.8 The Jurat-Fuentes Model

This model has recently been proposed to explain the mode of action of Cry1Ac in *H. virescens* (Jurat-Fuentes and Adang, 2006). The model suggests that cytotoxicity is due to the combined effects of osmotic lysis and cell signaling. It is proposed that first, activated monomeric Cry1Ac binds to the cadherin-like protein HevCaLP. This results in the activation of an intracellular signaling pathway regulated by phosphatases. Evidence to support the involvement of phosphatases in Cry1Ac-mediated signaling was provided by a proteomic analysis of BBMV that showed that resistant and susceptible strains of *H. virescens* had different levels of intracellular phosphatases (Jurat-Fuentes and Adang, 2004). Signaling might also be dependent on a direct interaction between Cry1Ac and actin (Jurat-Fuentes and

Adang, 2004; and McNalland Adang, 2003), a cytoskeletal protein that interacts with the cytosolic domain of cadherins through tyrosine phosphatases, catenin, and actinin (Lilien and Balsamo, 2005). It is hypothesized that after binding to HevCaLP, monomeric Cry1Ac oligomerizes and then binds to the GPI-anchored proteins APN and the ALP HvALP (Jurat-Fuentes and Adang, 2004). Either protein then drives Cry1Ac oligomers into DRMs, where pore formation results in osmotic shock and the activation of signaling pathways leading to cell death. While this model is intriguing, many steps are at present speculative and remain to be confirmed experimentally.

2.9 Applications of Cry Toxins

Three major applications of B.t. toxins have been achieved: (i) in the control of defoliator pests in forestry, (ii) in the control of mosquitoes that are vectors of human diseases, and (iii) in the development of transgenic insect-resistant plants. One of the most successful applications of B.t. has been the control of lepidopteran defoliators, which are pests of coniferous forests mainly in Canada and United States. In both countries, the control of forests defoliators relies mostly on the use of B.t. strain, HD-1, producing Cry1Aa, Cy1Ab, Cry1Ac and Cry2Aa toxins (Frankenhuyzen, 2000; and Bauce et al, 2004). Successful application of B.t. is highly dependent on proper timing, weather conditions and high dosage of spray applications. These factors combine to determine the probability of larvae ingesting a lethal dose (van Frankenhuyzen, 2000; and Bauce et al, 2004). The use of B.t. in the control of defoliators has resulted in a significant reduction in the use of chemical insecticides for pest control in the forests.

B.t. insecticides are highly active against disease vector mosquitoes like *Aedes aegypti* (vector of dengue fever), *Simulium damnosum* (vector of onchocerciasis) and certain *Anopheles* species (vectors of malaria). The lack of resistance to B.t. and the lack of toxicity to non-target organisms have resulted in a rapid implementation of B.t. as an alternative control method of mosquito and black fly populations (Becker, 2000). In 1983, a control program for the eradication of onchocerciasis was launched in eleven countries of Western Africa using B.t.i since *S. damnosum* populations had developed resistance to larvicidal organophosphates (Guillet et al, 1990). Furthermore, control of onchocerciasis has protected over 15 million

children without the appearance of black fly resistance to B.t.i (Guillet et al, 1990). This success of vector control using B.t.i will certainly increase its use around the world.

The development of transgenic crops that produce B.t. Cry proteins has been a major breakthrough in the substitution of chemical insecticides by environmental friendly alternatives. In transgenic plants the Cry toxin is produced continuously, protecting the toxin from degradation and making it reachable to chewing and boring insects. Cry protein production in plants has been improved by engineering cry genes with a plant biased codon usage, removal of putative splicing signal sequences and deletion of the carboxy-terminal region of the protoxin (Schuler et al, 1998). The use of insect-resistant crops has diminished considerably the use of chemical pesticides in areas where these transgenic crops are planted (Qaim and Zilberman, 2003). Interestingly, the use of B.t.-cotton in countries like China, Mexico and India has shown that the use of this B.t.-crop had a significant positive effect on the final yield and a reduction in the use of chemical pesticides, since in these countries the yield loss is mainly due to technical and economical constrains which are overcome in part by the use of insect-resistant crops (Qaim and Zilberman, 2003; Toenniessen et al, 2003).

2.10 Developing New Cry Biopesticides Based On *B.thuringiensis*

B.t. has evolved to produce large quantities of crystal proteins, making it a logical host for developing improved Cry biopesticides. Certain combinations of Cry proteins have been shown to exhibit synergistic effects (Crickmore et al, 1995; Lee et al, 1996; and Poncet et al, 1995). Accordingly, genetic manipulation of B.t. to create combination of genes more useful for a given purpose than those known to occur in natural isolates is desirable.

A conjugation-like system has been used to transfer Cry encoding plasmids from one strain to another, but most cry genes are not readily transmissible by this process. A breakthrough development for engineering *B.thuringiensis* and *B.cereus* came in 1989 when several groups independently applied electroporation technology to transform vegetative cells with plasmid DNA (Lereclus et al, 1989; Mahillon et al, 1989; and Masson et al, 1989). These protocols differed in various ways but each could achieve frequencies of 10^2 to 10^5 transformants per μg of plasmid DNA with a wide variety of hosts and vectors. The use of unmethylated DNA with the Macaluso and Mettus (1991) protocols allows transformation frequencies as high as

$3x10^6$ to be achieved. A variety of shuttle vectors, some employing B.t. plasmid replicons has been used to introduce cloned cry genes into B.t. Alternatively, integrational vectors have been used to insert cry genes by homologous recombination into resident plasmids or the chromosome. Application of this technique has included disruption of *cry* and *cyt* genes to assess their contribution to pesticidal activity (Poncet et al, 1993; and Delecluse et al, 1993) and inactivation of protease production genes to increase crystal production and stability (Donovan et al, 1997; and Poncet et al, 1993).

2.11 Insect Resistance

Given the multiple steps in processing the crystal to an active toxin, it is not surprising that insect populations might develop various means of resisting intoxication. It has been shown that B.t. based bioinsecticides are liable to resistance development both in the field and laboratory conditions. However, selection in the laboratory may be very different from resistance in the field due to lower level of genetic diversity in the lab maintained populations. In addition, the natural environment may contain factors affecting the viability and fecundity of resistant insects, factors excluded from the controlled environment of the laboratory. Resistance mechanisms can be associated with fitness costs that can be deleterious under natural conditions (Trisyono and Whalon, 1997). Natural enemies, such as predators, can influence the development of resistance to B.t. by preferring either the intoxicated, susceptible or the healthy, resistant insects. In the former case one would expect an increase in resistance development, while in the latter, natural enemies can help to retard resistance development to B.t.

Studies of B.t.-susceptible and -resistant insects suggest several mechanisms by which insects develop resistance. Altered or inadequate processing of Cry toxins has been suggested as a possible factor in the resistance mechanism. Oppert et al (1994) reported that the midgut proteolytic activity of *B.thuringiensis* (*B.t. entomocidus* HD-198)-resistant *Ploidia interpunctella* larvae was significantly reduced compared to midgut juice from susceptible insects. Studies with a Malaysian strain of *P.xylostella,* simultaneously highly resistant to the *kurstaki* subspecies and the *aizawai* subspecies suggested that a single locus, perhaps encoding a common receptor for many of the Cry1A toxins, can mutate to multitoxin resistance (Wright et al, 1997). Receptor binding studies with ^{125}I-labeled toxins and BBMVs prepared from susceptible and resistant insects (*P.interpunctella and P.xylostella*) have

indicated that the toxin binding sites could be altered or modified in the resistant strains (Van Rie et al, 1990; and Ferre et al, 1991). However, in the case of a Cry toxin resistant *H.virescens* strain, neither receptor neither binding affinity nor binding site concentration was found to be affected (Gould et al, 1992). These data suggest that mechanism of resistance is complex and probably a post binding event, such as integration into the membrane or ion-channel activity, could be altered in the above example.

Table 2 lists some of the insects that have developed resistance to Cry toxins. Injudicious use of Cry toxins could rapidly render them ineffective against other major crop pests, squandering a precious resource at time when synthetic organic pesticides are already increasingly ineffective.

Table, 2 Examples of Resistance to B.t.-endotoxins

Insect	Cry toxin or source	Resistance ratio	Reference
Lepidoptera			
Heliothis virescens	Cry1Ac	>10,000	Gould et al, 1992
Plutella xylostella	*Bacillus thuringiensis var.kurstaki*	2800	Ferre et al, 1991
Plodia interpuctella	*Bacillus thuringiensis var.kurstaki*	140	McGaughey et al, 1994
Spodoptera exigua	Cry1C	100	Moar, 1993
Trichoplusia ni	Cry1Ab	26	Estada and Ferre, 1992
Coleoptera			
Leptintarsa decemlineata	Cry3A	400	Whalon et al, 1993
Diptera			
Aedes aegypti	*Bacillus thuringiensis var.israelensis*	1.1	Goldman et al, 1986
Culex quinquefasciatus	*Bacillus thuringiensis var.israelensis*	6.0	Georghiou, 1990
Culex quinquefasciatus	Cry10A	70	Gill et al, 1992

The Goals and Strategies of Resistance Management

The goal of what is known as "resistance management" is to slow the development of resistance and extend an insecticide's useful lifespan as long as possible (Comins, 1977). There are three goals of resistance management: avoiding resistance where and if possible, delaying resistance as long as possible, and making resistant populations revert to susceptibility (Croft, 1990).

2.11.1 Keeping a Susceptible Population for Mating with Resistant Individuals

2.11.1.a The Release of Susceptible Insects Into an Exposed Population: This method is best used on populations of insects such as mosquitoes, in which insecticides generally target females (Wood, 1981).

2.11.1.b Refugia: This program involves the nearby placement of a susceptible population which, it is hoped, will diffuse into the treated population to mate. The success of refugia depends upon four conditions: that the resistance trait is recessive, that there is random mating, that adults will travel sufficiently between toxic plants, and that there is complete lack of insecticidal action in the refugia. If refugia are subjected to any sort of insecticide, the available susceptible population available for mating with B.t.-exposed individuals will decrease (Tabashnik, 1997).

2.11.1.c Seed Mixtures: A field planted using this strategy will result in a random mix of B.t. and toxin-free plants. Working in favor of seed mixtures is the potential behavioral pattern of some insect species to preferentially choose toxin-free plants over B.t. plants, thus cutting down on feeding exposure of susceptible insects to B.t.

2.11.1.d Mosaics: Mosaics are like refugia but differ in that they involve a patchwork of B.t. areas and non-B.t. areas are treated with some other insecticide-a different strain of B.t. or something completely unrelated. For this strategy to be effective, it is important that there is no cross-resistance between the two insecticides (Tabashnik, 1994b)

2.11.1.e Rotations: Here, a pattern of two or more insecticides is arranged temporally rather than spatially. Using two or more insecticides sequentially rather than simultaneously has been shown to delay resistance relatively (Wood, 1981).

2.11.2 Tissue-specific and Time-specific Toxin Expression

This strategy seeks to minimize B.t. overexposure by engineering transgenic plants to express toxin genes only at times when needed or only in parts of the plant which are most economically important or most vulnerable (Frutos et al, 1999).

2.11.3 Combining Insect Control Methods

2.11.3.a Use of Multiple Insecticides: The first of these strategies is a simple combination of two or more insecticides on the same field, such as a B.t. and non-B.t. toxin. Necessary to this approach is a lack of cross-resistance between the two.

2.11.3.b Combining B.t. with Natural Enemies: The success of this strategy depends on the use of those natural enemies which are not affected by B.t. toxins.

2.11.4 High Dose

The high dose strategy hopes to delay resistance by using a high enough dosage of toxin to kill heterozygous insects. The success of the high dose strategy depends on rare and recessive or partially recessive resistance alleles (Huang et al, 1999). Additionally, heterozygotes must have no advantage over insects homozygous for the susceptibility allele, or the development of resistance can be hastened (Curtis 1981). Resistance has generally been found to be a recessive or partially recessive trait, though some evidence indicates that some resistance alleles may be dominant or codominant (Tabashnik et al, 2000).

2.11.5 Trap Plants

A patch of B.t. crop is planted as a trap, and a non-B.t. productive crop is grown nearby. The B.t. patch is planted earlier and matures earlier than the crop, luring in insects, which are then killed by the toxin (Andow and Alstad, 1995).

Much research has been done in the areas of resistance inheritance, pest behavior within the ecosystem, frequency of resistance alleles in natural populations, toxin cross-resistance, and even economic impacts of these strategies. However, it still has not been nearly enough to guarantee or even successfully predict what will happen in the field. In this sense, none of the strategies are completely acceptable if we are serious about preserving B.t. as a safe and effective insect control method.

Mapping the Cry1C binding epitope(s) of Aminopeptidase N receptor of Spodoptera litura

4.1 INTRODUCTION

Insecticidal crystal proteins (ICPs) produced by *Bacillus thuringiensis* (B.t.) have been used as safer alternatives to chemical insecticides. These proteins are deployed for crop protection against insect predation either as formulation sprays or expressed in crop plants. The mechanism of action of these crystal (Cry) proteins involves their solubilisation and proteolytic activation inside the insect midgut. The activated toxin then binds to a specific receptor at the brush border membrane of midgut epithelial cells. In a susceptible larva, the interaction is followed by the insertion of the insecticidal protein into the membrane, creating an ionic imbalance, which results in the death of the insect. Binding of the toxin to the receptor is a two-stage process involving reversible and irreversible steps. The initial binding of the toxin to the receptor eventually facilitates its insertion into the membrane. The mechanism of pore formation has largely remained unresolved partly due to non-availability of adequate amount of the receptor ligand in relatively pure form.

Aminopeptidase N (EC 3.4.11.2) is one of the membrane proteins identified as a receptor to Cry proteins in different insects. Phylogenetic analysis of the lepidopteran midgut APN sequences led to categorization of APNs in five different classes (Herrero et al, 2005). The average sequence identity within a class varies from 56% (class 5) to 67% (class 4). Several common features of many different APNs are : the genes encode approximately 1,000 amino acids that undergo various forms of posttranslational modification to produce mature proteins of between 90 and 170 kDa in size, the proteins have a cleavable N-terminal signal peptide and the mature protein is attached to the membrane by a GPI anchor (Piggot and Ellar, 2007). The role of aminopeptidase N (APN) as a functional receptor for Cry toxins has been established by both in vitro (Agrawal et al, 2002; Lee et al, 1996; Luo et al, 1997; and Sangadala et al, 1994) and in vivo toxicity experiments (Gill et al, 2002; and Rajagopal et al, 2002). Attempts to solubilise membrane anchored APN with different detergents have lead to various degrees of solubilisation. The yield of the recovered aminopeptidase upon solubilisation varied with detergent's nature. The pattern of detergent solubilisation of GPI-anchored proteins is different from that of proteins anchored with a single membrane spanning polypeptide. A general paradigm seems to be that detergents with a high critical micellar concentration (cmc) are capable of solubilising GPI-anchored proteins more efficiently than the detergents with a low cmc (Hooper and Turner, 1988). In contrast, the

proteins anchored by a polypeptide are solubilised efficiently by all the detergents. Several groups have reported the purification of APNs from CHAPS-solubilised brush border membrane vesicle (BBMV) proteins by using a combination of toxin affinity chromatography and ion exchange chromatography and studied their binding kinetics with several Cry proteins (Lee et al, 1996; Jenkins and Dean, 2001; and Oltean et al, 1999). However, the yields from such protocols have been low (Denolf et al, 1997). To obtain APN protein in large amounts, *apn* from different insects have been cloned and expressed in *E. coli*, insect and mammalian cell cultures (Yaoi et al, 1999; Luo et al, 1999; and Simpson et al, 2000). However, efficient purification of the heterologously expressed receptor has not been reported yet.

Additionally, although some APN binding epitopes in the toxin have been characterized, little is known about the APN domains involved in Cry toxin binding. Previous work in the lab has demonstrated that a 110 kDa recombinant APN is a receptor for Cry1C toxin in *Spodoptera litura* midgut and is responsible for Cry1C toxin sensitivity in the larvae (Agrawal et al, 2002). The mapping of binding epitopes of APN is a challenging problem since, in contrast to toxins, no 3-D structure of insect APN is available and no mutagenesis studies of the receptor domains have been performed. In order to map the precise regions involved in APN-Cry1C interactions, phage-display technique has been used in the present work. This technology is highly successful in receptor identification (Fernandez et al, 2005) and epitope mapping (Gomez et al, 2001).

To date, 17 different APNs have been reported that bind to Cry toxins, and yet only two have been shown to mediate toxin susceptibility (Piggot and Ellar, 2007). One of these is the class 4 APN isolated from *S. litura* whose role in Cry1Ca susceptibility was demonstrated by gene silencing experiments (Rajagopal et al, 2002).

In this background of availability of the cloned receptor APN for the insecticidal Cry1C toxin, the present study was initiated to map the binding epitopes of the recombinantly expressed *Spodoptera litura* APN and to investigate the role of crucial receptor residues in Cry1C toxicity.

4.2 MATERIALS AND METHODS

4.2.1 Recombinant DNA Techniques for Cloning and DNA Analysis

4.2.1.a Polymerase Chain Reaction

Rapid amplification of the DNA fragments was done using Taq DNA polymerase and a set of convergent primers. All the Polymerase Chain Reactions (PCR) reactions included standard denaturation, annealing and extension steps. The temperature of denaturation was 94°C, and of extension was 72°C but that of annealing varied with the melting temperature (T_m) of the primers used. The time for which each step was carried out also varied depending on the size of the fragment to be amplified. The reaction was carried out for 32 cycles and the reaction product was electrophoresed on 1% or 0.8% agarose gel to check for the amplification. For performing colony PCR, a few cells tooth picked from each of the isolated colonies were resuspended in sterile water and boiled for 5 min. The lysed cells were centrifuged at 12,000 X g for 5 min and the supernatant was used as the DNA template for PCR reaction.

4.2.1.b Purification of DNA Fragments from Agarose Gel

Restriction enzyme digested plasmid or PCR amplified products were electrophoresed on 0.8% agarose gel in TAE buffer (40 mM Tris-acetate; 1 mM EDTA). The desired fragment was identified using standard molecular weight marker (1 kb ladder from MBI Fermentas) and purified using the QIAquick gel extraction column (Qiagen). The DNA fragment was excised from the agarose gel and collected in an eppendorf tube. Three volumes of solubilization buffer (Buffer QG) were added to 1 volume of gel and the gel slice was dissolved by heating at 55°C for 20 min. The mixture was loaded onto QIAquick spin column and centrifuged for 1 min. The flow through was discarded and the bound DNA was washed with wash buffer (Buffer PE). The bound DNA fragment was eluted with 50 µl of elution

buffer (Buffer PB: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0). All the buffers used were supplied with QIAquick gel extraction kit.

4.2.1.c Preparation of Competent Cells

E. coli DH5α cells were subcultured in LB (Luria Broth) medium (50 ml) from an overnight grown culture and incubated further at 37°C till the OD_{600} reached 0.4 or 0.6. Cells were harvested by centrifugation at 2,200 X g for 10 min at 4°C. The pellet was resuspended in 20 ml of ice-cold 100 mM $CaCl_2$ and incubated on ice for 30 min. Cells were collected by centrifugation at 1,500 X g for 20 min at 4°C and resuspended gently in 1.5 ml of ice-cold 100 mM $CaCl_2$. To this cell suspension 100% glycerol was added to a final concentration of 10%. Cell suspension (0.1 ml) was aliquoted into chilled eppendorf tubes, frozen immediately in liquid nitrogen and stored at –80°C.

4.2.1.d Ligation and Transformation

Fragments amplified by PCR were cloned in pGEM-Te vector carrying 3'-T overhangs using pGEM-Te vector system of Promega. The amplified DNA fragment was ligated to the linearized vector using T4 DNA ligase, the ligation reaction was carried out in water bath set at 15°C for 5 h or 4 °C for overnight. After stipulated incubation, the ligation mix was added to 100 µl of competent cells, mixed gently and incubated on ice for 30 min. Cells were subjected to heat shock at 42°C for 60 sec followed by addition of 900 µl of LB medium to the tube and incubated further at 37°C for 1 h with slow shaking. The revived cells were centrifuged at 750 X g for 5 min. After discarding 900 µl of LB medium, the cells were resuspended in the remaining 100 µl and plated on Luria agar plate containing the suitable antibiotic (depending on the vector used). If the vector allowed blue/white selection, 100 µl of 100 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 20 µl of 50 mg/ml X-Gal was spread over the surface of agar plate before plating. The plates were incubated overnight at 37°C. In order to subclone an insert from one vector to another, either suitable restriction sites present in the multiple cloning site (MCS) of both the vectors were chosen or the restriction sites were included in the primer itself. Transformed cells containing recombinant plasmids were identified by performing colony PCR.

4.2.1.e Isolation of Plasmid DNA

Plasmid DNA was isolated by alkaline lysis method as described in Sambrook et al. (1989). Bacterial cells containing the desired clone were grown overnight at 37°C in LB medium containing suitable antibiotic depending upon the vector and the host strain used [ampicillin (100 μg/ml) or kanamycin (20 μg/ml) or both]. The cells were harvested by centrifugation at 5,000 X g for 5 min at room temperature. The pellet was resuspended in 200 μl of solution I (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0 and 50 mM glucose) and subsequently 400 μl of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added and mixed by inversion. To the lysed cells, 300 μl of ice-cold solution III (7.5 M ammonium acetate) was added, mixed vigorously by inversion and incubated on ice for 20 min. The mixture was centrifuged at 22,000 X g for 15 min at 4°C. Supernatant (650 μl) was collected in a fresh tube and centrifuged again at 12,000 X g for 5 min at room temperature to remove any bacterial debris. Again, supernatant (550 μl) was collected in a fresh tube, to which 450 μl of isopropanol was added and mixed by inversion. The mixture was incubated at room temperature for 5 min and centrifuged at 12,000 X g to pellet the DNA. The pellet was washed with 70% ethanol and then air-dried. The pellet of nucleic acid was then dissolved in Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 50 μg/ml RNase A and incubated at 37°C for 30 min.

4.2.1.f Spectrophotometric Estimation of Nucleic Acids

Quantity and purity of nucleic acids in solution was determined by measuring the absorbance at 260 and 280 nm. Concentration of DNA was calculated by taking A_{260} = 50 μg/ml for DNA and 40 μg/ml for RNA. The purity of nucleic acid solutions was checked by taking the A_{260}/A_{280} ratio.

4.2.2 Detection and Analysis of Proteins Expressed from Cloned Genes

4.2.2.a Polyacrylamide Gel Electrophoresis (PAGE) of Proteins

Polyacrylamide Gel Electrophoresis (PAGE) was performed according to the protocol of Laemmli (1970). Gels were prepared and electrophoresed in the presence of 0.1% SDS (denaturing). The composition of the separating and stacking gel mixtures are given in Table 3. Protein samples were prepared by adding 4X SDS sample buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 4% β-mercaptoethanol {β-ME}, 0.01% bromophenol blue). Samples were boiled for 5 min, centrifuged at 12,000 X g and loaded on the gel. Gels were electrophoresed at 100 V till the proteins were stacked properly and thereafter gels were electrophoresed at a constant voltage of 180 V. Gels were stained with Coomassie Brilliant Blue (CBB) (0.05% Coomassie blue R-250, 25% isopropanol and 10% acetic acid) and destained with 10% acetic acid.

Table, 3. Composition for 7.5% resolving and 5% stacking gel.

REAGENTS	RESOLVING GEL (6 ml)	STACKING GEL (1.5 ml)
Acrylamide Stock Solution (30%)	1.5 ml	0.25 ml
4X Resolving Gel buffer (1.5 M Tris-HCl, pH 8.8)	1.5 ml	-
4X Stacking Gel buffer (0.5 M Tris-HCl, pH 6.8)	-	0.375 ml
Water	2.875 ml	1.1 ml
SDS (10%)	0.06 ml	0.015 ml
Ammonium persulphate (10%)	0.06 ml	0.015 ml
TEMED	0.005 ml	0.0025 ml

4.2.2.b Western Blotting

Western blotting was done according to the method described by Towbin et al. (1979). Mini Trans-blot Electrophoretic Cell (Bio-Rad) was used to transfer the proteins from the gel to the nitrocellulose membrane (Hybond-C, Amersham, England). The apparatus for electroblotting was assembled according to the manufacturer's instructions. For western analysis, the proteins were electrophoresed in SDS-PAGE along with prestained marker (broad range or low molecular weight or high molecular weight; Bio-Rad) and then transferred to the nitrocellulose (NC) membrane electrophoretically in transfer buffer (39 mM glycine, 48 mM Tris base, and 20% methanol) at a constant current of 100 mA for 2 h at 4°C. The membrane was rinsed briefly in Tris-buffered saline containing Tween-20 (TBST) (10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween-20) and then incubated in blocking solution (3% BSA in TBST) for 1 h with gentle shaking at room temperature. The membrane was washed twice for 5 min each with TBST. The washing was followed by 1 h incubation with primary antibodies [anti-APN antibody (1:5,000) or anti-His antibodies (1:10,000) (Clontech) in TBST containing 0.5% BSA] at room temperature with gentle shaking. Thereafter, the blots were washed thrice with TBST for 5 min each. After washing, blots were transferred to alkaline-phosphatase conjugated secondary antibody (Sigma) solution [goat anti-rabbit (1:5,000) or goat anti-mouse (1:10,000) in TBST containing 0.5% BSA] and further incubated for 1 h. The blots were washed again as described above. The protein-antibody complex was developed with alkaline phosphatase buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$) containing 150 μg/ml of p-nitroblue tetrazolium chloride (NBT) and 75 μg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidine (BCIP) (Life Technologies). The reaction was stopped by rinsing the blot with distilled water.

4.2.3 Preparation and purification of recombinant B.thuringiensis δ-endotoxins

E. coli strain JM103 expressing the recombinant protoxin Cry1Ac was kindly provided by D. H. Dean, Ohio State University, Columbus. The gene for this protoxin has been cloned in the pKK 223-3 expression vector. The Cry1C-expressing vector pTZ19R in *E. coli* strain DH5α was obtained from the *Bacillus* Genetic Stock Center, Ohio State University. Delta-

endotoxins were purified from *E. coli* by a modification of the procedure described by Lee et al. The crystal protein was solubilized in 50 mM sodium carbonate buffer (pH 10.5) containing 10 mM dithiothreitol at 37°C for 2 h with vigorous shaking. The unsolubilized proteins were removed by centrifugation at 15,000 × *g* at 4°C for 10 min. The solubilized crystal protein was digested with trypsin (United States Biochemical) at a trypsin/protoxin ratio of 1:10 (by mass) at 37°C. The activated Cry1Ac and Cry1C toxins were purified by ion-exchange liquid chromatography using a Q-Sepharose anion exchanger (Pharmacia Biotech Inc.) equilibrated with 50 mM sodium carbonate buffer (pH 10.5). Bound toxin was eluted with a 50 to 500 mM NaCl linear gradient prepared in the sodium carbonate buffer, and individual fractions were analyzed by SDS-PAGE. Fractions containing the purified toxin were pooled and dialyzed against PBS buffer and stored at -20°C.

4.2.4 Generation of Polyclonal antibodies against Cry1C

Fractions containing active Cry1C toxin protein after ion exchange chromatography were run on SDS-PAGE. The 65 kDa protein band was excised and mashed with saline and emulsified with Freund's adjuvant. Antiserum was raised by immunization of a New Zealand White (NZW) rabbit with 0.5 mg of the toxic fragment administered in Freund's complete adjuvant (Sigma). The rabbit was boosted once with the active toxin protein administered in Freund's incomplete adjuvant (Sigma). The rabbit serum was collected 10 days after the boost. The reactivity of the rabbit serum was assessed by western blotting.

4.2.5 Ligand Blotting

Purified SlAPN or BR-APN protein was heated in SDS sample buffer containing β-ME, resolved by SDS-PAGE and electrotransferred to NC membrane. After being blocked with 3% BSA in TBST, the membrane was incubated with 1 μg/ml (or otherwise mentioned in results section) of purified Cry toxins in TBS containing 0.2% BSA at room temperature (RT) for 1 h. The unbound toxins were removed by three washes with TBST. The membrane was then incubated with antibodies against respective overlaid toxins at room temperature for 1 h. After three washes with TBST, the blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit antibodies (Calbiochem) for 1 h at room temperature. The immunoblots were

washed again with TBST and developed using NBT/BCIPsubstrates according to manufacturer's instructions.

4.2.6 Expression, Purification and Characterisation of *S. litura apn* in *Sf*21 Cells

4.2.6.a Maintenance of Sf21 Cell Culture

The IPLB-*Sf*21 cell line, originally derived from the fall armyworm, *Spodoptera frugiperda* (Vaughn et al, 1977), was used to propagate AcMNPV-based expression vectors. The *Sf*21 cells were grown and maintained at 27°C in TNM-FH medium (Grace's basic medium with yeastolate, lactalbumin hydrolysate and L-glutamine) supplemented with 10% FBS and 50 μg/ml gentamycin (Pharmingen). Cells were grown as a monolayer in tissue culture flasks (Nunc). Cell viability was determined by the trypan blue exclusion method.

4.2.6.b Plaque Assay

The recombinant virus expressing the full length recombinant *Spodoptera litura* APN protein (SlAPN) was serially diluted to give final dilutions of 10^{-1}, 10^{-2}, and 10^{-3}, TNM-FH medium was used as the diluent. One million cells were seeded in each well of six-welled tissue culture plate. The cells were infected with 100 μl of each of the dilution, incubated at room temperature for 1 h with gentle shaking in between. During this incubation, 2% SeaPlaque agarose (in water, previously autoclaved) was melted and cooled to 37°C. The virus inoculum was removed from the cells after the stipulated incubation period. Equal volume of warm TNM-FH medium was added to the 2% agarose and mixed properly. The infected cell monolayer was overlaid with 1.5 ml of this 1% agarose solution. Once the agarose overlay had set, 1.5 ml of TNM-FH medium was added to each well. The plate was incubated at 27°C for 4-5 days.

Each well was overlaid with 0.03% of neutral red. Viral plaques were identified as clear (unstained) areas and were further confirmed by observing under a microscope. The plaques

were lifted by pushing the 1 ml pipette tip through the agarose overlay into the plaque and gently sucking the agarose plug into the tip. The plug obtained by plaque pick method was released into the microcentrifuge tube containing 0.5 ml of TNM-FH medium, vortexed and stored at 4°C overnight to allow viruses to diffuse out of the agarose.

4.2.6.c *Expression of S. litura apn in Sf21 Cells*

The recombinant virus expressing the *S. litura apn* was provided by Dr. Neema Agrawal. The virus which was positive by PCR and western analysis was further amplified following the instructions given in Clontech manual. The infection was carried out at a multiplicity of infection (MOI) of 0.1 at 27°C for 5 days. The cells were removed by centrifugation and the supernatant containing the amplified virus was stored at 4°C for further use. The titer of the amplified virus was calculated to be 1 X 10^8/ml. This amplified virus was used for expressing the recombinant APN (SlAPN). Expression was achieved by infecting the *Sf*21 cells at a MOI of 5 at 27°C for 4 days. The cells were pelleted down by centrifugation at 8000 r.p.m. for 15 minutes. Total cell extract of infected cells was prepared by resuspending 1 X 10^6 cells in 200 µl of 1X SDS sample buffer containing β-ME and boiling for 5 min. Ten microliters of the cell extract was resolved on a 7.5% SDS-PAGE. The expression of the protein was analyzed by staining the gel with CBB and destaining with acetic acid as well as by electrotransferring the proteins to nitrocellulose membrane. Western analysis of the transferred proteins was carried out by using polyclonal anti-APN antibodies (provided by Dr. Neema Agrawal).

4.2.6.d Solubilisation of recombinantly expressed aminopeptidase N from membranes of Sf21 cells

The *Sf*21 cells were grown and maintained at 27°C in TNM-FH medium as a monolayer. The aminopeptidase N was expressed using Baculovirus expression system as described earlier. The *Sf*21 cells expressing *S. litura* APN and uninfected cells were solubilised at 4°C for 4 h in solubilisation buffer (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT (1,4-Dithio-DL-threitol), 1 mM PMSF (Phenylmethanesulfonyl fluoride), 10 µg/ml leupeptin and 10 µg/ml aprotinin) containing 1% (w/v) nonionic detergent, CHAPS (3-{(3-Cholamidopropyl) diethylammonio}-1-propanesulfonate (USB)/ NP-40 (Nonidet P-40)

(USB)/ TX-100 (Triton X-100 (USB) or ionic detergent, NLS (N-lauryl sarcosine) (Sigma). For APN purification *Sf*21 cells expressing recombinant SlAPN were solubilised using 0.4% NLS. The cell suspension was incubated on ice for 4 h, intermittently vortexed and then sonicated (15 sec continuous on, 1.5 min cooling; 15 pulses). The sonicated suspension was centrifuged at 100,000 X g for 60 min at 4°C in 50Ti rotor (Beckman). Supernatant containing the solubilised SlAPN was used for enzyme assays and subsequent purification.

4.2.6.e Refolding of NLS-solubilised SlAPN

NLS-solubilised APN was refolded to catalytically active form by slow exchange of ionic detergent with milder non-ionic detergents. The solubilised APN was dialysed against buffer containing 1% (w/v) non-ionic detergent (Tween-20/NP-40/Triton-X100/CHAPS). One milliliter solubilised sample was dialysed against 2 L buffer for 8 h at 4°C with four changes. Catalytic activity of the dialysed sample was assayed as described below. For all APN characterization experiments, purified APN containing 0.2% NLS was similarly dialysed against buffer containing 0.2 % CHAPS.

