LOCALIZATION AND FUNCTIONAL STUDIES OF THE CADHERIN BT-R_1 AS A *BACILLUS THURINGIENSIS* CRY1A-BINDING PROTEIN IN THE MIDGUT OF TOBACCO HORNWORM (*MANDUCA SEXTA*)

by

JIANG CHEN

(Under the Direction of Michael J. Adang)

ABSTRACT

The members in *Bacillus thuringiensis* (Bt) Cry1A family have specific toxicity toward lepidopteran larva. A critical step in Bt-mediated insecticidal action is the binding of Bt toxin to its target molecules on insect midgut epithelium. Three Cry1A-binding proteins, aminopeptidase MsAPN1, the cadherin Bt-R_1 and the membrane-type alkaline phosphatase (m-ALP), had been identified in the midgut of tobacco hornworm (*Manduca sexta*). Immunohistochemistry illustrated that these binding proteins had different localization on *M. sexta* larval midgut epithelia. To compare with the immunolocalization of Cry1A-binding proteins, Cry1A toxins showed co-localization on insect midgut microvilli.

CR12-MPED, which is the membrane proximal part of Bt-R_1, was identified as a critical region for Cry1Ab binding. This fragment was over-expressed and purified as a peptide from *Escherichia coli*. Unexpectedly, the Cry1Ab-induced *M. sexta* larval mortality was not blocked, but significantly enhanced by the addition of CR12-MPED. Further bioassays indicated that CR12-MPED could also potentiate Cry1Ac toxicity against tobacco budworm (*Heliothis virescens*) and corn earworm (*Helicoverpa zea*). When a putative Cry1A-binding epitope was

removed from CR12-MPED, the derivative peptide (CR12-MPED/Del) lost binding to Cry1A toxins and the ability of toxicity enhancement. Far-UV circular dichroism (CD) and ^{1}H-NMR spectroscopy showed that CR12-MPED was mainly composed of β-strands and random coils in unfolded state. Histomicroscopy illustrated that CR12-MPED bound to midgut microvilli, but did not change Cry1A binding localization at these places. The CR12 sub-truncation was identified as the minimal region in CR12-MPED to enhance Cry1Ac toxicity against *H. zea* larvae. A proposed mechanism of this synergism was that the unfolded CR12-MPED accumulated onto microvilli through hydrophobic interactions, and Cry1A toxins could be attracted to these places by toxin-peptide binding, hence the Cry1A toxicity was increased. This discovery enables Cry1A toxins to effectively control lepidopteran pests even at low dosages, which can prevent insect resistance from high toxin selection pressure.

INDEX WORDS: *Bacillus thuringiensis*, Cry1A, lepidopteran, *Manduca sexta*, *Heliothis virescens*, *Helicoverpa zea*, midgut, aminopeptidase N, cadherin, alkaline phosphatase, synergist

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DEDICATION

This work is dedicated to my mentor the late professor Fu-Chun Yu (1914-2003), and my first biology teacher Mr. Yi-Nong Ni who introduced me into the gorgeous insect world when I was a middle school student.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Bacillus thuringiensis (Bt) is a soil-borne Gram-positive bacterium that produces parasporal crystalline inclusions composed of Cry insecticidal proteins. Most Cry proteins are highly toxic to insects in a narrow spectrum (Lepidoptera, Diptera or Coleoptera), but not harmful to non-target animals and humans (Clark et al. 2005, Crickmore 2005, de Maagd et al. 2001). Bt has been a valuable bio-pesticide although its usage is limited relative to chemical insecticides. Since 1996, Bt Cry proteins have become major tools for insect control through their expression in genetically modified plants (e.g. cotton and corn). Primarily due to the increased planting of Bt cotton and corn, scientists face a potential problem that insects may generate Bt-resistant properties (Kain et al. 2004, McGaughey 1985, Van Rie et al. 1990). Although the generation of Bt-resistance in insects is complicated, genetic modification of Bt-binding molecules on the microvilli of insect midgut epithelia has been regarded as a key mechanism of Bt-resistance (Gahan et al. 2001, Morin et al. 2003, Xu et al. 2005).

1. *Bacillus thuringiensis* Cry Protein

1.1 Cry Protein Classification and Nomenclature

The Cry insecticidal proteins (formerly called δ-endotoxins) are encoded by *cry* genes in Bt, many of which have been cloned and sequenced. The total number of recognized Cry proteins to date is above 300 (http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/index.html). Cry proteins are classified according to primary protein structure in a hierarchical fashion that indicates the degrees of phylogenetic divergence (Crickmore et al. 1998).

1.2 Cry Protein Structural Composition

1.2.1 Structure Overview

The crystals of the Bt strains are bi-pyramidal in shape and composed of 130-138 kDa Cry1A proteins (i.e. protoxins). Bt Cry1A protoxins are released in the basic lumen environment (pH 10-11) of lepidopteran larval midgut where they are digested by serine proteases, which are commonly trypsin and chymotrypsin-like proteases, to approximately 60 kDa activated toxins (de Maagd et al. 2003, Schnepf et al. 1998).

Three dimensional structures of five Cry toxins (Cry1Aa, Cry3Aa, Cry2Aa, Cry3Bb1 and Cry4Ba) have been resolved by X-ray diffraction method (http://www.pdb.org). The crystal structure of Cry1Aa, a 65 kDa lepidopteran-specific Bt toxin, was resolved at 2.25 Å resolution (Grochulski et al. 1995). Cry1Aa is composed of three domains. From amino to carboxy-terminus, domain I (residues 33 to 253) is a bundle of eight anti-parallel α helices, domain II (residues 265 to 461) is a "Greek key" β barrel, and domain III (residues 463 to 609) is a β sandwich (Grochulski et al. 1995). The following comments on Cry structure pertain specifically to Cry1Aa, but in general Cry toxin structures are highly conserved.

1.2.2 Domain I: pore-formation domain

Domain I is involved in membrane contact, insertion and pore formation. In domain I, helix α5 is in the center surrounded by other α helices (Grochulski et al. 1995). After the toxin binds to a molecule (i.e. "receptor" as commonly referred to) on the microvilli of insect midgut columnar cells, a conformational change will be induced in domain I. According to one model after toxin binding, helices α4 and α5 penetrate the epithelial membrane while other helices lie on the membrane surface like the ribs of an umbrella (Gazit et al. 1998).

1.2.3 Domain II: toxin-binding domain

Domain II recognizes molecules on midgut epithelia, which serve as docking sites for toxin. Three β-sheets build up domain II. The first sheet contains four strands β2 to β5, and similarly the second sheet contains strands β6 to β9. The connection of these two β sheets forms the typical "Greek-key" topology (Grochulski et al. 1995). The third sheet is composed of three β strands from two sequentially separated fragments. The first fragment is composed of two strands β10 and β11 from the C-terminus of domain II, while the second fragment is composed of strand β1b from N-terminus of domain II. Three long β hairpin extensions are formed in each β sheet of domain II. The loop-like β hairpin extensions contain residues 310 to 313, 367 to 379, and 438 to 446 from sheets 1, 2, and 3, respectively (Grochulski et al. 1995). Mutational analyses show that specific amino acids in the loop "tips" in domain II mediate receptor binding, and are determinants of insecticidal host range.

1.2.4 Domain III: toxin-binding and domain I- stabilization domain

The lectin-like domain III contains two twisted antiparallel β sheets, of which each is composed of five strands. Domain III of Cry1Ac contains a site that binds N-acetyl galactosamine (GalNAc), a moiety of Cry1Ac-binding aminopeptidase N (Burton et al. 1999). The existing GalNAc recognition site suggested that domain III might involve the interaction of toxins and their binding molecules on insect midgut. For instance, Cry1Ac binds to a 120 kDa aminopeptidase N from tobacco hornworm (*Manduca sexta*) larval midgut, but Cry1C does not. When domain IIIs of Cry1Ac and Cry1C are exchanged, the hybrid toxin of Cry1Ac (domains I and II) + Cry1C (domain III) lost binding to aminopeptidase N, whereas the hybrid toxin of Cry1C (domains I and II) + Cry1Ac (domain III) displayed binding (de Maagd et al. 1999). On

the other hand, since an area of 560 $Å^2$ on the surface of domain III contacts with domain I, it is thought that domain III is also important for toxin stability and integrity (Grochulski et al. 1995, Li et al. 1991). Thus, domain III might have dual functions.

2. Cry Toxin Binding Molecule on *M. sexta* Larval Midgut Epithelium

2.1 Aminopeptidase N

2.1.1 Molecular characters and classification

Aminopeptidase N (APN, EC 3.4.11.2) is a transmembrane metalloproteinase that digests amino acids from N-termini of peptides or proteins. APN has a peptide at the C-terminus for glycosylphosphatidyl inositol (GPI) anchor, which tethers the protein in the cell membrane. APN contains the Zn^{2+}-binding motif $HE(X)_2H(X)_{18}E$ for its enzymatic activity and several residual sites for glycosylation (Sato 2003). In insects, APN can be found mainly on the brush border membrane of midgut epithelia, which is the terminal site of food digestion (Terra and Ferreira 2005). A 120 kDa aminopeptidase N (MsAPN1) from *M. sexta* larval midgut was recognized and cloned as the first Cry-binding protein in insects, which is specific for Cry1Ac (Knight et al. 1994, Sangadala et al. 1994). To date, over twenty APN isoforms have been identified that serve as Cry1A-binding proteins in over 10 lepidopteran species (Agrawal et al. 2002, Nakanishi et al. 2002, Wang et al. 2005). Different APN isoform may play a distinct role in protein digestion, which allows insects to adapt various host plants. In Lepidoptera, APN isoforms could be grouped into four classes according to phylogenetic analysis (Nakanishi et al. 2002, Oltean et al. 1999). The silkworm (*Bombyx mori*) and the diamondback month (*Plutella xylostella*) contain all four classes of APN isoforms simultaneously in larval midguts (Nakanishi

et al. 2002). Among all lepidopteran APN isoforms, the 120 kDa MsAPN1 from *M. sexta* has been well studied as a representative Cry1A-binding protein.

2.1.2 MsAPN1: cry1A-binding site and affinity

Two Cry1A toxin-binding sites have been found on MsAPN1. One is a common binding site shared by Cry1Aa, Cry1Ab and Cry1Ac; the other is a specific binding site for Cry1Ac (Masson et al. 1995). Compared with the binding properties of Cry1Aa and Cry1Ab, a 2:1 molar ratio of monomeric Cry1Ac bound to MsAPN1 confirmed the existence of two Cry1Ac-binding sites on MsAPN1 in light scattering and surface plasmon resonance (SPR) experiments when a 115 kDa MsAPN1 with the GPI anchor removed (Masson et al. 1995, Masson et al. 2002). The two Cry1Ac-binding sites model was also supported by experiments showing that MsAPN1 bound to Cry1Ac with domain II and III mutations (Jenkins et al. 2000). However, when 120 kDa MsAPN1 was anchored in a lipid monolayer, a 1: 1 molar ratio of Cry1Ac bound to MsAPN1 (Cooper et al. 1998). This difference was explained to be a reversed insertion orientation of MsAPN1 into lipid monolayer, which could mask one Cry1Ac-binding site on MsAPN1 (Masson et al. 2002).

The binding affinity of Cry1Ac to soluble MsAPN1 without GPI anchor tail was measured to be 95 nM by SPR analysis (Masson et al. 1995). However, when MsAPN1 was anchored on a lipid monolayer mimicking cell membrane, its binding affinity to Cry1Ac was calculated as an initial rapid affinity constant of 110 nM followed by a slower higher-affinity interaction (0.028 nM) and an overall affinity of 3.0 nM (Cooper et al. 1998). These authors explained that this two-step process is due to an initial low affinity binding via GalNAc followed by a second higher-affinity interaction with membrane.

In bioassays, Cry1Ac toxicity to *M. sexta* larvae was reduced when toxin was mixed with GalNAc (Knowles et al. 1991), suggesting that GalNAc binding was relevant to Cry1Ac toxicity. Subsequently it was shown that Cry1Ac, but not Cry1Aa or Cry1Ab, binding to MsAPN1 could by specifically inhibited by competitor GalNAc (Masson et al. 1995). This was evidence that Cry1Ac binding to MsAPN1 was through protein-glycan interactions, whereas Cry1Aa or Cry1Ab binding to MsAPN1 was through protein-protein interactions. There are 10 predicted O-glycosylation sites at the Thr/Pro rich C-terminal region of MsAPN1, of which 5-6 are putatively occupied by GalNAc glycans, the potential carbohydrate binding sites for Cry1Ac (Knight et al. 2004).