4.2.6.f APN Assay

The amount of APN released by four different nonionic/zwitterionic detergents, CHAPS, NP-40, Tween-20 and Triton X-100 at 1% concentration and ionic detergent, NLS at 0.4% concentration was estimated by calculating the specific activity of aminopeptidase N in each treatment. A linearity of reaction was established by assaying APN activity with respect to different protein concentrations using CHAPS solubilised APN. For all experiments, total protein (20 μg) released in the supernatant after solubilisation of infected and uninfected membranes with different detergents was used for APN assay using L-leucine-p-nitroanilide (LpNA) as the substrate at a final concentration of 2 mM. The APN assay was carried out at 37°C in 1 ml of reaction volume using 67 mM Na_2HPO_4, 67 mM KH_2PO_4, pH 7.4 as substrate buffer. The amount of product (p-nitroaniline) released was measured spectrophotometerically at 405 nm. The APN activity was calculated using an extinction coefficient of 9.9 $mM^{-1}cm^{-1}$ for p-nitroaniline at 405 nm. For APN inhibition studies, stock solution of amastatin hydrochloride [(2S, 3R)-3-Amino-2-hydroxy-5-methylhexanoyl-Val-Val-Asp hydrochloride] (Sigma) was prepared in methanol. APN was pre-incubated with

different concentrations of amastatin for 3 h at room temperature on an end-over-end mixer. The complex was then used for activity determination. To map the difference in APN's catalytic site and Cry1C binding site, 100 nM pure SlAPN was pre-incubated with 350 nM pure Cry1C in the presence or absence of 1 μM amastatin for 3 h at room temperature before assaying the catalytic activity of the enzyme.

4.2.6.g Purification of NLS solubilised SlAPN

For purification, *Sf*21 cells (1.5 X 10^7) expressing *S. litura* APN were washed once with PBS and resuspended in solubilisation buffer containing 0.4% (w/v) NLS. The solubilisation protocol as described in earlier section was followed to release APN from membranes. The supernatant obtained after ultracentrifugation was analyzed by SDS-PAGE and total protein was estimated with Bradford assay kit (Bio-Rad) using BSA (Sigma) as the standard.

The supernatant obtained after ultracentrifugation was dialyzed against buffer A (20 mM Tris-HCl, pH7.5, 0.2% (w/v) NLS and 1 mM PMSF) for 8 h at 4°C with three changes. During dialysis the concentration of NaCl was gradually reduced from 300 mM to 50 mM.

In the two-step purification protocol, APN was initially subjected to batch purification by anion-exchange chromatography with Q-sepharose (Amersham-Pharmacia Biotech). The total solubilised protein (2.5 mg in 10 ml) was loaded onto 3 ml resin equilibrated with buffer A. Binding was done for 2 h at 4°C with slow agitation on end-over-end mixer. The resin was washed with 10 bed volumes of buffer A containing 50 mM NaCl followed by 10 bed volumes of buffer A containing 450 mM NaCl. Bound proteins were eluted using a salt gradient (500 mM to 700 mM NaCl with steps of 50 mM) prepared in buffer A and fractions of 3 ml each were collected. Fractions were analyzed on SDS-PAGE and those containing the partially purified APN were pooled and dialyzed against buffer B (20 mM Tris-HCl, pH7.5, 0.5 mM EDTA, 150 mM NaCl, 0.2% (w/v) NLS, 1 mM PMSF) for 6 h at 4°C with 3 changes.

The dialyzed sample was further purified by size exclusion chromatography. The S200 HR matrix (Amersham-Pharmacia) was packed in a column to a bed height of 25 cm and the column was run at 4°C. Buffer B was used as the mobile phase and the flow rate was 100

µl/min. 115 fractions of 0.3 ml each were collected and analyzed for homogeneity by SDS-PAGE. The gels were stained with CBB and the fractions containing purified APN were pooled and also analyzed by silver staining.
For Western blot analysis, electroblotted purified APN was probed with anti-APN antibodies (1:5000). The blot was then incubated with alkaline phosphatase-conjugated goat anti-rabbit Ig-G and NBT-BCIP as described earlier.

Pure refolded SlAPN was used to determine Michaelis constant for the substrate L-Leucine *p*-nitroanilide. The concentrations of the substrate used were from 0.5 mM to 10 mM.

4.2.6.h Ligand blot analysis and estimation of affinity of Cry1C and APN by indirect ELISA

Toxin binding property of purified and refolded APN was checked by ligand blotting experiment. Briefly, purified Cry1C and Cry1Ac proteins (1 µg each) were resolved on 7.5% SDS-PAGE and electrotransferred to NC membrane. After blocking, the blot was overlaid with 1 µg/ml of purified and refolded APN in 20 mM Tris-HCl pH 7.5, containing 0.2% BSA at room temperature for 1 h. The bound APN was observed by incubating the blot with polyclonal antibodies raised against APN. After three washes, the blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit antibodies (Calbiochem). The immunoblots were developed using NBT/BCIP substrates.

To measure the kinetics of association of APN to Cry1C toxin in solution, 0.2 µM to 1.2 µM Cry1C toxin was mixed with APN (40 nM) and aliquots of 100 µl after different time periods, were transferred to a 96-well ELISA plate, pre-coated with 2 µg of Cry1C toxin in coating buffer (50 mM sodium carbonate pH 9.6). The amount of unbound APN was estimated as described by Hardy et al (1997). At the end of the kinetic analysis, the ELISA plate was washed three times with washing buffer (PBS supplemented with 0.5% Tween-20) and incubated with anti-APN antibody (1:5000). The plate was washed again and the binding signal was detected using anti-rabbit antibody coupled to alkaline phosphatase using 7 mM *p*-nitrophenyl phosphate (PNPP, Sigma) as the substrate.

4.2.7 Immunoprecipitation of Cry1C and Aminopeptidase N

Purified SlAPN (100 nM) was incubated with 350 nM pure Cry1C active toxin in binding buffer (50 mM sodium phosphate, pH 7.5, 50 mM NaCl and 3 mM $MgCl_2$) at 4°C on an end-over-end mixer for 3 h. To this mixture anti-Cry1C antiserum (5 µl) was added and then incubated further at 4°C. After 3 h incubation, 100 µl of protein A-Sepharose 4B equilibrated with binding buffer was added. The reaction mixture was incubated at 4°C for 2 h with rotation. The protein A-sepharose beads were pelleted at room temperature and washed once with wash buffer (10 mM Tris-HCl, pH 7.5, 2 mM EDTA and 0.2% NP-40) containing 150 mM NaCl and once with wash buffer containing 300 mM NaCl and twice with the same buffer (without NaCl and NP-40). The beads were resuspended in SDS sample buffer containing β-ME and heated at 100°C for 5 min to dissociate the bound protein-toxin-anti-toxin antiserum complex. After centrifugation at 12,000 X g for 5 min, the supernatant was resolved on a 7.5% SDS-PAGE and electrotransferred to nitrocellulose membrane. The membrane was incubated with anti-APN antibodies followed by alkaline phosphatase conjugated goat anti-rabbit antibodies and developed with NBT/BCIP.

4.2.8 Phage-Display Experiments

4.2.8.a Phage Titering

A single colony of ER2738 was inoculated in 5-10 ml of LB and incubated with shaking until mid-log phase (O.D.600 ~ 0.5). While cells were growing, Agarose Top (Per liter: 10 g Bacto-Tryptone, 5 g yeast extract, 5 g NaCl, 1 g MgCl2•6H2O, 7 g agarose. Autoclaved, dispensed into 50 ml aliquots) was melted in microwave and dispensed into sterile culture tubes (3 ml each), one per expected phage dilution. Tubes were equilibrated at 45°C until ready for use. One LB plate per expected dilution was prewarmed at 37°C until ready for use and 10-fold serial dilutions of phage in LB were made. Dilution ranges used were: for amplified phage culture supernatants, 10^{8}-10^{11}; for unamplified panning eluates, 10^{1}-10^{4}. Once culture reached mid-log phase, 200 µl culture was dispensed into microfuge tubes, 1 for each phage dilution and then 10 µl of each dilution was added to each tube, vortexed quickly, and incubated at room temperature for 5 minutes. This was followed by transfer of infected

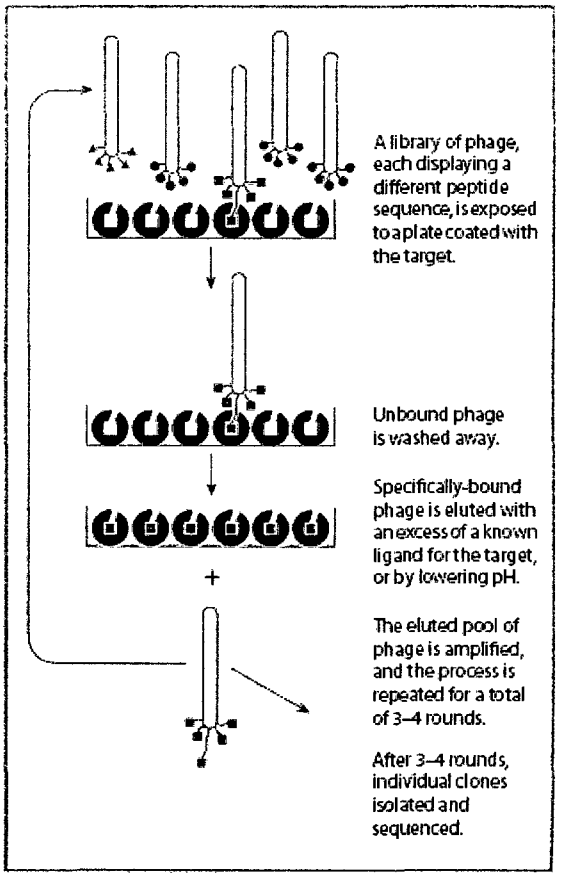
cells (one at a time) to a culture tube containing 45°C Agarose Top which was then vortexed quickly and immediately poured onto a pre-warmed LB/IPTG/Xgal plate. The plates were allowed to cool 5 minutes, inverted and incubated overnight at 37°C. Phage titer in terms of plaque forming units (pfu) per 10 µl was calculated by counting plaques on plates having ~10^2 plaques and multiplying each number by the dilution factor for that plate.

4.2.8.b Panning Procedure

A solution of 50 µg/ml of the ligand in 0.1 M $NaHCO_3$ (pH 8.6) was prepared and 150 µl of this solution was added to a well of a 96-well plate and swirled repeatedly until the surface was completely wet. The plate was incubated overnight at 4°C with gentle agitation in a humidified container (a sealable plastic box lined with damp paper towels). Inoculated 20 ml LB (in a 250 ml Erlenmeyer flask) and 10 ml LB (in a culture tube) with ER2738 and incubated both cultures at 37°C with vigorous shaking for 16 h. The coating solution from each plate was poured off and firmly slapped face down onto a clean paper towel to remove residual solution. The well was then filled completely with Blocking Buffer (0.1 M $NaHCO_3$ (pH 8.6), 5 mg/ml BSA, 0.02% NaN_3. Filter sterilized, stored at 4°C).and incubated for 1 h at 4°C. Following blocking, each well was washed rapidly 6X with TBST (TBS + 0.1% (v/v) Tween-20). Diluted 2 x 10^{11}phage (10 µl of original library) with 100 µl of TBST and pipeted onto coated plate and rocked gently for 60 min at room temperature. Following binding, nonbinding phages were discarded by pouring off and slapping plate face-down onto a clean paper towel. The wells were washed 10 times with TBST and bound phages were eluted with 100 µl of elution buffer [0.2 M Glycine-HCl (pH 2.2), 1 mg/ml BSA]. The eluate was pipeted into a microcentrifuge tube and neutralized with 15 µl of 1 M Tris-HCl (pH 9.1). Titered a small amount (1 µl) of the eluate as described earlier. The rest of the eluate was amplified by inoculating it in 20 ml of early log-phase ER2738 culture and incubated at 37°C with vigorous shaking for 4.5 h. The virus was harvested by centrifuging the culture for 10 min at 10,000 rpm (Sorvall SS-34) at 4°C. Upper 80% of the supernatant was transferred to a fresh tube and the virus precipitated by adding 1/6 volume of PEG/NaCl and incubating the tube at 4°C for overnight. The amplified virus was harvested by spinning PEG precipitation 15 min at 10,000 rpm, at 4°C. The pellet was suspended in 1 ml TBS and re-precipitated with 1/6 volume of PEG/NaCl for 60 min. The final amplified virus was obtained by centrifuging the

suspension for 10 min at 4°C and suspending. The amplified eluate was again tittered as described previously.

For subsequent rounds of panning, virus from previous selection round was used for binding and Tween-20 concentration was slowly increased (to 0.5% in third round)



Figure, 8. Panning with PhD peptide library

4.2.8.c Rapid purification of sequencing templates

After the first centrifugation step of plaque amplification, 500 µl of the phage-containing supernatant was transferred to a fresh microfuge tube and mixed with 200 µl PEG/NaCl. The mixture was allowed to stand at room temperature for 10 min and then centrifuged. The pellet was suspended in 100 µl Iodide Buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 4 M NaI. Stored at room temperature in the dark) and incubated for 10 min in the presence of 250 µl

ethanol to precipitate single-stranded phage DNA, leaving most phage protein in solution. The DNA pellet was then washed, dried and finally suspended in 30 µl TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). Ten microliter of the resuspended template was used for sequencing using -28 primer (5′-GTA TGG GAT TTT GCT AAA CAA C -3′) provided in the kit.

4.2.8.d DNA Sequencing

The sequencing of the insert cloned in pGEM-Te vector was carried out by Sanger's dideoxy termination method (Sanger et al, 1977) with the help of T7 sequencing kit (Amersham). Vector specific primers were used for sequencing. Briefly, 2-5 µg of the single-stranded M13 DNA and the primer were annealed by quick annealing method. The sequencing reaction was carried out in the presence of ^{35}S-dATP at 21°C and then terminated as described in the protocol. The samples were boiled for 5 min and cooled on ice before loading. The labeled fragments were separated on a 6% polyacrylamide gel containing 8M urea. Electrophoresis was carried out at constant power (40 watts) using 1X TBE buffer (90 mM Tris-borate, 2 mM EDTA) and the temperature of the gel was maintained at 50°C. After completion of the run, the gel was transferred to Whatmann 3MM paper, dried and autoradiographed. The nucleotide sequence was read manually and the protein sequence was deduced based on the reduced genetic code given in the manual of the phage display kit.

4.2.8.e Assaying selected peptides for target binding by Enzyme Linked Immunosorbent Assay (ELISA)

One row of ELISA plate wells for each clone to be characterized was coated with 100-200 µl of 50 µg/ml of target in 0.1 M $NaHCO_3$ (pH 8.6) and incubated at 4°C overnight in an air-tight humidified box (a sealable plastic box lined with wet paper towels). Excess target solution was discarded and each well was filled completely with Blocking Buffer. Additionally, one row of uncoated wells per clone to be characterized was also blocked in order to test for binding of each selected sequence to BSA-coated plastic. A second microtiter plate was also blocked, which was used for serial dilutions of phage before addition to the target-coated plate. The blocked plates were incubated at 4°C for 1 h and washed 6 times with

1X TBS/Tween. The percentage of Tween was same as the concentration used in the panning wash steps. In the separate blocked plate, serial dilutions of the phage in 200 µl of TBS/Tween per well were made. Using a multichannel pipettor, each row of diluted phage was transferred to the plate with the target and incubated at room temperature for 1 h with agitation. The plates were then washed and incubated with 200 µl of diluted HRP-conjugated anti-M13 antibody (1:5000) at room temperature for 1 h with agitation. This was followed by washings with TBST and developing the reaction by adding 200 µl substrate solution [36 µl 30% H_2O_2, 21 ml of ABTS stock solution (22 mg ABTS (Sigma #A1888) in 100 ml of 50 mM sodium citrate, pH 4.0] to each well, and incubating at room temperature for 15 minutes. The plate was read using a microplate reader set at 405-415 nm.

4.2.9 Cloning, Expression and Purification of BR-APN (Binding Region of SlAPN)

Phage display experiments were performed to identify the SlAPN region involved in Cry1C binding. It was observed that 70% of the Cry1C binding phages were similar to a 7 amino acid region present near the N-terminus of SlAPN (the APN-CRY region: ^{128}HLHFHLP^{134}). In order to study this region with respect to Cry1C binding and toxicity, *br-apn* a deletion derivative of *slapn*, was made by PCR approach. A 339 bp fragment from 174 bp to 513 bp (amino acids 51 to 162) was amplified using Sp9F (5'-TgT gTA CCC TAC TgA TgT CA-3') and BR-Rev (5'-CgA CgC CgC ACC TCT gTA gAA gCC-3') primers and cloned into pGEM-Te vector. To subclone this gene into prokaryotic expression vector, pQE 30 (Qiagen), the *br-apn* gene cloned in pGEM-Te vector was excised by digestion with *Sph*I and *Pst*I restriction enzymes. The excised fragment was ligated to *Sph*I/*Pst*I digested pQE 30 vector at 18°C for 6 h and transformed into competent cells of *E. coli* M15 strain, the expression host for pQE series of vectors. Plating was done on agar plates containing 100 µg/ml of ampicillin and 20 µg/ml of kanamycin and incubated overnight at 37°C. The positive clones were identified by carrying out colony PCR using gene specific primers.

The clone harboring the recombinant pQE30 plasmid was grown in LB medium to an OD_{600} of 0.6 and production of soluble BR-APN protein was achieved by incubating the log-phase M15 culture with 1mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 16°C for 16 h.

Cells were collected by centrifugation and resuspended in 20 mM Tris buffer (pH 8) containing 150 mM NaCl (TBS). Total proteins were obtained by sonicating the cell suspension on ice [four 30-second pulses at an output of 50 W using LABSONIC (Braun) sonicator] and observed under microscope for complete lysis. Both the pellet and the supernatant were resolved on 7.5% SDS-PAGE, stained with CBB, destained with acetic acid and checked for the expression of the recombinant protein.

The supernatant obtained after centrifuging the homogenate at low speed (5000 rpm at 4°C for 10 min) was dialysed against 50 mM sodium phosphate buffer (pH 8) (binding buffer) and loaded on Talon resin (Amersham) to purify the His-tagged BR-APN protein. The bound proteins were eluted in elution buffer containing 250 mM imidazole (according to manufacturer's instructions), resolved by SDS-PAGE and fractions containing the purified toxin were pooled and dialyzed against TBS for raising binding studies.

4.2.10 Competition Ligand Binding Assays

A total of 1 μg purified SlAPN or BR-APN was resolved on SDS-PAGE and electro blotted onto a nitrocellulose membrane. The blot was incubated with toxin and processed as described earlier. Following Cry1C overlaying, the membrane was washed with TBST (20 mM Tris buffer (pH 8) containing 150 mM NaCl and 0.5% Tween20) and incubated for 1 h in rabbit anti-Cry1C serum (diluted 1:20,000 in dilution buffer {TBS containing 0.2% BSA}). For competition assays, strips containing transferred SlAPN or BR-APN were cut from the NC membrane and overlaid with the toxin previously incubated with different concentrations of peptide competitors (APN-CRY or ϕ-71).

4.2.11 Insect Bioassays

Toxicity assay was performed on neonate larvae of *S. litura* and estimation of lethal concentration 50 (LC_{50}) of different toxins was carried out. LC_{50} is the concentration of toxicant that is sufficient enough to kill 50% of the population in a specified time. The LC_{50} for different toxins was calculated by probit analysis (Finney, 1971). The concentration of Cry1C toxins was estimated by dye-binding method of Bradford (1976). Five different concentrations of the toxin was tested, namely, 50, 100, 500, 1000 and 2000 ng/cm^2. The

toxin was serially diluted in 50 mM Tris-HCl, pH 8.0. A total of 200 µl (each side) of toxin dilutions (diluted in PBS) was layered on a leaf disc placed in a petri dish and allowed to dry. Ten larvae were placed on each petri-dish and mortality was analysed after two days of incubation. Bioassays were repeated 3-5 times. For bioassays performed in the presence of competitors the toxin was pre incubated with the competitor for 1h at RT and then coated on the leaf disc.

4.3 RESULTS

4.3.1 Expression and Purification of Cry1C and Cry1Ac δ-endotoxins

Both toxins were expressed as inclusion bodies after 4 h of induction with 1mM IPTG at 37°C. Solubilisation of inclusion bodies in the presence of 10 mM DTT resulted in the generation of 120-kDa solubilised toxins (Figure 9a). The solubilised supernatant was digested by trypsin to generate active toxic fragments. Though Cry1C soluble protein was nearly completely activated at a trypsin/protoxin ratio of 1:10 (by mass) at 37°C, incomplete activation of Cry1Ac was obtained under similar conditions (Figure 9b). The activated toxins were purified by ion-exchange chromatography (Figure 9c and 9d). Pure active toxins eluted at 300-400 mM NaCl concentration step. The fractions containing purified toxin were pooled and dialysed against PBS for binding studies. The purified proteins were stored in small aliquots at -20°C.

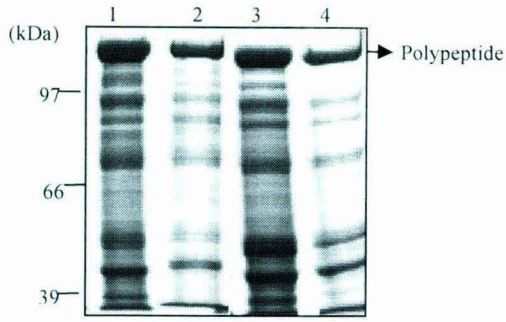


Figure 9, a. SDS-PAGE profile of Cry1C and Cry1Ac inclusion bodies and solubilised proteins. Inclusion bodies prepared from *E.coli* cells carrying recombinant Cry1Ac (lane 1) and Cry1C (lane 3) protoxins. The inclusion bodies were solubilised with 10 mM DTT containing buffer for 2h at 37°C. Soluble proteins obtained from Cry1Ac (lane 2) and Cry1C (lane 4) containing inclusion bodies.

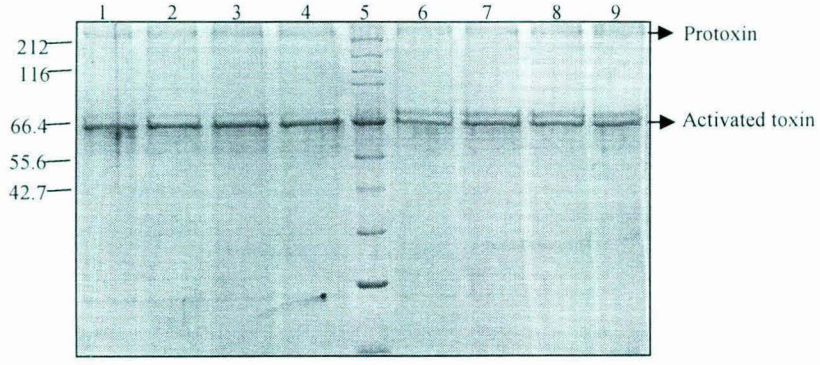


Figure 9, b. Standardisation of proteolytic activation of solubilised Cry1C (lanes 1-4) and Cry1Ac (lanes 6-9) toxins by trypsin. Toxins were incubated with trypsin at 1:5 (wt/wt) (lanes 1, 2, 6 and 7) or 1:10 (wt/wt) trypsin/protoxin ratio for 15 min (lanes 1, 3, 6 and 8) and 30 min (lanes 2, 4, 7 and 9). Lane 5, Marker.

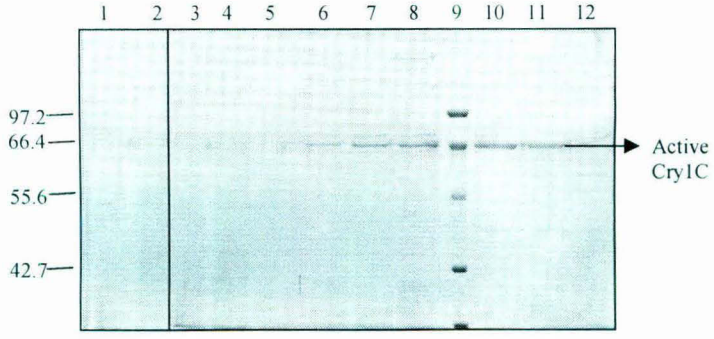


Figure 9, c. Purification profile of active Cry1C toxin. The trypsin activated protein sample was loaded onto anion exchange resin (Q-sepharose). The bound proteins were eluted using a salt gradient (100 mM to 500 mM NaCl with steps of 100 mM) and resolved on a 10% SDS-PAGE. Flow through (lane 1), wash fraction (lane 2), fractions eluted with 100 mM NaCl (lanes 3 and 4), 200 mM NaCl (lanes 5 and 6), 300 mM NaCl (lanes 7 and 8), 400 mM NaCl (lanes 10 and 11) and 500 mM NaCl (lane 12). The gel was stained with Coomassie blue.

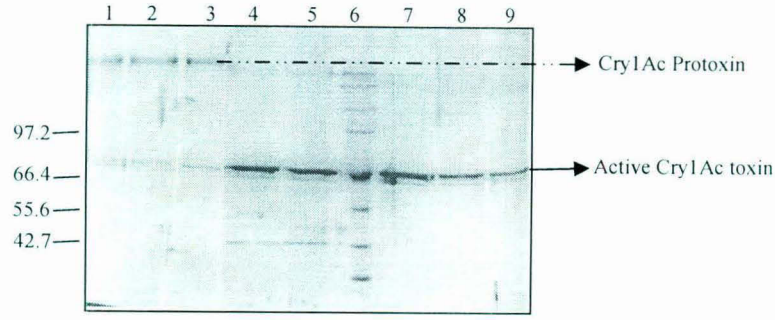


Figure 9, d. Purification profile of active Cry1Ac toxin. The trypsin activated protein sample was loaded onto anion exchange resin (Q-sepharose). The bound proteins were eluted using a salt gradient (100 mM to 500 mM NaCl with steps of 100 mM) and resolved on a 7.5% SDS-PAGE. Flow through (lane 1), wash fraction (lane 2), fractions eluted with 100 mM NaCl (lane 3), 200 mM NaCl (lane 4), 300 mM NaCl (lane 5), 400 mM NaCl (lane 7) and 500 mM NaCl (lanes 8 and 9). The gel was stained with Coomassie blue.

4.3.2 Solubilisation of recombinant APN and its Refolding (regeneration of catalytically active form)

In the present study, a number of ionic and nonionic/zwitterionic detergents were used to recover recombinantly expressed APN from the membrane of insect cells. As shown in SDS-PAGE, the *S. litura* APN showed differential solubilisation with the detergents used (Figure 10 a). In the absence of any other directly measurable activity for conformation and catalytic fidelity upon solubilisation, specific activity of aminopeptidase was taken as a measure of correct conformation. The CHAPS-solubilised proteins from APN expressing cells hydrolyzed LpNA with a specific activity of 42 nmol/h/mg whereas the proteins solubilised from uninfected cells hydrolyzed LpNA with a specific activity of 2 nmol/h/mg (Figure 10 b). The difference in the activity showed that the expressed APN was active after solubilisation with nonionic detergent. The relative amount of APN released from the expressing *Sf*21 cells by different nonionic detergents was determined by assaying the respective supernatants for APN activity (Figure 10 b). The ability of different nonionic detergents to release APN was in the following order:

CHAPS> TX-100 = NP-40 >Tween-20

The recombinant APN was almost completely released from *Sf*21 cells by the ionic detergent, NLS (Figure 10 a) but the catalytic activity of the enzyme was lost. The premise that NLS is a

mild ionic detergent and may not induce major structural and conformational anomalies was examined as follows. APN was solubilised from expressing cells with NLS and refolded by exchanging the ionic detergent with milder non-ionic detergents through slow dialysis. Different non-ionic detergents at 1% (w/v) concentration were used for dialysis and the catalytic activity of the refolded protein was assayed. The denatured APN showed maximum regain of activity with CHAPS (Figure 10 c). Hence, for subsequent experiments, NLS/CHAPS combination was used i.e., NLS-solubilised APN was used for purification and the purified protein was slowly dialysed against 0.2% CHAPS to regain its catalytic activity.

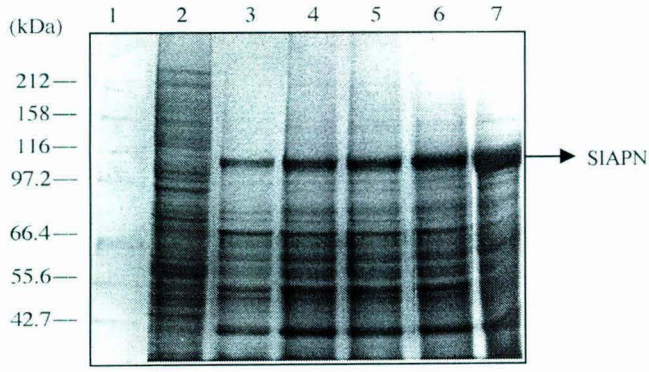


Figure 10, a. Differential solubilization of membrane-anchored recombinant S. litura aminopeptidase expressed in Sf21 cells. Total proteins from *Sf*21 cells were solubilized by using different detergents, insoluble material was removed by ultracentrifugation (100,000g) and equal amounts of solubilized proteins present in supernatant were resolved on a 10% SDS-PAGE. Molecular marker (NEB) (lane 1), CHAPS-solubilized proteins of uninfected *Sf*21 cells (lane 2), Tween-20-solubilized (lane 3), TX-100-solubilized (lane 4), NP-40-solubilized (lane 5), CHAPS-solubilized (lane 6) and NLS-solubilized (lane 7) proteins of APN expressing *Sf*21 cells. The gel was stained with Coomassie blue.

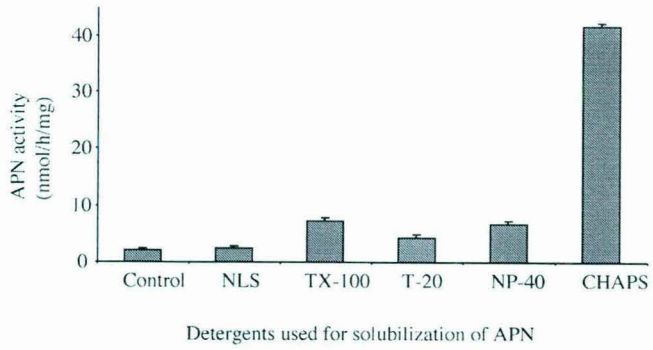


Figure 10, b. Amount of SlAPN released from Sf21 cells by different nonionic detergents. The supernatants obtained after solubilization of APN expressing *Sf*21 cells were assayed for aminopeptidase activity using 2 mM Leucine-p-nitroanilide as the substrate. The specific activity of APN was calculated in each treatment and plotted in the form of bar diagram. Control refers to the specific activity of supernatant of CHAPS solubilized non-expressing *Sf*21 cells. Activities were given as the mean values from three independent experiments.

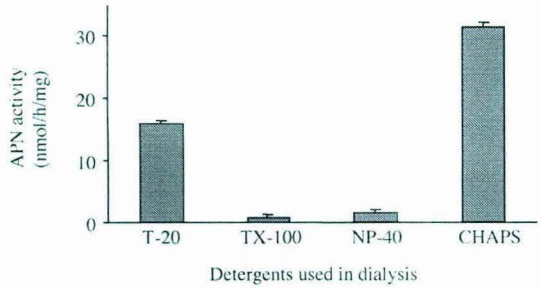


Figure 10, c. Recovery of catalytic activity of NLS –solubilized APN. SlAPN expressing *Sf*21 cells were solubilized with 0.4% NLS and the supernatant obtained after ultracentrifugation was dialyzed against different non-ionic/zwitterionic detergents at 1% concentration. The dialyzed sample was assayed for APN activity using 2 mM LpNA as the substrate. The specific activity of APN was calculated in each treatment and plotted in the form of bar diagram. Activities were given as the mean values from three independent experiments.

4.3.3 Purification of Spodoptera litura aminopeptidase N

In this study, a purification strategy in which the recombinant SlAPN was initially solubilised from *Sf*21 cells and subsequently purified by using two-step chromatography protocol was devised. Since the recombinant GPI-anchored SlAPN showed maximum solubilisation with ionic detergent NLS, NLS was used to release membrane-anchored APN from the cells. Being

a mild ionic detergent it does not induce major structural and conformational anomalies and could be removed by dialysis. Different concentrations of NLS were used to find the concentration that releases the maximum amount of APN (Figure 11 a). At 0.4% NLS, nearly complete solubilisation of SlAPN was achieved.

Since the recombinant APN carried a negative charge (pI = 5.5 (predicted)) in solubilisation buffer of pH 7.5, the initial purification was done by anion exchange chromatography which removed major contaminating proteins giving a 60% pure APN (Figure 11 b). The fractionation of partially purified protein by gel filtration chromatography resulted in > 90% purification of the recombinant APN protein (Figure 11 c). The purity of APN was further confirmed by silver staining in which only the APN band was observed (Figure 11 d). This band reacted positively with the anti-APN antibodies confirming the identity of the purified protein (Figure 11 e).

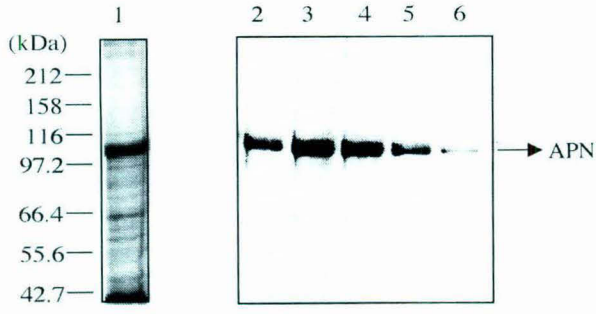


Figure 11, a. SlAPN solubilisation with different NLS concentrations. *Sf* cells expressing recombinant SlAPN were solubilised with buffer containing 0.04% (lane 1), 0.06% (lane 2), 0.08% (lane 3), 0.2% (lane 4), 0.4% (lane 5), 0.6% (lane 6), 0.8% (lane 7) and 1% (lane 8) NLS. Solubilised proteins were resolved on 12% SDS-PAGE and the gel stained with Coomassie blue.