2.1.3 Lipid raft associated with aminopeptidase N

Lipid rafts are membrane microdomains formed by cholesterol and sphingolipid which are more tightly packed in a phospholipid-rich environment than surrounding non-raft lipid bilayer (Alonso and Millan 2001, London 2005). Rafts are resistant to solubilization with cold non-ionic detergent, whereas the rest of the membrane lipids are soluble (Brown and London 2000, Kusumi and Suzuki 2005). Some membrane proteins, including the GPI-anchored ones, preferentially partition into the ordered raft microdomains (Danielson and Hansen 2003). In tobacco budworm (*Heliothis virescens*), two GPI-anchored Cry1A-binding proteins, the 120 kDa and 170 kDa APN, were mainly distributed in lipid rafts of midgut epithelial membranes. Similarly, in *M. sexta*, 120 kDa MsAPN1 was found exclusively in lipid rafts (Zhuang et al. 2002). In vitro assays demonstrated that Cry1A toxins were specifically associated with lipid rafts, and the integrity of rafts was essential for Cry1Ab pore forming activity (Zhuang et al.

2002). Thus, lipid rafts as well as their associated APN are critical for Cry1A binding and toxicity.

2.2 Cadherin

2.2.1 Molecular character of Bt-R_1

Concurrently with the discovery that APN bound Cry toxins, a 210 kDa glycoprotein, named Bt-R_1, was also identified as a Cry1Ab-binding molecule in *M. sexta* (Vadlamudi et al. 1993). The sequence of Bt-R_1 shares 30-60 % similarity and 20-40 % identity to proteins in the cadherin superfamily (Vadlamudi et al. 1995). Including Bt-R_1, the cadherin Cry1A-binding proteins have been characterized and cDNA sequenced from eleven lepidopteran species: *Bombyx mori*, *Chilo suppressalis*, *Helicoverpa armigera*, *H. zea*, *Heliothis virescens*, *Lymantria dispar*, *M. sexta*, *Ostrinia nubilalis*, *Pectinophora gossypiella*, *Plutella xylostella*, and *Spodoptera frugiperda* (http://www.ncbi.nlm.nih.gov).

Cadherins are adhesion molecules involved in cell-cell adhesion, cell morphogenesis and mortality, wound healing, and the maintenance of organized tissues (Takeichi 1995). From N to C-terminus, Bt-R_1 is composed of twelve tandem similar cadherin repeats (CR), one membrane-proximal extracellular domain (MPED), a single transmembrane region (TM) and a cytoplasmic domain (CYTO) like the structure of classical cadherins (Dorsch et al. 2002). The cadherin Cry1A-binding proteins from other lepidopterans may contain variant number of CR domains, but their basic structural components are same as Bt-R_1, although sometimes the MPED domain was regarded as the last repeat of CR domains.

For cadherins, the CR domains of the extracellular portion mediate cell-cell adhesion through conserved His-Ala-Val motifs (Overduin et al. 1996), two of which are located in CR4

and CR10 domains of Bt-R_1 (Dorsch et al. 2002). Bt-R_1 also contains two putative binding motifs for integrins, which are heterodimeric cell-surface receptors having functions as cell adhesion, proliferation, migration, survival, and intercellular mechnical signaling transduction (Ingber 1991, Stupack and Cheresh 2004). These putative integrin-binding motifs, Arg-Gly-Asp and Lys-Asp-Val, are located in CR2 and CR8 domains of Bt-R_1, respectively (Dorsch et al. 2002).

The MPED domain of Bt-R_1 is a functionally unique domain existing in nonchordate cadherins but absent in chordate cadherins (Oda and Tsukita 1999). *Drosophila* E-cadherin (DE-cadherin) mutants in the MPED domain (also called the "primitive classic cadherin domain") did not eliminate cell-cell adhesion but hindered the effective transport of DE-cadherin to the cell membrane in epithelial cells of the developing embryo (Oda and Tsukita 1999).

The CYTO domain is the most conserved portion of cadherins (Nagafuchi and Takeichi 1988, Takeichi 1995). The functional role of the CYTO domain is to connect cadherins to cytoskeletal structures. Three independent proteins, termed α, β and γ-catenin, respectively, mediated the connection of cadherin CYTO domain to actin, the protein of cytoskeleton (Kobielak and Fuchs 2004). Since the CYTO domain of Bt-R_1 does not appear to be directly exposed to Bt toxins, this portion has not been studied yet. However, it still retains interests if Bt toxin-binding is found to mediate signal transductions within cytoplasm via Bt-R_1.

2.2.2 Toxin-Binding Region on Bt-R_1

The cadherin Bt-R_1 is a high-affinity (K_d = 0.7 nM) binding molecule for Cry1Ab. Up to now, three Cry1Ab-binding regions have been reported on the extracellular portion of Bt-R_1. The first Cry1Ab-binding epitope (TBR1) was reported to be a fragment of eight amino acid

residues (^{869}HITDTNNK^{876}) located in the CR7 domain through phage mapping (Gomez et al. 2001). The binding affinity of this fragment to Cry1Aa, Cry1Ab, and Cry1Ac toxins was in the range of 20-51 nM (Gomez et al. 2001). Hydropathic complementary analysis indicated that this epitope on Bt-R_1 could bind the second β hairpin extension (i.e. loop 2) in domain II of Cry1Aa and Cry1Ab (Gomez et al. 2002a).

The second Cry1Ab-binding region (TBR2) contained 67 amino acid residues (L1296 to G1362) located in the CR11 domain of Bt-R_1 (Dorsch et al. 2002). This toxin-binding fragment was recognized through toxin-overlay assays on different Bt-R_1 truncations expressed in *Escherichia coli*. Subsequent analysis narrowed this toxin-binding epitope to 12 amino acid residues (^{1331}IPLPASILTVTV^{1342}) within the above fragment, and demonstrated that the α-8 loop of Cry1Ab domain II specifically bound to this fragement (Gomez et al. 2003). However, no Cry1Ab-binding was observed in toxin-overlay assays when Bt-R_1 truncations contained only TBR1 in CR7 (Gomez et al. 2001) but no TBR2 in CR11 (Dorsch et al. 2002).

The third Cry1Ab-binding region (TBR3) was localized to the CR12 domain (Hua et al. 2004). In this research, a series of Bt-R_1 truncations were expressed on *Drosophila* S2 cell surfaces allowing comparisons of Cry1Ab binding and Cry1Abmediated cytotoxicity properties. Toxin binding and cytotoxicity assays demonstrated that the membrane proximal CR12 domain was the only region required for Cry1Ab binding and Cry1Ab-binding induced S2 cell death (Hua et al. 2004). However, it is important to note that when CR11 was inserted between CR12 and MPED, the CR12-CR11-MPED truncation lost binding to Cry1Ab although the CR12 domain still existed. This suggests that the MPED domain might participate in Cry1Ab binding to CR12, and that the relative position of these two domains in space is relevant to toxin binding. In *H. virescens* larva, a Bt-R_1 homologue (called HevCaLP or HvCad) is a binding protein for

Cry1Ac (Gahan et al. 2001). An 8-residue fragment (^{1423}GVLTLNFQ^{1431}) was identified as Cry1Ac-binding epitope in HevCaLP CR11 domain, which corresponds to CR12 in Bt-R_1, and this epitope specifically bound to loop 3 of Cry1Ac domain II (Xie et al. 2005). Two single amino acid mutations within ^{1423}GVLTLNFQ^{1431} resulted HevCaLP lost binding to Cry1Ac (Xie et al. 2005). In Bt-R_1, this toxin-binding epitope exists as ^{1416}GVLTLNIQ^{1423} which has only one residue difference (F → I) from that in HevCaLP.

In summary, three TBRs on cadherin were identified by different binding methodologyies. The effect of Bt-R_1 conformation on Cry1Ab-binding has been illustrated through comparing toxin-binding abilities between toxin-overlay assay and *Drosophila* S2 cell assay (Hua et al. 2004). In toxin-overlay assays, Bt-R_1 truncations were blotted on the surface of polyvinyl difluoride (PVDF) filter with denatured and unfolded states (Dorsch et al. 2002). However, in *Drosophila* S2 cell assays Bt-R_1 truncations were expressed on the surface of *Drosophila* S2 cells with native and folded states, which was expected to approximate the native conformation of Bt-R_1 on the brush border of *M. sexta* larval midgut epithelia (Hua et al. 2004). For example, the CR12-MPED truncation bound to Cry1Ab with the later method, but did not with the former one (Hua et al. 2004). It is worthy to notice that neither Dorsch et al. (2002) nor Hua et al. (2004) assigned Cry1Ab binding properties to CR7, where TBR1 was located by Gomez et al. (2001). While TBR2 does bind to Cry1Ab, the results of Hua et al. (2004) suggest that TBR2 may only function as an assistant region for increasing Cry1Ab-TBR3 binding.

2.3 Alkaline Phosphatase

Another Cry1Ac-binding protein was identified as alkaline phosphatase (ALP) from *M. sexta* larval brush border membrane vesicles (BBMV) proteins through a two-dimensional (2D)

SDS-PAGE based toxin-overlay assay and a combined proteomic analysis (McNall and Adang 2003). The apparent mass of ALP on 2D SDS-PAGE was 65 kDa which suggested that it was the same protein as a Cry1Ac-binding band with same molecular weight observed on 1D SDS-PAGE (Sangadala et al. 1994), and the phosphatidylinositol-specific phospholipase C (PIPLC) treatment indicated that this 65 kDa ALP was a GPI-anchored membrane protein (McNall and Adang 2003). The toxin overlay assay illustrated that biotinylated Cry1Ac binding to 65 kDa ALP could be competed by unlabeled Cry1Ac with 100-fold molar excess, pointing to the specificity of this Cry1Ac-ALP binding (McNall and Adang 2003). Since the gene coding this 65 kDa ALP has not been cloned from *M. sexta*, its specific Cry1A-binding properties are still unknown.

2.4 Glycolipid

Glycolipids were recently reported as receptors for Cry toxins in nematodes and insects. (Griffitts et al. 2005). In *Caenorahbditis elegans*, the core tetrasaccharide, GalNAc β1-4 GlcNAc β1-3 Man β1-4 Glc, on glycosphingolipids determined their Cry5Ba-binding properties. This specific tetrasaccharide is conserved in nematodes and insects but is lacking in vertebrates (Mucha et al. 2004). Consistent with the hypothesis that glycolipids are also involved in Cry toxin action against insects, Cry1Ab and Cry1Ac bound to glycoplipids from *M. sexta* (Griffitts et al. 2005).

3. Cry1A Mode-of-Action Based on Bt-Binding Protein

During the process of toxicity to susceptible insect, Bt Cry1A crystals undergo solubilization, protease digestion, and conformational changes from a soluble protein to a

membrane-inserted form that generates non-specific ion channels on insect midgut epithelia. In *M. sexta* larvae, Cry1Ab protoxin is digested by trypsin and chymotrypsin-like serine proteases into a Cry1Ab monomer that binds the cadherin Bt-R_1. Subsequently, a membrane-associated protease removes the helix α1 in domain I of Cry1Ab enabling toxin monomers to form tetrameric oligomers, which are competent for membrane insertion (Gomez et al. 2002b). After this the oligomeric Cry1Ab bound to MsAPN1 in lipid rafts, which drives toxin to form pores on the midgut epithelial cells (Bravo et al. 2004).

Although glycolipids were identified as functional receptors for Bt toxins in both nematodes and insects, the mode of action for glycolipid-mediated Cry1A toxicity is not known. Glycolipids and protein binding molecules may play roles sequentially or simultaneously in anchoring Bt toxins on cell membranes properly for insertion (Griffitts et al. 2005).

4. Binding Molecule Related Insect Resistance to Bt

The application of Bt Cry insecticidal proteins could induce target insects to generate resistant strains during pest management (Christou et al. 2006, Griffitts and Aroian 2005). Insect resistance to Bt was first reported in Indianmeal moth (*Plodia interpunctella*) after selection on arficial diet with the commercial Bt powder Dipel (McGaughey 1985). A 30-fold resistance to Dipel was generated in this insect only after two generations, and the resistance reached 100-fold after 15 generations (McGaughey 1985). The roads to resistance are complicated and multiple in insects (Griffitts and Aroian 2005). Since the binding of Bt toxins to their target molecules on midgut is a critical step in Bt toxicity, insect could generate resistance associated with this process. The primary mechanism of this resistance mode is due to the reduction of toxin binding affinity or loss of toxin harboring sites on binding molecules (Ferre et al. 1991, Van Rie et al.

1990). Cross-resistance could also appear when two or more Cry toxins share same binding molecules (Sayyed et al. 2000, Tabashnik 1994, Tabashnik et al. 1997, Tabashnik et al. 2000).

4.1 Resistance Associated with APN

Two point mutations on APNs were found after comparing their cDNAs between Bt resistant and susceptible strains of *P. interpunctella*, which is the only insect species reported resistance to Bt in the field (McGaughey 1985, Zhu et al. 2000). One conserved point mutation in the APN of Bt resistant *P. interpunctella* strains was D185 to G185, which was located in a proposed binding region for Cry1Aa, thus contributing to resistance due to altered toxin binding (Zhu et al. 2000). Beet armyworm (*S. exigua*) possesses four isoforms of APN on larval midgut. The analysis of APN encoding cDNA indicated that the lack of one APN isoform (SeAPN1) conferred resistant to Cry1Ca in an *S. exigua* strain (Herrero et al. 2005). The RNA interference (RNAi) was used to 'knock-down' an APN (SlAPN) expression in *S. litura* larvae, which resulted in reduced susceptibility to Cry1Ca in these insects (Rajagopal et al. 2002). By contrast, genetic mapping illustrated that resistant to Cry1Ac in *H. virecens* was not linked to 120 kDa and 170 kDa APNs, both of which were identified as Cry1Ac-binding proteins. Resistance to Cry1Ac in *H. virescens* was associated with loss of a cadherin-like protein HevCaLP (Gahan et al. 2001).

4.2 Resistance Associated with Cadherin

The retrotransposon insertion mediated cadherin gene *BtR-4* (or called *HevCaLP*) disruption, which correlated with high levels of resistance to Cry1Ac in *H. virecens* from laboratory selection (Gahan et al. 2001). This disruption induced the *BrR-4* gene coded cadherin

HevCaLP lacking some membrane proximal CR domains and the transmebrane and cytoplasmic portions; thus the incomplete HevCaLP proteins were released into the lumen and not anchored on the microvilli for Cry1Ac binding. The resistance to Cry1Ac in larvae of *P. gossypiella* was associated with three cadherin alleles (Morin et al. 2003). Like *BtR-4* gene disruption in *H. virecens*, any combination of these alleles also caused cadherin to form truncation deletions in *P. gossypiella*, hence resistance to Cry1Ac.

4.3 Resistance Associated with Sugar Moiety on ALP and Glycolipid

Cry1Ac could bind to the N-linked sugar moieties on the membrane-type ALP (m-ALP) on insect BBMV (Jurat-Fuentes et al. 2002). However, the resistance to Cry1Ac in *H. virescens* strain YHD2-B was associated with the reduced amount, but not altered glycosylation of m-ALP (Jurat-Fuentes and Adang 2004). Similarly, resistance to Cry1Ac in *P. xylostella* larvae was due to reduced amounts of neutral glycolipids on insect midgut epithelia (Kumaraswami et al. 2001). For another aspect, the lack of certain glycotransferases, which would alter the carbohydrate complex structures on glycolipids, was proposed to be another mechanism in *P. xylostella* Cry1Ac-resistant strain, which has been illustrated in nematode (Griffitts et al. 2005). Thus, insects might contain multiple pathways to overcome the binding of Bt toxin to its target molecules on midgut, hence the resistant insect strains generate.

5. Synergist of Bt Cry Protein

A synergist as relates to a pesticide is defined as "Substance, which, while formally inactive or weakly active, can significantly enhance the activity of the active ingredient in a formulation." by International Union of Pure and Applied Chemistry (IUPAC) (Holland 1996).

The use of synergist has become one of the methods to increase the toxicity of Cry insecticidal proteins and to overcome insect resistance (Tabashnik 1992, Tabashnik 1994).

Synergists of Cry toxins have been broadly reported. Although there is no generalized mechanism that applies to these synergists, several of them affect toxin docking and membrane insertion. For instance, Cyt1Aa protein from Bt subsp. *israelensis* synergized Cry11Aa toxicity against yellow fever mosquito (*Aedes aegypti*) larvae by functioning as a receptor, which increased the capability of toxin to bind and insert into the microvilli of insect midgut (Perez et al. 2005). For a lepidopteran, Cyt1Aa could synergize the activity of Cry1Ac toward a Cry1Ac resistance strain of *P. xylostella* larvae (Sayyed et al. 2001). A synergistic effect was also reported when different Cry toxins were mixed. The mixture of Cry1Aa and Cry1Ac exhibited higher toxicity to gypsy month (*L. dispar*) larvae than when a single toxin was used. According to voltage clamping assay results, a proposed explanation was that the hetero-oligomer of Cry1Aa and Cry1Ac had better insertion ability than Cry1Ac homo-complex on insect midgut (Lee et al. 1996).

After Bt insecticidal spore crystals are solubilized and activated in the midgut lumen, they need to transport across the peritrophic matrix (PM) to reach epithelial cells. The PM is a protein-chitin matrix, which protects the fine structured epithelial microvilli in lepidopteran larval midgut (Wang and Granados 2001). Enhancin is a 104 kDa metalloprotease from granulosis virus (Lepore et al. 1996). This metalloprotease could damage the ledipopteran PM, hence increase the permeability of PM for Bt toxins (Granados et al. 2001). With the addition of enhancin, the toxicity of a commercial Bt formulation, Dipel, was increased 3 to 6-fold against *Trichoplusia ni*, *Pseudoplusia includens*, *Anticarsia gemmatalis*, *H. virescens*, *H. zea* and *S. exigua* larvae (Granados et al. 2001). Similar to enhancin, the endochitinase ChiAII from

bacterium *Serratia marcescens* results in perforation on lepidopterous PM. The growth of *S. littoralis* larvae was obviously stunted after insects fed the combination of Cry1Ca and ChiAII (Regev et al. 1996).

The target site for a synergist may not be restricted to the midgut epithelia and PM, like that of Cyt1A and enhancin. A synergistic effect was reported when *L. dispar* larvae fed the mixture of zwittermicin A and Bt subsp. *kurstaki* (Broderick et al. 2000). Zwittermicin A is an antibiotic which inhibits the growth of eubacteria with a broad spectrum. A midgut microbial community is essential for insect growth and development. The population of gut microflora decreased after insects fed zwittermicin A. The disrupted microflora may increase the potency of Bt toxicity toward *L. dispar* larvae (Broderick 2000).

6. Dissertation Rationale

There were two major goals of the research presented in this dissertation: (1) determine the localization of Cry1A-binding proteins on *M. sexta* larval midgut, and (2) define the synergistic effect of an *E. coli* expressed CR12-MPED peptide, which is a truncation of Bt-R_1, on Cry1A toxicity against lepidopteran larvae.

Towards the first goal, I examined the localization of three Cry1A-binding proteins (viz. MsAPN1, Bt-R_1 and m-ALP) on the midgut of *M. sexta* larva, which is a model insect for Bt toxin mode-of-action research. All three proteins were recognized by Cry1A binding in insect midgut BBMV, which was a pool containing multiple membrane proteins on the brush border side of midgut epithelia (Knight et al. 1994, Sangadala et al. 1994, McNall and Adang 2003, Vadlamudi et al. 1993). Bt-R_1 is a member of the cadherin super-family, whose members mainly localize at cell-cell containing areas for adhesion and signaling transduction (Goodwin and Yap

2004, Takeichi 1995). However, to be a binding protein for Bt toxins, Bt-R_1 must be exposed toward the midgut lumen. Thus, it is unlikely that Bt-R_1 has a function for cell-cell adhesion. Because the role of Bt-R_1 contradicted with general functions of traditional cadherins, it had been questioned for a long time whether or not Bt-R_1 is a Cry1A-binding protein under *in vivo* conditions. Localization of Bt-R_1 on *M. sexta* larval midgut was established by the method of immunohistochemistry. Concurrently, the immunolocalization of MsAPN1 and m-ALP was studied in same insect. The polyclonal antisera against MsAPN1 and Bt-R_1 were available in our laboratory. As shown by McNall and Adang (2003), antiserum prepared against m-ALP of *B. mori* was cross-reactive with m-MLP from *M. sexta*. These antisera allowed the immunolocalization of Bt-R_1, MsAPN1 and m-ALP on *M. sexta* larval midgut.

Bravo et al. (1992a, 1992b) demonstrated that Cry1Ab and Cry1Ac had different binding localization on the microvilli of *M. sexta* larval midgut. In my research, three major Cry1A toxins (Cry1Aa, Cry1Ab and Cry1Ac) were labeled by a rhodamine derivative. This labeling approach allowed the direct localization of toxin on sectioned insect midgut. The binding localization of three Cry1A toxins was compared with the immunolocalization of three Cry1A-binding proteins (MsAPN1, Bt-R_1 and m-ALP). This comparison enabled me to ascertain whether toxins and their binding proteins co-localized on *M. sexta* larval midgut. Since the midgut of *M. sexta* larva could be morphologically divided into three regions (Cioffi 1979), I probed the binding localization of each Cry1A toxin, as well as the immunolocalization of each binding protein, in different regions (anterior, middle and posterior) of insect midgut.

The second goal in my dissertation research was to investigate synergism of Cry1A toxicity to larvae by CR12-MPED peptide. Hua et al. (2004) observed that when expressed on *Drosophila* S2 cell surfaces, the CR12 domain of Bt-R_1 was a critical region for Cry1Ab binding

and toxin mediated cytotoxicity. To study the function of Bt-R_1 as a Cry1A-binding protein *in vivo*, I cloned the cDNA region of Bt-R_1 encoding CR12-MPED and expressed and purified the peptide from *E. coli*. Our preliminary experiments showed that the purified CR12-MPED peptide neutralized Cry1Ab binding to Bt-R_1 on *Drosophila* S2 cell. Since Bt-R_1 is a critical molecule for Cry1Ab binding on *M. sexta* larval midgut, we proposed that Cry1Ab induced insect mortality would be inhibited when insects were fed mixtures of Cry1Ab combined with CR12-MPED. However, bioassay results demonstrated that the toxicity of Cry1Ab, as well as Cry1Ac, was not blocked, but was significantly potentiated by CR12-MPED. These results suggested that when mixed with CR12-MPED peptide, Cry1A toxins could effectively control lepidopteran pests even at lower dosages. Based on this finding, I planned to investigate whether CR12-MPED was able to enhance Cry1Ac toxicity to *H. virescens* and *H. zea* larvae, which are important lepidopteran pests on crop and vegetable. Since the over-expressed CR12-MPED peptides formed inclusion bodies in *E. coli*, denaturants [6 M guanidine hydrochloride (GuHCl) and 8 M urea] were used to dissolve the inclusion bodies prior to CR12-MPED purification. Because this process could modify the folding state of CR12-MPED, I utilized far-UV circular dichroism (CD) and ^{1}H-NMR to determine the secondary structure and folding state of CR12-MPED. Dot-blot binding assays were used to investigate the relationship between CR12-MPED binding to Cry1A toxins and toxicity enhancement. Histochemistry was used to determine whether Cry1A-binding localization on *M. sexta* larval midgut was modified by the addition of CR12-MPED.

Another objective in the second goal was to identify the minimal region in CR12-MPED with the maximal peroperty to enhance Cry1Ac toxicity against *H. zea* larvae. The 23 kDa CR12-MPED peptide was composed of two domains with 206 residues. Since 1:10 and 1:100

mass ratios of Cry1A vs. CR12-MPED were necessary to significantly increase Cry1A toxicity, I planed to identify a sub-truncation of CR12-MPED possessing synergistic ability equivalent to the full length CR12-MPED. If such an active shorter peptide was identified, this synergist could be used with less mass than the full-length one. To approach this objective, I designed and constructed a series of CR12-MPED sub-truncations and tested their synergistic abilities with Cry1Ac toxin. *H. zea* larvae would be used in bioassays because this lepidopteran is a major insect pest on cotton with tolerance to Cry1Ac. The overall goal of my CR12-MPED research was to assist in making Cry1Ac more effective in *H. zea* management.

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CHAPTER 2

COMPARING THE LOCALIZATION OF *BACILLUS THURINGIENSIS* CRY1A δ-ENDOTOXINS AND THEIR BINDING PROTEINS ON LARVAL MIDGUT OF TOBACCO HORNWORM, *MANDUCA SEXTA* [1]

[1] Chen, J., M.R. Brown, G. Hua and M.J. Adang MJ. 2005. *Cell Tissue Res*. 321: 123-129. Reprinted here with permission of publisher.

Summary:

Tobacco hornworm, *Manduca sexta*, is a model insect for studying the action of *Bacillus thuringiensis* (Bt) Cry toxins on lepidopterans. The Bt-binding proteins on midgut epithelial cells are key factors involved in the insecticidal functions of Bt toxins. With immunohistochemistry, three Cry1A-binding proteins from larval *M. sexta*, aminopeptidase N (MsAPN1), the cadherin-like Bt-R_1, and membrane-type alkaline phosphatase (m-ALP), were localized on sections from anterior, middle, and posterior regions of midguts from second instar *M. sexta* larvae. MsAPN1 was distributed predominantly along microvilli in the posterior region and to a lesser extent on the apical tip of microvilli in anterior and middle regions. Bt-R_1 was localized at the base of microvilli along the entire midgut region and at the apex of microvilli also in middle and posterior regions. The immunolocalization of m-ALP was at the apex of microvilli and the basal lamina of epithelial cells in all three midgut regions. The localization of rhodamine-labeled Cry1Aa, Cry1Ab, and Cry1Ac binding was determined on sections from the same midgut regions. Cry1Aa and Cry1Ab bound to the apical tip of microvilli almost equally in all midgut regions. Binding of Cry1Ac was much stronger in the posterior region than in the anterior and middle ones. With these results, we demonstrated that Bt-binding proteins and Cry1A toxins binding sites co-localized on the microvilli of *M. sexta* midgut epithelial cells.