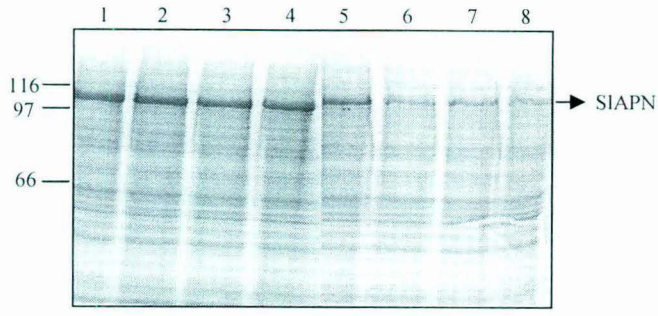


Figure 11, b. Partial purification of solubilised SlAPN by Anion exchange chromatography. *Sf*21 cells expressing APN were solubilized with 0.4% NLS and the solubilized proteins were loaded onto anion exchange resin (Q-sepharose). The bound proteins were eluted using a salt gradient (500 mM to 700 mM NaCl with steps of 50 mM) and resolved on a 7.5% SDS-PAGE. The protein sample loaded on Q-sepharose (lane 1), flow through (lane 2), wash fraction (lane 3), fraction eluted with 600 mM NaCl (lane 4) and 650 mM NaCl (lane 5). The gel was stained with Coomassie blue. The partially pure fractions (lanes 4 and 5) were pooled, concentrated by centricon column and resolved further by gel filtration chromatography.

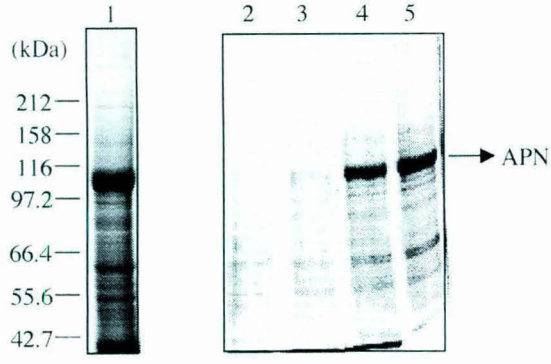


Figure 11, c. Gel filtration chromatography of partially purified SlAPN sample. Partially purified SlAPN obtained from anion exchange chromatography was further purified by gel filtration using S200HR matrix. Every fourth fraction out of 115 fractions collected was resolved on a 7.5% SDS-PAGE. The fractions containing purified APN are only shown in figure. Protein sample loaded on gel filtration column (lane 1) and fraction no. 85, 89, 93, 97 and 101 (lanes 2 to 6) were resolved on 7.5% SDS-PAGE. The gel was stained with Coomassie blue.

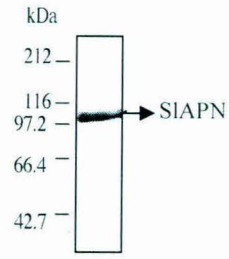


Figure 11, d. Silver staining of purified APN. Gel filtration fractions (85 to 104) containing purified APN were pooled and resolved on 10% SDS-PAGE. The gel was silver stained to check for purity of APN.

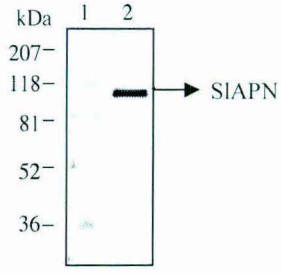


Figure 11, e. Western analysis of purified APN. Pool of fractions (85 to 104) containing pure APN (lane 2) were resolved on 10% SDS-PAGE along with broad range prestain marker (Bio Rad) (lane 1) and electrotransferred to nitrocellulose membrane. The identity of the purified protein was confirmed by western blot analysis using anti-APN antibodies.

After the two-step purification strategy, 270 µg pure APN was obtained from 15 X 10^6 *Sf*21 cells. The recovery of pure APN based on total protein estimation was 11% (starting from 2.5 mg total protein). Activity of the purified, refolded protein was 360 nmol/h/mg which corresponded to a 9-folds purification of the enzymatically active APN (Table 4).

While the NLS-solubilised APN could be purified to > 90% purity, the enzyme was catalytically inactive. The slow removal of NLS from purified receptor by dialyzing it against CHAPS allowed reversal from catalytically inactive to an active state. Adoption of this protocol resulted in the recovery of large amounts of catalytically active APN in pure form. The availability of pure aminopeptidase in abundance will greatly facilitate the elucidation of mechanism of action of insecticidal proteins of *B. thuringiensis.*

Table, 4. Evaluation of purification of recombinant APN expressed in Sf21 cells. At each step protein concentration was determined by Bradford (Bio-Rad) and enzymatic activity by using 2 mM LpNA as the substrate.

Sample	Protein Concentration (µg/ml)	Total Protein (mg)	Yield	Specific Activity (nmol/h/mg)	Purification fold
Solubilised Sample	250	2.5	100%	40	1
Ion Exchange Chromatography	200	1.2	48%	250	6
Gel Filtration Chromatography	100	0.27	11%	360	9

4.3.4 Characterization of purified aminopeptidase N

The conformation competence of purified and refolded aminopeptidase was verified by examining its kinetic constant, its interaction with insecticidal protein Cry1C and by calculating its dissociation constant for Cry1C protein.

The affinity of pure SlAPN for LpNA substrate was determined by calculating its activity at different substrate concentrations ranging from 0.5 mM to 10 mM. Maximum velocity (Vmax) of the reaction was calculated to be 590 nM/h and Km for LpNA was 1 mM (Figure 12 a).

Since purified SlAPN was refolded from an inactive to active form, its ability to bind to bioactive toxin Cry1C after refolding was also investigated. Importantly, the toxin-binding characteristic displayed by the 108 kDa purified and refolded APN receptor was similar to that of membrane-anchored APN. It interacted with bioactive toxin, Cry1C but showed no interaction with Cry1Ac toxin on ligand blots (Figure 12 b).

The equilibrium dissociation constant (K_D), the association rate constant (k_{on}) and the dissociation rate constant (k_{off}) are the basic parameters for characterizing protein-protein interactions. To obtain quantitative data for the interaction of the Cry1C toxin with the APN, the interaction of the toxin with the receptor in solution was analyzed using an indirect enzyme-linked immunosorbent assay (ELISA). In this methodology, the association rate of Cry1C toxin and APN receptor was measured by mixing both molecules in solution and at different time intervals aliquots were withdrawn to determine by indirect ELISA the amount of free APN that remained in solution. The reduction in the amount of free APN represents the association reaction. To have a pseudo-first order reaction, the concentration of the APN was at least 5-fold lower than the concentration of Cry1C toxin in solution. Also, the concentration of the Cry1C toxin in solution was 10-fold higher than the reported K_D values for APN. The kinetics of association of the APN to Cry1C is shown in figure 12 c. The plot is the logarithm of relative absorbance versus time where the slope of line suggests a pseudo-first order rate constant of association (k_{obs}). The association constant (k_{on}) was obtained by dividing the k_{obs} constant with the concentration of the Cry1C toxin and is shown in Table 4. The ordinate at the origin corresponded to a first order dissociation rate constant (k_{off}) and is shown in Table 5. The equilibrium dissociation constant (K_D) was then calculated as the ratio of dissociation and association rate constants (k_{off} /k_{on}). The results demonstrated that the APN possessed a high affinity site for Cry1C with K_D value of 90 nM. The toxin exhibited a moderate on rate (~2.2×10^3 $M^{-1}s^{-1}$) and a slow reversible off rate ($0.2 \times 10^{-3}s^{-1}$).

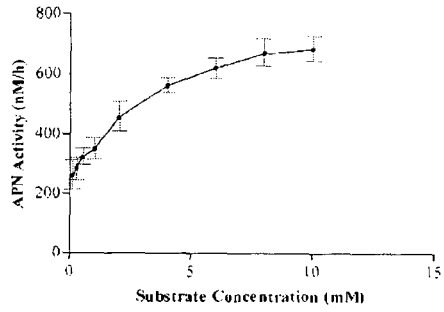


Figure 12, a. Substrate saturation profile of purified SlAPN. Michaelis-Menton constants were calculated by incubating purified SlAPN with different concentrations of substrate, L-leucine-p-nitroanilide.

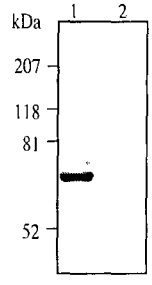


Figure 12, b. Ligand blot analysis of purified SlAPN. Purified Cry1C (lane 1) and Cry1Ac (lane 2) were resolved on 7.5% SDS-PAGE and electrotransferred to nitrocellulose membrane. The nitrocellulose membrane was probed with purified APN (1 μg/ml). Binding of the receptor was detected by using anti-APN antibodies followed by AP-conjugated goat anti-rabbit IgG.

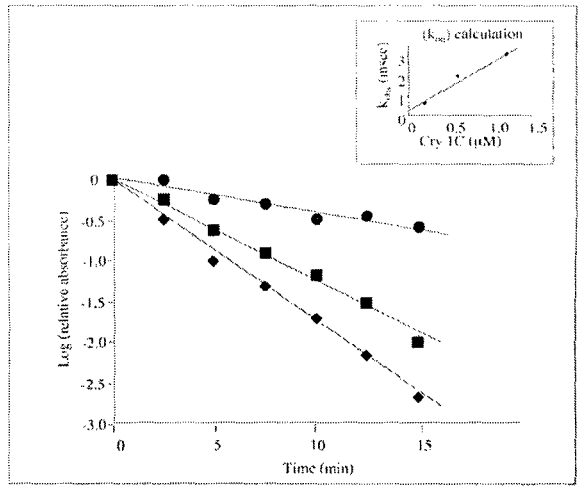


Figure 12, c. Association rate kinetics of Cry1C toxin/APN complex. Logarithm of relative absorbance was plotted versus time. The measured signal was the absorbance at 405 nm obtained in the last step of competitive ELISA. Relative absorbance represents the ratio $(A_t\text{-}A)/(A_0\text{-}A)$ where A is the ELISA signal on completion of

the reaction, A_t, the signal at time t, A_0, the signal at time zero and t corresponds to the time elapsed between initiation of the association and transfer of the aliquots into the wells. The complex was formed by mixing 40 nM APN to 0.2 μM (λ), 0.6 μM (■) and 1.2 μM (♦) Cry1C. The pseudo-first order rate constants (k_{obs}) is given by slope of each straight line

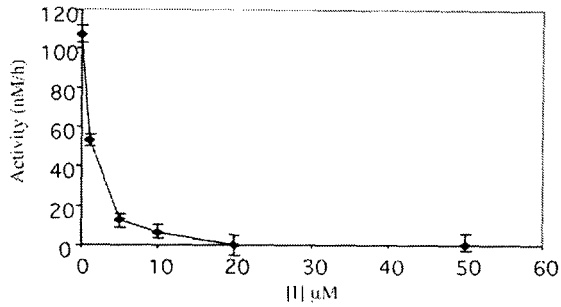
Inset. Determination of the association rate constant (k_{on}). The (k_{obs}) obtained from independent experiments were plotted versus the Cry1C concentrations. The second-order rate constant (k_{on}) is given by the slope of the straight line obtained. The extrapolation to zero gives the (k_{off}) values.

Table, 5. Kinetics of binding of Cry1C to APN receptor. Binding constants were determined by indirect ELISA. K_D is calculated from the equation $K_D = k_{on}/k_{off}$

k_{on}	k_{off}	K_D
$(M^{-1}s^{-1})$	(s^{-1})	(nM)
$2.2x10^3$	$0.2x10^{-3}$	90

4.3.5 Distinct catalytic and toxin binding sites of aminopeptidase N

Amastatin, an active site inhibitor of aminopeptidase, was used in binding experiments to examine the catalytic and toxin-binding sites of pure SlAPN. A linearity of inhibition reaction was established using different concentrations of the inhibitor. The pre-incubation of APN with 20 μM amastatin inhibited the enzyme completely while 1 μM amastatin reduced the activity to half (Figure 13). The aminopeptidase activity of the APN-Cry1C complex was determined by immunoprecipitation experiments. APN was pre-incubated with Cry1C toxin for 3 h and checked for its enzymatic activity. The resulting APN-Cry1C complex hydrolyzed leucine-p-nitroanilide as efficiently as native APN (Table 6). The effect of binding of Cry1C toxin on inhibition of APN with amastatin was also investigated. The aminopeptidase activity of the Cry1C-APN complex in presence of 1mM amastatin was reduced to half as compared with free APN (Table 6). This suggested that the binding of Cry1C toxin does not block the active site of APN and it remains available for substrate or inhibitor (amastatin) binding.



Figure, 13. Effect of amastatin inhibitor on the catalytic activity of APN. 100 nM APN was incubated with different concentrations of amastatin hydrochloride for 3 h at RT and then assayed for enzymatic activity using LpNA as the substrate.

Table, 6. SlAPN has distinct catalytic and toxin binding sites. Pure APN was pre-incubated with Cry1C in the presence or absence of amastatin for 3 h at room temperature before assaying the catalytic activity of the enzyme. The activity of APN as well as its inhibition by amastatin was not affected by Cry1C binding.

Sample	Activity (nM/h)
100 nM APN	333
100 nM APN+350 nM Cry1C	333
100 nM APN+1 μM amastatin	160
100 nM APN+350 nM Cry1C+1 μM amastatin	150

4.3.6 Identifying the SlAPN epitope involved in Toxin- Receptor interaction by phage display

To identify the SlAPN region involved in Cry1C binding, Ph.D.-C7C phage display library was used to select a population of phages that bound immobilized purified Cry1C. Three rounds of panning were done and phages from each round were analyzed to determine enrichment of SlAPN binding clones (Figure 14 a, panel A). Ten random phages obtained from third panning round were sequenced and their amino acid sequence determined using the reduced genetic code of the randomized library. Approximately 70% phages were found to

represent the same amino acid sequence (ϕ71: HPSFHWK) while all other phages encoded unique peptides. The ϕ71 bound Cry1C in a dose dependent manner (suggesting specific interaction) (Figure 14 a, Panel B) and shared 71% similarity to a 7 amino acid region present near the N-terminus of SlAPN (APN-CRY: ^{128}HLHFHLP^{134}). To determine if the APN region that shared sequence similarity with ϕ71 played any role in toxin binding, competition binding experiments using synthetic peptides corresponding either to ϕ71 peptide sequence (ϕ71) or to the corresponding region in APN (APN-CRY) as competitors were performed. Figure 14 b shows that binding of Cry1C to SlAPN was efficiently competed by both peptides suggesting that ^{128}HLHFHLP^{134} epitope is responsible for Cry1C binding.

A

Panning Round	Phage Output (pfu/ml)	Tween-20 Concentration (%)	Absorbance (490nm)
0	$2x10^{13}$	-	-
1	$3x10^{6}$	0.2	0.25
2	$7.5x10^{7}$	0.35	1
3	$1.8x10^{8}$	0.5	2

B

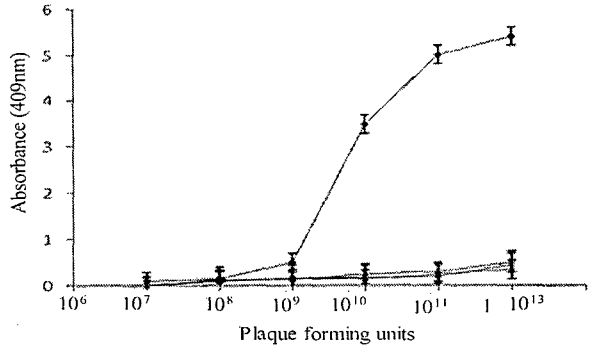


Figure 14, a. Phage display assay with Cry1C as ligand. Assaying the enrichment of clones (panel A) for binding with Cry1C. Equal number of phages after each round of selection was assayed for toxin binding by ELISA. Tween20 concentrations used in the ELISA washing steps were same as that used during individual biopanning step. The titer output and the absorbance value of each panning round are shown. Assaying the binding affinities (panel B) of phages selected after third round of panning (ϕ-71=phage clone 71% homologous to ^{128}HLHFHLP^{134} sequence of SlAPN, ϕ-1 to ϕ-3= other three phage clones obtained after third round of panning). Equal numbers of individual peptide displaying phages were allowed to bind to 2.5 μg Cry1C and relative affinities determined by standard ELISA protocol.

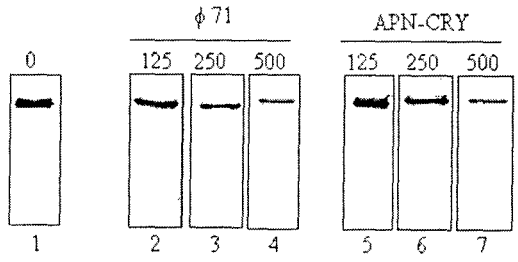


Figure 14, b. Synthetic peptides homologous to ϕ-71 & BR- APN compete with Cry1C binding to SlAPN. Toxin overlay assays of Cry1C to SlAPN. Purified SlAPN (1 μg) was loaded on SDS- polyacrylamide gel and transferred to NC membrane which was probed for toxin binding. Pure active Cry1C toxin (2.5 μg /ml) was pre incubated with increasing concentrations of peptides for 1 h at RT and then overlaid on the receptor containing NC membrane. Binding was visualized by standard NBT-BCIP assay using anti-Cry1C antibodies. Lane1, binding of Cry1C to SlAPN; lanes 2-4, competition of Cry1C binding with a 125-, 250- and 500- fold molar excess of peptide ϕ-71 respectively; lanes 5-7, competition of Cry1C binding with a 125-, 250- and 500- fold molar excess of peptide APN-CRY respectively.

4.3.7 Cloning, Expression, Purification and Characterisation of recombinant BR-APN (Binding region of SlAPN carrying the mapped ^{128}HLHFHLP^{134} region of the receptor)

In attempt to subclone a small region of SlAPN that retains the Cry1C binding capability, a 340 bp *br-apn* gene fragment was amplified by PCR (Figure 15 a) and cloned as described in Materials and methods. The expression of the 12 kDa recombinant protein (BR-APN) in *E.coli* M15 host cells, was analysed by SDS-PAGE. Incubation of recombinant pQE harboring M15 culture with 1mM IPTG for 16 h at 16°C resulted in production of the BR-APN protein in soluble form (Figure 15 b). The recombinant BR-APN protein reacted with anti-His antibodies indicating the expression of His-tag (Figure 15 c). The protein eluting from the Ni-NTA was more than 95% pure as shown by SDS-PAGE (Figure 15 d) and the yield of the purified protein was 4 mg per 100 ml of culture. This mutant (BR-APN) cloned and expressed in *E.coli,* was able to bind to Cry1C in toxin overlay assays (Figure 15 e, lane 1). Since this mutant expresses the APN-CRY region (^{128}HLHFHLP^{134}) of SlAPN which has previously been shown to be involved in ligand binding, competition binding assays between BR-APN and Cry1C were performed in the presence of APN-CRY peptide as the competitor

(Figure 15 e). The result shows that the BR-APN binding to Cry1C is competed by APN-CRY peptide thus reinforcing the specific binding capability of BR-APN peptide to Cry1C.

Figure 15, a. PCR Amplification of 340 bp BR-APN DNA fragment. Lane 1, 1kb DNA marker; lane 2, 340 bp *br-apn* gene.

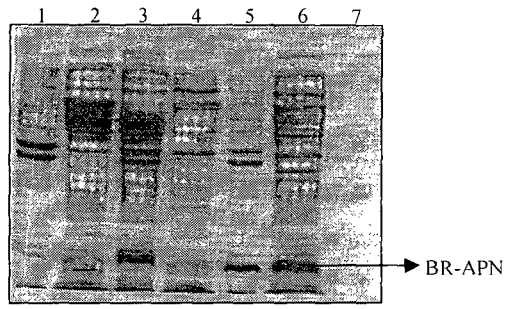


Figure 15, b. Expression of 12 kDa BR-APN protein. Recombinant pQE vector carrying *br-apn* gene was transformed in M15 cells. The transformed cells were incubated with 0.1 mM (lanes 3 and 4) or 1 mM (lanes 5 and 6) IPTG for 16 h at 25°C. The induced cell pellet was suspended in PBS buffer and sonicated to obtain the soluble proteins. The sonicated sample was centrifuged and both the pellet (lanes 3 and 5) and supernatant (lanes 4 and 6) was checked for the presence of BR-APN protein. Lane 1, pellet fraction of 1 mM IPTG induced M15 cells; lane 2, soluble protein fraction of 1mM IPTG induced M15 cells; and lane 7, marker.

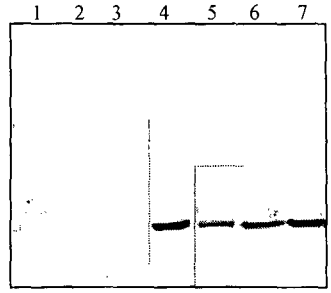


Figure 15, c. Western Blot of recombinant BR-APN. Lane 1, marker; lane 2, pellet fraction of 1 mM IPTG induced M15 cells; lane 3, supernatant fraction of 1 mM IPTG induced M15 cells; lanes 4 and 5, pellet and supernatant fractions of 0.1 mM IPTG induced M15 cells expressing the recombinant BR-APN protein; lanes 6 and 7, pellet and supernatant fraction of fraction of 1 mM IPTG induced M15 cells expressing the recombinant BR-APN protein.

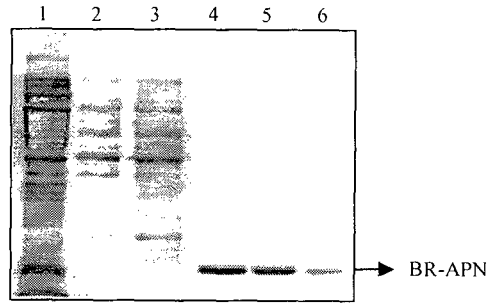


Figure 15, d. Purification profile of BR-APN protein. Lane 1, soluble protein sample loaded on Ni-NTA resin; lane 2, Flow through; lane 3, Wash fractions; lanes 4-6, 1st, 2nd and 3rd elution fractions.

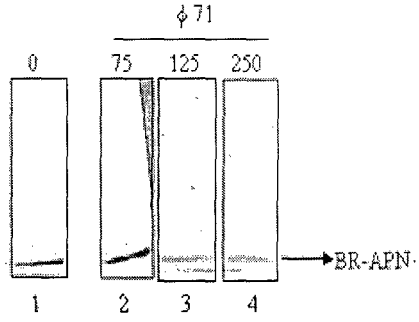


Figure 15, e. The deletion mutant BR-APN has binding capability similar to SlAPN. Synthetic peptide APN-CRY compete Cry1C binding to BR-APN. Lane1, binding of Cry1C to BR-APN; lanes 2-4 binding of Cry1C to BR-APN with a 75-, 125- and 250- fold molar excess of ϕ-71 peptide.

4.3.8 Involvement of BR-APN protein in Cry1C toxicity

Phage display experiments suggested involvement of APN-CRY (^{128}HLHFHLP^{134}) region of SlAPN in binding to Cry1C toxin. Attempts to clone a small binding region of SlAPN led to expression of BR-APN protein that carries the APN-CRY region. Ligand blots with the toxin and the purified BR-APN protein showed that the deletion receptor mutant was able to bind to the active toxin. Furthermore, binding of BR-APN to Cry1C was competed efficiently by APN-CRY peptide which also competed the binding of full length SlAPN protein and Cry1C. To ascertain the implication of this putative receptor binding region (BR-APN) in Cry1C toxicity to *S.litura*, bioassays were performed on first instar larvae fed on Cry1C toxin either alone or in combination with 100-fold molar excess of BR-APN protein. Table 7 shows that

incubation of the toxin with BR-APN reduces the toxicity of Cry1C by 50%. Additionally, incubating the toxin with APN-CRY peptide also reduced the mortality to similar levels (45%). At the same time incubation with a non specific scrambled peptide did not affect the toxicity of Cry1C. This showed that not only the APN-CRY region was an important binding epitope but also affected the toxicity of Cry1C. In this regard, the BR-APN mutant retained the specific Cry1C binding capability which led to death of the target insect. Thus the BR-APN protein served as the Cry1C receptor protein that had the ease of prokaryotic expression and purification and retained the binding property of the full length receptor protein.

Table, 7. Toxicity of Cry1C to S.litura first instar larvae in the presence or absence of competitors. A total of 200 µl (each side) of toxin was layered on a leaf disc placed in a petri dish and allowed to dry. Ten larvae were placed on each petri-dish and mortality was analysed after two days of incubation. For competition assays, toxin was pre-incubated with 100-fold molar excess of competitor for 1h at room temperature and then fed to the larvae.

Treatment	Mortality [a] (%)
Cry1C (80ng/cm^2)	100 ± 2.2
Cry1C+BR-APN	45 ± 3.1
Cry1C+APN-CRY	50 ± 1.9
Cry1C+scr	1 ± 1.5

[a]40 larvae per treatment. ± S.D. of three experiments

4.4 DISCUSSION

This section was aimed at the identification of SlAPN epitope(s) involved in binding with the Cry1C toxin. Recombinantly expressed and membrane anchored aminopeptidase N (SlAPN) showed differential solubilisation with various ionic and nonionic detergents. The amount of protein solubilised correlated directly with the cmc of the detergents. Detergents with a high cmc (e.g., CHAPS) were more effective in solubilising APN as compared to the detergents with a low cmc (e.g., Tween-20). Amongst the non-ionic detergents tried for solubilisation, CHAPS with highest cmc released maximum amounts of APN from the membranes. It has been reported that the GPI-anchored proteins exhibit differential solubilisation by range of detergents (Hooper et al, 1998). In contrast, proteins anchored by a transmembrane sequence of hydrophobic amino acids are efficiently solubilised by all the detergents. Thus, the

recombinant GPI-anchored SlAPN displayed the same property of differential solubilisation as shown by native GPI-anchored proteins.

The N-lauryl sarcosine (NLS)-solubilised SlAPN was purified to near homogeneity by anion exchange and gel filtration chromatography and refolded to its catalytically active form. Native APNs purified from BBMVs of insect midgut have been studied for their ability to interact with different Cry proteins by various in vitro and in vivo experiments, however; in all experiments incisive analysis of the binding of insecticidal protein was limited due to the lack of availability of APN in sufficient amount (Denolf et al, 1997). In this study, the recombinant SlAPN was initially solubilised from *Sf*21 cells and subsequently purified by using two-step chromatography protocol. While the NLS-solubilised APN could be purified to > 90% purity, the enzyme was catalytically inactive. The slow removal of NLS from purified receptor by dialyzing it against CHAPS allowed reversal from catalytically inactive to an active state. Adoption of this protocol resulted in the recovery of large amounts of catalytically active APN in pure form. The availability of pure aminopeptidase in abundance would greatly facilitate the elucidation of mechanism of action of insecticidal proteins of *Bacillus thuringiensis*.

The optimized purification regimen led to >90% purification of the catalytically active SlAPN with 11% recovery and 9-folds purification.

The interaction of purified SlAPN with biologically active Cry1C protein has been qualitatively and quantitatively characterized. By ligand blotting experiment, the linearity of interaction of the two purified proteins and lack of interaction of SlAPN with structurally divergent nontoxic Cry1Ac protein was demonstrated. The equilibrium dissociation constant (K_D) of purified SlAPN for Cry1C was calculated by ELISA (90 nM). The toxin exhibited a moderate on rate (~2.2 x 10^3 $M^{-1}s^{-1}$) and a slow reversible off rate (0.2 x $10^{-3}s^{-1}$). This suggested that though the initial binding of APN with Cry1C is moderate but once the complex is formed it was quite stable and dissociated very slowly thus explaining the high affinity of APN for Cry1C. Previous surface plasmon resonance studies with pure APN have shown that APN possesses a high affinity binding site for various Cry1A toxins whose K_D values are in the range of 40 nM to 280 nM (Luo et al, 1997 and Lee et al, 2000).

Amastatin was used in binding experiments to examine the catalytic and toxin-binding sites of pure SlAPN. Interaction of enzymatically inactive SlAPN with Cry1C and catalytic activity of APN-Cry1C complex suggested that the binding of Cry1C toxin did not block the active site of APN and it remained available for substrate or inhibitor (amastatin) binding. These results provided strong evidence that the receptor and catalytic functions associated with SlAPN involved distinct domains of the molecule.

The toxin-receptor interaction is a complex multi step process involving multiple epitopes on both interacting molecules. The identification of epitopes involved in Cry toxin-receptor interaction will be important for the characterization of resistant insect populations in nature and to develop novel toxin formulations useful in insect pest management.

In the present section, a detailed look at the SlAPN toxin binding epitope(s) was taken. Using phage display technique, a putative receptor epitope (APN-CRY) including residues ^{128}HLHFHLP^{134} was identified and demonstrated to be involved in toxin binding by competition experiments. Biopanning experiments with active Cry1C toxin resulted in the selection of binding phages predominantly localized in the same region. This phage clone was ϕ-71, whose randomized hepta-peptide region (HPSFHWK) shared 71% homology to a region in SlAPN (^{128}HLHFHLP^{134}). Synthetic peptides corresponding to this epitope inhibited binding of Cry1C toxin to SlAPN. The data suggested that this region played important role in the binding of SlAPN to Cry1C toxin. To test this, a deletion derivative of *slapn* carrying the putative binding region of 7 amino acids (^{128}HLHFHLP^{134}) was cloned. This mutant named BR-APN (binding region of SlAPN) bound efficiently with Cry1C on ligand blots and the binding was competed efficiently by ϕ-71 peptide demonstrating the specificity of binding.

Moreover, bioassays in the presence of the synthetic peptide corresponding to the mapped SlAPN epitope (APN-CRY) and the BR-APN protein significantly reduced the toxicity of Cry1C, suggesting a critical function of ^{128}HLHFHLP^{134} moiety of SlAPN in insect toxicity.

Mapping the SlAPN Binding Epitope(s)of Cry1C

5.1 INTRODUCTION

Most of the B.t. Cry toxins are synthesized as inactive protoxins which upon ingestion by a susceptible larvae, dissolve in the alkaline and reducing environment of the larval midgut, thereby releasing soluble proteins. The soluble protoxin is processed proteolytically by midgut enzymes, yielding 60-70 kDa protease-resistant toxic fragments. The activated toxin then binds specific receptor molecules located in the midgut epithelial cell brush border membranes of the host insect. The specific binding involves two-steps, a reversible followed by an irreversible one leading to insertion of the toxin oligomer into the cell membrane. This disrupts the transepithelial potential difference leading to the formation of pores in the membrane resulting in a net influx of ions and an accompanying influx of water, so that the cells swell and lyse. However, the exact involvement of each toxin domain in the overall toxic process is still unclear.

The toxin-receptor interaction is a complex process and regarding APN, both domain II and domain III are suggested to be involved in receptor recognition. The domain III of Cry toxins is involved in the APN-Cry interaction, as shown by the interchange of domain III between Cry1Ac and Cry1Ab toxins (de Maagd et al, 1999). A sequential-binding mechanism was proposed in the interaction of Cry1Ac with APN. Cry1Ac domain III initiates docking through recognition of an N-acetylgalactoseamine moiety on APN facilitating the subsequent interaction of domain II loop regions with a higher affinity region in this receptor (Jenkins and Dean, 2001).

The prediction that domain II is involved in receptor binding (Ge et al, 1989 and Li et al, 1991) has led to extensive substitution of loop residues in this domain in Cry3A, Cry1A and Cry1C by mutagenesis. Mutations located in loop 1 of Cry1Aa and Cry1Ab toxins demonstrated that the residues in this region are essential for binding to the midgut brush border membranes of *B. mori* and *M. sexta* and *H. virescens* respectively. Smith and Ellar (1994) showed that mutations in loops 1 and 2 of Cry1C toxin were able to modulate toxicity and specificity. It has been suggested (Rajamohan et al, 1996 and Rajamohan et al, 1995) that the two arginine residues at the first position of the loop 2 affect the earliest toxin-receptor interactions (initial binding) whereas the remaining hydrophobic residues affect irreversible binding. The results of these studies provided evidence that positive charges might be

required to orient the toxin to the receptor, and that binding is strengthened by a hydrophobic aromatic side-chain after initial recognition of the receptor. Substitution mutations in loop a-8 in Cry1Ac (R281A and R289A) resulted in loss of toxicity which correlated with reduction in binding affinity to APN receptor suggesting the involvement of this loop in initial receptor recognition (Lee et al, 2001).

Substitution studies (Rajamohan et al, 1996) suggested that the loop 3 residues of Cry1Aa and Cry1Ab these toxins establish hydrophobic interactions with the receptor molecule, and mutations at these hydrophobic residues affect initial binding. In Cry3A, substitution of loop 3 residues with alanines resulted in an increase in toxicity in the beetle *T.molitor* (Wu and Dean, 1996). Interestingly, heterologous competition with the mutant protein showed a loss of initial binding, but dissociation kinetic analysis showed that the mutant toxin has better irreversible binding, which could explain its greater toxicity.

One of the many speculated roles of domain III is its involvement in receptor binding. Several groups (Ge et al, 1989 and Schnepf et al, 1998) have suggested a role for domain III of Cry1Ac in *H.virescens* specificity. Lee et al (1995) analyzed homolog scanning mutants that exchanged domain III between Cry1Aa and Cry1Ac. Hybrid proteins containing the Cry1Aa domain III bound a 210-kDa receptor while hybrid proteins containing the Cry1Ac domain III bound a 120-kDa receptor in gypsy moth. Domain switching experiments have also suggested a role for Cry1ab domain III in binding to *S.exigua* (de Maagd et al, 1996). Finally, there is one report (Du and Nickerson, 1996) suggesting a biotin-binding activity for domain III, although a role for this activity in receptor binding has not been demonstrated directly.

The major concern regarding the sustained application of B.t.-crops is the possibility of generation of insect populations resistant to Cry toxins. Different mechanisms of insect resistance in laboratory selected insect populations have been characterized (Oppert et al, 1994; Gahan et al 2001; and Darbaux et al 2002). Among them, the most frequent mechanism has been the selection of insects affected on receptor-toxin interaction. Therefore, it is crucial to determine the critical residues involved in the initial receptor recognition event and in the eventual pore formation activity of Cry toxin-receptor interactions. Identification of such residues could be useful in designing new toxins that would overcome the potential generation of resistant insect populations in nature.