Keywords: *Manduca sexta*, Cry1A, aminopeptidase N, cadherin, alkaline phosphatase

Introduction:

The bacterium *Bacillus thuringiensis* (Bt) produces crystalline inclusions composed of Cry proteins. The Cry1A-type proteins are highly active against some species of Lepidoptera and are widely used in genetically engineered cotton and corn (Whalon and Wingerd 2003). After ingestion by insect larvae, Cry1A protoxins (~ 130 kDa) are digested by proteases in the midgut to form a protease-resistant core toxin of 60-65 kDa. Soluble toxin binds to specific molecules on midgut brush border membranes and form pores that cause cytolysis resulting in cell death (Aronson and Shai 2001).

A 120 kDa aminopeptidase N (MsAPN1) (Knight et al. 1994; Sangadala et al. 1994) and a 210 kDa cadherin-like protein Bt-R_1 (Vadlamudi et al. 1995) are high affinity Cry1A-binding proteins located in the brush border epithelium of the tobacco hornworm, *Manduca sexta*, a widely used model insect for studying the toxicity of Cry1A. MsAPN1 is an N-acetyl-D-galactosamine (GalNAc) bearing glycoprotein that is linked to the cell membrane by a glycosyl-phosphatidylinositol (GPI) anchor through its carboxyl-terminus (Garczynski and Adang 1995). The mature cadherin-like protein Bt-R_1 is also a glycoprotein whose structure is composed of twelve cadherin units, one membrane-proximal extracellular domain, a single transmembrane region, and one cytoplasmic domain from amino-terminal to carboxyl-terminal (Dorsch et al. 2002). MsAPN1 and Bt-R_1 bind Cry1Aa, Cry1Ab and Cry1Ac (Masson et al. 1995; Keeton and Bulla 1997; Hua et al. 2004). While MsAPN1 binding to Cry1Ac is mediated through its GalNAc residues, the toxin binding properties of Bt-R_1 do not involve sugars. Recently, a 62 kDa membrane-type alkaline phosphatase (m-ALP), which is GPI-anchored on *M. sexta* brush border membrane vesicle (BBMV), was identified by its binding to Cry1Ac through proteomic analysis (McNall and Adang 2003).

The role of binding proteins in toxin action has evolved from arguments about whether aminopeptidases or cadherins are functional receptors to a model that integrates multiple binding proteins into toxin action. Cry1A toxin binding to Bt-R_1 cadherin induces a proteolytic cleavage in toxin at the α-1 helix of domain 1 followed by toxin oligomerization into a pre-pore structure (Gomez et al. 2002). The pre-pore toxin has increased affinity for MsAPN1 that presumably leads to insertion in lipid raft domains in the epithelium membrane (Bravo et al. 2004). Lipid rafts are microdomains known to be enriched in GPI-anchored aminopeptidase N (APN) and alkaline phosphatase (ALP) (Zhuang et al. 2002).

The cellular localizations of MsAPN1, Bt-R_1, and m-ALP in insect midgut epithelium have not been reported yet. Only the immunolocalization of Cry1Ab and Cry1Ac on midgut epithelium of *M. sexta* has been reported (Bravo et al. 1992a), and the relationship between the localization of Cry1A toxins and their binding proteins has not been established. The midgut of *M. sexta* can be morphologically separated into anterior, middle, and posterior regions by epithelium folding, columnar and goblet cell structures, and pH gradient (Cioffi 1979; Dow 1984). In this study, we used immunohistochemistry to determine the localization of MsAPN1, Bt-R_1, and m-ALP on tissue sections from different regions of *M. sexta* larval midgut. In addition, the binding locations of Cry1A toxins on similarly prepared midgut were detected through fluorophore labeled toxins.

Materials and Methods:

Experimental insect

M. sexta eggs were purchased from Carolina Biological Supply Co. (Burlington, NC), hatched, and reared on an artificial diet (Southland Products, Lake Village, AR) at 30°C with a photoperiod of 12:12 h (L:D).

Tissue preparation and sectioning

Six second instar larvae were dissected 10 days after hatching. Midguts were cut into anterior, middle, and posterior fragments of approximately same length and were immediately fixed with a 4% paraformaldehyde phosphate-buffered saline (PBS) solution (pH 7.4) for 2 h on ice. Midguts were dehydrated in the same solution containing 30%, 50%, 70%, 80% and 90% ethanol, respectively for 10-15 min each, 100% ethanol for the same time twice, and then 100% acetonitrile for 5 min. Midguts were infiltrated in the 2:1 and 1:1 (v:v) mixed solutions of acetonitrile and plastic mixture [6.3 ml Epon 812 (Electron Microscopy Sciences, Washington, PA), 3.3 ml Araldite 6005, 13.8 ml dodecenylsuccinic anhydride (Polysciences, Inc., Warrington, PA) and 25-30 drops of 2,4,6-tri (dimethylaminomethyl) phenol (Polysciences, Inc., Warrington, PA)] for 1-3 h, respectively, and then the 1:2 mixed solution for overnight. Midgut regions were placed in molds and covered with plastic mixture for overnight at room temperature, and then polymerized for 3 d at 60 °C. For each midgut region, three larval tissues were randomly chosen for sectioning. Cross or longitudinal sections (3 μm thick) of embedded midguts were cut on a Sorvall Porter-Blum MT2-B Ultra-Microtome (Sorvall Inc., Newtown, CT), then were placed onto gelatin-coated glass slides and baked at 45 °C for 1-2 h. Twelve adjacent sections of anterior, middle, or posterior midgut fragment were placed onto each slide.

Antibodies of Cry1A-binding proteins

The Bt-R_1 antiserum was produced in rabbit against a truncated form of Bt-R_1 expressed in *Eschericia coli* and provided by Dr. Ronald D. Flannagan (Pioneer Hi-Bred International Inc., Johnston, IA). Rabbit anti-MsAPN1 serum was produced in the laboratory of Dr. Michael J. Adang (Luo et al. 1999b). The m-ALP antiserum against *Bombyx mori* m-ALP (Ikezawa et al. 1976; Takesue et al. 1989) was kindly provided by Dr. Masanobu Itoh (Department of Applied Biology, Faculty of Textile Science, Kyoto Institute of Technology, Sakyo-ku, Matsugasaki, Kyoto, Japan).

Rhodamine-labeled Cry1A toxins

Cry1Aa and Cry1Ac were produced by Bt strains HD-37 and HD-73, respectively, obtained from the *Bacillus* Genetic Stock Center (Columbus, OH). An *E. coli* strain (Dr. Luke Masson, Biotechnology Research Institute, Montreal, Canada) was used to produce Cry1Ab. Bacterial growth and toxin purification and activation were as described previously (Luo et al. 1999a). Cry1A toxins were quantified with a protein assay using bovine serum albumin (BSA) as standard (Bradford 1976). Cry1A toxins were labeled with the rhodamine derivative, 5-(6)-carboxy-tetramethylrhodamine (TAMRA), as described (Banks et al. 2003), and stored at -80 °C. The concentration of rhodamine-labeled Cry1A toxins was approximately 1 mg/ml after separation through a Sephadex G-50 (Sigma) column (25 ml).

Immunohistochemistry

Slides containing midgut sections were sunk into saturated NaOH-ethanol solution (35 g NaOH in 250 ml ethanol) for 4 min to dissolve plastic, then rehydrated in sequence with 95%

and 70 % ethanol, dH_2O, and PBS (pH 7.4) with 0.2 % Tween-20 (PBST) for 1 min, respectively. To block non-specific protein binding sites, sections on slides were covered with 400 µl PBST buffer with 5 % BSA (PBST-5 % BSA) for 1 h at room temperature. After blocking buffer was decanted, sections were incubated in 400 µl PBST-5 % BSA with 1:500 diluted antisera overnight at 4 °C. Unbound primary antibodies were washed off by PBST four times each for 10-15 min. Sections then were incubated in 400 µl PBST-5 % BSA with 1:2000 diluted Alexa Fluor 488 conjugated goat-anti-rabbit IgG $F(ab')_2$ fragment (Molecular Probes, Eugene, OR) overnight at 4°C in dark. Control sections were incubated with secondary antibody after the blocking step. After washing as described above, a 1:1 (v:v) glycerol and PBST medium was dropped onto sections, and a cover slip was mounted on the slide for further observation.

Detection of Cry1A binding to midgut sections

After blocking with BSA as described above, sections were treated with 1 µg/µl rhodamine-labeled Cry1Aa, Cry1Ab, or Cry1Ac in 400 µl PBST-5 % BSA at 4 °C overnight in the dark.

Light microscopy

Midgut sections were observed with an Olympus BX60 microscope (Olympus Optical Co., Ltd, Tokyo, Japan) equipped with epifluorescence and the appropriate filters. Images were collected by Auto-Montage software (Synoptics Ltd, Cambridge, UK) and assembled by Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA).

Results:

Immunolocalization of Cry1A-binding proteins

The immunolocalization of Cry1A-binding proteins was observed in anterior, middle, and posterior portions of *M. sexta* larvae midguts. Immunostaining for MsAPN1, Bt-R_1, and m-ALP was localized generally on the apical portion of microvilli (i.e. brush border) in three midgut regions, but their localization varied in different portions of the midgut. MsAPN1 was only immunostained weakly on the apical tip of microvilli in the anterior and middle midgut regions (Fig. 1A-B) and strongly stained over the entire microvilli areas in the posterior midgut region (Fig. 1C). With anti-Bt-R_1 serum, immunostaining was observed at the base of microvilli along the whole midgut (Fig. 1D-F) and at the apex of microvilli in the middle and posterior regions of the midgut (Fig. 1E-F). In addition, Bt-R_1 was detected at the basal lamina along the whole midgut (Fig. 1D-F) and at the lateral basal labyrinth in the anterior region (Fig. 1D). Organelles in the columnar cells of the posterior midgut region were immunostained for Bt-R_1 thus suggesting protein trafficking toward microvilli and basal lamina (Fig. 1F). The immunostaining of m-ALP was observed almost equally at the apex of microvilli in all three midgut regions (Fig. 1G-I). Unlike MsAPN1 and Bt-R_1, the m-ALP staining detected at the basal lamina was much stronger than that on the microvilli along the entire midgut regions (Fig. 1G-I). For all three midgut regions, the control sections probed with the secondary antibody alone showed no background immunostaining (Fig. 1J-L).

Localization of Cry1A toxins

All three rhodamine-labeled Cry1A toxins bound to the microvilli and peritrophic matrix of the midgut, but their localization on the microvilli was variable among different midgut

regions. Both Cry1Aa and Cry1Ab bound to the apex of microvilli along the midgut regions (Fig. 2A-F). In addition, Cry1Ab bound to the base of microvilli in middle and posterior regions (Fig. 2E-F). Cry1Ac bound to the apical tip of microvilli only in anterior and middle region (Fig. 2G-H), but it bound to the entire length of microvilli in the posterior region (Fig. 2I). The intensities of bound Cry1Aa and Cry1Ab on the apex of microvilli were almost the same along the whole midgut. In contrast, the intensity of Cry1Ac on microvilli was much stronger in posterior region (Fig. 2I) than that in other midgut regions (Fig. 2G-H). Neither Cry1Aa nor Cry1Ab appeared to bind to basal lamina, but Cry1Ac did weakly (Fig. 2G-I). All three Cry1A toxins bound to the peritrophic matrix along the whole midgut (Fig. 2A and 2C for Cry1Aa; data not shown for Cry1Ab and Cry1Ac). As a control, the rhodamine-labeled BSA did not bind to any portion of *M. sexta* midgut (Fig. 2J-L).

Discussion:

Our immunohistochemistry results demonstrated that all three Cry1A-binding proteins, MsAPN1, Bt-R_1, and m-ALP, localized specifically on the microvilli of *M. sexta* midgut epithelial cells, but the patterns of their localization are different from each other. In addition, the patterns were different for the same binding protein in different midgut regions. For each specified binding protein (MsAPN1, Bt-R_1 or m-ALP), its localization pattern on the microvilli and along the whole midgut is very similar to the binding pattern of rhodamine-labeled Cry1A toxins (Cry1Aa, Cry1Ab, or Cry1Ac), thus verifying the interaction and relationship of Cry1A toxins and their binding proteins at the morphological level on *M. sexta* midgut sections.

Aminopeptidase N is an exopeptidase found in the midgut of insects that digests peptides or protein substrates by removal of the amino acid residues from N-termini (Terra et al. 1996).

In *M. sexta*, this enzyme binds three Cry1A toxins used in this study, with Cry1Ac binding mediated primarily by a GalNAc moiety (Masson et al. 1995). In our study the antiserum of MsAPN1 did not stain the microvilli uniformly along the entire length of *M. sexta* midgut. The predominant localization occurred on the whole microvilli in the posterior region (Fig. 1C), which coincides with the results of enzymatic assays and Cry1Ac ligand blots showing that MsAPN1 was enriched in BBMV from the posterior regions, but not the anterior or middle region of midguts from the fifth instar larvae of *M. sexta* (Wolfersberger 1996; Carroll et al. 1997).

Phylogenetic analyses classify lepidopteran APNs into four groups (Nakanishi et al. 2002; Adang 2004) and the anti-MsAPN1 serum used in this study was prepared against a fragment of MsAPN1 expressed in *E. coli* (Luo et al. 1999b). Unlike MsAPN1, *B. mori* APN1 (BmAPN1), which is a Cry1Aa binding protein (Yaoi et al. 1997; Hua et al. 1998), was detected on all microvilli areas uniformly along the whole midgut of the third and fifth instars *B. mori* larvae by immunohistochemistry (Hara et al. 2003), thus APN isoforms in the same class may be distributed differently along the midgut among different insect species.

Bt-R_1 from *M. sexta* is a cadherin-like molecule shown to bind Cry1Aa, Cry1Ab and Cry1Ac toxins on ligand blots (Francis and Bulla 1997; Keeton and Bulla 1997) and in solution (Hua et al. 2004). This protein is expressed specifically in the midgut during *M. sexta* larval stage but not in eggs or adults (Midboe et al. 2003). To date, all cadherins found in either chordates or nonchordates are known to be adhesion molecules that interact homotypically with cadherin on adjacent cells (Takeichi 1995; Gumbiner 1996; Humphries and Newham 1998). The immunostaining of mammal neural-cadherin (Tamura et al. 1998), vascular epithelial-cadherin (Ismail et al. 2003) and insect *Drosophila* epithelial- and neural-cadherins (Oda et al. 1998)

showed that these cadherin molecules are distributed at cell-cell contact areas. In contrast, our research indicated that Bt-R_1 is localized on the microvilli exposed to the lumen, but not at contact areas of adjacent midgut epithelial cells. This pattern of localization would allow interaction between Bt-R_1 and molecules in larval midgut juice, thereby enabling the function of Bt-R_1 as a Cry1A receptor.

The cadherin-like protein BtR175 from *B. mori* binds Cry1Aa (Nagamatsu et al. 1998a; 1998b) and has a similar distribution in *B. mori* larval midgut (Hara et al. 2003). BtR175 stained the whole microvilli areas uniformly in the larval midguts of the third and fifth instars of *B. mori* larvae, whereas immunostaining of Bt-R_1 occurred in our study at the apex and base of microvilli. This immunolocalization pattern varied slightly among different midgut regions of the second instar *M. sexta* larvae. In our research, the base of microvilli was a common place where Bt-R_1 was localized in anterior to posterior regions (Fig. 1D-F). However, due to the low resolution of light microscopy we could not determine whether the localization was at the base of microvilli or the "hammock" area between adjacent microvilli (Lindberg et al. 1981). Bt-R_1 immunostaining localized at the apical microvilli in middle and posterior regions (Fig. 1E-F), but not in the anterior region (Fig. 1D). Midboe et al. (2003) indicated that Bt-R_1 was distributed differentially through the larval midgut of *M. sexta* and was localized primarily in the posterior half of the fifth instar larval midgut. The expression level of Bt-R_1 varied during larval development, and mRNA and protein of Bt-R_1 were detected at higher levels in the third through the fifth instars relative to the first and the second instars *M. sexta* larvae (Midboe et al. 2003). Since the midguts used in our research were from second instar larvae, this might explain the relative weak immunostaining signals of Bt-R_1 on microvilli.

The other common site of Bt-R_1 immunolocalization was the basal lamina along the entire midgut (Fig. 1D-F). This indicates that the synthesized Bt-R_1 protein might be transported across the basal labyrinth of midgut epithelium cells to the basal lamina, although Bt-R_1 contains a transmembrane region, which could anchor the protein on the cell membrane. The localization of Bt-R_1 on the basal labyrinth of midgut epithelium is not clear due to the tight contact of basal labyrinth and basal lamina, but we did observe the immunostaining of Bt-R_1 on the lateral basal labyrinth (Fig 2D). The immunostaining of Bt-R_1 was observed on the peritrophic matrix as well (data not shown), suggesting the transport of Bt-R_1 across the apical midgut membranes. The localization of Bt-R_1 on microvilli, peritrophic matrix, and basal lamina was also supported by the observation of small immunostained organelles in the cytoplasm, thus suggesting transport to apical and basal regions (Fig. 2F).

Alkaline phosphatase has recently received attention as a Cry1Ac binding protein in *M. sexta* (McNall and Adang 2003) and *H. virescens* (Jurat-Fuentes and Adang 2004). In *B. mori,* there are membrane-anchored and soluble forms of ALP in midgut epithelium of larvae (Eguchi 1995), and the similarity between their gene sequences is 60-79% (Itoh et al. 2003). The membrane anchored form (m-ALP) is tethered to the membrane by a GPI-anchor while the soluble form is localized primarily in the cavity of goblet cells (Eguchi 1995). In McNall and Adang (McNall and Adang 2003), antiserum against *B. mori* m-ALP recognized 62 kDa and 65 kDa forms of ALP in BBMV isolated from *M. sexta*. Although those authors proposed that the 62 and 65 kDa forms were membrane-anchored and soluble forms, the same anti-m-ALP serum detects only membrane anchored ALP in *B. mori* (Azuma and Eguchi 1989). In the present study, the same *B. mori* m-ALP antiserum uniformly stained the apex of microvilli and basal lamina along the whole midgut region (Fig. 1G-I). Staining of the apex of microvilli is evidence

that detected ALP is membrane bound and that the protein is localized in regions of microvilli quite distinct from the GPI-anchored APN. We did not detect localization of ALP in the cavity of goblet cells, which is the primary site for soluble ALP in *B. mori* midgut (Eguchi 1995). Detection of m-ALP on basal lamina was not expected. Basal lamina with collagens as the main component (Lane et al. 1996) separates midgut epithelium from hemolymph. The staining of basal lamina by *B. mori* m-ALP antiserum may be due to ALP isoforms or cross-reaction with collagens sharing the same epitopes as *B. mori* m-ALP.

Bravo et al. (1992a; 1992b) first reported the localization of Cry1Ab and Cry1Ac on larval midgut of *M. sexta* fifth instar larvae by immunohistochemistry with antibodies against toxins. Rhodamine-labeled toxins were applied directly to detect the binding localization of Cry1Aa, Cry1Ab, and Cry1Ac on sections from second instar *M. sexta* larvae. In fifth instar *M. sexta*, Cry1Ab bound only to the apical tip of microvilli along the length of the midgut, while Cry1Ac bound to the entire microvilli area (Bravo et al. 1992a). Our results indicated that Cry1Ab bound to the apex of microvilli with almost equal intensity from anterior to posterior midgut regions (Fig. 2D-F), but Cry1Ac bound to the entire length of microvilli only in the posterior midgut region (Fig. 2J). In anterior and middle midgut regions, Cry1Ac only bound to the apical tip of microvilli with very weak intensity (Fig. 2G-H). This might explain the observations of Carroll et al. (1997) and Wolfersberger (1996) that the binding intensity of Cry1Ac was stronger in the posterior midgut than the anterior midgut region.

The immunostaining pattern with MsAPN1 antiserum in different *M. sexta* midgut regions (Fig. 1A-C) was similar to the binding pattern of Cry1Ac in corresponding midgut regions (Fig. 2G-I), thus suggesting that MsAPN1 might be the specific binding protein detected for Cry1Ac. Considering the denatured state of the midgut sections, it is likely that Cry1Ac

binds via GalNAc on MsAPN1. This conclusion is supported by the results of Knight et al. (2004) showing that elimination of sugars, specifically GalNAc, abolished Cry1Ac binding to APN. Cry1Aa bound to the apex of microvilli along the three midgut regions (Fig. 2A-C); an observation that coincided with the immunostaining pattern of m-ALP on microvilli. However, while m-ALP antiserum stained basal lamina (Fig. 1G-I), no Cry1Aa binding was detected to this tissue (Fig. 2A-C). Cry1Ab bound not only to the apical microvilli (Fig. 2D-F), as reported (Bravo et al. 1992a; 1992b), but also the base of microvilli in middle and posterior midgut regions (Fig. 2E-F). This specific binding localization of Cry1Ab coincided with the immunolocalization of Bt-R_1 in the same midgut regions (Fig. 1E-F), as expected for the major Cry1Ab binding protein in *M. sexta* (Vadlamudi et al. 1995). However, it is worthy of notice that the binding intensity of Cry1Ab was much stronger at the apex of microvilli than at the base of microvilli in the middle and posterior midgut regions (Fig. 2E-F), but the intensity of immunostained Bt-R_1 was almost equal at the apex and base of microvilli in these midgut regions (Fig. 1E-F), suggesting that another specific Cry1Ab-binding molecule might commonly localize at apical microvilli.

For all three Cry1A toxins used in our research, we observed binding on the peritrophic matrix, whereas Bravo et al. (1992a; 1992b) only found Cry1Ab, but not Cry1Ac, on the peritrophic matrix of the fifth instar *M. sexta* larval midguts. Some differences in results between Bravo et al. (1992a; 1992b) and the present study may be accounted for by the different instars used in the studies. Cry1Aa, Cry1Ab and Cry1Ac bind peritrophic matrix in *B. mori*, with only Cry1Ac being subject to inhibition by GalNAc (Hayakawa et al. 2004). Those authors' results are evidence that the peritrophic matrix can have a trapping effect on Cry toxins.

This study presents a pattern of localization for each binding protein (MsAPN1, Bt-R_1, or m-ALP) and each Cry1A toxin (Cry1Aa, Cry1Ab, or Cry1Ac) on *M. sexta* larval midgut that is unique for each binding protein and toxin. However, the possibilities that other unknown Cry1A-binding molecules or MsAPN1, Bt-R_1, or m-ALP, including isoforms, simultaneously contribute toxin-binding likely exist and should not be ignored. Additional studies are needed to determine how this spatial relationship of protein localization fits with the recently proposed model (Bravo et al. 2004) of Cry1A toxin action.

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Figure Legends:

Fig. 1 Localization of three Cry1A-binding proteins on different regions of *M. sexta* larval midgut. MsAPN1 was stained weakly on the apical tip of microvilli in anterior and middle regions (A-B), but stained strongly over the entire microvilli areas in the posterior region (C). Bt-R_1 immunostaining was observed at the base of microvilli along the whole midgut (D-F) and at the apical tip of microvilli in middle and posterior regions (E-F). Lateral basal labyrinth (D) and basal lamina (D-F) were immunostained also. The boxed stained organelles in panel F are presumable newly synthesized Bt-R_1 trafficking toward microvilli and basal lamina. The immunolocalization of m-ALP was at the apical tip of microvilli and basal lamina along the whole midgut (G-I). As a control, secondary antibody alone probed midgut sections were not immunostained (J-L). (Scale bar = 10 μm; AMv = apical tip of microvilli; BL = basal lamina; BMv = base of microvilli; LBL=lateral basal labyrinth; Nu = nucleus)

Fig. 2 Binding localization of three Cry1A toxins on different regions of *M. sexta* larval midgut. Cry1Aa bound to the apical tip of microvilli along the whole midgut (A-C), and this toxin also bound the peritrophic matrix (A and C). Cry1Ab bound to the apical tip of microvilli along the whole midgut (A-C) and to the base of microvilli in middle and posterior regions (E-F). Cry1Ac bound to the apical tip of microvilli weakly in anterior and middle midgut regions (G-H), but to the entire microvilli areas in the posterior region (I). Neither Cry1Aa (A-C) nor Cry1Ab (D-F) bound to the basal lamina, but Cry1Ac did weakly (G-I). As a control, rhodamine-labeled BSA did not bind to any portion of the midgut (J-L). (Scale bar = 10 μm; AMv = apical tip of microvilli; BL = basal lamina; BMv = base of microvilli; Nu = nucleus; PM = peritrophic matrix)