5.2 MATERIALS AND METHODS

5.2.1 Cloning, Expression and Purification of Cry1C domains

Domain II, and Domain III of Cry1C were cloned based on PCR approach. The primers used and details of the clones are reported in table 8.

Table, 8. Summary of domain II and domain III clones used in the study

Name	Abbreviation	Primers	Base pairs	Amino acids	Mass (kDa)
Cry1C domain II	1C-II	(primer 1) AAC TAT GAC AAT AGG AGA TAT (primer 2) AAG CTT TCA CTA GTG GTG GTG GTG GTG GTG AAGAGT GCA CTA CGA	591	261-457	22
Cry1C domain III	1C-III	(primer 3) GGA TCC TAT GAC AAA TAC AAT TGA TCC AGA (primer 4) AAG CTT TCA CTA GTG GTG GTG GTG GTG GTG ATT CAC CGC CTT TTTG TGC TCT TTC	450	467-616	17

To subclone the above genes into prokaryotic expression vector, pQE 30 (Qiagen), the complete gene cloned in pGEM-Te vector was excised by digestion with *Sph*I and *Pst*I restriction enzymes. The excised fragment was ligated to *Sph*I/*Pst*I digested pQE 30 vector at 18°C for 6 h and transformed into competent cells of *E. coli* M15 strain. Transformants were plated on agar plates containing 100 µg/ml ampicillin and 20 µg/ml kanamycin and incubated overnight at 37°C. The positive clones were identified by colony PCR using gene specific primers.

The clone harboring the recombinant pQE30 plasmid was grown in LB medium to an $O.D._{600}$ of 0.6 and over expression of Cry1C domains (1C-II and 1C-III) was induced by the addition of 1mM IPTG at 37°C for 4h. The inclusion bodies, expressing the domain proteins, were solubilized in the solubilisation buffer (50 mM sodium carbonate buffer (pH 10.5) containing

10 mM dithiothreitol) at 37°C for 12 h with vigorous shaking. The solubilised proteins were dialysed against binding buffer and loaded on Talon resin (Amersham), pre-equilibrated with same buffer, to purify the His-tagged Cry1C domain II and domain III proteins. The bound proteins were eluted in elution buffer containing 250 mM imidazole (according to manufacturer's instructions), resolved by SDS PAGE and fractions containing the purified toxin were pooled and dialyzed against TBS for binding studies.

5.2.2 Specificity of Cry1C domain interaction by Competition binding assay and immunoprecipitation

Competition assays with individual Cry1C domain proteins and purified SlAPN were performed to evaluate specificity of interactions between Cry1C domains and SlAPN receptor. The full length receptor was purified from the *Sf* membranes expressing the recombinant protein as described in previous section (Section 4.2.6.g). Briefly, *Sf* cells expressing the recombinant protein were solubilised in 0.4% NLS containing solubilisation buffer. The supernatant obtained after ultracentrifugation of the solubilised sample was dialysed to lower the NLS concentration to 0.2% and NaCl concentration to 50 mM. The resulting sample was subjected to batch purification by anion-exchange chromatography with Q-sepharose. The partially purified protein samples eluting from the resin were pooled and resolved further by gel-filteration chromatography. The purified APN protein was dialysed against PBS and used for binding assays. For domain competition assays, 500 ng SlAPN was incubated with 500 nM domain III protein for 30 min at RT for saturation binding, following which increasing concentrations (0-500 nM) of domain II protein was added. To this mixture anti-Cry1C antiserum (5 µl) was added and then incubated further at 4°C. After 2 h incubation, the SlAPN-domain-Cry1C complex was pulled down using 100 µl of protein A-sepharose 4B beads. After 2 h of incubation at 4°C, the beads were pelleted at RT and washed once with wash buffer (50 mM sodium phosphate buffer containing 0.2% Tween20) containing 150 mM NaCl followed by a second washing with wash buffer containing 300 mM NaCl, and finally with the same buffer (without NaCl and Tween20). The beads were then resuspended in SDS-sample buffer containing β-ME and heated at 100°C for 5 min (to dissociate the bound domain-SlAPN-anti APN antiserum complex). After centrifugation at 12,000xg for 5 min at RT, the supernatant was resolved by SDS-PAGE and electro transferred

to NC membrane. The membrane was incubated with anti-Cry1C antiserum (diluted 1:10,000) followed by AP-conjugated secondary antibody and developed by NBT-BCIP.

5.3 RESULTS

5.3.1 Cloning, Expression and Purification of domain II and domain III of Cry1C

The domain II, and domain III gene fragments were amplified by PCR and cloned as described in Materials and Methods (Figure 16 a). Both domains were expressed as inclusion bodies after 4 h of induction with 1mM IPTG at 37°C (Figure 16 b). Incubation of inclusion bodies with 10 mM DTT resulted in the generation of solubilised proteins. The expressed recombinant domain proteins reacted with anti-His antibodies (Figure 16 c). This facilitated the purification of the expressed proteins using Ni-NTA resin. The domain II and III proteins eluting off the Ni-NTA resin were more than 95% pure as shown by SDS-PAGE (Figure 16 d) and the yield of the purified proteins was 5-7 mg per 100 ml of culture. The fractions containing purified domains were pooled and dialysed against PBS for use in binding studies. The purified proteins were stored in small aliquots at -20°C.

Figure 16, a. Agarose gel (0.8%) showing PCR amplification of domain II and domain III DNA fragments. Lane 1, 1 kb marker; lane 2, 590 bp domain II fragment; and lane 3, 450 bp domain III fragment

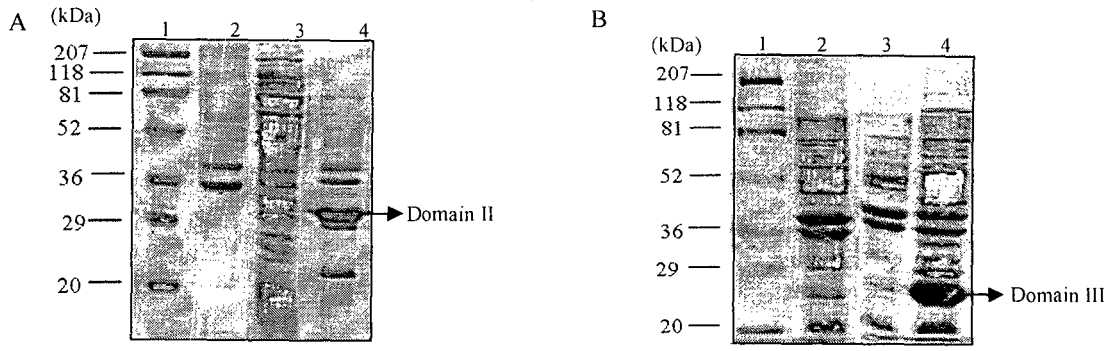


Figure 16, b. SDS-PAGE (12% SDS-polyacrylamide gel) Expression profiles of domain II (A) and domain III (B) proteins. Lane 1, marker; lane 2, induced pQE-31 vector containing M15 cells; lane 3, uninduced recombinant pQE-31 carrying M15 cells; and lane 4, induced recombinant (A) domain II and (B) domain III pQE-31 carrying M15 cells. The gel was stained by CBB dye.

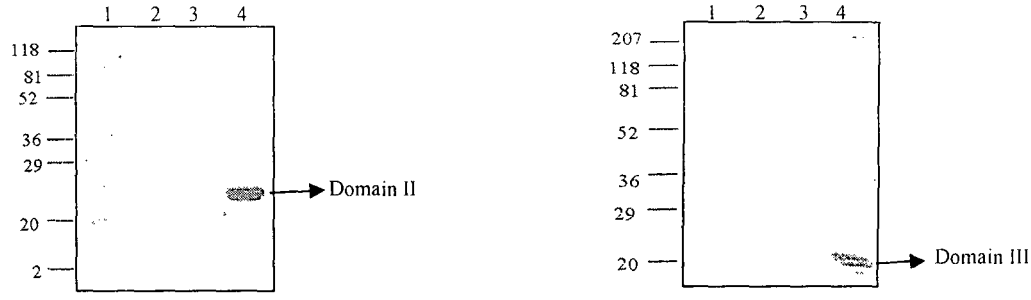


Figure 16, c. Western blot profiles of domain II and domain III proteins. Proteins were resolved by SDS-PAGE and transferred onto nitro cellulose membrane. The expressed protein was detected by anti His antibody at 1:5000 dilution. Lane 1, marker; lane 2, induced pQE-31 vector containing M15 cells; lane 3, uninduced recombinant pQE-31 carrying M15 cells; and lane 4, induced recombinant pQE-31 carrying M15 cells.

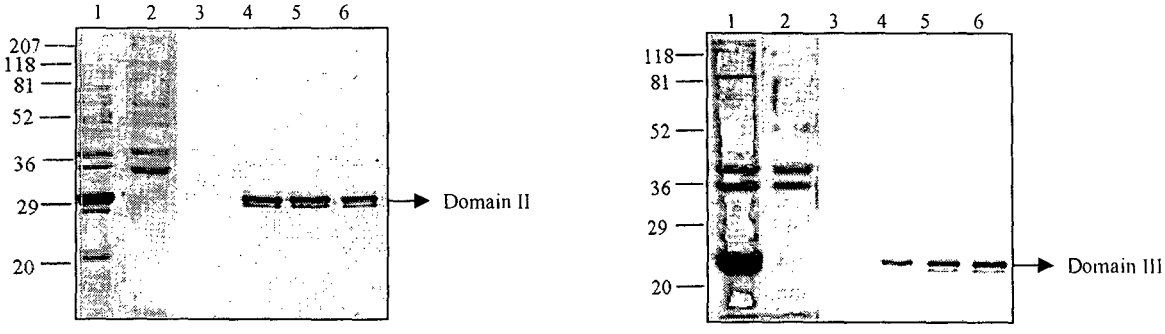
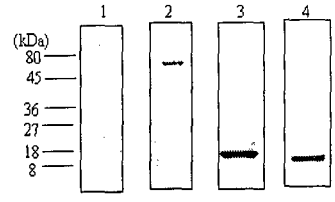


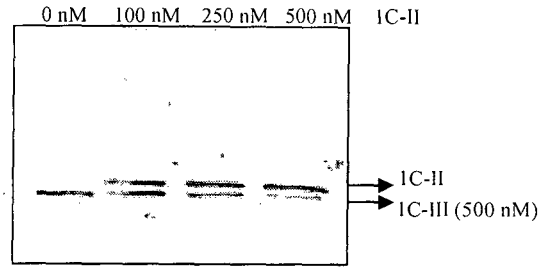
Figure 16, d. Purification profiles of domain II and domain III proteins. Solubilised domain protein samples were loaded on Ni-NTA resin and bound His-tagged domain proteins were purified by step-wise gradient of 100-250 mM imidazole. Lane 1, solubilised domain protein sample loaded on Ni-NTA resin; lane 2, Flow through; lane 3, wash fraction; lanes 4-6, 1st, 2nd and 3rd elution fractions with 250 mM imidazole.

5.3.2 Cry1C domain II has a higher binding affinity for SlAPN

Reports have suggested that all three individual domains of a toxin molecule can interact specifically with the gut epithelial membrane as separate entities. Studies with chimeric toxins and individual domains have indicated that Cry1C domain III plays an important role in determining insect specificity (de Maagd et al, 2001 and Lovell et al, 2003). It has been shown that SlAPN interacts with Cry1C on ligand blots (Agrawal et al, 2002). To investigate whether SlAPN-Cry1C binding involves both domains II and III of Cry1C, individual purified domains were employed in binding experiments with purified SlAPN. The borders of each domain were determined by multiple amino acid sequence alignment of Cry1A(a) with Cry1C, PCR-amplified using specific primers and expressed in M-15 cells harboring the recombinant pQE vector. Binding assays with purified 1C-II and 1C-III showed that both domains could interact with SlAPN as individual molecules (Figure 17a). To determine the binding affinity of the individual domains, competition binding experiments were performed in which 1C-III binding with SlAPN was competed by increasing concentrations of 1C-II. These assays showed a gradual decrease of 1C-III binding with increasing 1C-II concentrations (Figure 17b) suggesting that 1C-II had a higher affinity for SlAPN than 1C-III.



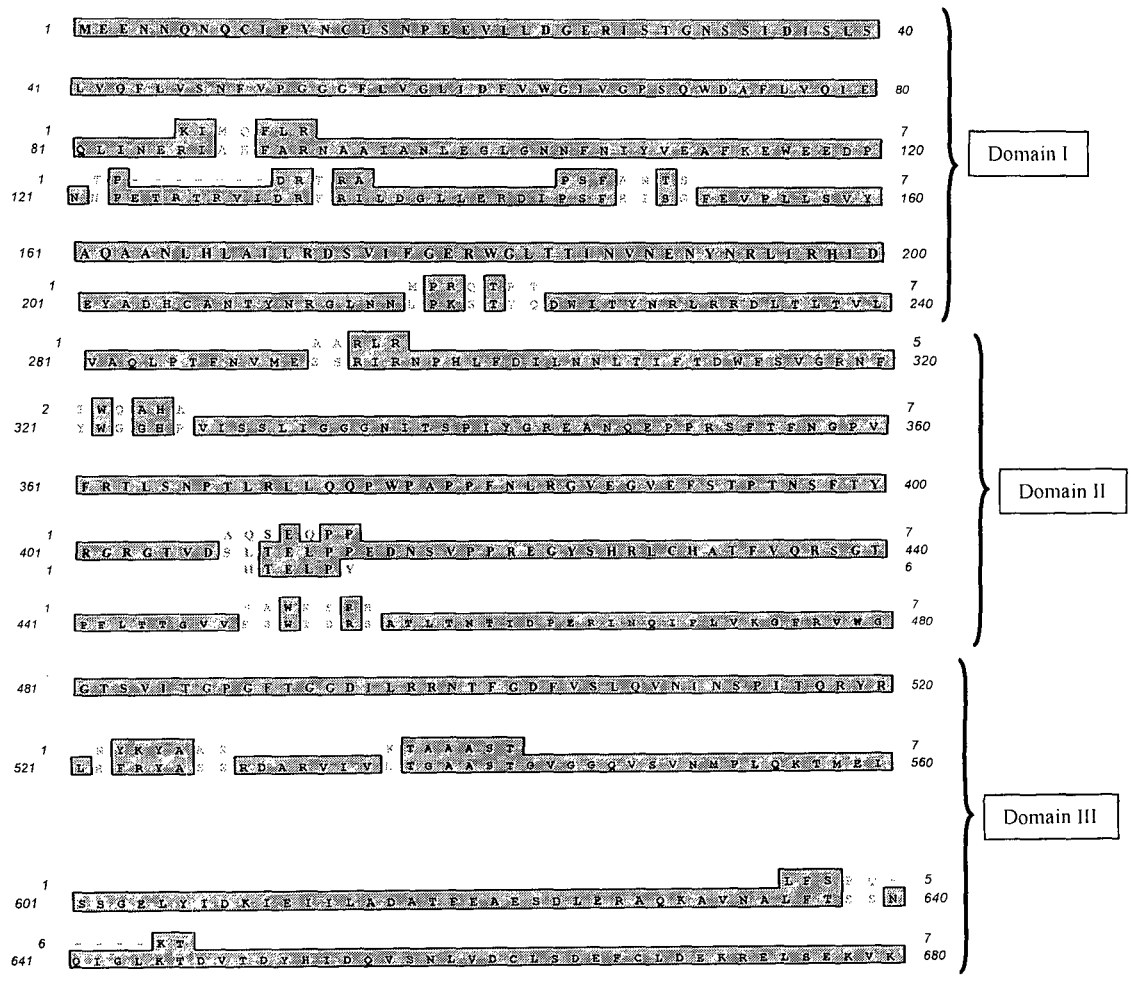
Figure, 17 a. Binding of the individual Cry1C domains to SlAPN. Purified toxins and domains were resolved on SDS-PAGE. The 65-kDa toxic fragment of biologically inactive Cry1Ac (lane 1), bioactive Cry1C (lane 2), Cry1C domain II (lane 3) and Cry1C domain III (lane 4) and electro-transferred to NC membrane. The membrane was then overlaid by pure APN (1 µg/ml in TBS) for 1h at RT to visualise binding, which was probed by anti-APN antibody (raised in Rabbit) followed by ALP conjugated anti-Rabbit antibody.



Figure, 17 b. Binding competition between D-II and D-III. Immunoblot of bound D-II and D-III, resulting from APN incubation with 500 nM D-III and increasing concentrations of D-II. APN was incubated with 500 nM D-III for 30 min for saturation binding following which increasing concentrations of D-II were added. The bound APN-domain complex was further incubated for h with anti-APN antibodies before being pulled down by protein A sepharose 4B beads. The washed beads were then resuspended in SDS-sample buffer, boiled, centrifuged and resolved by SDS PAGE. After electro blotting, the NC membrane blot was probed by anti Cry1C antibodies and the bands visualized by NBT-BCIP assay.

5.3.3 Mapping the Cry1C epitope involved in SlAPN binding by phage display

To map the Cry1C epitope implicated in toxin-receptor binding, a combinatorial phage display library of random hepta peptides was used as described in Material and Methods section of previous chapter (Section 4.2.8). After three rounds of panning against purified SlAPN, ten random phages were sequenced which revealed that all of them encoded unique amino acid sequences homologous to different regions of Cry1C toxin. Amino acid sequence of 20% clones shared similarity to domain I of Cry1C while 50% and 30% clones shared similarity to different regions of domain II and domain III respectively (Figure 18a). Though a representative phage clone homologous to a unique site in Cry1C could not be obtained, all phages obtained from final panning round showed similar apparent binding affinities to Cry1C determined by ELISA (Figure 18b). These and domain competition results suggested that multiple regions of Cry1C might be involved in binding with SlAPN.

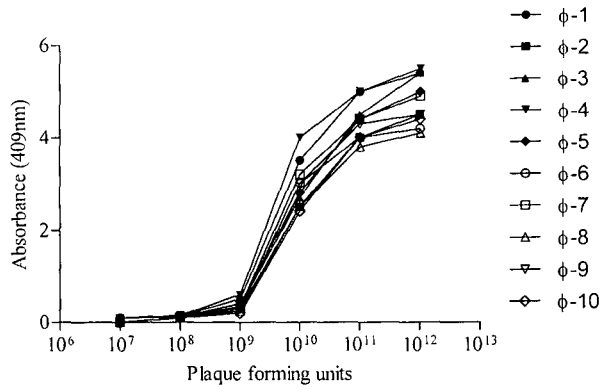


Figure, 18 a. Amino-acid sequence alignment of the selected phage clones with SlAPN. One microgram purified active APN was coated in one well of a 96-well plate and the hepta-peptide phage display library was screened for binding clones. Ten phage clones were sequenced after fourth round of panning and the sequences obtained were compared with the amino acid sequence of Cry1C. The amino acid sequence of the representative phage clones share similarities with multiple regions of Cry1C.

(A)

Panning Round	Phage Output (pfu/ml)	Tween-20 Concentration (%)	Absorbance (490nm)
0	$2x10^{13}$	-	-
1	$3x10^{5}$	0.2	0.2
2	$6x10^{6}$	0.35	0.8
3	$8x10^{7}$	0.5	2

(B)



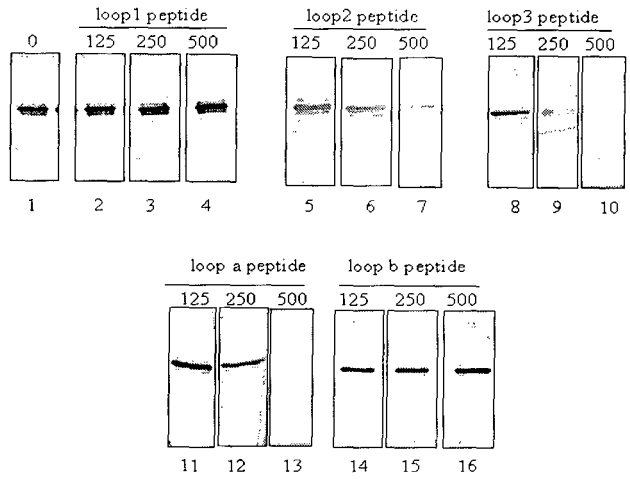
Figure, 18 b. Characterization of selected peptides for SlAPN binding. *Assaying the enrichment of bound clones (a).* Equal numbers of phages, after each round of selection, were assayed for receptor binding by ELISA. Two microgram of the toxin was coated in each well of an ELISA plate and incubated with phage clones resuspended in TBS. Tween20 concentrations used in the ELISA washing steps were same as that used during individual biopanning step. The titer output and the absorbance value of each panning round are shown. *Assaying the binding affinities of individual phages (b).* Ten random peptide-displaying phages (labeled ϕ-1 to ϕ-10) were eluted after last round of selection. All of them displayed similar apparent binding affinities to the receptor as determined by ELISA. Two microgram of the toxin was coated in each well of an ELISA plate and increasing concentrations of individual phage clones resuspended in TBS were used to study their binding. Tween20 concentrations used in the ELISA washing steps were same as that used during final panning step. Binding was analysed using HRP-conjugated anti-M13 antibody.

5.3.4 Mapping the Cry1C epitope involved in SlAPN binding by competition binding experiments

The structure of the toxin provided the basis to study the potential receptor binding segments by elucidating the sequence and structure of the loops of domain II and domain III (discussed later in section 6.3.2). The three exposed loops of domain II- loop 1(313-321), loop 2 (373-380) and loop 3 (436-443) and, two loops of domain III- loop a (533-544), loop b (592-602) were chosen for this study. To test results of phage display assays, peptides corresponding to these putative loop regions were synthesized and used as competitors in Cry1C-SlAPN binding assays (Table 9). Purified SlAPN was incubated with different concentrations of peptides in TBS for 1h before incubating the receptor with Cry1C toxin electro transferred on NC membrane. Figure 19 shows that loop 2, loop 3 and loop a peptides (lanes 5-13) competed the binding of Cry1C to SlAPN while no significant competition was shown by loop1 and loop b peptides (lanes 2-4 and lanes 14-16). This suggests that loop 2 and loop 3 of domain II and loop a of domain III are involved in interaction with SlAPN.

Table, 9. Synthetic Peptide sequences used in the study

Name	Description	Sequence
L1-1C	Amino acid sequence of Cry1C domain II loop 1	WFSVGRNFY
L2-1C	Amino acid sequence of Cry1C domain II loop 2	QQPWPAPPRN
L3-1C	Amino acid sequence of Cry1C domain II loop 3	RSGTPFL
La-1C	Amino acid sequence of Cry1C domain II loop a	DARVIVLTGAASTGVGGQ
Lb-1C	Amino acid sequence of Cry1C domain II loop b	QPLFGAGSISS
ϕ-71	Amino acid sequence of Cry1C binding phage clone	HPSFHWK
APN-CRY	Amino acid sequence of residues 128-134 of SlAPN	HLHFHLP
Scrambled (Scr)	Amino acid sequence of scrambled peptide	LRFGATP

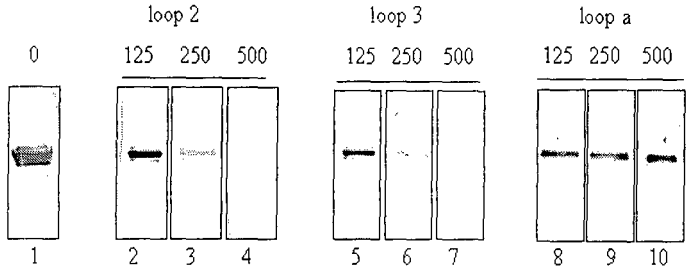


Figure, 19. Synthetic peptides homologous to DII loops 2, 3 and a compete with SlAPN binding to Cry1C. Binding assays of Cry1C with SlAPN in the presence of increasing concentrations of peptide competitors. Purified Cry1C active toxin (1μg) was loaded on SDS- polyacrylamide gel and transferred to NC membrane which was probed for receptor binding. Purified SlAPN receptor (2.5 μg /ml) was pre incubated with increasing concentrations of peptides for 1 h at RT and then overlaid on the toxin containing NC membrane. Binding was visualized by standard NBT-BCIP assay using anti-APN antibodies. Lane1, binding of Cry1C to APN; lanes 2-4, competition of binding with a 125-, 250- and 500- fold molar excess of loop1 peptide; lanes 5-7, competition of binding with a 125-, 250- and 500- fold molar excess of loop 2 peptide; lanes 8-10, competition of binding with a 125-, 250- and 500- fold molar excess of loop 3 peptide; lanes 11-13 binding of Cry1C to SlAPN with a 125-, 250- and 500- fold molar excess of loop a peptide and ; lanes 14-16 binding of Cry1C to APN with a 125-, 250- and 500- fold molar excess of loop b peptide.

5.3.5 Identifying toxin binding regions of BR-APN

The mutant BR-APN, cloned and expressed in *E.coli,* was able to bind to Cry1C in toxin overlay assays and the binding was competed by ϕ-71 peptide (as described previously in section 4.3.7, figure 15e). BR-APN was used as the ligand to screen for binding clones of the random phage display library. Seventy percent of the clones sequenced after third round of panning encoded the same amino acid sequence (ϕ72: PSPNIPI) while all others represented unique peptide sequences. ϕ-72 shared 72% similarity with a region involving loop 3 of domain II of Cry1C (^{432}ATFVQRS^{438}) suggesting that loop 3 might be the Cry1C epitope involved in binding with BR-APN region. No phage peptide homologous to any domain III

region was obtained suggesting that BR-APN-Cry1C interaction might not involve domain III. To test the hypothesis further, BR-APN-Cry1C binding in the presence of loop 2, loop 3 and loop a peptide competitors was analyzed. Figure 20 shows that while loop2 and loop3 peptides (lanes 2-7) competed the toxin-receptor binding, loop a peptide had no effect in this interaction (lanes 8-10).



Figure, 20. Synthetic peptides homologous to DII loops 2 & 3 competed with BR-APN binding to Cry1C while that of loop a did not. Purified BR-APN (1 μg) was loaded on SDS- polyacrylamide gel and transferred to NC membrane which was probed for toxin binding. Pure active Cry1C toxin (2.5 μg /ml) was pre incubated with increasing concentrations of peptides for 1 h at RT and then overlaid on the receptor containing NC membrane. Binding was visualized by standard NBT-BCIP assay using anti-Cry1C antibodies followed by ALP-conjugated anti Rabbit antibodies. Lane1, binding of Cry1C to BR-APN; lanes 2-4, binding of Cry1C to BR-APN with a 125-, 250- and 500- fold molar excess of loop 2 peptide; lanes 5-7, binding of Cry1C to BR-APN with a 125-, 250- and 500- fold molar excess of loop 3 peptide; lanes 8-10, binding of Cry1C to BR-APN with a 125-, 250- and 500- fold molar excess of loop a peptide.

5.4 DISCUSSION

Most of the toxin-receptor binding events have been studied using full length toxic region of the Cry toxins and BBMVs of the susceptible insects. This approach is based on the possibility of inter domain interactions of the toxin and presence of accessory proteins in the mid gut epithelium that might contribute to toxicity. However, an in-depth analysis of individual toxin domains participating in these interaction events that determine the binding specificity of the toxin is essential. In this study, interactions of Cry1C were evaluated with purified SlAPN receptor for individual domains. It was found that, both domain II and domain III of the toxin were interacting with the receptor as revealed by the binding experiments. The results suggested that the toxin-receptor interaction might involve multiple binding sites in both molecules. It is found to be in agreement for Cry1C interactions in *P.xylostella* (Zhao et

al, 2003 and Atsumi et al, 2005) and *S.exigua* (Lovell et al, 2003). In the former case, this is supported by the observation that two separate linkage groups contribute to Cry1C resistance. Similarly, the higher estimated Kd values for Cry1C domain II binding with respect to the whole toxin in *S.exigua* suggests the presence of more than one binding site. By using individual domains and purified SlAPN receptor, this study showed that Cry1C-SlAPN interaction involves both domain II and domain III of the toxin molecule. Furthermore, the observation that domain II could compete with domain III binding while the latter was still able to bind to the receptor in reverse competition experiments, suggests the presence of two different but structurally close SlAPN epitopes, involved in binding.

To facilitate the identification of toxin and receptor epitopes phage display technology was utilized. The preliminary findings from mapping the toxin epitope using full length SlAPN as the ligand further suggested the existence of multiple toxin epitopes. All of the eluted phage clones showed specific binding with Cry1C in ELISA and yet were found to be homologous to various regions of all three domains of the toxin. This led to the identification of the putative loop regions in the toxin molecule by structural studies (Chapter 3, section 6.3.2) which were studied for their involvement in specific binding to SlAPN. The involvement of multiple Cry epitopes in APN interaction has also been suggested in *Bombyx mori* and *Manduca sexta*. While in *B. mori*, the Cry1Aa-APN interacting epitopes have been mapped to residues of domain III β16 (^{508}STLRVN^{513}) and β22 (^{582}VFTLSAHV^{589}) (Jenkins and Dean, 2000), in *M. sexta* loop 2 and loop 3 of domain II of Cry1Ab were shown to be involved in APN recognition (Atsumi et al, 2005). Therefore to assess the contribution of the loops in receptor interactions, synthetic peptides corresponding to various loop regions were used in competition binding experiments. It was interesting to note that loop 2 and 3 of domain II and loop a of domain III had similar binding capabilities since synthetic peptides corresponding to these loop regions competed with the binding of SlAPN to Cry1C toxin. However, loops a and b of domain III were also evaluated critically since the corresponding regions are absent in the template sequence (Cry1A(a)). Based on the results of phage display experiments and competition binding experiments employing synthetic peptides, three putative SlAPN binding epitopes (loop 2, loop 3 and loop a) in Cry1C protein were identified.

Since loops of domain II and domain III of Cry1C toxin do not lie very close to each other in its 3-dimensional structure (Section 6.3.2), it is speculated that domain II of Cry1C interacts

with the APN-CRY epitope (^{128}HLHFHLP^{134}) of SlAPN while the other domain might be interacting with some other epitope of the SlAPN protein.

Homology based Modeling of Cry1C Toxin and SlAPN Receptor AND In-silico Docking of the Derived Structures

6.1 INTRODUCTION

The primary, secondary and 3-D structure of several insecticidal proteins has been solved and analysed in great detail. Solution of 3-D structure of Cry toxins (Cry1Aa, Cry1Ac, Cry 2Aa, Cry3Aa, Cry3Bb, Cry 4Aa and Cry 4Ba) by x-ray crystallography has been an important advancement in the understanding of the mode of action of these toxins (Grochulski et al, 1995; Derbyshire et al, 2001; Morse et al, 2001; Li et al, 1991; Galitsky et al, 2001; and Boonserm et al, 2005 and 2006). The insecticidal polypeptide is grouped into three distinct domains; domain I, domain II and domain III (Piggot and Ellar, 2007). Domain I consists of an alpha helical bundle in which six helices surround a central helix. Each of the outer helices is amphipathic in nature; polar or charged residues are generally solvent exposed and hydrophobic residues, typically aromatic in nature, project towards the central helix. Polar groups are present in interhelical spaces, but all are either hydrogen bonded or involved in salt bridges. These properties, and an overall structural similarity to the pore forming domain of colicin, led to the hypothesis that domain I was the major determinant of pore formation in Cry toxins. Domain II has a pseudo three-fold symmetry and is formed by three antiparallel β-sheets each terminating in a loop, packed around a triangular hydrophobic core (Grochulski et al, 1995 and Morse et al, 2001). Two of the sheets are composed of four strands in a Greek-key motif and are solvent exposed. The third sheet is arranged in a Greek-key like motif with three strands and a short alpha-helix (Peggot and Ellar, 2007). Mutagenesis studies have suggested that this domain participates in receptor recognition and hence insect specificity (Baalester et al, 1999 and Jurat-Fuentes and Adang, 2001). Lastly, domain III is a 'jelly roll' of 2 antiparallel β-sheets. Both sheets are composed of five strands with the outer sheet facing the solvent and the inner sheet packing against domain II. Two long loops extend from one end of the domain and interact with domain I (Piggot and Ellar, 2007). While the functional roles have been assigned to various domains the precise contact residues that affect the insecticidal protein activity have not been identified or evaluated.

The receptors of the Cry toxins have been classified into five major groups: a cadherin like protein (CADR), a glycosyl phosphatidyl-inositol (GPI)-anchored aminopeptidase-N (APN), a GPI-anchored alkaline phosphatase (ALP), a 270 kDa glycoconjugate and glycolipids (Jurat-Fuentes and Adang, 2004; Griffits et al, 2005; and Valaitis et al, 2001). Since 1994, more than 60 different APNs from different Lepidopterans have been sequenced and

registered in databases showing the high diversity in isoforms (Herrero et al, 2005). This exopeptidase is believed to be anchored in lipid rafts and serves as a binding molecule for Cry1A, Cry1C, Cry1F and Cry1J toxins in different Lepidopteran species (Jurat-Fuentes and Adang, 2001; Luo et al, 1996; and Agrawal et al, 2002). Identification and characterization of receptors to insecticidal proteins has been achieved by three independent strategies. Ellar et al (2001) created transgenic *Drosophila* with APN of *Manduca sexta* and demonstrated simultaneous recruitment of sensitivity to Cry1Ac by transgenic *Drosophila* flies. Rajagopal et al (2002) silenced the APN in *Spodoptera litura* by RNAi resulting in abrogation of insecticidal activity of Cry1C protein. On the other hand Barrows et al (2007) resorted to genetic screening of *C.elegans* and correlated action of Cry5B protein with the mutants that lacked specific glycolipids in their midgut. However, the details of APN-toxin interaction are controversial and require extensive research. The mapping of binding epitopes of APN is a challenging problem since, in contrast to toxins, no 3-D structure of insect APN is available and no mutagenesis studies of the receptor domains have been performed.