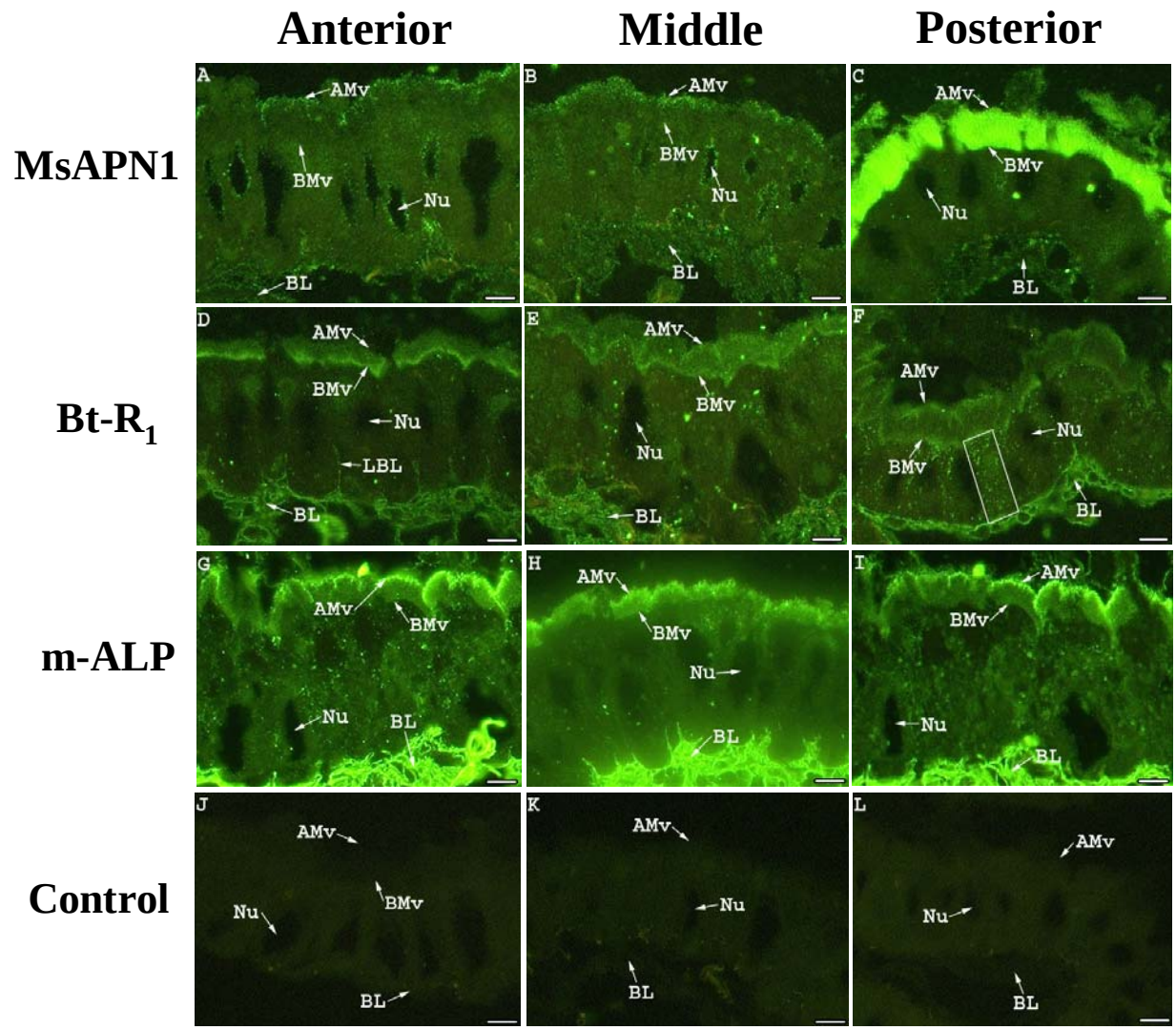
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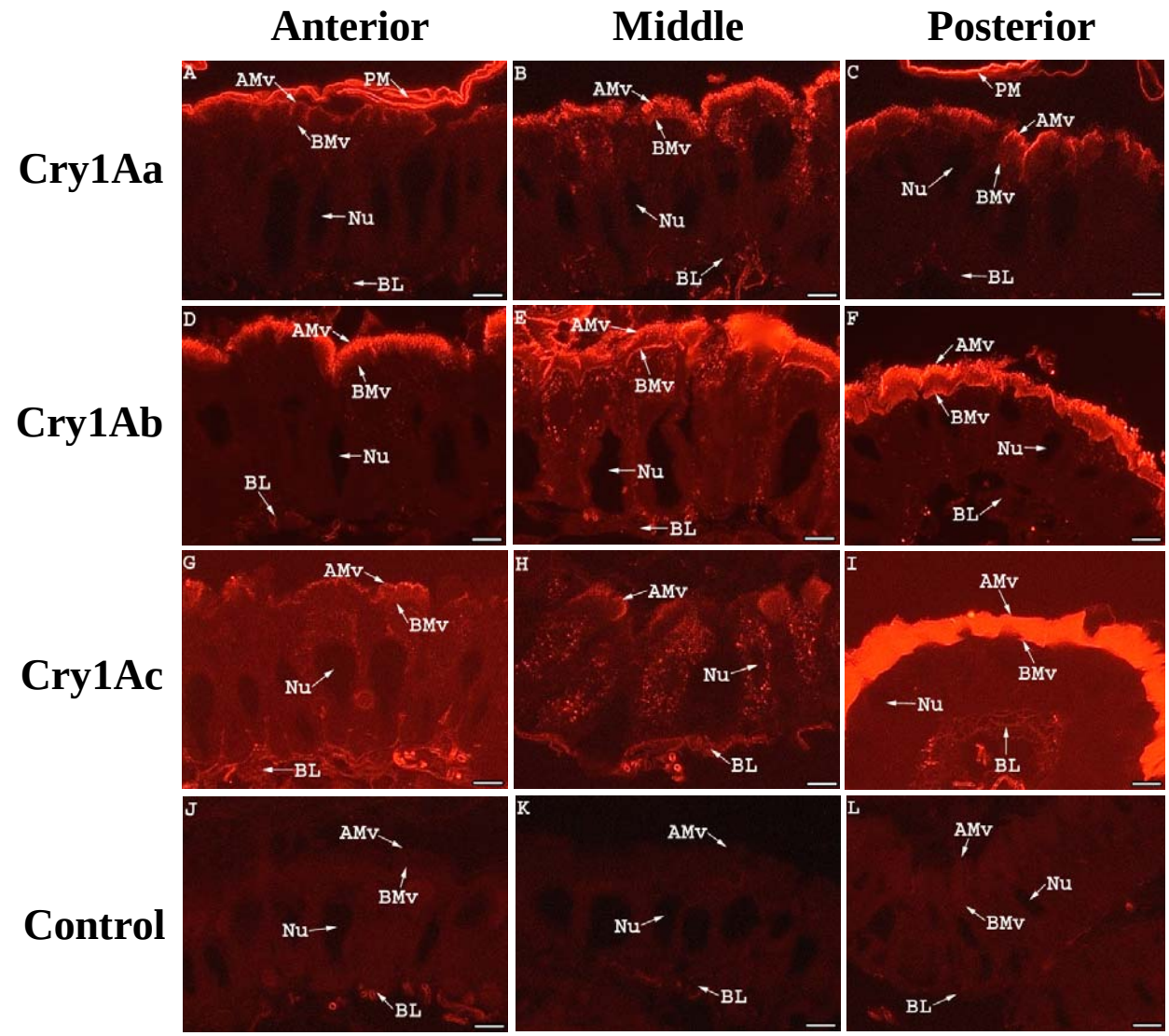
Fig. 1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Nu
BL

Fig. 2

CHAPTER 3

THE CR12-MPED FRAGMENT OF THE CADHERIN BT-R_1 IS A SYNERGIST OF CRY1A INSECTICIDAL PROTEINS AGAINST LEPIDOPTERAN LARVAE [1]

[1] Chen, J., G. Hua, J.L. Jurat-Fuentes, M.R. Brown and M.J. Adang. To be submitted to *Proc. Natl. Acad. Sci. USA*.

Abstract:

The insecticidal crystal proteins produced by *Bacillus thuringiensis* (Bt) are broadly used to control insect pests with agricultural importance. The cadherin Bt-R_1 is a binding protein for Bt Cry1A toxins in midgut epithelia of tobacco hornworm (*Manduca sexta*). We previously identified the Bt-R_1 region most proximal to the cell membrane (CR12-MPED) as the essential binding region required for Cry1Ab-mediated cytotoxicity (1). Here we report that a peptide containing this region expressed in *Escherichia coli* functions as a synergist of Cry1A toxicity against lepidopteran larvae. Far-UV circular dichroism (CD) and ^{1}H-NMR spectroscopy confirmed that our purified CR12-MPED peptide mainly consisted of β-strands and random coils with unfolded structure. CR12-MPED peptide bound to insect midgut microvilli and brush border membrane vesicles (BBMV), but did not change Cry1Ab or Cry1Ac binding localization in the midgut. CR12-MPED-mediated Cry1A toxicity enhancement was significantly reduced when a putative Cry1A-binding epitope (^{1416}GVLTLNIQ^{1423}) within the peptide was altered. Based on these results, we propose that the synergistic mechanism of CR12-MPED involves accumulation of the peptide on insect midgut microvilli to promote attachment of the Cry1A toxins to the epithelium. Because the mixtures of low Bt toxin dose and CR12-MPED peptide effectively control target insect pests, our discovery has important implications related to the use of this peptide to enhance insecticidal activity of Bt toxin-based biopesticides and transgenic Bt crops.

Keywords: *Bacillus thuringiensis*, Cry insecticidal protein, synergist, insect, cadherin

Introduction:

Bacillus thuringiensis (Bt) Cry1A proteins are pore-forming toxins that are specifically toxic to insect larvae in the order Lepidoptera. This family of proteins is widely used for insect control with Bt-transgenic crops, particularly cotton, and Bt microbial pesticides. One of the main issues on the deployment of Bt crops is the evolution of resistance in target pests due to increased selection pressure and lower level of control of specific target pests. For example, Bt cotton is highly effective in controlling tobacco budworm (*Heliothis virescens*) populations, while the control of corn earworm (*Helicoverpa zea*) larvae is only achieved after additional insecticide treatment due to Bt-insensitivity in this species (2).

The mode of action for Cry1A toxins includes sequential steps that determine their specificity. Following ingestion by the lepidopteran larvae, Cry1A proteins are solubilized and activated to a toxic form by the insect digestive fluids. After crossing the peritrophic matrix, activated toxins bind to specific proteins on the midgut microvilli. According to a current model (3), monomeric toxin binds a cadherin, facilitating further processing necessary for toxin oligomerization. Toxin oligomers display high affinity binding to proteins that are attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor, such as aminopeptidase or alkaline phosphatase. This binding and the localization of GPI-anchored proteins in specific membrane regions called lipid rafts results in oligomer insertion, formation of a pore and cell death by osmotic shock. An alternative model (4) proposes the activation of intracellular apoptotic pathways by toxin monomer binding to cadherin. After enterocyte death, septicemia induced by midgut bacteria leads to insect death (5).

The 210 kDa cadherin Bt-R_1 in the midgut of *Manduca sexta* larvae is a transmembrane glycoprotein containing twelve cadherin repeats (CR) and one membrane-proximal extracellular

domain (MPED) in its extracellular portion (6). Unlike other members of the cadherin superfamily that localize to the intercellular adhesion points (7), BtR_1 is most abundant on the microvilli of midgut epithelia (8). Bt-R_1 is a high-affinity binding protein for Cry1Aa, Cry1Ab and Cry1Ac toxins (9). Three Cry1Ab-binding regions, CR7 (10), CR11 (6) and CR12-MPED (1), have been identified on the Bt-R_1 extracellular portion. However, only the CR12-MPED fragment was reported as the functional receptor region for Cry1Ab binding and cytotoxicity (1).

Homologs of Bt-R_1 cadherin are identified as Cry1A binding proteins in other lepidopteran species including several important agricultural pests (11-13). The loss of cadherin proteins is known to cause target site resistance to Cry1Ac in tobacco budworm (*H. virescens*) (14), pink bollworm (*Pectinophora gossypiella*) (15) and cotton bollworm (*Helicoverpa armigera*) (16).

The original goal of this study was to establish the relevance of the CR12-MPED fragment in Cry1Ab-intoxication. This peptide fragment was expressed and purified from *Escherichia coli* and fed with Cry1Ab toxin to *M. sexta* larvae. We expected that this peptide would block Cry1Ab binding to Bt-R_1 in the midgut, hence neutralizing Cry1Ab toxicity. However, the addition of CR12-MPED resulted in dramatically enhanced Cry1Ab-induced insect mortality. Further bioassays demonstrated that the CR12-MPED peptide could also potentiate Cry1Ac toxicity against other lepidopteran pest species, indicating that this peptide may have practical utility for insect pest control.

Materials and Methods:

Insect and bacterial strains

Eggs of *M. sexta* were purchased from Carolina Biological Supply Co. (Burlington, NC). *H. virescens* and *H. zea* eggs were purchased from Benzon Research Inc. (Carlisle, PA). Bt strain HD-37 producing Cry1Ac was obtained from the *Bacillus* Genetic Stock Center (Columbus, OH), while an *E. coli* strain producing Cry1Ab was kindly provided by Dr. Luke Masson (Biotechnology Research Institute, Montreal, Canada).