6.2 MATERIALS AND METHODS

6.2.1 Modeling of Cry1C and SlAPN and Docking of the derived structures

A standalone version of BLAST 2.2.4 (Altschul et al, 1990) was used to blast the target sequences against PDB database to retrieve the top hits to select respective templates for 3D-homology modeling. Using ClustalX 1.83 (Thompson et al, 1997), an alignment was obtained between the best hit and the target sequence. Extensive manual inspection and curation of alignments was done and *alignment.check* script of MODELLER (Sali and Blundell, 1993) was used to find any trivial alignment mistakes. These alignment files (after successful run of *alignment.check*) were given as input to the MODELLER 8v2, one at a time for model building. Using WHATIF programs (Vriend, 1990, missing side chains were added to the respective models. Further, removal of bumps, if any, was carried out. Energy minimization (EM) was accomplished by AMBER FF02 of SYBYL 7.2 as force field and applying AMBER charges (formal charges method). Both steepest descent and conjugate gradient methods with the termination gradient of 0.05 kcal/mol was applied to the models. EM was done *in vacuo* to relieve steric interactions within the model structure. The quality of energy

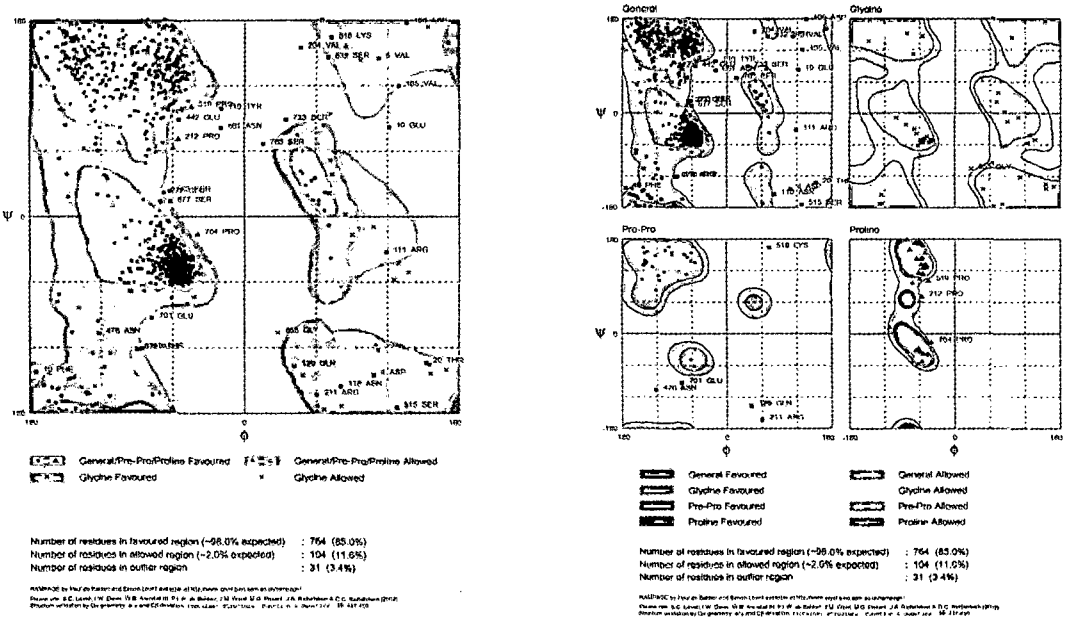
minimized models was checked by various WHATIF programs. Coarse and fine packing of amino acids, bond lengths, torsion angles and planarity were all observed to be in regular fashion and within the allowed standard deviations for both the models. Stereochemical parameters of the model structures were analyzed with the PROCHECK program (Laskowski et al, 1993). Local environment of atoms, packing quality and folding states of models were further studied using more advanced methods like PROSA II (Sippl, 1993), Verify3D (Luthy et al, 1992) and ANOLEA (Melo et al, 1997). Finally, to access the deviation of modeled structure with their respective templates, an analysis of model and template structures superimposition was performed. STAMP based 3d-SS (Sumathi et al, 2006) and CE/CL based Compare3D methods were used for this analysis. Using GRAMM v1.03 (Vakser, 1995), the docking process was carried out in two modes, mode1: provided the HLHFHLP sequence moiety of APN receptor protein as interface residue constraints for consideration during docking and mode2: without any residue constraint mode. For each mode, first 2000 predicted docked complexes were ranked and rescoring functions during terminal runs of simulations were applied. This resulted in top 10 complexes for each mode for final analysis.

6.3 RESULTS

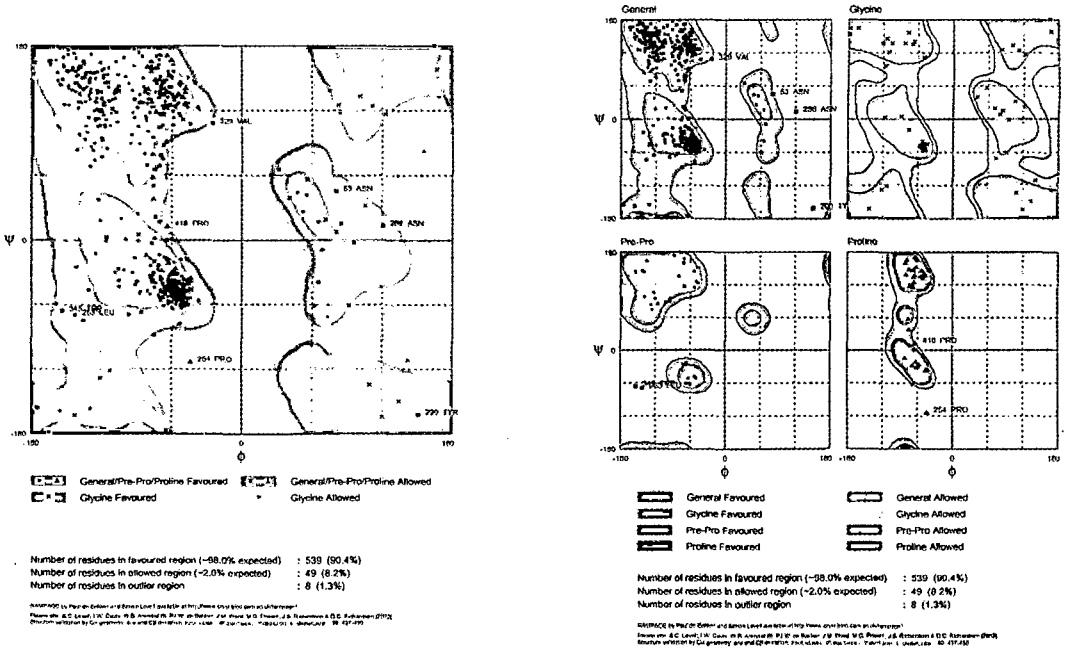
6.3.1 Validation of Cry1C and APN models

The results of secondary structure prediction by PSIPRED coincide with the secondary structure of the final models which indicates that the homology models are consistent with current understanding of protein structure. Moreover, Ramachandran plots generated using RAMPAGE (Lovell et al, 2003) and iMolTalk (Diemand and Scheib, 2004) web servers suggests that >96% of the residues of the APN are in core plus allowed regions. For Cry1C structure this was true for more than 98% of the residues. The PROSA II gave a Z-score of -8.58 for Cry model (template: 1CIY= -9.47) and -7.66 for APN model (template: 1Z5H= -13.04), which suggests their acceptable structure. Similarly, using ANOLEA and Verify3D, majority of the structure of both models were found to be confined within the acceptable range of energy calculations and atom packing. 3d-SS gave an rmsd of 0.672Å for Cry model whereas 1.142Å for APN model when compared to their respective template structures. A highly comparable result was also obtained by Compare3D which gave an rmsd of 0.73Å for Cry model while 1.1Å for APN model. After a rigorous refinement and extensive analysis of

the homology models of Cry and APN proteins, their 3D structures are believed to be in agreement to most of the structural features and constraints. Thus, these acceptable models were then used for docking purpose.

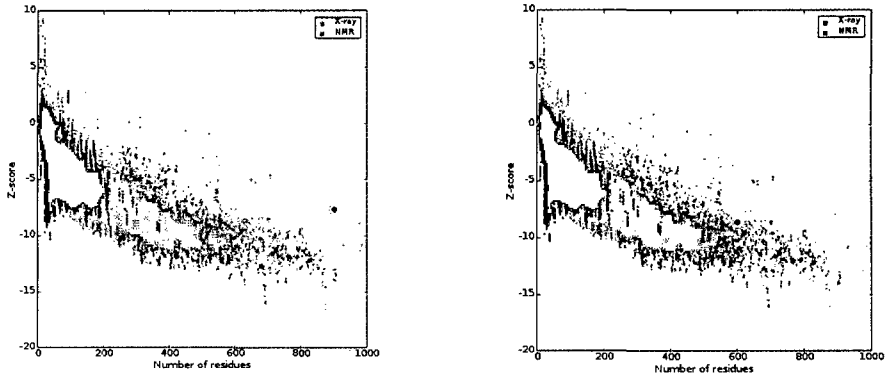


Figure, 21. Ramachandran plot of SlAPN.



Figure, 22. Ramachandran plot of Cry1C.

(a) (b)

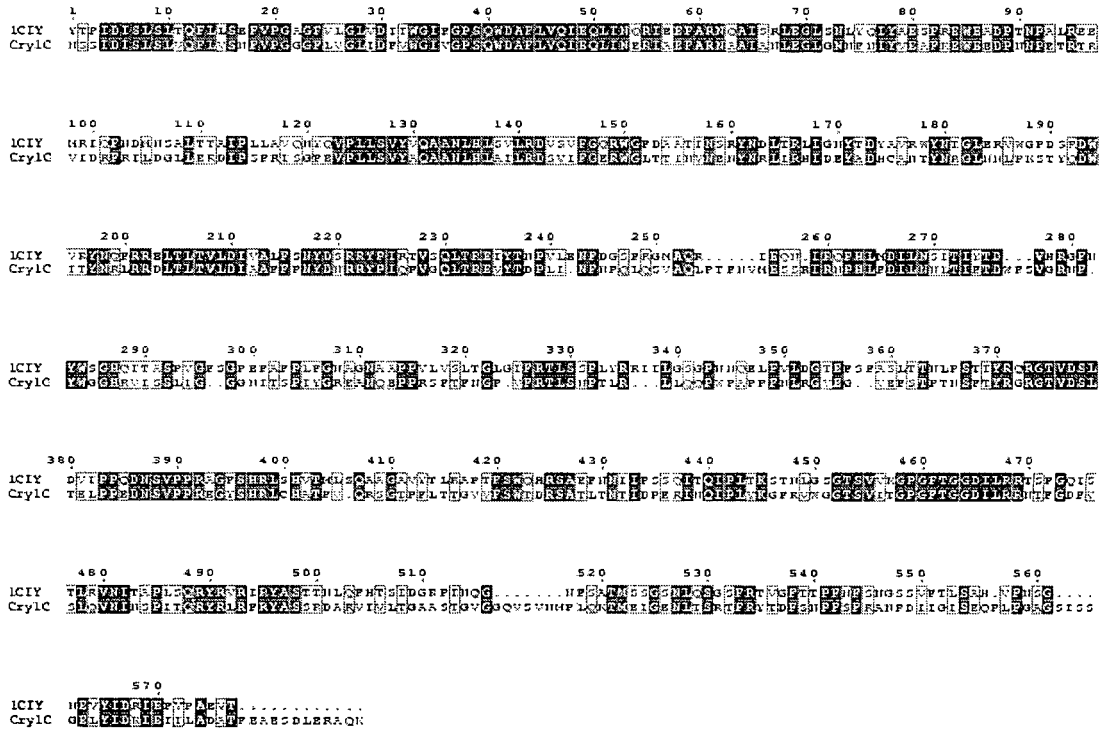


Figure, 23. Z-plots of (a) SlAPN and (b) Cry1C.

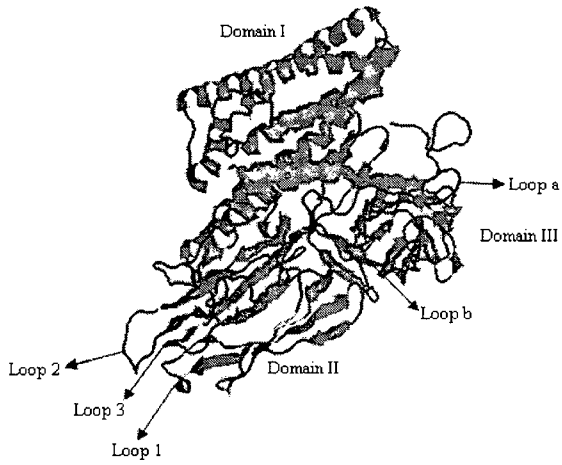
6.3.2 Description of Cry1C structure

The BLAST showed 46% identity (60% similarity) with its template, 1CIY protein (an insecticidal toxin) at E-value 2.3E-145 as closest homologue (Figure 24). The final identity of 48.5% was achieved after refinement of obtained alignment. This covered a sequence length of 598 residues of Cry1C protein for a region of 32-630 residues. The three endotoxin domains of both target and template sequences are perfectly overlapped along the alignment region. Other than these domains, Cry protein has an additional domain; CBM_6 domain (Carbohydrate binding module (family 6)), as a part of endotoxin_C domain (residues 474-582). The derived 3-dimensional structure of Cry1C revealed a folding pattern similar to those of known crystal structures of Cry toxins (Figure 25). The N-terminal domain (I) (32-253) is completely α-helical in nature. The central helix H5 of the anti parallel helix bundle is entirely encircled by 7 outer helices. The central domain (II) (261-457) has mainly β strands and two short α-helices. It has 3 sheets (Figure 26a). The first sheet is very similar to that of Cry1A(a). It is formed by four strands which are connected according to the typical "Greek-key" topology. Search for second sheet revealed only 2 β -strands in Cry1C. The third sheet also resembles Cry1A(a) and is formed by two separate fragments made by 3 strands: the C-terminus of domain II donates 2 central strands while the outer strand comes from N-terminus. The C-terminal domain (III) (467-616) is all β -strands except one short α-helix (α9=F314-V316) which is not present in the template toxin structure. Domain III consists of two twisted, anti parallel β sheets forming a jelly-roll topology. Each sheet (Figure 26b) is

made of five main beta strands. Loop b joins the outer and the inner sheet. The structure of the toxin provided the basis to identify the potential loop regions. A close inspection of the alignment with respect to the loop regions revealed that besides being in low conservation, the regions near the loops contain many mutations either in the template or the target sequence thus implying their importance as specificity determining regions. SPPIDER (Porollo and Meller, 2007) analysis of Cry1C showed that all the residues of domain II loops were interfacial while those of domain III loops were either in interfacial or buried regions. Prediction probabilities for interacting sites revealed that loop 2 had the highest probability (>0.9) closely followed by loop 3 (>0.8). While loop a and loop 1 had lower probabilities of being in interaction site (0.5 and 0.3 respectively), only 4 out of 11 loop b residues had significant interacting probability (>0.55). Structural analyses of Cry1C showed that the domains of the toxin are closely packed together with 500 hydrogen bonds. Domain II makes the maximum number of contacts- 20 H bonds with domain III and 13 with domain I. Domain I and III are connected by only 7 hydrogen bonds. None of the inter-domain contacts involve loop regions.

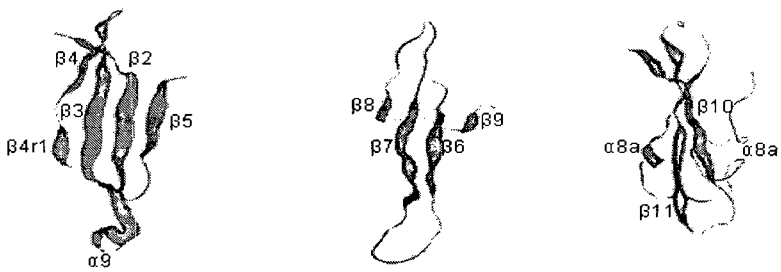


Figure, 24. Sequence alignment of the 65-kDa active form of Cry1C with the crystal structure of Cry1Aa. The degree of conservation among the sequences is represented by background shading of the residues.

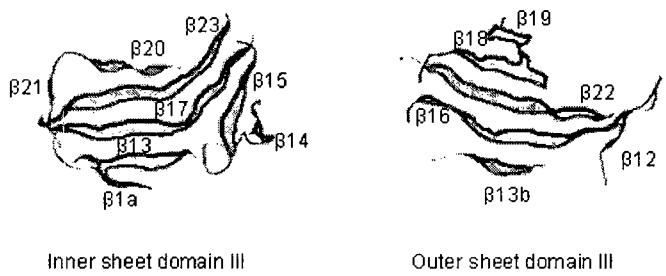


Figure, 25. Homology-based model for Cry1C. Schematic ribbon presentation of 65-kDa Cry1C toxin illustrating the three-domain organization of the protein molecule. Loops analyzed in the study are labeled.

(a)



(b)

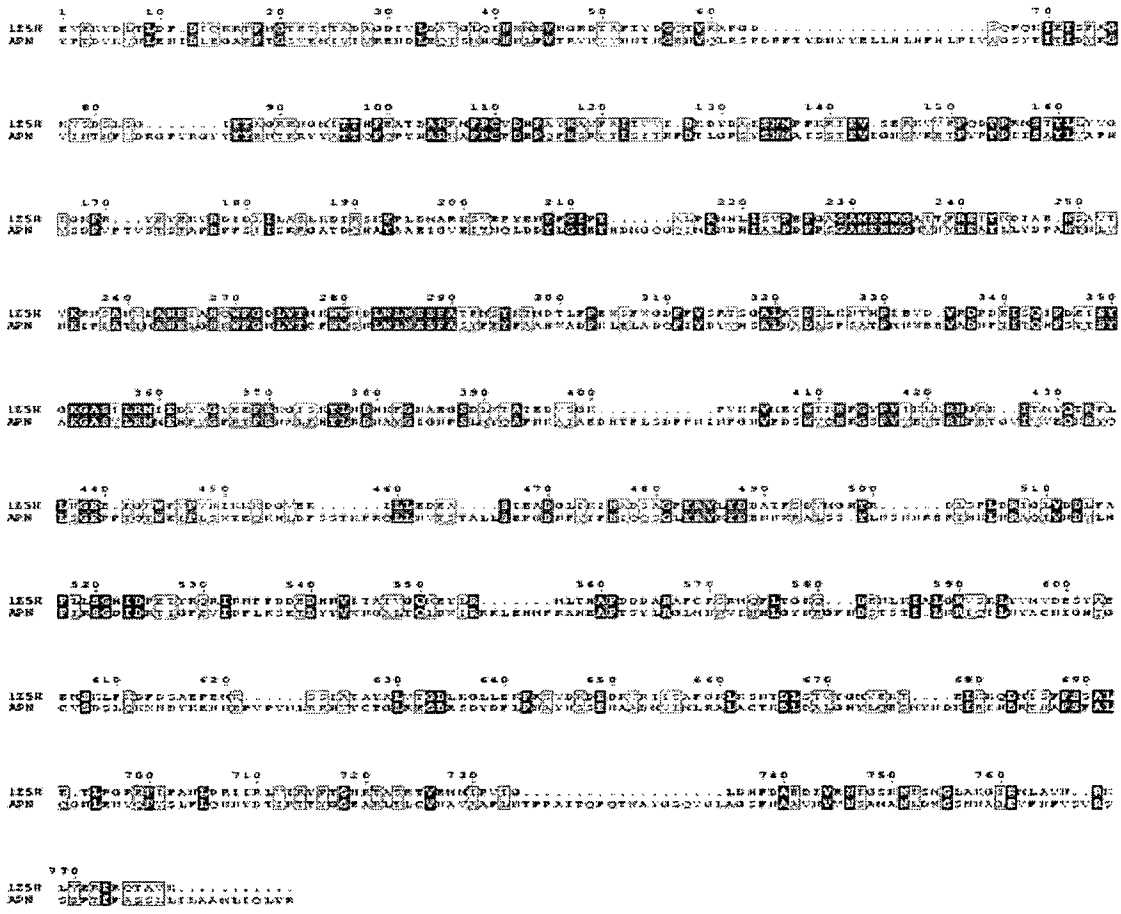


Figure, 26. Ribbon representations of individual β-sheets of domain II (a) and domain III (b) of Cry1C. Residue numbers in the predicted secondary structural elements are: β1a=P258-V259, β1b=V266-T268, α8a=P270-I272, β2=D302-N313, α9=N314-V316, β3=F320-L321, β4=N336-T338, β4r=Y342-G343, β5=R352-F355, β6=L364-L369, β7=P380-V386, β8=T400-Y401, β9=V406-D407, β10=H427-F435, β11=L443-D453, β12=T461-I462, β13=I467-P471, β13b=R477-V478, β14=S483-I486, β15=I495-R498, β16=G502-I511, β17=Y519-S527, β18=D531-T537, β19=A540-L545, β20=R570-D574, β21=F578, β22=D584-Q592, β23=L605-L614.

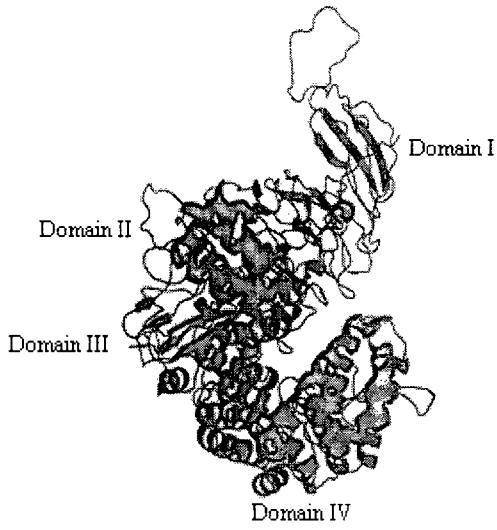
6.3.3 Description of SlAPN structure

The BLAST of SlAPN showed 29% identity (47% similarity) with 1Z5H protein at E-value 1.1E-50 as top hit (Figure 27). 1Z5H is a zinc aminopeptidase (tricorn interacting factor F3) from *Thermoplasma acidophilum* (Kyrieleis et al, 2005). An extensive refinement of alignment was carried out for a total of nine changes which lead to the increase in identity to 31%. This covered a sequence length of 901 residues of APN protein for a region of 51-952 residues. The two aminopeptidase (F3 and SlAPN) share the conserved HEXXH zinc binding motif. The third zinc binding ligand in the motif NEXFA is at a conserve distance of 18 amino acid residues from the second zinc binding ligand, histidine. This makes SlAPN a member of gluzinicin family of aminopeptidase (Hooper, 1994). However, SlAPN deviates at the third anchoring glutamate residue corresponding to Glu 101in factor F3, where it carries a glutamine residue (Gln 179). Like F3, SlAPN has a hook like structure formed by four domains (Figure 28). The longer left lobe of the hook is constituted by domain I (residues 51-251) and domain II (residues 252-518) while the right lobe is composed entirely of domain IV (residues 603-952) helices. Fifth helix of domain II, entire domain III (residues 519-602) and first three helices of domain IV form the connecting turn of the helix, joining the two lobes. Adjacent domains are connected to each other by hydrogen bonds (3 between domain I & II, 8 between domain II & III and 13 between domain III & IV). Domain I (Figure 29) is formed of two beta sheets. The four stranded upper sheet has a Greek-key topology while the lower sheet has five strands that run anti parallel to each other. The catalytic domain, Domain II (Figure 29), shows the maximum structural similarity with the template structure. The first part of this domain is a mixed α/β structure. The outer strands are parallel to each other but anti parallel to the inner strands. The third and fourth strands are interrupted by a helix α1. The second part of the domain carries the conserved HEXXH and NEXFA motifs on two separate helices α-3 and α-4 (respectively) which are connected by a beta strand β-21. The domain III (Figure 29) of SlAPN is a beta-sandwich formed of eight beta strands. Region corresponding to template's β25 (545PPTQ548) is in a loop region instead of forming a beta sheet. The predominantly helical domain IV (Figure 29) is organized into a super helix consisting of two modules approximately perpendicular to each other. A SPPIDER analysis for interacting residue prediction identified three high probability (>0.9) regions in SlAPN – region I involving residues 123-135, region II of residues 881-889 and region II of residues 936-941. These were also the longest stretches of amino acids predicted to be in interfacial

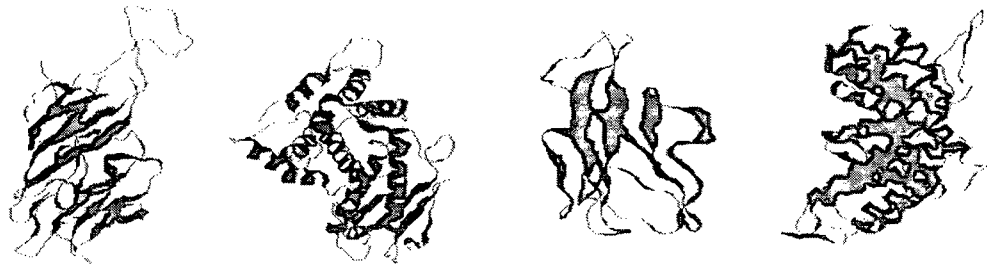
region. While regions I and II were free from any intra-molecular hydrogen bonding, residues from region III were involved in multiple hydrogen bonding with each other.



Figure, 27. Sequence alignment of the Spodoptera litura Aminopeptidase N with the crystal structure of Tricon Interacting Factor F3 of Thermoplasma acidophilum. The degree of conservation among the sequences is represented by background shading of the residues.



Figure, 28. Homology based model for SlAPN. Schematic ribbon presentation of the hook shaped structure of SlAPN, illustrating the four-domain organization of the receptor molecule.



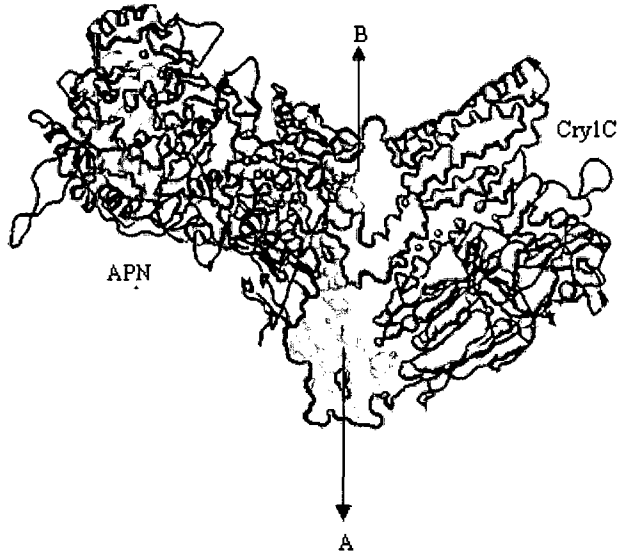
Figure, 29. Ribbon representations of individual domains of SlAPN. Domain I. Residue numbers in the predicted secondary structural elements are- β1=P52-D54, β2= T70-V77, β3=M89-V96, β4=V99-V101, β5=N103-N104, β6=E108-V110, β7=Y140-G148, β8=Y173-T175, β9=P188-F190, β10=I201-R205, β11=S212-S214, β12=I218-V223, β13=V228-F232, β14=I238-S239, β15=F245-V247. ***Domain II.*** Residue numbers in the predicted secondary structural elements are: β16=P252-T258, β17=S264-R268, α1=Y277-L293, β18=G307-M313, β19=G319-M321, β20=M326-R330, α2=E331-Y333, α3=L343-H359, β21=V366-C368, α4=W371-A392, α5=L398-D405, α6=V407-D414, α7=T439-V456, α8=P458-D471, β22=G475-G477, α9=P479-A489, α10=F505-Q514. ***Domain III.*** Residue numbers in the predicted secondary structural elements are- β23=S518-R524, β24=I532-Y539, β26=I552-E558, β27=L562-L573, β28=T576-S577, β29=F588-F591, β30=Y599-K602. ***Domain IV.*** Residue numbers in the predicted secondary structural elements are- α11=E605-L611, α12=K625-S641, α13=R646-L656, α14=Y662-I675, α15=F689-L704, α16=S715-K742, α17=R759-E768, α18=R771-N779, α19=S784-A796, α20=T800-E812, α21=I821-L832, α22=L836-Y857, α23=A861-F873, α24=A900-N906, α25=A910-A937

6.3.4 Description of the docked complex M1.8

Earlier phage display and competition binding assay results suggested that the APN-CRY (Cry binding sequence of SlAPN) sequence ^{128}HLHFHLP^{134} of SlAPN is involved in binding with the Cry1C toxin. Using GRAMM v1.03, the docking process was carried out in two modes, mode 1: provided the APN-CRY sequence (HLHFHLP) of APN receptor protein as interface residue constraints for consideration during docking and mode 2: without any residue constraint mode. Using contact map analysis (CMA) tool (Vladimir et al, 2005), the interface residues of each of the ten final docked complexes of both modes 1 and 2 was studied. It was found that seven complexes of mode 1 and six complexes of mode 2 were having the complete APN-CRY region along with flanking residues conserved within the interaction surface. Other complexes were having at least 70% of this region as interface residues. Most importantly, using model, seven complexes where the APN-CRY region was found to be interacting with loop 3 or / and loop 2 of Cry protein were obtained. The reliability of this prediction is enhanced as four of these complexes were also observed in mode 2 outputs, where no interface residue constraints were given. The remaining three complexes obtained from mode 1 showed APN-CRY region to be interacting with other regions of domain II. The models were also subjected to the interface analysis by SPPIDER. The SPPIDER analysis was found to be in consensus with most of the interface interacting residues, as predicted by CMA. In most of the complexes two regions of contact were identified: one involving interaction of APN-CRY region of APN and domain II of Cry1C and second involving C-terminus of domain I of APN and domain I of Cry1C. Since the aim of this study was to identify the APN-CRY cognate binding epitope in Cry1C, one of the complexes (complex M1.8) obtained from mode 1 was chosen as the representative model to study APN-Cry1C interaction in detail.

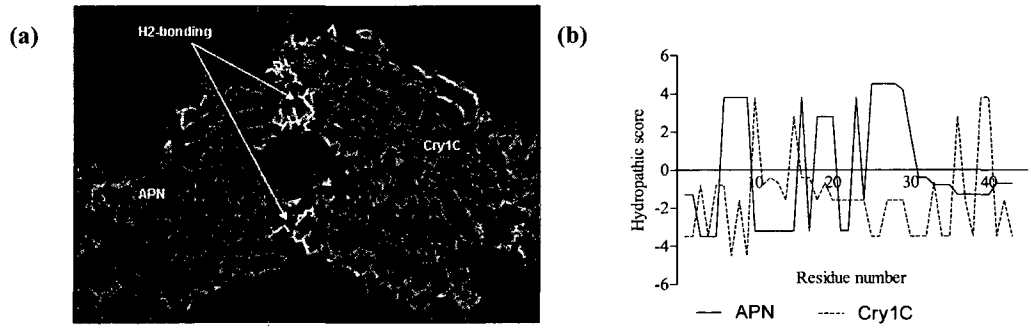
The docked complex (complex M1.8) showed two regions of contact between the receptor and the toxin (Figure 30). While region A involves interactions between domain I of Cry1C and latter half of domain I and 3 residues of domain II of APN, region B showed multiple interactions involving loop 2 and loop3 of Cry1C domain II and first half of domain I of APN (including the APN-CRY sequence ^{128}HLHFHLP^{134}). CMA analysis shows that the maximum area of contact involves loop 3 of Cry1C and a region involving APN-CRY of (128-134) SlAPN. The specific interaction showed significant contact area between His-128::Arg-437 (49.5 Å^2), His-128::Gly-439 (42.5 Å^2), Leu-129::Gly-439 (36.7 Å^2) and Leu-133::Pro-375 (30

$Å^2$) amino acid pairs of APN:Cry1C. It is interesting to note that while β-carbon atom of His-128 of APN was having exclusive interaction with oxygen atom of Gly-439 of the toxin, other atoms were having multiple interactions with atoms of Arg-437 of Cry1C. An analysis of the putative forces involved in the receptor toxin interaction revealed both hydrogen bonding (Figure 31 a) and Van der Waal overlaps in the region B of contact while the region A lacked any overlaps. Residues Thr-97, Val-99, Thr-141, His-128, Leu-129, Ile-135 and Ser-139 of SlAPN and residues Asn-275, Pro-276, Gln-277, Leu-371, Gln-373 and Gly-439 of Cry1C are engaged in Van der Waal overlaps. CMA of the docked complex revealed that each residue in the interacting regions is capable of multiple interactions; for example, His 128 of APN interacts with Leu 278, Arg 437, Ser 438, Gly 439, Thr 440, Pro 441 and Phe 442 of Cry1C. Therefore, the hydropathicity of individual interaction events was analyzed and not just the linear amino acid sequence of the two proteins. Since, it has been demonstrated that ^{128}HLHFHLP^{134} region of APN is involved in interaction with loops 2 and 3 of Cry1C and complete CMA predicted more than hundred interactions between the two molecules, the focus was on the interactions near the APN-CRY epitope. The hydropathy profiles of interactions involving Tyr123-Thr143 region of APN were determined and compared. Figure 31b shows that the hydropathy profiles of the interacting epitopes of Cry1C and APN are reverse to each other.



Figure, 30. Ribbon representation of docked complex M1.8. The two regions of contact (A and B) of the interacting toxin (blue) and receptor (green) molecules are shown. The interacting residues are: region A= residues E74, I76, I78, V79, V96-V101, R113, P115, D116, Y123-T143 of SlAPN and S220, T221, Q223,

N273-S280, L284, P285, L369-P375, R437-F442 of Cry1C, region B= residues R198, A217-S220, T231, Y233, P234, P262, D299, M306 of SlAPN and D73, V77, E80, Q81, N84-F90, R92 of Cry1C. The amino acid residues in the binding epitopes are in sphere representation.



Figure, 31. Analysis of docked complex M1.8. *(a) Stick representation of the binding epitopes of Cry1C and APN.* The residues engaged in hydrogen bonding are Ser-139 and Ser-219 of SlAPN and Gln-277 and Arg-86 of Cry1C (colored green).Image generated by iMoltalk. *(b) Comparison of hydropathy profiles of APN (full line) and Cry1C (broken line) interacting residue pairs as obtained from CMA of the docked complex.*

6.4 DISCUSSION

To further validate the binding experiment results and to facilitate mapping of the second Cry1C binding site of SlAPN, in silico docking of the two protein molecules was attempted. GRAMM v1.03 was used which performs an exhaustive 6-D search through the relative rotations and translations of the molecules. The advantage with this docking program is that no prior information about the binding or interacting sites is mandatory and its ability to smoothen the surface representation to account for possible conformational changes upon binding within the rigid body docking approach. The docking studies identified two regions of binding in both APN and Cry molecules. Consistent with the competition binding experiments, in silico study also suggested that the ^{128}HLHFHLP^{134} region of SlAPN interacts with loop3 of domain II of Cry1C toxin. The second interaction site probably involves domain I of Cry1C and domain I of SlAPN. Though the APN epitope interacting with loop a of domain III could not be identified, it is tempting to propose that this second epitope might lie in domain III or domain IV of SLAPN and is behaving like a weak affinity binding region. This proposition is based on following observations: i) from domain competition experiments, it is proposed that the two receptor epitopes are structurally close enough to bring about

significant interactions, ii) one of the two epitopes has been mapped to domain I of the receptor, iii) most of domain I region has been expressed in BR-APN mutant (deletion mutant of SlAPN carrying the BR-CRY sequence ^{128}HLHFHLP^{134} and expressed in *E.coli*) whose binding with the toxin was not affected by synthetic peptide homologous to loop a of domain III, and iv) domain II being the catalytic domain, can be excluded from toxin binding function thereby leaving domain III and domain IV as the possible second candidate epitope involved in toxin binding. However, further studies with individual domains of the receptor and the Cry toxin are required to validate this proposition.

However, this is the first study of homology modeling of an insect aminopeptidase and this will act as a useful benchmark in further studies of identification of receptor epitopes. Additionally, for the first time a Cry toxin-receptor docking has been attempted and shown to correlate with the experimental data. Analysis of docked complex suggests that binding of domain II loops to the APN-CRY epitope might be responsible in bringing domain I of Cry1C close to the lipid raft anchored receptor thereby facilitating its insertion into the membrane. However, extensive mutagenesis and substitution studies need to be employed in this direction to precisely elucidate the sequence of events leading to toxin insertion and pore formation.

Elucidating the Role of Critical Residues of Toxin and Receptor Epitopes in Receptor recognition and Toxicity

7.1 INTRODUCTION

B.t. insecticidal proteins of different specificities are believed to have similar mode of action. The complex process of B.t. toxicity includes multiple steps that include (i) solubilisation of the ingested inclusions to release protoxin proteins (ii) protease assisted activation of the protoxin in the reducing environment of the midgut to form active toxic core (iii) binding of the active toxin to receptor molecules on the brush border epithelium of insect midgut (Lee et al, 1992) (iv) ion-channel formation across the epithelial cell membrane ultimately killing the host insect (Schwartz et al, 1991). The susceptibility of a host insect to the toxin depends primarily on the association of the toxin to the receptor molecules. This makes toxin-receptor binding studies very crucial to determine the specificity, toxicity and eventually insect resistance mechanisms.

Toxin-receptor binding involves multiple epitopes on both molecules. It is now widely accepted that receptor-binding process of Cry1 toxins in several Lepidopteran insects involves two steps. At an early stage of binding there is a large accumulation of reversible receptor and toxin complex which is followed by the formation of an irreversibly associated complex either with the receptor or membrane (Liang et al, 1995 and Rajamohan et al, 1996). Membrane binding studies with brush border membrane vesicles (BBMV) isolated from susceptible insect mid guts have indicated both direct and indirect relationship between toxicity and binding affinity for Cry1 toxins (Garczynski et al, 1991 and Wolfersberger, 1990). Interestingly, only a direct correlation between irreversible binding and toxicity has been demonstrated for all insects (Liang et al, 1995, Rajamohan et al, 1995 and Rajamohan et al, 1996). The prediction that domain II is involved in receptor binding has led to extensive substitution of residues at this loop in Cry3A, Cry1A and Cry1C by mutagenesis (Wu and Dean, 1996; Lee et al, 2001; Herrero et al, 2004; and Rauf and Ellar, 1999). Deletion of hydrophobic residues of loop 3 of Cry1Ab and Cry1Aa toxins resulted in reduced toxicity to their target susceptible hosts *M.Sexta* and *H.virescens,* and *B.mori* and *M.Sexta* respectively (Rajamaohan et al, 1996). Reduced initial binding to midgut vesicles prepared from these insects was suggested to be responsible for the loss of toxicity in these cases. Substitution studies suggest that the loop 3 residues of these toxins establish hydrophobic interactions with the receptor molecule which affect initial binding. However, in Cry3A, substitution of loop 3 residues with alanines resulted in an increase in toxicity in the beetle *T.molitor* (Wu and Dean,

1996). Though it was shown that the mutant protein had lost initial binding, the greater toxicity of the mutant was explained by dissociation kinetic analysis which showed that the mutant toxin had better irreversible binding. These studies indicate that mutations may have either a negative or positive effect on binding and toxicity and that mutation in different loop regions can have a different effect on binding. It is apparent that the same mutation in a toxin can have quite different results on different insects.

Regarding Cry1C, substitution studies have suggested an important role for domain II and domain III in receptor binding (Smith and Ellar, 1994; and Herrero et al, 2004). It has been shown that certain residues at loops 2 and 3 in domain II of Cry1C are involved in initial specific binding to *S.littoralis* and *A.aegypti* BBMV. At the same time it has been speculated that R437 residue in loop3 might play a role in irreversible binding (Abdul Rauf and Ellar, 1999). Though various amino acids of loop2 and loop3 are implicated in reversible and irreversible binding of the toxin, involvement of domain III in initial receptor recognition or later binding events has not been extensively researched. Additionally, the role of individual amino acid residues in toxin binding epitopes of APN has also not been looked into detail. Such studies are likely to provide new insights in designing new improved toxins for pest management programmes.

Agrawal et al (2002) have earlier shown that Cry1C δ-endotoxin binds to an APN receptor on the mid gut of susceptible host *Spodoptera litura* which is responsible for killing the insect. It has been earlier shown (Section 5.3.2, figure 17 a), using independent domains, that both domain II and domain III of Cry1C are involved in APN recognition. Competition binding experiments with synthetic peptides homologous to various loop regions of domain II and domain III implicated a role of loop2 and loop3 of domain II and loop a of domain III in receptor binding. Additionally, a seven amino acid region (^{128}HLHFHLP^{134}) in C-terminus of SlAPN was identified as the Cry1C binding epitope. In the present section, role of individual amino acids of the Cry1C toxin and SlAPN receptor binding epitopes in determining Cry1C toxicity has been evaluated by introducing mutations in the putative interacting domains. The results revealed that while loop2 and loop3 residues are involved in initial receptor recognition, loop a of domain III affects the irreversible step of receptor binding.

7.2 MATERIALS AND METHODS

7.2.1 Construction and Expression of Cry1C and BR-APN (SlAPN derivative expressing the APN-CRY Region) mutants

The recombinant protoxin Cry1C expressing DH5α *E.coli* strain was obtained from the Bacillus Genetic Center, Ohio State University. In the previous sections (Section 5.3.4, figure 19) it has been shown that loop 2 & loop 3 of domain II and loop a of domain III are putative receptor binding epitopes of the Cry1C toxin. In order to study these loops in more detail, Cry1Ca mutants were constructed by introduction of deletions and alanine substitutions (as indicated in table 10) of residues in loop 2, loop 3 and loop a of *cry1Ca* gene. Phage display experiments with Cry1C toxin identified ^{128}HLHFHLP^{134} region (APN-CRY) of the receptor as the putative toxin binding region (Section 4.36). Attempts to clone the binding region of SlAPN led to the cloning and expression of BR-APN protein which is a deletion derivative of full length SlAPN receptor that expresses the putative toxin binding region (APN-CRY: ^{128}HLHFHLP^{134}). Additionally, BR-APN retains the binding ability of the full length receptor as shown by the competition binding experiments and also influences the insect mortality like a functional toxin receptor (Sections 4.3.7 and 4.3.8). In order to study the APN-CRY region of BR-APN in detail, alanine substitution at residues 128-134 in BR-APN (table 10) were introduced in the *br-apn* gene in expression plasmid pQE-31, using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene). Native and mutant proteins were expressed and purified as described earlier (Section 4.2.9).

Table, 10. Summary of various mutants of Cry1C toxin and BR-APN receptor generated by site-directed mutagenesis. Numbers in parentheses indicate the corresponding amino acids deleted while the residues substituted by alanines are underlined and are in bold.

Residue Mutated	Sequence Region of mutation
Residues mutated in SlAPN	
H128A	126LL**H**LHFHLPIV136
L129A	126LLH**L**HFHLPIV136
H130A	126LLHL**H**FHLPIV136
F131A	126LLHLH**F**HLPIV136
H132A	126LLHLHF**H**LPIV136
L133A	126LLHLHFH**L**PIV136
P134A	126LLHLHFHL**P**IV136
Residues mutated in Cry1C	
Loop 2del(373-380)	370RLL--------FNLR384
Loop 3del(436-443)	432ATFV--------TTGV447
Loop adel(533-544)	529RDAR----------GGQV548
QQ-AA	370LRLL**QQ**PWPAPPFNLR384
PW-AA	370LRLLQQ**PW**PAPPFNLR384
P-A	370LRLLQQPW**P**APPFNLR384
PP-AA	370LRLLQQPWPA**PP**FNLR384
QR-AA	432ATFV**QR**SGTPFLTTGV447
SG-AA	432ATFVQR**SG**TPFLTTGV447
TP-AA	432ATFVQRSG**TP**FLTTGV447
FL-AA	432ATFVQRSGTP**FL**TTGV447
VIVL-AAAA	529RDAR**VIVL**TGAASTGVGGQV548
TG-AA	529RDARVIVL**TG**AASTGVGGQV548
ST-AA	529RDARVIVLTGAA**ST**GVGGQV548
GV-AA	529RDARVIVLTGAAST**GV**GGQV548

7.2.2 Biotin labeling of toxin and receptor proteins and competition binding assays

Native and mutant toxin and receptor proteins were labeled with biotin using EZ-Link Sulfo-NHS-Biotin Reagents (Pierce) according to manufacturer's instructions. Binding assays were performed as described earlier (Section 4.2.10). For heterologous competition assays involving native Cry1C and mutant toxins, 1 μg of purified SlAPN or purified BR-APN protein was transferred on nitro-cellulose membrane and overlaid with 1nM of purified biotin-labeled Cry1C toxin in the presence of increasing concentrations (0.1nM, 1nM, 10 nM and 100 nM) of nonlabeled mutant toxins in PBS (pH 8.0). Bound, labeled toxin on the blot was detected with streptavidin/peroxidase in a standard Western-blot protocol. For heterologous competition assays involving native BR-APN and mutant proteins, 1 μg of purified Cry1C was transferred on NC-membrane and overlaid with saturating concentration of labeled BR-APN protein in the presence of increasing concentrations of nonlabeled mutant BR-APN proteins. Binding parameters (K_d and B_{max}) were calculated from homologous competition assays (involving binding competition of labeled proteins with the corresponding nonlabeled proteins), using GraphPad Prism version 5.0 programme.

7.2.3 Analysis of Irreversible Binding of Cry1C to SlAPN by Dissociation binding assays

Biotin-labeled native Cry1C toxin (1nM) was incubated with 20 μg of SlAPN expressing *Sf* cell membranes in 500 μl of binding buffer on end-on-end shaker at room temperature for 1 h (association reaction) to achieve saturation binding. After 1 h, 1000 nM of nonlabeled loop a mutant toxin was added to each sample tube, and the unbound toxin was removed at different time points (15 min, 30 min, 45 min and 60 min) by centrifugation. The membrane pellet containing bound toxin was washed twice with binding buffer containing 0.2% Tween-20 and once with only binding buffer. The pellet was then resuspended in SDS-sample dye, boiled, cooled and centrifuged and supernatant loaded on SDS-polyacrylamide gel. The resolved proteins were transferred to nitro-cellulose membrane and bound, labeled toxin was detected with streptavidin/peroxidase in a standard Western-blot protocol.

7.2.4 Cytotoxicity Experiments

Expression of *S.litura apn* in *Sf*21 sells was achieved as described earlier (Section 4.2.6.c). Cells were seeded in 24-well plates and allowed to grow as monolayer. For cytotoxicity assays, growth medium of uninfected *Sf*21 cells and BV-SlApn-infected *Sf* cells was replaced with fresh medium containing increasing concentrations (1 nM to 15 nM) of purified native and mutant Cry1C protein. After 4 h of incubation at 27°C, cell death was determined by trypan blue exclusion. In each well, 100 µl of trypan blue (0.4%, wt/vol) was added and incubated for 2 min. Stained cells were viewed immediately under a microscope and photomicrographs were taken with a camera. Stained dead cells and transparent viable cells were counted manually.

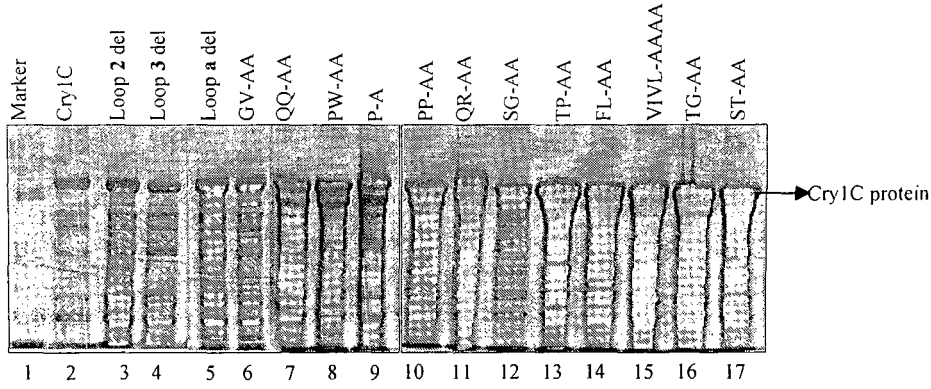
7.2.5 Insect Bioassays with mutant Cry1C and BR-APN proteins

Bioassays were performed with various toxin and BR-APN receptor mutants to study the contribution of critical toxin and receptor residues to insect mortality. Activity of native Cry1C and mutant toxins was determined with one day old *S.litura* larvae. For each toxin concentration, 200 µl of toxin resuspended in PBS was coated on each side of a leaf disc on which ten larvae were placed. Previous experiments have shown that 80 ng/cm^2 pure native toxin is sufficient to induce 100% mortality (Section 4.3.8, table 7). For mortality assays in the presence of BR-APN and mutant receptors as competitors, 80 ng/cm^2 of native Cry1C toxin was pre-incubated with 100-fold molar excess of purified native BR-APN receptor or mutant receptor proteins for 1h at room temperature on end-on-end shaker and then coated on leaf disc.

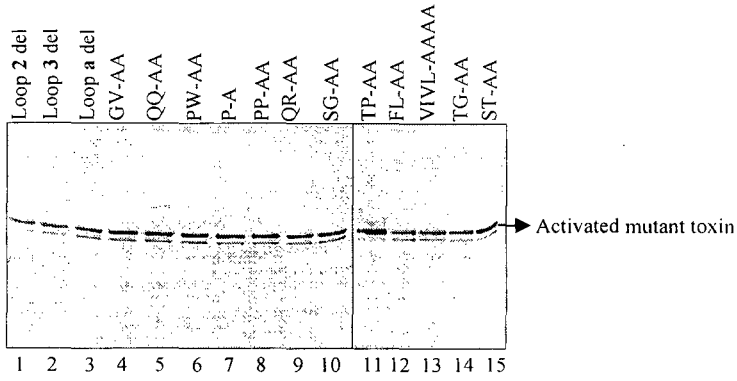
7.3 RESULTS

7.3.1 Construction and Expression of Cry1C mutants

Mutants with deletions or alanines substitutions at the predicted loop**2** (^{373}QQPWPAPP^{380}) and loop**3** (^{436}QRSGTPFL^{443}) of domain II and loop**a** (^{533}VIVLTGAASTGV^{544}) of domain III of Cry1C were constructed. The loop regions were identified on the basis of amino acid alignment with Cry1A(a) toxin as described previously. The positions of amino acids mutated in Cry1C are shown in table 10. All the toxin mutants were highly expressed as inclusion bodies in *E.coli*, similar to native Cry1Ca (Figure 32).Trypsin activation of all the solubilised mutant proteins yielded stable 65-kDa toxins when treated with trypsin/protoxin ratio of 1:10 (by mass) at 37°C for 15 min (Figure 33).



Figure, 32. Expression profile of Cry1C mutants. Recombinant vector carrying mutated *cry1C* was transformed into *E.coli* cells. The transformed cells were incubated with 1 mM IPTG for 4h at 37°C. The induced cell pellet was suspended in PBS buffer and sonicated. The sonicated suspension was centrifuged and the pellet was checked for the presence of Cry1C protein. Lane 1, marker; lane 2, native Cry 1C; lane 3, loop **2**del; lane 4, loop **3**del; lane 5, loop **a**del; lane 6, GV-AA; lane 7, QQ-AA; lane 8, PW-AA; lane 9, P-A; lane 10, PP-AA; lane 11, QR-AA; lane 12, SG-AA; lane 13, TP-AA; lane 14, FL-AA; lane 15, VIVL-AAAA; lane 16, TG-AA; and lane 17, ST-AA mutant protein.



Figure, 33. Activation of Cry1C mutant proteins by trypsin. Solubilised mutant inclusion bodies were treated with 1:10 trypsin/protoxin ratio (by mass) for 30 min at 37°C. The trypsin activity was stopped by immediate chilling of the samples on ice. The activated proteins were resolved by SDS-PAGE and the gel treated with Coomassie-blue dye solution to visualize the resolved protein bands. Lane 1, loop **2**del; lane 2, loop **3**del; lane 3, loop **a**del; lane 4, GV-AA; lane 5, QQ-AA; lane 6, PW-AA; lane 7, P-A; lane 8, PP-AA; lane 9, QR-AA; lane 10, SG-AA; lane 11, TP-AA; lane 12, FL-AA; lane 13, VIVL-AAAA; lane 14, TG-AA; and lane 15, ST-AA mutant activated protein.

7.3.2 Insecticidal activities of mutant Cry1C proteins against larvae of Spodoptera litura

The biological activities of native and mutant toxins on first-instar *S.litura* larvae were analysed and the results are in table 11. Some of the salient observations from feeding the mutant Cry1C proteins to larvae can be summed up as follows:

(a) All deletion mutants (loop**2**del, loop**3**del and loop**a**del) showed 500 times reduced insecticidal activity.

(b) In contrast, mutants PP-AA, QR-AA, ST-AA and TG-AA displayed 25 times reduction in toxicity.

(c) The LC_{50}s showed seventeen-fold difference in potency between native and QQ-AA or SG-AA mutants.

(d) On the other hand, the mutants P-A, PW-AA, FL-AA, TP-AA and GV-AA were similar or up to two times less potent when compared to the native toxin.

(e) Interestingly, ala substitution of residues 533VIVL536 in loopa nearly abolished (500 times lower) the toxicity of this mutant to *S.litura*.

Taken together, these results reinforce the role of loop a, loop 2 and loop 3 in the recognition event of toxin receptor interaction.

Table, 11. Insecticidal activity and binding efficiency of Cry1C and its loop mutants. LC_{50} values were measured for neonate *Spodoptera litura* larvae. Dissociation constant (K_d) and concentration of binding sites (B_{max}) are estimated from homologous competition experiments performed with biotin-labeled toxins and purified SlAPN or BR-APN receptors electro-blotted on nitro-cellulose membrane. The amount of bound toxin was calculated as follows:

A (Input) =B (Bound) + C(Unbound) where Bound Toxin amount was calculated from the western estimate

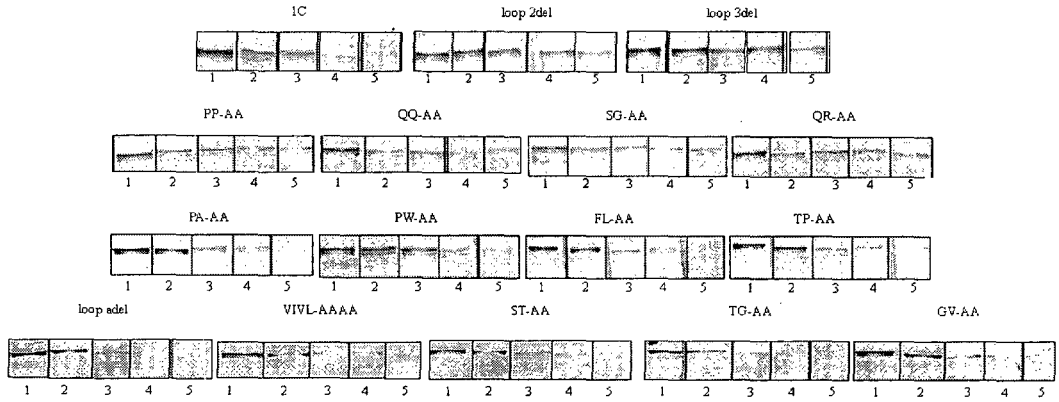
Toxin		SlAPN		BR-APN		
	LC_{50} (ng/cm^2)	K_d (nM)	B_{max} (pmol/mg)	K_d (nM)	B_{max} (pmol/mg)	Relative Potency[a]
Cry1C	20	90	219.5	9.008	139.2	1
Loop**2**del	>10000	UD[b]	UD	UD	UD	>0.002
Loop**3**del	>10000	UD	UD	UD	UD	>0.002
Loop**a**del	>10000	105	184.2	ND[c]	ND	>0.002
QQ-AA	350	325	174.9	27.5	133	0.057
PP-AA	500	370.5	179.4	27.86	141.1	0.04
P-A	25	183.3	237.1	10.73	141.3	0.8
PW-AA	25	171.8	222.5	10.16	140.8	0.8
QR-AA	500	337.8	176.2	28.74	152.3	0.04
SG-AA	350	324	171.7	26.94	151.4	0.057
FL-AA	25	207.7	225.2	9.482	139.5	0.8
TP-AA	25	194.4	232.5	10.27	145.7	0.8
VIVL-AAAA	>10000	175.2	220.5	ND	ND	>0.002
TG-AA	500	192.5	229.4	ND	ND	0.04
ST-AA	500	173.4	229.2	ND	ND	0.04
GV-AA	40	165.1	217.6	ND	ND	0.5

[a]Relative Potency, LC_{50} native /LC_{50} mutant.
[b]UD, Undetectable.
[c]ND, Not determined.

7.3.3 In vitro evaluation of interaction of mutant Cry1C proteins with Receptor ligand

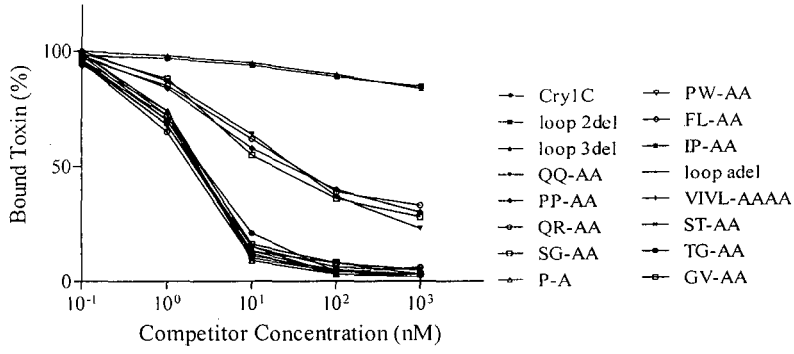
Homologous and heterologous competition experiments were performed to evaluate the binding affinity of each toxin mutant to SlAPN and to analyse whether the mutant toxins recognize the same binding site on SlAPN as the native toxin. Binding parameters such as dissociation constant (K_d) and binding site concentration (B_{max}) were obtained from homologous competition experiments and are reported in table 11. In the currently employed experimental regimen, Cry1C bound to SlAPN with K_d=90 nM which was similar to what was observed earlier using ELISA. Since the mutants loop**2**del and loop**3**del showed negligible binding to SlAPN, K_d values for these mutants could not be determined. On the other hand, loop**a**del bound to SlAPN nearly as efficiently (K_d=105 nM) as the native Cry1C toxin (Table 11 and Figure 34). In addition, all loop **a** mutants (VIVL-AAAA, ST-AA, TG-AA and GV-AA) and mutants PA-AA, PW-AA, FL-AA and TP-AA bound to the full receptor with similar binding affinities which were up to two times lower than that of Cry1C toxin. On the other hand, mutant toxins PP-AA, QQ-AA, QR-AA and SG-AA had comparatively lower (approximately 4-fold lower) binding affinities (Table 11 and Figure 34).

(a)



Native Loop 2= QQPWPAPP Native Loop 3= QRSGTPFL Native Loop a= QRSGTPFL

(b)

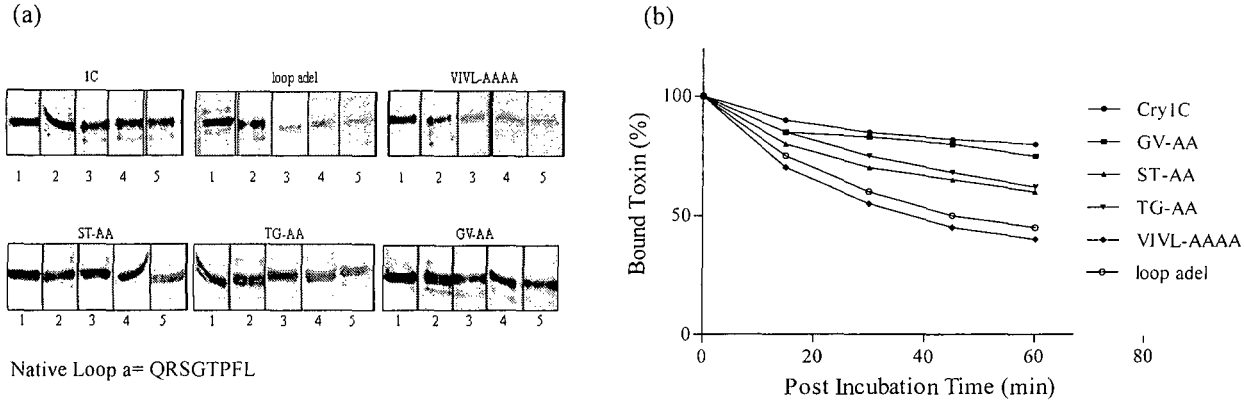


Figure, 34. Binding of biotin-labeled Cry1C in the presence of increasing concentrations of nonlabeled Cry1C, loop 2del, loop 3del, QQ-AA, PW-AA, QR-AA, FL-AA, P-A, PP-AA, SG-AA, TP-AA, loop adel, VIVL-AAAA, ST-AA, TG-AA and GV-AA toxins to SlAPN. (a) 200 ng SlAPN was electro blotted on NC membrane and overlaid with saturating concentration of purified biotin-labeled Cry1C in the presence of increasing concentrations (lane 1, no competitor; lane 2, 0.1X; lane 3, 1X; lane 4, 10X; and lane 5, 100X fold molar excess of competitor protein) of non labeled native toxin or mutant toxins as the competitors. Blots were developed using HRP-conjugated streptavidin labeled antibody. (b) Binding is expressed as a percentage of the amount bound upon incubation with labeled toxin alone. Average Integrated Density values (obtained from the above ligand blots) were taken as bound toxin values which were calculated as follows:

A (Input) =B (Bound) + C(Unbound) where Bound Toxin amount was calculated from the western estimate. These values were then plotted against competitor concentration using graph-pad software package.

Since all loop a mutants showed considerably reduced toxicities even when they bound SlAPN with affinities comparable to native Cry1C, further investigation of the effect of these mutations on irreversible step of receptor binding was also done. The mutant toxins were first allowed to bind saturably to SlAPN expressing *Sf*-membranes and were then chased with 1000-fold excess of corresponding non-labeled toxins. The dissociation binding assays

showed that 80% of native and 75% of loop a GV mutant toxins bound to *Sf* membranes could not be displaced by the addition of non-labeled toxin (i.e. they were irreversibly associated) (Figure 35). In contrast, only 62%, 60%, 45% and 42% of the TG-AA, ST-AA, loopadel and VIVL-AAAA-, respectively, mutants were able to associate irreversibly with receptor expressing *Sf*-membranes. The continued decrease in binding for these mutants was not due to toxin breakdown since the intact labeled toxins could be recovered even after incubation with membranes for 6 h.



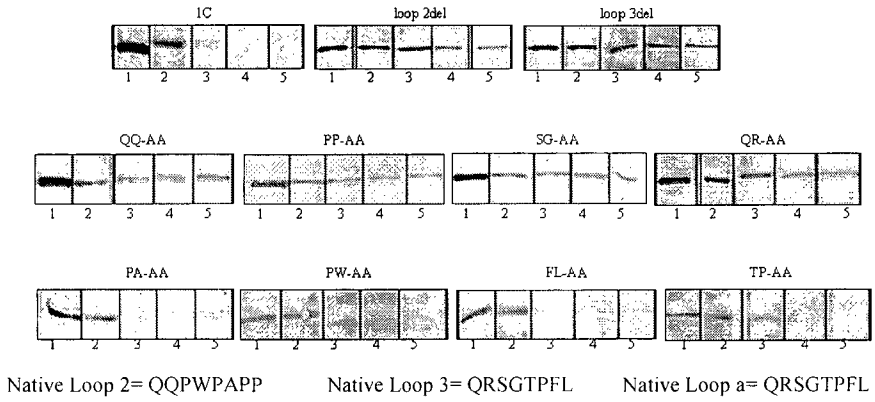
Figure, 35. Dissociation of bound biotin-labeled toxins from SlAPN expressing Sf membranes. *Sf* membranes expressing SlAPN were incubated with saturating concentrations of purified labeled Cry1C, loopadel, GV-AA, ST-AA, TG-AA and VIVL-AAAA toxins (association reaction). At 60 min after association reaction, 1000 fold of corresponding nonlabeled toxins were added to the test samples and incubation was continued (post binding incubation). (a) Ligand blots obtained at various time intervals (lane 1, 0 min; lane 2, 15 min; lane 3, 30 min; lane 4, 45 min; and lane 5, 60 min; of post binding incubation). Membranes were pelleted down, resuspended in SDS-sample dye, boiled and loaded on polyacrylamide gel which was the electro blotted on a nitro-cellulose membrane. The blot was developed using HRP-conjugated streptavidin antibodies. (b) Binding is expressed as a percentage of the amount bound compared with the amount bound at 0 min post-incubation. $A=A_0+A_t$

7.3.4 Effect of Cry1C mutations on binding to BR-APN

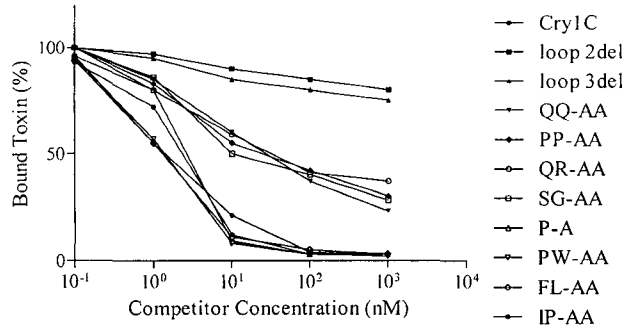
Previous Phage display and ligand binding results led to the identification of binding region of SlAPN (^{128}HLHFHLP^{134}). BR-APN, a deletion mutant of SlAPN expressing this region retained the binding ability with the insecticidal protein. It has been also demonstrated that the binding was competed by synthetic peptides homologous to predicted loop 2 and loop 3 regions but not by that of loop a region. Subsequently, the effect of mutations in loop 2 and loop 3 regions of Cry1C on binding to BR-APN receptor was analysed. Homologous and

heterologous competition binding experiments were performed to investigate the binding affinities of these mutants with respect to BR-APN receptor. Binding site parameters (K_d and B_{max}) as determined from homologous competition experiments are reported in table 11. Cry1C bound BR-APN with a considerably higher binding affinity (K_d=9 nM) than that with SlAPN (K_d=90 nM), though the binding site concentration value was higher for the full length SlAPN receptor. Dissociation constant values for loop**2**del and loop**3**del mutants could not be determined because of their negligible binding to the BR-APN receptor. Mutants PP-AA, QR-AA, SG-AA and QQ-AA bound BR-APN with considerably reduced affinities (K_d values up to 3 fold lower than that of native Cry1C) (Table 11 and Figure 36). On the other hand, mutants P-A, TP-AA, PW-AA and FL-AA had binding affinities similar to that of native toxin (Table 11 and Figure 36)

(a)



(b)

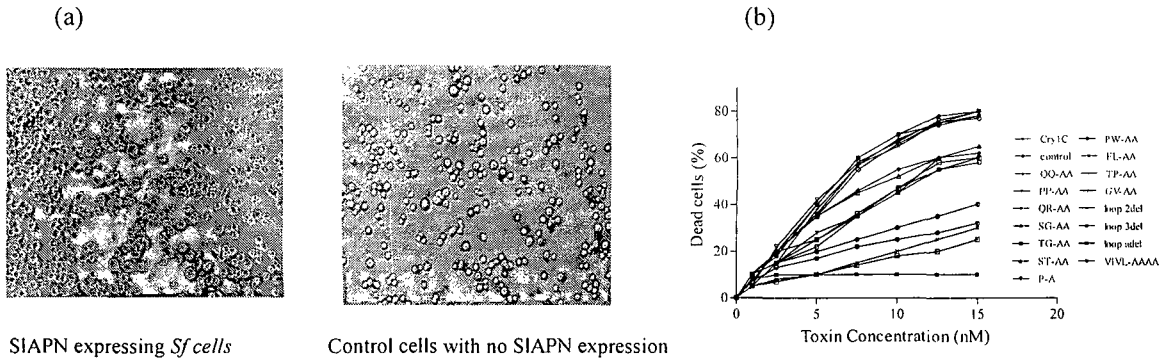


Figure, 36. Binding of biotin-labeled Cry1C in the presence of increasing concentrations of nonlabeled Cry1C, loop 2del, loop 3del, QQ-AA, PW-AA, QR-AA, FL-AA, P-A, PP-AA, SG-AA, and TP-AA toxins to BR-APN. (a) Purified BR-APN protein was electro blotted on NC membrane and overlaid with saturating concentration of purified biotin-labeled Cry1C in the presence of increasing concentrations (lane 1, no competitor; lane 2, 0.1X; lane 3, 1X; lane 4, 10X; and lane 5, 100X fold molar excess of competitor protein) of

non labeled native toxin or mutant toxins as the competitors. Blots were developed using HRP-conjugated streptavidin labeled antibody. (b) Binding is expressed as a percentage of the amount bound upon incubation with labeled toxin alone. Average Integrated Density Values (obtained from the above ligand blots) were taken as bound toxin values and plotted against competitor concentration using graph-pad software package.