Preparation of Bt cry1A toxins

Growth of bacterial strains and purification and activation of Cry1Ab and Cry1Ac were as described elsewhere (17). Purified toxin samples (as determined by SDS-10 % PAGE) were pooled, quantified (18) and stored at -80 ^{o}C.

Cloning, expression and purification of M. sexta CR12-MPED peptide

Cloning of the cadherin Bt-R_1 (GenBank AY094541) from *M. sexta* larvae has been described (19). The cDNA encoding Bt-R_1 cloned in the pIZT vector (Invitrogen Co., Carlsbad, CA) was used as template for subcloning the CR12-MPED fragment (amino acids G1362 to P1567) by PCR with primers: 5'-GTACCATATGGGGATATCCACAGCGGACTCCATCG-3' and 5'-GGCTCTCGAGAGGCGCCGAGTCCGGGCTGGAGTTG-3'. The resulting PCR fragment was gel purified and digested by Nde I and Xho I endonucleases, and then subcloned into the pET-30a (+) vector (Novagen, Inc., Madison, WI) to yield plasmid pET-CR12-MPED. The coding sequence and clone orientation were confirmed by sequencing at the Integrated Biotech Laboratories (University of Georgia, Athens, GA). The pET-CR12-MPED construct

was electrotransformed into *E. coli* strain BL21(DE3)/pRIL (Stratagene Co., La Jolla, CA), and positive clones were selected on LB plates containing kanamycin and chloramphenicol. A single bacterial clone was grown overnight in 50 ml LB containing kanamycin and chlorophenicol at 37 °C. This preculture was then inoculated in 1 l of the same media and incubated at 37 °C. When the bacterial culture reached an OD_{600} of 0.5-0.6, protein expression was induced by 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG), and the culture was harvested 6 hours after induction. Bacterial cells were collected by centrifugation. The CR12-MPED peptides formed inclusion bodies in bacteria, and were solubilized in buffer A (6 M GuHCl, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.0) by sonication on ice. Following centrifugation to eliminate cellular debris, soluble extracts were loaded on a HiTrap™ Ni^{2+}-chelating HP column (GE Biosciences Co., Piscataway, NJ) equilibrated with buffer A. After successive washing the column with buffer A and buffer B (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.0), proteins were eluted with buffer C (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 6.3) with increasing imidazole concentrations ranging from 0.01 M to 1.0 M. Purity of the eluted fractions was confirmed by running an 8-16 % gradient SDS-PAGE. High purity fractions were pooled and extensively dialyzed against buffer D (10 mM Tris-HCl, pH 8.0) at 4 °C. Protein concentration was quantified by the method of Bradford (18) with bovine serum albumin as standard. Purified CR12-MPED peptides were stored at -20 °C. Polyclonal antisera against purified CR12-MPED were produced in New Zealand white rabbits at the Animal Resources facility of the University of Georgia.

Deletion of Cry1A-binding epitope in CR12-MPED

To delete the putative Cry1A-binding epitope ^{1416}GVLTLNIQ^{1423} within the CR12-MPED peptide (20), a pair of primers containing EcoR I cleavage sites (underlined) were

designed, 5'-TAATGAATTCCCCACGGCCACGATGCATGGAC-3' and 5'-TCAGGAATTCGGTTTGAGCGTTCAGTACGAAAGCCG-3'. PCR was performed using the Expand High Fidelity PCR System kit (Roche Diagnostics Co., Indianapolis, IN) with pET-CR12-MPED as template. The amplicon was gel-purified, digested with EcoR I, cloned into the pET-30a (+) vector and expressed as described for CR12-MPED. The expressed peptide, CR12-MPED/Del, lacked the ^{1416}GVLTLNIQ^{1423} Cry1A-binding epitope (20). These 8 amino acids were replaced with E and L due to the introduced EcoR I sites in the DNA sequence. Purification of CR12-MPED/Del was as described above for CR12-MPED.

Insect bioassays

Cry1Ab or Cry1Ac was mixed with CR12-MPED peptide at different toxin:peptide mass ratios (1:0, 1:1, 1:10 and 1:100) in buffer D. The maximum dose of CR12-MPED peptide applied with toxin was used alone in buffer D as a negative control. Toxin and peptide samples were overlaid on bioassay tray wells filled with 1 ml artificial diet (Southland Products, Lake Village, AR). After drying, insect neonates were placed on the wells and reared at 28 °C with a 12L:12D photoperiod. Bioassays were repeated thrice for each treatment, and each replicate contained 32 larvae. Larval mortality was quantified after 7 days. Data were analyzed by the GLM method and Tukey's test at $\alpha = 0.05$ nominal criterion level using SAS 9.1 (SAS Institute Inc., Cary, NC).

To compare the bioactivity between CR12-MPED and CR12-MPED/Del, *M. sexta* neonates were fed Cry1A, Cry1A plus 100-fold (mass) CR12-MPED or CR12-MPED/Del peptides, or peptides alone. Bioassays were performed as described above except that 4 replicates were performed for each treatment.

Circular dichroism and NMR spectroscopy

Purified CR12-MPED peptide was prepared in 10 mM Tris-HCl (pH 8.0) at a concentration of 15 μM. Circular dichroism (CD) spectra were recorded with a J-815 instrument (JASCO Inc., Easton, MD). Data points were collected in far-UV wavelength (190 – 250 nm) using a 0.1 cm path length quartz cuvette at room temperature. The acquired CD spectroscopic data were analyzed by CDSSTR program (21, 22) on DICHROWEB (http://www.cryst.bbk.ac.uk/cdweb/html/home.html).

For ^{1}H-NMR spectroscopy, purified CR12-MPED peptide was concentrated to 0.2 mM in 1 mM Tris-HCl buffer (pH 8.0) containing 8 % D_2O. Spectra were acquired at 25 °C using a Varian INOVA 600 MHz spectrometer (Palo Alto, CA).

Labeling of Cry1A toxins and CR12-MPED peptide

Five μg Cry1Ab, Cry1Ac or CR12-MPED were radiolabeled with 0.5 mCi of $Na^{125}I$ using the chloramine-T method (23) and stored at 4 ºC. One μg Cry1Ab, Cry1Ac, CR12-MPED or BSA were labeled with the rhodamine derivative 5-(6)-carboxy-tetramethylrhodamine (TAMRA) as described elsewhere (24), purified by size exclusion chromatography, and stored at -80 ºC.

Dot-blot binding assays

CR12-MPED or CR12-MPED/Del peptide (1 μg) was dotted onto a polyvinylidene difluoride (PVDF) filter (Millipore Co., Bedford, MA). After blocking in PBS-T buffer (0.1 % Tween-20 in phosphate-buffered saline, pH 7.4) with 3 % BSA, filters were bathed in 3.3 ng/ml ^{125}I-Cry1Ab or ^{125}I-Cry1Ac for 1 h at room temperature. Unlabeled Cry1Ab or Cry1Ac (1000-

fold excess) were used in competition assays. After washing off the unbound ^{125}I-labeled toxins, bound toxins were detected by autoradiography at -80 ºC overnight.

For dot-blot experiments, brush border membrane vesicles (BBMV) were prepared from midguts which were dissected from *M. sexta* and *H. virescens* 5th instar larvae according to Wolfersberger et al. (25). To study the interaction between CR12-MPED and BBMV, 10 μg *M. sexta* or *H. virescens* BBMV were spotted onto a PVDF filter. After blocking as above, filters were probed with 3.3 ng/ml ^{125}I- CR12-MPED for 2 h at room temperature. Competition assays were performed by the addition of 500-fold unlabeled CR12-MPED. After washing, bound ^{125}I-CR12-MPED was detected by autoradiography at -80 ^{o}C for 3 days.

To study the effect of CR12-MPED on Cry1A toxin binding to BBMV, 5 μg proteins of BBMV from *M. sexta* larvae were dotted on PVDF filters. After blocking, filters were probed with 3.3 ng/ml ^{125}I-Cry1Ab or ^{125}I-Cry1Ac alone or in the presence of 100-fold mass excess of CR12-MPED, which was the highest toxin-to-peptide ratio used in bioassays, for 2 h at room temperature. The bound ^{125}I-labeled toxins on BBMV were detected by autoradiography at -80 ºC for 3 days.

Histomicroscopy

Insect midguts were dissected from 10-day old *M. sexta* larvae. Longitudinal sections (3 μm thick) of posterior midgut were prepared as described by Chen et al. (8). After blocking, sections were treated with TAMRA-labeled Cry1Ab (0.1 μg/ml), Cry1Ac (0.1 μg/ml), CR12-MPED (2 μg/ml), BSA (2 μg/ml) or the mixture of TAMRA-labeled Cry1A (0.1 μg/ml) with 20-fold (mass) excess of unlabeled CR12-MPED. Immunolocalization of Bt-R_1 in midgut sections was achieved with a polyclonal antiserum against CR12-MPED.

Results:

CR12-MPED peptide is structurally unfolded

The CR12-MPED region of Bt-R_1 formed inclusion bodies during expression in *E. coli*. After solubilization and purification, the resultant peptide had the expected size of 23.3 kDa on SDS-PAGE (Fig. 1A). Although the predicted structure of CR12-MPED based on homology modeling contains extensive β-strands (26), the far-UV CD spectra indicated the composition of CR12-MPED was 15 % helix, 37 % β-strands and 48 % random coils (Fig. 1B). ^{1}H-NMR spectroscopy showed the chemical shifts of amide protons on the CR12-MPED backbone clustering in a narrow range of around 7 to 8.5 ppm (Fig. 1C), confirming the unstructured and unfolded state of the peptide after purification.

CR12-MPED peptide enhances Cry1A toxicity to lepidopteran larvae

Because the CR12-MPED peptide contained the critical Cry1Ab-binding region (1), we predicted that this peptide would reduce the potency of Cry1Ab against *M. sexta* larvae. However, we observed a significant enhancement of Cry1Ab toxicity to *M. sexta* larvae when including CR12-MPED in our bioassays (Fig. 2A). This enhancing effect was dependent on both Cry1Ab dosage and toxin-to-peptide ratio used. For example, 4 ng/cm^2 Cry1Ab alone induced 4.2 ± 1.1 % insect mortality, but when the same amount of toxin was mixed with 10 or 100-fold of CR12-MPED, larval mortality reached 23.9 ± 4.2 % and 82.3 ± 6.8 %, respectively. At low Cry1Ab doses (1 ng/cm^2), addition of even 100-fold CR12-MPED did not increase Cry1Ab toxicity significantly. Similar toxicity enhancement effects were observed when testing Cry1Ac toxicity towards *M. sexta* larvae in the presence of CR12-MPED (Fig. 2B).

Because Cry1Ac is produced in Bt transgenic cotton to control *H. virescens* and *H. zea* we tested the synergistic effect of CR12-MPED on Cry1Ac toxicity against larvae from these species. In bioassays with *H. virescens* (Fig. 2C), the addition of 100-fold CR12-MPED resulted in an increase in the mortality observed with 3 ng/cm^2 of Cry1Ac from 5.2 ± 2.8 % to 83.4 ± 6.3 % mortality. When insect mortality was not significantly increased after addition of CR12-MPED peptide, the growth of surviving larvae was stunted (Fig. 1 in supporting information). *H. zea* is less susceptible to Cry1Ac than *H. virescens*, and consequently, more difficult to control with Bt transgenic cotton and sprayed Bt biopesticides (27, 28). In our bioassays (Fig. 2D), treatment with 120 ng/cm^2 of Cry1Ac caused 24.0 ± 2.8 % *H. zea* larval mortality, while the addition of 1 or 10-fold excess of CR12-MPED resulted in 47.9 ± 5.5 % and 85.4 ± 2.8 % mortality respectively. In controls, insecticidal activity was not observed when insects were fed with buffer or the highest CR12-MPED peptide concentration used (from 400 to 5000 ng/cm^2) alone, evidence that Cry1Ab and Cry1Ac toxicity enhancement was due to a synergistic rather than direct effect of CR12-MPED.

Toxicity enhancement is dependent on Cry1A binding to a region of CR12-MPED

Xie et al. (20) used alanine scanning mutagenesis to map a critical Cry1A-binding region (^{1423}GVLTLNFQ^{1430}) in the HevCaLP cadherin from *H. virescens*. The homologue to this region in Bt-R$_1$ (^{1416}GVLTLNIQ^{1423}) is contained within CR12-MPED. A peptide derivative, CR12-MPED/Del, was constructed to remove this toxin-binding region from CR12-MPED. As shown in Fig. 3A, ^{125}I-labeled Cry1Ab and Cry1Ac bound specifically to CR12-MPED on dot blots. In contrast, CR12-MPED/Del did not bind to either toxin, thus confirming that the removed residues in this peptide were essential determinants for toxin binding (Fig. 3A). In bioassays

with *M. sexta* larvae, the CR12-MPED/Del peptide did not exhibit the enhancing effect observed for CR12-MPED (Fig 3B). These results demonstrated that the binding of Cry1A toxins to the ^{1416}GVLTLNIQ^{1423} region was necessary for CR12-MPED-mediated synergism.