7.3.5 In vitro toxicity assays of Cry1C and its mutants using APN expressing Sf21 cells

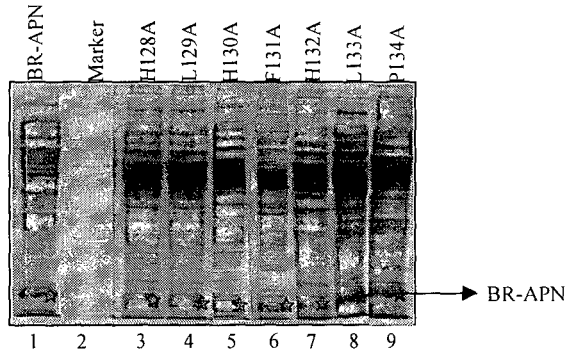
It has earlier been reported that the expression of *S.litura apn* in insect cell culture confers specific binding of bioactive Cry1C toxin to intact cells (Agrawal et al, 2002). In the present study, such SlAPN expressing *Sf*21 cells were used to investigate the effect of mutations in predicted loop regions on cytotoxicity of Cry1C. Trypan blue staining based cell viability test revealed that a vast majority of receptor expressing *Sf* cells lysed at 5nM native Cry1C toxin, (Figure 37). Control *Sf* cells (no SlAPN expression) underwent normal cell growth under similar conditions. All loop deletions (loop**2**del, loop**3**del and loop**a**del) and VIVL-AAAA mutant had very low toxic effect on SlAPN expressing *Sf* cells (20%-40% cell lysis) while mutants PA-AA, PW-AA, FL-AA, TP-AA and GV-AA were as toxic as native Cry1C. On the other hand, mutants QQ-AA, PW-AA, QR-AA, FL-AA, TG-AA and ST-AA showed reduced but considerable toxicity (55%-65% cell lysis) under similar conditions.



Figure, 37. Cytotoxicity of Cry1C on SlAPN expressing Sf cells. (a) Trypan blue staining of nuclei represents dead *Sf*21 cells expressing SlAPN under 15nM Cry1C toxin while the toxin had no lethal effect on normal *Sf*21 cells under similar conditions. (b) Effect of mutations on cytotoxic property of Cry1C. SlAPN expressing *Sf* cells were sensitive to Cry1C in dose-dependent manner, whereas control cells were insensitive to the toxin. Various mutants could be grouped into 3 categories depending upon their ability to lyse *Sf*21 cells. While loop deletions could only lyse 20-25% cells, some of the loop substitutions were as effective as the WT Cry1C while others were able to lyse only 55-60% *Sf* cells.

7.3.6 Construction and Expression of BR-APN (Binding Region of SlAPN) mutants

In the earlier section, (Section 4.3.6) the putative toxin binding epitope of SlAPN was mapped to a region of seven amino acids (^{128}HLHFHLP^{134}) in the N-terminus of the SlAPN molecule. In the present section, the analysis of receptor ligand interaction was focused on the epitope identified by phage display and deletion mutant data. Alanine substitutions of single amino acids in the region ^{128}HLHFHLP^{134} were introduced and positions of mutated amino acids are shown in table 12. All the mutants were highly expressed as soluble proteins in *E.coli*, similar to native BR-APN receptor (Figure 38).



Figure, 38. Expression of BR-APN mutant proteins. Alanine substitution mutations at residues 128-134 of BR-APN protein were introduced in the *br-apn* gene in expression plasmid pQE-31, using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene). Recombinant pQE vector carrying mutated *br-apn* gene was transformed in M15 cells. The transformed cells were incubated 1 mM IPTG for 16h at 25°C. The induced cell pellet was suspended in PBS buffer and sonicated to obtain the soluble proteins. The sonicated sample was centrifuged and the supernatant was checked for the presence of BR-APN protein. Lane 1, native BR-APN; lane 2, marker; lane 3, H128A; lane 4, L129A; lane 5, H130A; lane 6, F131A; lane 7, H132A; lane 8, L133A; and lane 9, P134A protein.

7.3.7 Effect of mutations in BR-APN on Cry1C toxicity

In the previous section (Section 4.3.7) it was shown that the BR-APN protein served as a small functional receptor of Cry1C whose binding to the toxin was specific and influenced the mortality of the target insect. Since this is a small protein expressing the toxin binding region

(APN-CRY: ^{128}HLHFHLP^{134}) and offers the ease of prokaryotic expression and purification system, various substitution mutations were carried out in the APN-CRY region of this protein to study the region in detail. Since the APN-CRY region has been shown to be involved in both toxin binding and mortality of the target insect, the significance of individual amino acid residues of this region for both these properties was analysed. This section focuses on the effects of BR-APN and its mutants on *S.litura* mortality caused by Cry1C. A hundred-fold excess of purified native BR-APN or mutated receptor protein was incubated with the bioactive Cry1C toxin before feeding the toxin to the *S.litura* larvae. Incubation of toxin with the native BR-APN reduced insect mortality by 55% (Table 12) while mutants H128A, P134A, H130A and H132A were not able to affect the mortality significantly (85%, 80%, 75% and 70% mortality). On the other hand, mutants L133A, L129A and F131A reduced Cry1C mortality to 50%, 55% and 60% respectively.

Table, 12. Effect of BR-APN and its mutants on toxicity of Cry1C protein to Spodoptera litura larvae. Toxicity of Cry1C is measured in the presence of 100-fold molar excess of purified BR-APN or mutant receptor proteins as the competitors.

Treatment	Mortality[a] (%)
Cry1C (80 ng/cm^2)	100 ± 2.2
Cry1C+BR-APN	45 ± 1.3
Cry1C+H128A	85 ± 3.4
Cry1C+L129A	55 ± 1.8
Cry1C+H130A	75 ± 2.6
Cry1C+F131A	60 ± 4.1
Cry1C+H132A	70 ± 3.1
Cry1C+L133A	50 ± 2.5
Cry1C+P134A	80 ± 3.7

[a]40 larvae per treatment. ± S.D. of three experiments

7.3.8 Effect of BR-APN mutations on toxin binding

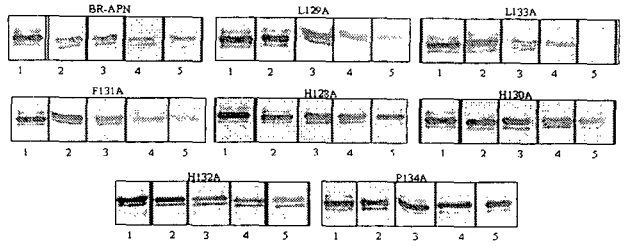
Insect bioassays with purified receptor BR-APN and Cry1C highlighted relevance of ^{128}HLHFHLP^{134} region of SlAPN in the recognition and toxicity events of insecticidal protein. To elucidate the role of individual residues of this region in toxin binding, in vitro competition binding experiments were performed with mutant BR-APN proteins. Binding constants determined from homologous competition experiments are reported in table 13.

Mutants L129A and L133A bound Cry1C with affinities similar to that of native BR-APN (Table 13 and Figure 39) while mutants H128A, H130A, H132A and P134A showed up to 4-fold lower affinity for the Cry1C toxin. Thus the residues shown to be involved in mortality of the target insect by in vivo assays were also found to influence the toxin binding by in vitro assays. Interestingly, mutant F131A which displayed a 60% mortality, showed reduced affinity (K_d =14 nM) for the Cry1C toxin.

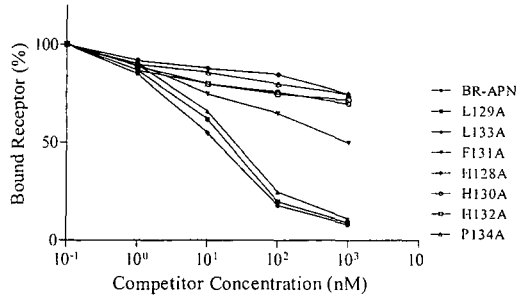
Table, 13. Binding efficiency of BR-APN and mutants to Cry1C toxin. Dissociation constant (K_d) and concentration of binding sites (B_{max}) are estimated from homologous competition experiments performed with biotin-labeled, purified BR-APN receptor or mutant receptor proteins and Cry1C toxin electro-blotted on nitro-cellulose membrane. Each value is the mean of thee experiments.

Receptor	**K_d** (nM)	**B_{max}**(pmol/mg)
BR-APN	9.318	138.7
H128A	24.63	95.09
L129A	9.239	140.4
H130A	39.69	105.4
F131A	14.66	122.8
H132A	25.45	74.4
L133A	10.42	135.7
P134A	25.11	92.33

(a)



(b)



Figure, 39. Binding of biotin-labeled BR-APN receptor protein in the presence of increasing concentrations of nonlabeled BR-APN, H128A, L129A, H130A, F131A, H132A, L133A, and P134A proteins to Cry1C. (a) Purified Cry1C toxin was electro blotted on NC membrane and overlaid with saturating concentration of purified biotin-labeled BR-APN in the presence of increasing concentrations (lane 1-no competitor, lane 2-0.1X, lane 3-1X, lane 4-10X and lane 5-100X fold molar excess of competitor protein) of non labeled native receptor or mutant proteins as the competitors. Blots were developed using HRP-conjugated streptavidin labeled antibody. (b) Binding is expressed as a percentage of the amount bound upon incubation with labeled receptor protein alone. Average integrated density values (obtained from the above ligand blots) were taken as bound toxin values and plotted against competitor concentration using graph-pad software package.

7.4 DISCUSSION

The Lepidopteran moth *Spodoptera litura* is a polyphagous pest affecting many economically important crops such as groundnut, maize, banana and many vegetables. The most recent application of Cry proteins for the control of *Spodoptera* larvae is the development of transgenic crops that express a synthetic *cry1Ca* gene. Cry1Ca has been shown to bind to an APN receptor expressed in midgut of *S.litura* larvae and tolerance to Cry1C generated by silencing of this receptor demonstrates the functional role of APN in Cry1C toxicity (Agrawal et al, 2002). The data reported in Section 5.3.4 suggested that amino acid residues

373QQPWPAPP380 (predicted loop**2**), 436QRSGTPFL443 (predicted loop**3**) and 533VIVlTGAASTGV544 (predicted loop**a**) of Cry1C toxin are essential for APN binding.

In the present study, investigations on the receptor binding epitopes of Cry1C toxin were continued and the role of individual amino acids of putative loop regions in receptor binding and toxicity of Cry1C to *S.litura* was analysed. Using site-directed mutagenesis techniques, various deletion and substitution mutations were performed in various loop regions of the toxin. Alanine substitutions were chosen because they do not introduce gross structural changes in the configuration of the mutant toxins. The results showed that while loop2 and loop3 of domain II were involved in initial receptor recognition, mutations in loop a of domain III affected irreversible association of the toxin to the receptor.

The insect bioassay data revealed that the deletion of region spanning loops (loop**2**/loop**3**/loop**a**) was sufficient to nearly abolish most of the toxicity to *S.litura* larvae which emphasize the importance of these loops in insecticidal activity. A series of alanines substitution mutants were generated to analyse the role of individual amino acids of loop regions in determining receptor binding and toxicity to *S.litura*. A change of polar, hydrophilic residues (373QQ374, 379PP380, 436QR437, 438SG439) to non polar, hydrophobic alanines substantially reduced toxicity to susceptible *S.litura* species (Table 11). While mutants PW-AA, P-A, FL-AA and TP-AA which did not involve significant changes in polarity or hydropathicity had toxicities similar to native Cry1C. In contrast all mutations in loop a of domain III (except GV-AA) had drastic effect on Cry1C toxicity. Alanine substitutions of a stretch of highly hydrophobic residues 533VIVL536 resulted in nearly complete loss of toxicity of Cry1C to *S.litura* larvae.

Binding assays between mutant proteins and receptor ligand were performed to investigate the correlation between toxicity and binding properties of the mutants. The data showed that except loop**2**del and loop**3**del, binding of all mutant Cry toxins to SlAPN was saturable like that of native toxin. In contrast deletion of residues 533 to 544 (predicted loop a of domain III) did not alter the initial binding of the mutant to the SlAPN receptor, suggesting the role of only domain II loop regions in initial receptor recognition. Competition binding studies suggested that the binding affinity and binding site concentration of toxic mutants PA-AA, PW-AA, FL-AA and TP-AA were similar to that of native toxin. The reduced larvicidal activity of loop2 and loop3 mutants (PP-AA, QQ-AA, QR-AA and SG-AA) can be explained

by their reduced ability to recognize and bind to the SlAPN receptor (Table 11 and Figure 34). While alanine substitution of Pro375 in Cry1C toxin has been shown to have no effect on toxicity to *S.littoralis* also, at the same time G439A mutation resulted in nearly complete loss of toxicity. The results with QR-AA mutant showed that alanine substitution of arginine at position 437 resulted in 4-fold lower initial binding affinity with the receptor as compared to the native toxin. In contrast to this data which suggested the involvement of Arg^{437} residue in initial receptor recognition, this residue has been implicated to play a role in irreversible binding in case of *S.littoralis* (Abdul Rauf and Ellar, 1999). Earlier results have shown that SlAPN-Cry1C interaction involves multiple epitopes on both binding molecules; therefore heterologous competition experiments were performed to investigate whether the mutant and native toxins recognize the same binding site on SlAPN. All the mutants had competition patterns corresponding to their binding affinities and competed with native Cry1C for binding with SlAPN (Figure 34 and table 11).

However, analysis of mutants generated in domain III yielded some interesting results. Deletion of loop a region or alanine substitution of a stretch of highly hydrophobic residues (VIVL) resulted in nearly complete loss of toxicity of these mutants. Mutants ST-AA and TG-AA showed highly reduced larvicidal activity. Interestingly, all loop a mutants had binding affinities comparable to native toxin. Binding data revealed that loss of toxicity of loop a mutants could not be due to their failure to recognize the APN receptor and so events after the initial receptor recognition might be involved in these cases. This also reinforced the role of loop2 and loop3 regions in initial binding events as the loop a mutants were having native loop2 and loop3 regions. Therefore, the effect of loop a mutations on irreversible binding of the toxin was investigated. Since such study necessitated mimicking conditions where membrane insertion of the toxin could take place, membranes of *Sf* cells expressing the recombinant SlAPN were used. Such a system gave the advantage of analyzing the effects of toxin mutations on specific full length APN binding. Results of dissociation binding assay correlated with the toxicity assay; the greater the irreversible association of the toxin to the membrane, the more the toxicity to the susceptible insect. About 80% to 75% of native and GV-AA toxins were irreversibly associated with the receptor expressing *Sf* cell membranes, whereas 40%-50% of the lesser toxic/non toxic mutants showed irreversible association under similar conditions (Figure 35). Taken together, these results suggest that Cry1C binding to SlAPN involves two steps. While domain II is involved in initial receptor recognition; domain III plays a more important role in determining the irreversible association of the toxin with the

receptor. It is therefore speculated that at least a part of the toxin might remain associated with the receptor at the time of toxin insertion. While the present analysis revealed that residues 533 to 544 of Cry1C were involved in irreversible binding to the SlAPN receptor, decrease in toxicity of alanine substituted residues 541-544 in *S.exigua* has been attributed to much greater reduction of the affinity of the mutant toxin to the host insect BBMV (Herrero et al, 2004). These data suggests that mutations in the same loop regions, sometimes even in the same amino acids, can bring about different effects in different insect species; thus making the toxin-receptor interaction a very complex system. Cytotoxicity experiments involving various toxin mutants showed results similar to bioassay, providing additional evidence of functional role of APN in determining toxicity of Cry1C. All non-toxic (as determined from bioassay experiments) Cry1C mutants could only lyse 20%-30% of APN expressing *Sf* cells while highly toxic mutants (PA-AA, PW-AA, FL-AA, TP-AA and GV-AA) lyse 80%-85% of *Sf* cells under similar conditions (Figure 37).

In the previous section (Section 4.3.7) the cloning and expression of a SlAPN derivative, BR-APN (expressed in *E.coli*) receptor which bound to the bioactive Cry1C toxin was discussed. Both loop2 and loop3 were shown to be involved in Cry1C binding to BR-APN protein. Through experiments described in previous sections and in this section, the aim was to determine whether Cry1C mutants affected in SlAPN binding were also affected in their ability to bind BR-APN receptor. Binding patterns (as determined from heterologous competition experiments) of Cry1C mutants for BR-APN were found to be similar to that for SlAPN. The more toxic mutants competed more efficiently than the lesser toxic ones (Table 11 and figure 36).

After establishing that BR-APN was similar to SlAPN with respect to the toxin binding property, the role of individual amino acid residues in the previously mapped APN-CRY epitope of the receptor molecule was analysed. Single point alanine substitutions were performed and the mutants studied for their Cry1C binding and toxicity affects on *S.litura* larvae. Bioassays were done by incubating the native Cry1C toxin with 100-fold molar excess of BR-APN receptor or its mutants before feeding the toxin to the susceptible larvae. Alanine substitution of residues H128, H130A, H132 and P134 resulted in considerable loss of their binding affinity to the toxin. These mutants neither competed efficiently with BR-APN for Cry1C binding (Figure 39 and table 12) nor were they able to affect *S.litura* mortality (Table

13). However, the mutants L129A and L133A were as efficient as native BR-APN in both mortality and competition binding experiments.

To conclude, in the present study it has been shown that residues ^{373}QQ^{374}, ^{379}PP^{380}, ^{436}QRSG^{439} of loop 2 and loop 3 of domain II of Cry1C are involved in initial receptor recognition while the residues ^{533}VIVLTG^{538}, ^{541}STGV^{544} of loop a of domain III of Cry1C are involved in irreversible association of the toxin to the SlAPN receptor. Additionally, mutational analysis of ^{128}HLHFHLP^{134} region of APN receptor reveals that basic residues at positions 128 and 130 and 132 are important for toxin binding.

Summary And Conclusions

1. The 135 kDa Cry1Ac and Cry1C protoxins were over-expressed in *E. coli* in the form of inclusion bodies. The inclusion bodies were solubilized and activated by trypsin to give an active 65 kDa and 60 kDa Cry1Ac and Cry1C toxins respectively. The activated toxins were purified by anion-exchange chromatography and then used for binding studies.

2. The full-length aminopeptidase of *Spodoptera litura* was successfully expressed in *Sf*21 cells using baculovirus expression system. The Recombinant virus (BV-SlApn) was used to express a 108 kDa protein (SlAPN) that reacted with anti-APN antibodies, thus demonstrating the expression of *S. litura* APN in insect cells.

3. Recombinant SlAPN expressing *Sf* cells were solubilised in 0.4% NLS containing solubilisation buffer to obtain the membrane bound APN protein in soluble form. Regeneration of the catalytically active form of solubilsed APN was achieved by slow dialysis against 0.2% CHAPS containing buffer.

4. After the two-step purification strategy involving anion exchange chromatography and gel filteration chromatography, 270 μg pure SlAPN was obtained from 15 X 10^6 *Sf*21 cells. The recovery of pure APN based on total protein estimation was 11% (starting from 2.5 mg total protein). Activity of the purified, refolded protein was 360 nmol/h/mg which corresponded to a 9-folds purification of the enzymatically active APN.

5. Maximum velocity (V_{max}) of the enzymatic reaction of SlAPN was calculated to be 590 nM/h and K_m for leucine-p-nitroanilide substrate was 1 mM.

6. The toxin-binding characteristic displayed by the 108 kDa purified and refolded SlAPN receptor was similar to that of membrane-anchored APN. It interacted with bioactive toxin, Cry1C but showed no interaction with Cry1Ac toxin on ligand blots.
7. Indirect ELISA results demonstrated that the purified SlAPN possessesed a high affinity site for Cry1C with K_D value of 90 nM. The toxin exhibited a moderate on rate (~2.2 x 10^3 $M^{-1}s^{-1}$) and a slow reversible off rate (0.2 x $10^{-3}s^{-1}$).

8. The APN-Cry1C complex hydrolyzed leucine-p-nitroanilide as efficiently as native APN. Binding of amastatin to free APN or APN-Cry1C complex reduced the activity of the aminopeptidase to same levels. Therefore, the binding of Cry1C toxin did not

block the active site of APN which remained available for substrate or inhibitor (amastatin) binding.

9. To find toxin binding epitopes on the receptor, phage display system was used. In experiments with Cry1C toxin as the ligand, approximately 70% phages were found to represent the same amino acid sequence (ϕ71: HPSFHWK) while rest 30% phages encoded unique peptides.

10. The ϕ71 bound to Cry1C in a dose dependent manner (suggesting specific interaction) and shared 71% similarity to a 7 amino acid region present near the N-terminus of SlAPN (APN-CRY: ^{128}HLHFHLP^{134}).

11. Binding of Cry1C to SlAPN was efficiently competed by both ϕ71 and APN-CRY synthetic peptides suggesting that ^{128}HLHFHLP^{134} epitope of the receptor was responsible for Cry1C binding.

12. In attempt to clone a small region of SlAPN that retained the Cry1C binding capability, a 340 bp *br-apn* gene fragment (encoding the ^{128}HLHFHLP^{134} epitope) was amplified by PCR and cloned and expressed in *E.coli* M15 cells.

13. Incubation of recombinant pQE harboring M15 culture with 1mM IPTG for 16 h at 16°C resulted in production of the BR-APN protein in soluble form.

14. The BR-APN protein was purified by Ni-NTA resin and was more than 95% pure and the yield of the purified protein was 4 mg per 100 ml of culture.
15. BR-APN was able to bind to Cry1C in toxin overlay assays and the binding was competed by APN-CRY peptide.

16. Incubation of the Cry1C toxin with pure BR-APN reduced the toxicity of Cry1C by 50%. Additionally, incubating the toxin with APN-CRY peptide also reduced the mortality to similar levels (45%). This showed that not only the APN-CRY region was an important binding epitope but also affected the toxicity of Cry1C. In this regard, the BR-APN mutant retained the specific Cry1C binding capability which led to death of the target insect.

17. The domain II and domain III gene fragments of Cry1C were amplified by PCR. Both domains were expressed as inclusion bodies in *E.coli* after 4 hrs of induction with 1mM IPTG at 37°C. Overnight incubation of inclusion bodies with 10 mM DTT containing solubilisation buffer resulted in the generation of solubilised proteins. The domain II and III proteins purified by Ni-NTA resin were more than 95% pure and the yield of the purified proteins was 5-7 mg per 100 ml of culture.

18. Binding assays with purified domain II and domain III showed that both domains could interact with SlAPN as individual molecules. Competition binding experiments showed a gradual decrease of 1C-III binding with increasing 1C-II concentrations suggesting that 1C-II had a higher affinity for SlAPN than 1C-III.

19. With SlAPN as the ligand, phage clones obtained from the final panning round showed sequence similarity to various regions of the toxin. These and domain competition results suggested that multiple regions of Cry1C might be involved in binding with SlAPN.

20. Cry1C-SlAPN binding assays with various synthetic peptide competitors demonstrated that loop 2, loop 3 and loop a peptides competed the binding of Cry1C to SlAPN while no significant competition was shown by loop1 and loop b peptides. This suggested that loop 2 and loop 3 of domain II and loop a of domain III were involved in interaction with SlAPN.

21. With BR-APN as the ligand to screen for binding clones of the random phage display library, 70% of the clones sequenced after third round of panning encoded the same amino acid sequence (ϕ72: PSPNIPI). The ϕ-72 clone shared 72% similarity with a region involving loop 3 of domain II of Cry1C (^{432}ATFVQRS^{438}). No phage peptide homologous to any domain III region was obtained indicating that BR-APN-Cry1C interaction might not involve domain III.

22. BR-APN-Cry1C binding was competed by loop2 and loop3 peptides but loop a peptide had no effect on this interaction.

23. All the toxin deletion and substitution mutants were highly expressed as inclusion bodies in *E.coli*, similar to wild-type Cry1Ca. Trypsin activation of all the solubilised mutant proteins yielded stable 60-kDa toxins when treated with trypsin/protoxin ratio of 1:10 (by mass) at 37°C for 15minutes.

24. All deletion mutants (loop**2**del, loop**3**del and loop**a**del) showed 500 times reduced insecticidal activity. In contrast, mutants PP-AA, QR-AA, ST-AA and TG-AA displayed 25 times reduction in toxicity. Interestingly, ala substitution of residues 533VIVL536 in loopa nearly abolished (500 times lower) the toxicity of this mutant to *S.litura*.

25. Mutants loop**2**del and loop**3**del showed negligible binding to SlAPN. loop**a**del bound to SlAPN nearly as efficiently (K_d=105 nM) as the wild-type Cry1C toxin. Toxins PP-AA, QQ-AA, QR-AA and SG-AA had comparatively lower (approximately 4-fold lower) binding affinities

26. The dissociation binding assays showed that 80% of wild-type and 75% of loop a GV mutant toxins bound to *Sf* membranes could not be displaced by the addition of non-labeled toxin (i.e. they were irreversibly associated). In contrast, only 62%, 60%, 45% and 42% of the TG-AA, ST-AA, loop**a**del and VIVL-AAAA, respectively, mutants were able to associate irreversibly with receptor expressing *Sf*-membranes.

27. Cry1C bound to BR-APN with a considerably higher binding affinity (K_d=9 nM) than that with SlAPN (K_d=90 nM). Mutants PP-AA, QR-AA, SG-AA and QQ-AA bound BR-APN with considerably reduced affinities (K_d values up to 3 fold lower than that of wild-type Cry1C).

28. All loop deletions (loop**2**del, loop**3**del and loop**a**del) and VIVL-AAAA mutant had very low toxic effect on SlAPN expressing *Sf* cells (20%-40% cell lyses). Mutants QQ-AA, PW-AA, QR-AA, FL-AA, TG-AA and ST-AA showed reduced but considerable cytotoxicity (55%-65% cell lysis) under similar conditions.

29. All the substitution mutants of BR-APN were highly expressed as soluble proteins in *E.coli*, similar to the native BR-APN receptor.

30. Incubation of Cry1C toxin with the native BR-APN reduced insect mortality by 55% while mutants H128A, P134A, H130A and H132A were not able to affect the mortality significantly (85%, 80%, 75% and 70% mortality).

31. Mutants L129A and L133A bound Cry1C with affinities similar to that of native BR-APN while mutants H128A, H130A, H132A and P134A showed up to 4-fold lower affinity for the Cry1C toxin. Thus the residues shown to be involved in mortality of the target insect by in vivo assays were also shown to influence the toxin binding by in vitro assays.

32. The BLAST of Cry1C against PDB database showed 46% identity (60% similarity) with its template, 1CIY protein (an insecticidal toxin) at E-value 2.3E-145 as closest homologue.

33. The BLAST of SlAPN showed 29% identity (47% similarity) with 1Z5H protein at E-value 1.1E-50 as top hit.

34. Ramachandran plots generated using RAMPAGE and iMolTalk web servers suggested that >96% of the residues of the APN were in core plus allowed regions. For Cry1C structure this was true for more than 98% of the residues.

35. The PROSA II gave a Z-score of -8.58 for Cry model (template: 1CIY= -9.47) and -7.66 for APN model (template: 1Z5H= -13.04), which suggested their acceptable structure.
36. The derived 3-dimensional structure of Cry1C revealed a folding pattern similar to those of known crystal structures of Cry toxins.

37. The N-terminal domain (I) (32-253) was completely α-helical in nature. The central helix H5 of the anti parallel helix bundle was entirely encircled by 7 outer helices.

38. The central domain (II) (261-457) had mainly β strands and two short α-helices. It had 3 sheets. The first sheet was formed by four strands which were connected according to the typical "Greek-key" topology. Search for second sheet revealed only 2 β - strands in Cry1C. The third sheet was formed by two separate fragments made by 3 strands: the C-terminus of domain II donated 2 central strands while the outer strand came from N-terminus.

39. The C-terminal domain (III) (467-616) was all β -strands except one short α-helix (α9=F314-V316) which was not present in the template toxin structure. Domain III consisted of two twisted, anti parallel β sheets forming a jelly-roll topology. Each sheet (Figure 26b) was made of five main beta strands.

40. Prediction probabilities for interacting sites revealed that loop 2 had the highest probability (>0.9) closely followed by loop 3 (>0.8).

41. Domain II made the maximum number of contacts- 20 H bonds with domain III and 13 with domain I. Domain I and III were connected by only 7 hydrogen bonds. None of the inter-domain contacts involved loop regions.

42. SlAPN carried the conserved HEXXH zinc binding motif. The third zinc binding ligand in the motif NEXFA was at a conserved distance of 18 amino acid residues from the second zinc binding ligand, histidine.

43. SlAPN had a hook like structure formed by four domains.

44. Domain I of SlAPN was formed of two beta sheets. The four stranded upper sheet had a Greek-key topology while the lower sheet had five strands that ran anti parallel to each other.

45. The first part of domain II of SlAPN was a mixed α/β structure. The second part of the domain carried the conserved HEXXH and NEXFA motifs on two separate helices α-3 and α-4 (respectively) which were connected by a beta strand β-21.

46. The domain III (Figure 29) of SlAPN was a beta-sandwich formed of eight beta strands.

47. The predominantly helical domain IV of SlAPN was organized into a super helix consisting of two modules approximately perpendicular to each other.

48. SPPIDER analysis for interacting residue prediction identified three high probability (>0.9) regions in SlAPN – region I involving residues 123-135, region II of residues 881-889 and region II of residues 936-941. These were also the longest stretches of amino acids predicted to be in interfacial region.

49. Using GRAMM v1.03, the docking process of the derived Cry1C and SlAPN structures was carried out in two modes, mode 1: provided the APN-CRY sequence (HLHFHLP) of APN receptor protein as interface residue constraints for consideration during docking and mode 2: without any residue constraint mode.

50. Seven complexes of mode 1 and six complexes of mode 2 were having the complete APN-CRY region along with flanking residues conserved within the interaction surface. Other complexes were having at least 70% of this region as interface residues.

51. Using model, seven complexes where the APN-CRY region was found to be interacting with loop 3 or / and loop 2 of Cry protein were obtained.

52. The docked complex (complex M1.8) showed two regions of contact between the receptor and the toxin. While region A involved interactions between domain I of Cry1C, latter half of domain I and 3 residues of domain II of APN, region B showed multiple interactions involving loop 2 and loop3 of Cry1C domain II and first half of domain I of APN (including the APN-CRY sequence ^{128}HLHFHLP^{134}).

53. CMA showed that the maximum area of contact in the docked complex M1.8 involved loop 3 of Cry1C and a region involving APN-CRY of (128-134) SlAPN.

54. An analysis of the putative forces involved in the receptor toxin interaction in complex M1.8 revealed both hydrogen bonding and Van der Waal overlaps in the region B of contact while the region A lacked any overlaps.

55. An analysis of interactions involving Tyr123-Thr143 region of APN showed that the hydropathy profiles of the interacting epitopes of Cry1C and APN which involved the loop 2 and loop 3 of Cry1C and APN-CRY region of SlAPN were reverse to each other.

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Purification and characterization of aminopeptidase N from *Spodoptera litura* expressed in Sf21 insect cells

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Abstract

Insecticidal crystal proteins produced by strains of *Bacillus thuringiensis* cause larval death upon interaction with specific receptors located at the midgut epithelium of susceptible insects. Large quantities of easily purified aminopeptidase and cadherin-like Cry toxin receptors can facilitate the further study of Cry toxin binding and pore formation. Here, we report the solubilisation and purification of aminopeptidase N from *Spodoptera litura* (SlAPN). Recombinantly expressed and membrane anchored aminopeptidase N showed differential solubilisation with various ionic and nonionic detergents. The *N*-lauryl sarcosine (NLS)-solubilised SlAPN was purified to near homogeneity by anion exchange and gel filtration chromatography and refolded to its catalytically active form. The optimized purification regimen lead to >90% purification of the catalytically active SlAPN with 11% recovery and 9-folds purification. The interaction of purified SlAPN with biologically active Cry1C protein has been qualitatively and quantitatively characterized. By ligand blotting experiment, we demonstrated the linearity of interaction of the two purified proteins and lack of interaction of SlAPN with structurally divergent nontoxic Cry1Ac protein. The equilibrium dissociation constant (K_D) of purified SlAPN for Cry1C was calculated by ELISA (90 nM). Interaction of enzymatically inactive SlAPN with Cry1C and catalytic activity of APN–Cry1C complex suggested that the catalytic site and toxin-binding sites of SlAPN do not overlap.

Keywords: Aminopeptidase N; Purification; Sf21 insect cells; Detergent; Solubilisation; *Spodoptera litura*

Insecticidal crystal proteins (ICPs)[1] produced by *Bacillus thuringiensis* (Bt) have been used as safer alternatives to chemical insecticides. These proteins have been deployed for crop protection against insect predation either as formulation sprays or expressed in crop plants [1]. The mechanism of action of Cry proteins involves their solubilisation and proteolytic activation inside the insect midgut. The activated toxin then binds to a specific receptor at the brush border membrane of midgut epithelial cells. In a susceptible larva, the interaction is followed by the insertion of the insecticidal protein into the membrane, creating an ionic imbalance, which results in the death of the insect [2]. Binding of the toxin to the receptor is a two-stage process involving reversible [3] and irreversible steps [4–6]. The initial binding of the toxin to the receptor eventually facilitates its insertion into the membrane. The mechanism of pore formation has largely remained unresolved partly due to non-availability of adequate amount of the receptor ligand in relatively pure form.

Aminopeptidase N (EC 3.4.11.2) is one of the membrane proteins identified as a receptor to Cry proteins in different insects. The role of APN as a functional receptor for Cry toxins has been established by both *in vitro* [7–10] and *in vivo* experiments [11,12]. It is found

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[1] *Abbreviations used:* NLS, *N*-lauryl sarcosine; ICP, insecticidal crystal protein; Bt, *Bacillus thuringiensis*; GPI, glycosyl phosphatidylinositol; cmc, critical micellar concentration; BBMV, brush border membrane vesicle; SlAPN, *Spodoptera litura* aminopeptidase N; PMSF, phenylmethanesulfonyl fluoride; LpNA, L-leucine-*p*-nitroanilide; CBB, coomassie brilliant blue.

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abundantly in the midgut of insects where it is anchored to the membrane of epithelial cells by glycosyl phosphatidylinositol (GPI) moiety [13–15]. Attempts to solubilise membrane anchored APN with different detergents have lead to various degrees of solubilisation. The yield of the recovered aminopeptidase upon solubilisation varied with detergent's nature. The pattern of detergent solubilisation of GPI-anchored proteins is different from that of proteins anchored with a single membrane spanning polypeptide. Detergents with a high critical micellar concentration (cmc) are capable of solubilising GPI-anchored proteins more efficiently than the detergents with a low cmc [16]. In contrast, the proteins anchored by a polypeptide are solubilised efficiently by all the detergents [16]. Several groups have reported the purification of APNs from CHAPS-solubilised brush border membrane vesicle (BBMV) proteins by using a combination of toxin affinity chromatography and ion exchange chromatography and studied their binding kinetics with several Cry proteins [8,17–21]. Though, the yield of purified receptor from such protocols is good for binding studies, the isolation of BBMVs in large amounts is labour-intensive. APN-encoding cDNA has been cloned from different insects and expressed in heterologous expression systems, *Escherichia coli*, insect and mammalian cell cultures [7,22–25]. However, purification of the heterologously expressed receptor has not been reported yet.

We have earlier reported the isolation of a 2.8 kb aminopeptidase N-encoding cDNA from the midgut of a polyphagous pest, *Spodoptera litura* [7]. The *apn* was expressed in Sf21 (*Spodoptera frugiperda*) insect cells using Baculo-Virus Expression system and the recombinant *S. litura* APN protein (SlAPN) was localized in the membrane of the Sf21 insect cells. A significant proportion of heterologously expressed APN was anchored to the membrane by GPI moiety [7]. In this communication, we report the differential solubilisation of SlAPN with the ionic and nonionic/zwitterionic detergents used. The solubilised APN was purified to near homogeneity by ion exchange and gel filtration chromatography and the purified APN was refolded to catalytically active form. Though, the efficiency of recovery of receptor from BBMVs or recombinantly expressed protein from insect cell culture are comparable, the ease for obtaining purified protein through baculovirus system makes it a method of choice. The purified, refolded APN exhibited toxin-binding characteristics similar to that shown by membrane-anchored APN, as it interacted with Cry1C but showed no interaction with structurally divergent Cry1Ac protein. The equilibrium dissociation constant (K_D) of pure APN for Cry1C was 90 nM while the Michaelis–Menten constant (K_m) for L-leucine-*p*-nitroanilide substrate was 1 mM. We have also shown that the Cry1C binding and aminopeptidase activities found to be associated with SlAPN probably involve distinct domains of the molecule.

Materials and methods

Solubilisation, purification and assay of recombinantly expressed aminopeptidase N (EC 3.4.11.2)

The Sf21 cells were grown and maintained at 27 °C in TNM-FH medium as a monolayer. The aminopeptidase N was expressed using Baculovirus expression system as described earlier [7]. The Sf21 cells expressing *S. litura* APN and uninfected cells were solubilised at 4 °C for 4 h in solubilisation buffer [20 mM Tris–HCl, pH 7.5, 300 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT (1,4-dithio-DL-threitol), 1 mM PMSF (phenylmethanesulfonyl fluoride), 10 μg/ml leupeptin and 10 μg/ml aprotinin] containing 1% (w/v) of different ionic or nonionic detergents. NLS was used in the category of ionic detergents and among nonionic detergents, CHAPS (3-{(3-cholamidopropyl) diethylammonio}-1-propanesulfonate (USB) or NP-40 (Nonidet P-40) (USB) or TX-100 (Triton X-100) (USB) were used. For APN purification Sf21 cells expressing recombinant APN were solubilised using 0.4% NLS. The cell suspension was incubated on ice for 4 h, intermittently vortexed and then sonicated (15 s continuous on, 1.5 min cooling; 15 pulses). The sonicated suspension was centrifuged at 100,000*g* for 60 min at 4 °C in 50Ti rotor (Beckman). Supernatant containing the solubilised SlAPN was used for enzyme assays and subsequent purification.

NLS-solubilised APN was refolded to catalytically active form by slow exchange of ionic detergent with milder nonionic detergents. The solubilised APN was dialyzed against buffer containing 1% (w/v) nonionic detergent (Tween 20/NP-40/Triton-X100/CHAPS). One millilitre solubilised sample was dialyzed against 2 L buffer for 8 h at 4 °C with four changes. Catalytic activity of the dialyzed sample was assayed as described below. For all APN characterization experiments, purified APN containing 0.2% NLS was similarly dialyzed against buffer containing 0.2% CHAPS.

The amount of APN released by four different nonionic/zwitterionic detergents, CHAPS, NP-40, Tween 20 and Triton X-100 at 1% concentration and ionic detergent, NLS at 0.4% concentration was estimated by calculating the specific activity of aminopeptidase N in each treatment. A linearity of reaction was established by assaying APN activity with respect to different protein concentrations (data not shown) using CHAPS-solubilised APN. For all experiments, total protein (20 μg) released in the supernatant after solubilisation of infected and uninfected membranes with different detergents was used for APN assay using L-leucine-*p*-nitroanilide (LpNA) as the substrate at a final concentration of 2 mM. The APN assay was carried out at 37 °C in 1 ml of reaction volume using 67 mM Na_2HPO_4, 67 mM KH_2PO_4, pH 7.4 as substrate buffer. The amount of product (*p*-nitroaniline) released was measured spectrophotometerically at 405 nm. The APN activity was calculated using an extinction coefficient of 9.9 mM^{-1} cm^{-1} for *p*-nitroaniline at 405 nm. For APN

inhibition studies, stock solution of amastatin hydrochloride (2S,3R)-3-amino-2-hydroxy-5-methylhexanoyl-Val-Val-Asp hydrochloride (Sigma) was prepared in methanol. APN was pre-incubated with different concentrations of amastatin for 3 h at room temperature on an end-over-end mixer. The complex was then used for activity determination. To map the difference in APN's catalytic site and Cry1C binding site, 100 nM pure APN was pre-incubated with 350 nM pure Cry1C in the presence or absence of 1 μM amastatin for 3 h at room temperature before assaying the catalytic activity of the enzyme.

For purification, Sf21 cells (1.5×10^7) expressing *S. litura* APN were washed once with PBS and resuspended in solubilisation buffer containing 0.4% (w/v) NLS. The solubilisation protocol as described in earlier section was followed to release APN from membranes. The supernatant obtained after ultracentrifugation was analyzed by SDS–PAGE and total protein was estimated with Bradford assay kit (Bio-Rad) using BSA (Sigma) as the standard.

The supernatant obtained after ultracentrifugation was dialyzed against buffer A (20 mM Tris–HCl, pH 7.5, 0.2% (w/v) NLS and 1 mM PMSF) for 8 h at 4 °C with three changes. During dialysis the concentration of NaCl was gradually reduced from 300 to 50 mM.

In the two-step purification protocol, APN was initially subjected to batch purification by anion-exchange chromatography with Q-sepharose (Amersham-Pharmacia Biotech). The total solubilised protein (2.5 mg in 10 ml) was loaded onto 3 ml resin equilibrated with buffer A. Binding was done for 2 h at 4 °C with slow agitation on end-over-end mixer. The resin was washed with 10 bed volumes of buffer A containing 50 mM NaCl followed by 10 bed volumes of buffer A containing 450 mM NaCl. Bound proteins were eluted using a salt gradient (500–700 mM NaCl with steps of 50 mM) prepared in buffer A and fractions of 3 ml each were collected. Fractions were analyzed on SDS–PAGE and those containing the partially purified APN were pooled and dialyzed against buffer B (20 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 150 mM NaCl, 0.2% (w/v) NLS, 1 mM PMSF) for 6 h at 4 °C with 3 changes.

The dialyzed sample was further purified by size exclusion chromatography. The S200 HR matrix (Amersham-Pharmacia) was packed in a column to a bed height of 25 cm and the column was run at 4 °C. Buffer B was used as the mobile phase and the flow rate was 100 μl/min. Hundred and fifteen fractions of 0.3 ml each were collected and analyzed for homogeneity by SDS–PAGE. The gels were stained with coomassie brilliant blue (CBB) and the fractions containing purified APN were pooled and also analyzed by silver staining [26].

For Western blot analysis, electroblotted purified APN was probed with anti-APN antibodies (1:5000) [7]. The blot was then incubated with alkaline phosphatase-conjugated goat anti-rabbit Ig-G and NBT (*p*-nitroblue tetrazolium chloride)-BCIP (5-bromo-4-chloro-3-indolyl phosphate toluidine) (Life Technologies) as described earlier [7].

Pure refolded SlAPN was used to determine Michaelis constant for the substrate L-leucine-*p*-nitroanilide. The concentrations of the substrate used were from 0.5 to 10 mM.

Ligand blotting analysis and estimation of affinity of Cry1C and APN by indirect ELISA

Toxin-binding property of purified and refolded APN was checked by ligand blotting experiment as described earlier [7]. Briefly, purified Cry1C and Cry1Ac proteins (1 μg each) were resolved on 7.5% SDS–PAGE and electrotransferred to NC membrane. After blocking, the blot was overlaid with 1 μg/ml of purified and refolded APN in 20 mM Tris–HCl pH 7.5, containing 0.2% BSA at room temperature for 1 h. The bound APN was observed by incubating the blot with polyclonal antibodies raised against APN [7]. After three washes, the blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit antibodies (Calbiochem). The immunoblots were developed using NBT/BCIP substrates.

To measure the kinetics of association of APN to Cry1C toxin in solution, 0.2–1.2 μM Cry1C toxin was mixed with APN (40 nM) and aliquots of 100 μl after different time periods, were transferred to a 96-well ELISA plate, precoated with 2 μg of Cry1C toxin in coating buffer (50 mM sodium carbonate pH 9.6). The amount of unbound APN was estimated as described [28]. At the end of the kinetic analysis, the ELISA plate was washed three times with washing buffer (PBS supplemented with 0.5% Tween 20) and incubated with anti-APN antibody (1:5000). The plate was washed again and the binding signal was detected using anti-rabbit antibody coupled to alkaline phosphatase using 7 mM *p*-nitrophenyl phosphate (PNPP, Sigma) as the substrate.

Results and discussion

Solubilisation of recombinant APN and its refolding (regeneration of catalytically active form)

In the present study, a number of ionic and nonionic/zwitterionic detergents were used to recover recombinantly expressed APN from the membrane of insect cells. As shown in SDS–PAGE, the *S. litura* APN showed differential solubilisation with the detergents used (Fig. 1a). In the absence of any other directly measurable activity for conformation and catalytic fidelity upon solubilisation, we have taken specific activity of aminopeptidase as a measure of correct conformation. The CHAPS-solubilised proteins from APN expressing cells hydrolyzed LpNA with a specific activity of 42 nmol/h/mg whereas the proteins solubilised from uninfected cells hydrolyzed LpNA with a specific activity of 2 nmol/h/mg (Fig. 1b). The difference in the activity showed that the expressed APN is active after solubilisation with nonionic detergent. The relative amount of APN released from the expressing Sf21 cells by different

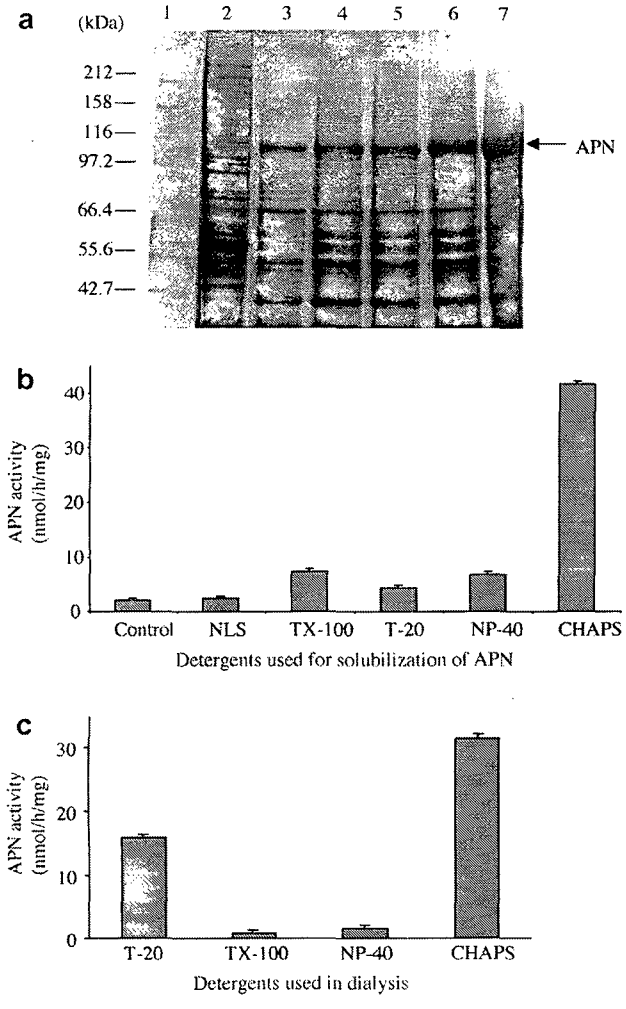

Fig. 1. (a) Differential solubilisation of membrane-anchored recombinant *S. litura* aminopeptidase expressed in Sf21 cells. Total proteins from Sf21 cells were solubilised by using different detergents, insoluble material was removed by ultracentrifugation (100,000*g*) and equal amounts of solubilised proteins present in supernatant were resolved on a 10% SDS–PAGE. Molecular marker (NEB) (lane 1), CHAPS-solubilised proteins of uninfected Sf21 cells (lane 2), Tween 20-solubilised (lane 3), TX-100-solubilised (lane 4), NP-40-solubilised (lane 5), CHAPS-solubilised (lane 6) and NLS-solubilised (lane 7) proteins of APN expressing Sf21 cells. The gel was stained with coomassie blue. (b) Amount of SlAPN released from Sf21 cells by different nonionic detergents. The supernatants obtained after solubilisation of APN expressing Sf21 cells were assayed for aminopeptidase activity using 2 mM leucine-*p*-nitroanilide as the substrate. The specific activity of APN was calculated in each treatment and plotted in the form of bar diagram. Control refers to the specific activity of supernatant of CHAPS-solubilised non-expressing Sf21 cells. Activities were given as the mean values from three independent experiments. (c) Recovery of catalytic activity of NLS-solubilised APN. SlAPN expressing Sf21 cells were solubilised with 0.4% NLS and the supernatant obtained after ultracentrifugation was dialyzed against different nonionic/zwitterionic detergents at 1% concentration. The dialyzed sample was assayed for APN activity using 2 mM LpNA as the substrate. The specific activity of APN was calculated in each treatment and plotted in the form of bar diagram. Activities were given as the mean values from three independent experiments.

nonionic detergents was determined by assaying the respective supernatants for APN activity (Fig. 1b). The ability of different nonionic detergents to release APN was in the following order:

CHAPS > TX-100 = NP-40 > Tween 20

The amount of protein solubilised correlated directly with the cmc of the detergents. The cmc (in mM) of detergents used is CHAPS (6–10), TX-100 (0.2–0.9), NP-40 (0.05–0.3) and Tween 20 (0.059). Detergents with a high cmc (e.g., CHAPS) were more effective in solubilising APN as compared to the detergents with a low cmc (e.g., Tween 20). Amongst the nonionic detergents tried for solubilisation, CHAPS with highest cmc released maximum amounts of APN from the membranes. It has been reported that the GPI-anchored proteins exhibit differential solubilisation by range of detergents [16]. In contrast, proteins anchored by a transmembrane sequence of hydrophobic amino acids are efficiently solubilised by all the detergents [16]. Thus, the recombinant GPI-anchored SlAPN displayed the same property of differential solubilisation as shown by native GPI-anchored proteins. The recombinant APN was almost completely released from Sf21 cells by the ionic detergent, NLS (Fig. 1a) but the catalytic activity of the enzyme was lost. The premise that NLS is a mild ionic detergent and may not induce major structural and conformational anomalies was examined as follows. APN was solubilised from expressing cells with NLS and refolded by exchanging the ionic detergent with milder nonionic detergents through slow dialysis (molecular weight cut-off of dialysis membrane was 10 kDa). Different nonionic detergents at 1% (w/v) concentration were used for dialysis and the catalytic activity of the refolded protein was assayed. The denatured APN showed maximum regain of activity with CHAPS (Fig. 1c). Hence, for subsequent experiments, NLS/CHAPS combination was used i.e., NLS-solubilised APN was used for purification and the purified protein was slowly dialyzed against 0.2% CHAPS to regain its catalytic activity.

Purification of S. litura aminopeptidase N

Aminopeptidases have been identified as receptor for a number of Cry proteins. Native APNs purified from BBMVs of insect midgut have been studied for their ability to interact with different Cry proteins by various *in vitro* and *in vivo* experiments, however; in all experiments incisive analysis of the binding of insecticidal protein was limited due to the lack of availability of APN in sufficient amount [21].

In this study, we devised a purification strategy in which the recombinant SlAPN was initially solubilised from Sf21 cells and subsequently purified by using two-step chromatography protocol. Since the recombinant GPI-anchored SlAPN showed maximum solubilisation with ionic detergent NLS, it was used to release membrane-anchored APN from the cells. NLS, being a mild ionic detergent does

not induce major structural and conformational anomalies and could be removed by dialysis. Different concentrations of NLS were used to find the concentration that releases the maximum amount of APN (data not shown). At 0.4% NLS, nearly complete solubilisation of SlAPN was achieved (Fig. 1a).

Since the recombinant APN carried a negative charge [pI = 5.5 (predicted)] in solubilisation buffer of pH 7.5, the initial purification was done by anion-exchange chromatography which removed major contaminating proteins giving a 60% pure APN (Fig. 2a). The fractionation of partially purified protein by gel filtration chromatography resulted in >90% purification of the recombinant APN protein (Fig. 2b). The purity of APN was further confirmed by silver staining in which only the APN band was observed (Fig. 2c). This band reacted positively with the anti-APN antibodies confirming the identity of the purified protein (Fig. 2d).

After the two-step purification strategy, 270 μg pure APN was obtained from 15×10^6 Sf21 cells. The recovery of pure APN based on total protein estimation was 11% (starting from 2.5 mg total protein). Activity of the purified, refolded protein was 360 nmol/h/mg which corresponded to a 9-folds purification of the enzymatically active APN (Table 1).

While the NLS-solubilised APN could be purified to >90% purity, the enzyme was catalytically inactive. The slow removal of NLS from purified receptor by dialyzing it against CHAPS allowed reversal from catalytically inactive to an active state. Adoption of this protocol resulted in the recovery of large amounts of catalytically active APN in pure form. The availability of pure aminopeptidase in abundance will greatly facilitate the elucidation of mechanism of action of insecticidal proteins of *B. thuringiensis*.

Characterization of purified aminopeptidase N

The conformation competence of purified and refolded aminopeptidase was verified by examining its kinetic constant, interaction with insecticidal protein Cry1C and by calculating its dissociation constant for Cry1C protein.

The affinity of pure SlAPN for LpNA substrate was determined by calculating its activity at different substrate concentrations ranging from 0.5 to 10 mM. Maximum velocity (V_{max}) of the reaction was calculated to be 590 nM/h and K_m for LpNA was 1 mM.

Since purified SlAPN was refolded from an inactive to active form, we investigated its ability to bind to bioactive toxin Cry1C after refolding. Importantly, the toxin-binding characteristics displayed by the 108 kDa purified and refolded APN receptor were similar to that of membrane-anchored APN. It interacted with bioactive toxin, Cry1C but showed no interaction with Cry1Ac toxin on ligand blots (Fig. 3a). This is in agreement with our previous report where we showed that APN expressed on Sf21 cells interacted with Cry1C but not with Cry1Ac [7].

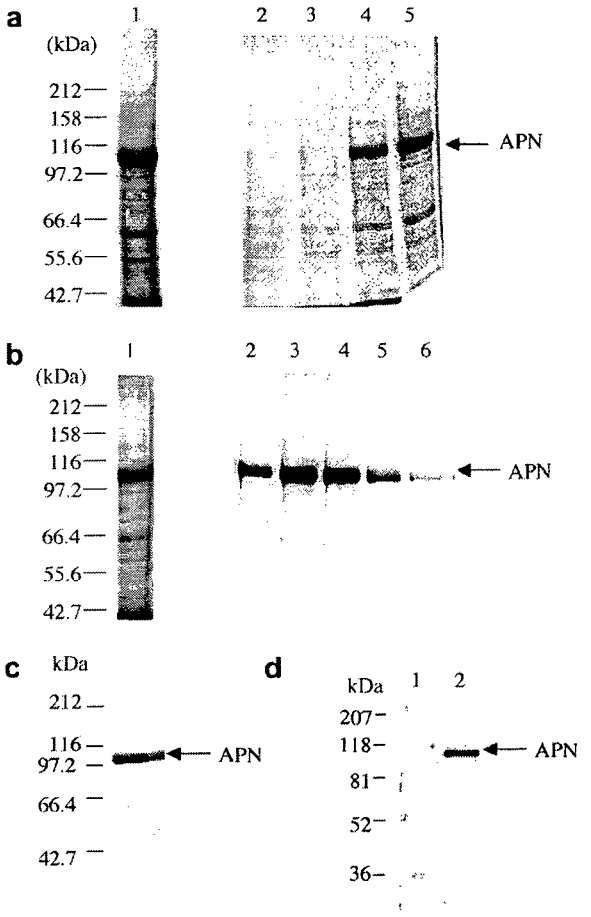

Fig. 2. Purification of NLS-solubilised APN. (a) Anion-exchange chromatography. Sf21 cells expressing APN were solubilised with 0.4% NLS and the solubilised proteins were loaded onto anion-exchange resin (Q-sepharose). The bound proteins were eluted using a salt gradient (500–700 mM NaCl with steps of 50 mM) and resolved on a 7.5% SDS-PAGE. The protein sample loaded on Q-sepharose (lane 1), flow through (lane 2), wash fraction (lane 3), fraction eluted with 600 mM NaCl (lane 4) and 650 mM NaCl (lane 5). The gel was stained with coomassie blue. The partially pure fractions (lanes 4 and 5) were pooled, concentrated by centricon column and resolved further by gel filtration chromatography. (b) Gel filtration chromatography (S200HR). Partially purified APN obtained from anion-exchange chromatography was further purified by gel filtration using S200HR matrix. Every fourth fraction out of 115 fractions collected was resolved on a 7.5% SDS–PAGE. The fractions containing purified APN are only shown in figure. Protein sample loaded on gel filtration column (lane 1) and fraction no. 85, 89, 93, 97 and 101 (lanes 2–6) were resolved on 7.5% SDS–PAGE. The gel was stained with coomassie blue. (c) Silver staining of purified APN. Gel filtration fractions (85–104) containing purified APN were pooled and resolved on 10% SDS–PAGE. The gel was silver stained to check for purity of APN. (d) Western analysis of purified APN. Pool of fractions (85–104) containing pure APN (lane 2) were resolved on 10% SDS–PAGE along with broad range prestain marker (Bio-Rad) (lane 1) and electrotransferred to nitrocellulose membrane. The identity of the purified protein was confirmed by western blot analysis using anti-APN antibodies.

The equilibrium dissociation constant (K_D), the association rate constant (k_{on}) and the dissociation rate constant (k_{off}) are the basic parameters for characterizing protein-protein interactions. To obtain quantitative data for the

Table 1
Evaluation of purification of recombinant APN expressed in Sf21 cells

Sample	Total protein (mg)	Yield (%)	Specific activity (nmol/h/mg)	Purification fold
Solubilised sample	2.5	100	40	1
Ion-exchange chromatography	1.2	48	250	6
Gel filtration chromatography	0.27	11	360	9

At each step protein concentration was determined by Bradford (Bio-Rad) and enzymatic activity by using 2 mM LpNA as the substrate.

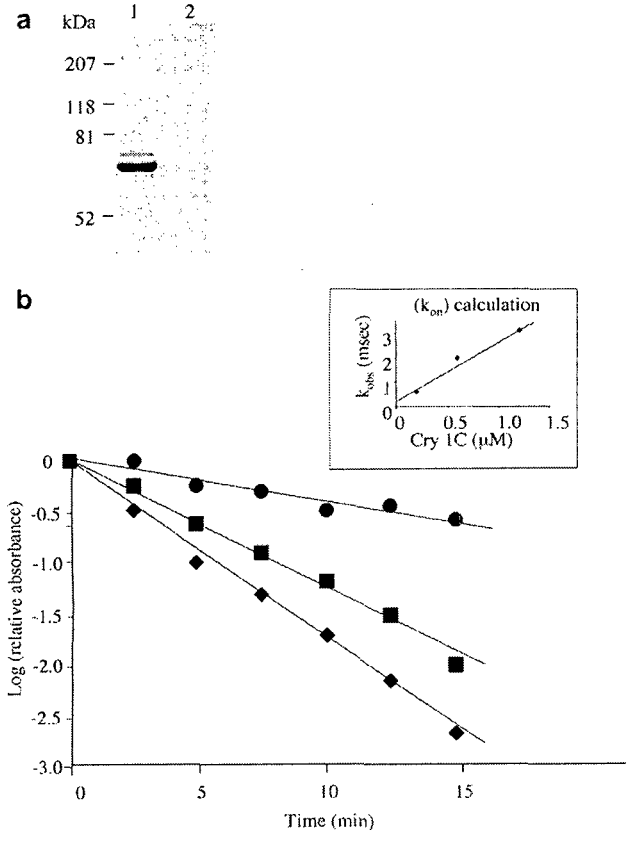

Fig. 3. Characterization of pure APN. (a) Ligand blot analysis of purified SlAPN. Purified Cry1C (lane 1) and Cry1Ac (lane 2) were resolved on 7.5% SDS–PAGE and electrotransferred to nitrocellulose membrane. The nitrocellulose membrane was probed with purified APN (1 μg/ml). Binding of the receptor was detected by using anti-APN antibodies followed by AP-conjugated goat anti-rabbit IgG. (b) Association rate kinetics of Cry1C toxin/APN complex. Logarithm of relative absorbance was plotted versus time. The measured signal was the absorbance at 405 nm obtained in the last step of competitive ELISA. Relative absorbance represents the ratio $(A_t - A)/(A_0 - A)$ where A is the ELISA signal on completion of the reaction, A_t, the signal at time t, A_0, the signal at time zero and t corresponds to the time elapsed between initiation of the association and transfer of the aliquots into the wells. The complex was formed by mixing 40 nM APN to 0.2 μM (●), 0.6 μM (■) and 1.2 μM (♦) Cry1C. The pseudo-first-order rate constants (k_{obs}) is given by slope of each straight line. (Inset) Determination of the association rate constant (k_{on}). The (k_{obs}) obtained from independent experiments were plotted versus the Cry1C concentrations. The second-order rate constant (k_{on}) is given by the slope of the straight line obtained. The extrapolation to zero gives the (k_{off}) values.

interaction of the Cry1C toxin with the APN, we analyzed the interaction of the toxin with the receptor in solution using an indirect enzyme-linked immunosorbent assay (ELISA) [27]. In this methodology, the association rate of Cry1C toxin and APN receptor was measured by mixing both molecules in solution and at different time intervals aliquots were withdrawn to determine by indirect ELISA the amount of free APN that remained in solution. The reduction in the amount of free APN represents the association reaction [27]. To have a pseudo-first-order reaction, the concentration of the APN was at least 5-fold lower than the concentration of Cry1C toxin in solution. Also, the concentration of the Cry1C toxin in solution was 10-fold higher than the reported K_D values for APN [28,29]. The kinetics of association of the APN to Cry1C is shown in Fig. 3b. The plot is the logarithm of relative absorbance versus time where the slope of line suggests a pseudo-first-order rate constant of association (k_{obs}). The association constant ($k_{on} = 2.2 \times 10^3\ M^{-1}\ s^{-1}$) was obtained by dividing the k_{obs} constant with the concentration of the Cry1C toxin. The ordinate at the origin corresponded to a first-order dissociation rate constant (k_{off}) and was found to be $0.2 \times 10^{-3}\ s^{-1}$. The equilibrium dissociation constant ($K_D = 90$ nM) was then calculated as the ratio of dissociation and association rate constants (k_{off}/k_{on}). Our results demonstrated that the APN possessed a high affinity site for Cry1C with K_D value of 90 nM. The toxin exhibited a moderate on rate ($\sim 2.2 \times 10^3\ M^{-1}\ s^{-1}$) and a slow reversible off rate ($0.2 \times 10^{-3}\ s^{-1}$). This suggested that though the initial binding of APN with Cry1C is moderate but once the complex is formed it is quite stable and dissociated very slowly thus explaining the high affinity of APN for Cry1C. Previous surface plasmon resonance studies with pure APN have shown that APN possesses a high affinity binding site for various Cry1A toxins whose K_D values are in the range of 40–280 nM [28,29]. This is the first report describing the kinetics of association of recombinant APN with Cry1C class of proteins.

Distinct catalytic and toxin-binding sites of aminopeptidase N

The role of pig aminopeptidase N as a virus receptor does not appear to depend on its proteolytic activity [30]. It has been previously shown that amastatin, a competitive active site inhibitor, did not block Cry1C binding to a 106 kDa form of APN from *Manduca sexta*, suggesting that the toxin did not bind to the active site of the APN molecule [31]. We also used amastatin in binding experiments to examine the catalytic and toxin-binding sites of pure SlAPN. APN activity was inversely proportional to inhibitor concentration. The pre-incubation of APN with

20 μM amastatin inhibited the enzyme completely while 1 μM amastatin reduced the activity to half (Fig. 4). We have previously demonstrated APN–Cry1C interaction by immunoprecipitation experiment [7]. Using a similar approach, we determined the aminopeptidase activity of the APN–Cry1C complex. APN was pre-incubated with Cry1C toxin for 3 h and checked for its enzymatic activity. The resulting APN–Cry1C complex hydrolyzed leucine-*p*-nitroanilide as efficiently as native APN (Table 2). We also investigated the effect of binding of Cry1C toxin on inhibition of APN with amastatin. The aminopeptidase activity of the Cry1C-APN complex in presence of 1 mM amastatin was reduced to half as compared with free APN (Table 2). This suggested that the binding of Cry1C toxin does not block the active site of APN and it remains available for substrate or inhibitor (amastatin) binding. These results provide strong evidence that the receptor and catalytic functions associated with SlAPN involve distinct domains of the molecule.

Diverse models describing consequences of receptor–toxin interaction have been proposed. Specific elucidation of mechanism of pore formation is being limited due to the non-availability of receptor protein in adequate amount. It is rather intriguing to note that different insecticidal proteins seem to predominantly interact with different biomolecules at the midgut of susceptible insect. Insecticidal protein Cry1C interacts with aminopeptidase N of *S. litura*, on the other hand Cry1Ac and CryV protein interact with cadherin and glycoconjugates of *M. sexta* and *Caenorhabditis elegans*, respectively [32,33]. Similarly, the subsequent events of post-toxin–receptor interaction are also apparently divergent. While oligomerization has been speculated to be essential for pore formation in *M. sexta* [34], oligomerization independent events have been suggested by Bulla et al. [35] to be responsible for pore formation and lysis of transgenic cell lines. The availability of a binding-competent pure recombinant APN receptor in large amounts would help in elucidating the mode of action of Bt toxins that recognize APN as their receptor. The purified SlAPN was not only catalytically active but also competent to bind to biologically active Cry1C protein. Such an APN protein would give us a handle to study the mode of action of APN-interacting Cry1C class of proteins. By incorporating pure SlAPN in phospholipid vesicles, the events following binding of Cry1C to APN can be elucidated. We speculate that binding of Cry protein to a receptor, cadherin or APN, is an independent event and both lead to lysis of midgut epithelial cells followed by death of the insect.

To conclude, we have optimized a rapid and efficient purification protocol for recombinantly expressed aminopeptidase. The availability of this system would permit critical evaluation of interacting ligands.

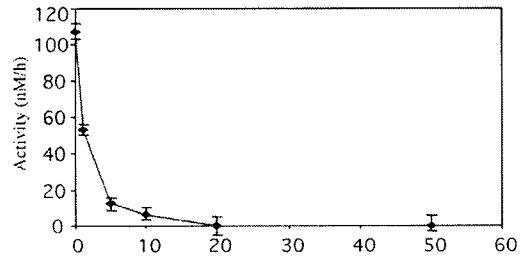

Fig. 4. Effect of amastatin inhibitor on the catalytic activity of APN. 100 nM APN was incubated with different concentrations of amastatin hydrochloride for 3 h at RT and then assayed for enzymatic activity using LpNA as the substrate.

Table 2
APN has distinct catalytic and toxin-binding sites

Sample	Activity (nM/h)
100 nM APN	333
100 nM APN + 350 nM Cry1C	333
100 nM APN + 1 μM amastatin	160
100 nM APN + 350 nM Cry1C + 1 μM amastatin	150

Pure APN was pre-incubated with Cry1C in the presence or absence of amastatin for 3 h at room temperature before assaying the catalytic activity of the enzyme.The activity of APN as well as its inhibition by amastatin was not affected by Cry1C binding.

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