CR12-MPED did not change Cry1A binding localization on M. sexta midgut epithelia

Antisera against CR12-MPED stained the apex and base of midgut microvilli (Fig. 4A) as described before (8). To test whether CR12-MPED altered the localization of Cry1A toxin binding in the larval gut epithelium, we treated midgut sections with TAMRA-labeled toxins. As previously reported (8), Cry1Ab bound to the peritrophic matrix, apical tip and base of epithelial microvilli (Fig. 4B), while Cry1Ac bound trough the entire length of microvilli (Fig. 4D). The addition of CR12-MPED peptide did not change the binding localization of Cry1Ab in *M. sexta* midgut, but it induced formation of Cry1Ab aggregates that accumulated on the apical tips of microvilli and peritrophic matrix (Fig. 4C). However, since similar aggregates were also observed when mixing Cry1Ab with CR12-MPED/Del peptide (data not shown), which did not potentiate Cry1Ab toxicity to *M. sexta* larvae, these Cry1Ab aggregates were probably not associated with the enhanced Cry1Ab toxicity. These aggregates were not observed when mixing 20-fold CR12-MPED and Cry1Ac (Fig. 4E), and only a reduction in Cry1Ac bound to microvilli was detected.

In dot-blot binding assays using *M. sexta* BBMV (Fig. 4H), no effects on Cry1Ab or Cry1Ac toxin binding were observed using 100-fold CR12-MPED. This discrepancy could be due to the removal of some lipids present in BBMV from the microvilli membranes during tissue section preparations for histomicroscopy.

Compared with Cry1Ab and Cry1Ac, TAMRA-labeled CR12-MPED bound with low intensity to the entire length of microvilli (Fig. 4G). As a control, TAMRA-labeled BSA showed no binding on microvilli (Fig. 4F). The ability of CR12-MPED to bind microvilli was confirmed by using ^{125}I-labeled peptide and BBMVs from *M. sexta* and *H. virescens* (Fig. 4I). Radiolabeled CR12-MPED bound to BBMVs from both insects, and these bindings were inhibited by 500-fold unlabeled peptide, suggesting binding specificity. Binding of CR12-MPED to midgut sections and BBMV was considerably less intense than Cry1A toxin binding. These results suggest that even though CR12-MPED does not change the localization of Cry1A binding, it might alter toxin binding and insertion abilities at those sites so that enhanced toxicity is observed.

Discussion:

The cadherin Bt-R_1 serves as the primary binding protein for Cry1A toxins in *M. sexta* (3). Multiple toxin binding regions (TBRs) are present on Bt-R_1, including the one located in CR12-MPED (1, 6, 10, 20). An *in vivo* approach to correlate binding with insecticidal activity for a specific TBR has been to inhibit toxicity by mixing toxin with a Bt-R_1 truncation containing a specific TBR (6, 10, 20). The expectation is that a peptide containing a relevant toxin-binding region will form a Cry1A-TBR complex and block toxin binding to Bt-R_1 on insect microvilli. When we attempted to use this approach to validate CR12-MPED as an essential region involved in Cry1A toxicity to *M. sexta* larvae, we unexpectedly discovered that this peptide synergized Cry1A toxicity.

It is interesting that like the TBR-induced toxicity inhibition (6, 10, 20), CR12-MPED-synergized Cry1A toxicity was also relevant to toxin-peptide binding, which was dependent on

the existence of a previously reported Cry1A-binding epitope (^{1416}GVLTLNIQ^{1423}) (20). Xie et al. (20) demonstrated that Cry1Ac binding to HevCadLP, a Bt-R_1 homolog in *H. virescens*, was inhibited when one of these residues was modified. In agreement with this report, CR12-MPED also lost binding to Cry1A, as well as its synergistic ability, when the ^{1416}GVLTLNIQ^{1423} fragment was removed. These results support the correlation between Cry1A-peptide binding and synergism.

Dorsch et al. (6) reported a 25 kDa Bt-R_1 truncation (TBR2), which was able to bind Cry1A toxins and completely inhibit Cry1Ab toxicity to *M. sexta* larvae. TBR2 contains the last 66 residues of CR11 (L1296 to G1362) and the first 103 residues of CR12 (I1363 to F1465). The Cry1A binding fragment (^{1416}GVLTLNIQ^{1423}) is located in CR12, which is the overlap region between TBR2 and CR12-MPED (G1362 to P1567). Thus, we initially hypothesized that the enhanced Cry1Ab toxicity observed with CR12-MPED was due to the lack of additional toxin-binding sites in CR11 (6). When we over-expressed TBR2 following our methodology, these peptides formed inclusion bodies in *E. coli*. After solubilization and purification, our TBR2 peptide bound Cry1A toxins, yet in bioassays it enhanced toxicity against *M. sexta* and *H. zea* larvae as CR12-MPED (Chen et al., unpublished data).

Since the function of a peptide is determined not only by its primary sequence but also its folding state, we hypothesized that the later is one of the critical factors leading to the remarkably different bioassay results. Far-UV CD and ^{1}H-NMR spectroscopy demonstrated that CR12-MPED, which enhanced Cry1A activity, was an unfolded peptide with more hydrophobic residues being exposed to the surrounding environment than in a folded state. In previous reports testing TBR2 and a fragment of HevCaLP, these peptides were purified from soluble fractions of bacterial cultures (6, 20). Although the folding state of these peptides was not

experimentally determined, it is quite possible that folded structures existed in both cases. We propose that the unfolded state of CR12-MPED with more exposed hydrophobic residues is associated with the synergistic capability to potentiate Cry1A toxicity against lepidopteran larvae. In support of this hypothesis, Cry1Ac-binding to the aminopeptidase MsAPN1 in *M. sexta* is also dependent on the folding state of the protein (29). Although speculative, it is possible that the unfolded conformation of CR12-MPED could modify interactions with Cry1A toxins and other molecules on insect midgut epithelium.

Both dot-blot binding assay and histomicroscopy observation suggest that CR12-MPED binds weakly to epithelial microvilli. This binding could be due to hydrophobic interactions promoted by exposed hydrophobic residues in CR12-MPED. Since toxin-CR12-MPED binding and the unfolded state of CR12-MPED are two critical factors in our observed Cry1A toxicity enhancement, one explanation of this synergism is that CR12-MPED peptides could attract Cry1A molecules to microvilli, increasing the probability of toxin interaction with Cry1A receptors such as Bt-R_1, GPI-anchored aminopeptidase N (30-32) and alkaline phosphatase (31, 33, 34), or sphingoglycolipids (35). This hypothesis is consistent with the model proposed by Bravo et al. (3), whereby binding of Cry1A monomer to the cadherin Bt-R_1 induces structural changes in the toxin that result in further processing and formation of a toxin oligomer. As a Bt-R_1 truncation for Cry1A binding, the addition of CR12-MPED might promote the switch of toxin from monomer to oligomer, a form which primarily binds to GPI-anchored receptors resulting in oligomer insertion in the cell membrane (36)

An alternative explanation for toxicity enhancement is that CR12-MPED modifies the pathological site of Cry1A toxins. However, according to microscopic observations, no significant differences in pathology were detected along the alimentary channel of *M. sexta*

larvae which were fed Cry1Ab alone or the mixture of Cry1Ab with CR12-MPED peptides (Chen et al., unpublished data).

The use of synergists has become one of the methods to increase Bt Cry toxicity and to overcome and delay insect resistance to this biopesticide (37, 38). The CR12-MPED peptide is the first Cry-synergist originating from an insect protein. This peptide is able to enhance Cry1A toxicity against lepidopteran pests in diet overlay bioassays. Other Cry protein synergists have been reported, and can be categorized according to their synergistic mechanisms, including (i) improvement of toxin docking and membrane insertion (39, 40), (ii) destruction of midgut peritrophic matrix to increase toxin permeability (41, 42), and (iii) disruption of midgut bacterial community which is essential for insect growth and development (5). As a synergist, CR12-MPED may accumulate on midgut microvilli, and its mediated toxicity enhancement relates to synergist-toxin binding. Although the synergistic mechanism of CR12-MPED is not fully characterized, it seems that this peptide could be grouped in the first synergist category Another member in this category, Bt var. *israelensis*, produced Cyt1Aa protein which could synergize Cry11Aa toxicity against yellow fever mosquito (*Aedes aegypti*) larvae by functioning as a binding site, and increasing toxin binding and insertion into midgut cells (40). Cyt1Aa is a cytolysin which is highly toxic not only to mosquito larvae but also to other vertebrate and invertebrate cells (43). In comparison, insects were healthy after fed CR12-MPED alone, suggesting that CR12-MPED may be an environmentally safe Cry1A synergist.

In summary, acting as a Bt Cry1A synergist, CR12-MPED peptide has the potential to augment the control of lepidopteran pests by Cry1A toxins. This synergist is a good candidate for development of a more effective strategy to control lepidopteran pests that are currently not being efficiently controlled by Bt crops, such as the case of *H. zea*. The activity of this synergist

towards other insect groups and organisms needs to be established. Because homologues of Bt-R_1 cadherin are present in other lepidopteran species, it is likely that CR12-MPED may also enhance Cry1A toxicity towards alternative lepidopteran pests.

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Figure Legends:

Fig. 1 Purified CR12-MPED has unfolded structure. (A) SDS-PAGE of lysates of induced pET-CR12-MPED in *E. coli* and purified CR12-MPED obtained from a Ni^{2+}-column. (B) Far-UV CD spectrum (190 – 250 nm) of CR12-MPED peptide, indicative of a mixture of 15 % helix, 37 % β-strands and 48 % random coils. (C) Chemical shifts of amide protons (red boxed) on CR12-MPED backbone clustered within a narrow range of around 7 to 8.5 ppm in 600 MHz ^{1}H-NMR spectroscopy, evidence of the unfolded state of this peptide.

Fig. 2 CR12-MPED peptides enhanced Cry1A toxicity towards lepidopteran larvae in diet surface bioassays. Cry1A toxins were mixed with purified CR12-MPED at toxin:peptide ratios of 1:0, 1:1, 1:10 and 1:100. Control treatments included buffer and CR12-MPED alone. (A) Cry1Ab plus CR12-MPED against *M. sexta* larvae. (B) Cry1Ac plus CR12-MPED against *M. sexta*. (C) Cry1Ac plus CR12-MPED against *H. zea*. (D) Cry1Ac plus CR12-MPED against *H. virescens*. Each column presents data for the mean ± SE from tri-replicate bioassays with 32 larvae per treatment. For each species, an asterisk above the column indicates that the mortality of Cry1A plus CR12-MPED treatment showed significant difference from Cry1A alone treatment within same Cry1A dosage (Tukey's test, $\alpha = 0.05$).

Fig. 3 Correlation between toxin-synergist binding and toxicity enhancement. (A) CR12-MPED or CR12-MPED/Del (5 μg for each) were spotted in duplicate on a PVDF filter and probed with ^{125}I-Cry1Ab or ^{125}I-Cry1Ac alone or in the presence of a 1000-fold excess of unlabeled homologous Cry1A. (B) Cry1Ab and Cry1Ac toxicity to *M. sexta* larvae when combined to either CR12-MPED or CR12-MPED/Del at a toxin:peptide mass ratio of 1:100. Each column

represents the mean ± SE from 4 replicates of 32 individuals per treatment. Significantly different mortalities for each Cry1A dosage are indicated by an asterisk above the column (Tukey's test, $\alpha = 0.05$).

Fig. 4 Cry1A binding localization on *M. sexta* larval midgut epithelia and the effect of CR12-MPED on toxin binding to brush border membrane vesicles. Antisera against CR12-MPED specifically immunostained the apex and base of microvilli (A). Midgut sections were treated with TAMRA-labeled Cry1Ab (B) or Cry1Ac (D) alone or in the presence of 20-fold excess of CR12-MPED (C and E, respectively). Binding of TAMRA-labeled CR12-MPED is shown in (G). Control treatment with TAMRA-labeled BSA is shown in (F). Five μg of *M. sexta* or *H. virescens* BBMVs were dotted as a spot on the PVDF filter with two replicates. Both ^{125}I-Cry1Ab and ^{125}I-Cry1Ac (3.3 ng/ml) bound to *M. sexta* BBMV, and their bindings were not blocked by in the addition of 100-fold unlabeled CR12-MPED (H). ^{125}I-CR12-MPED (3.3 ng/ml) bound *M. sexta* and *H. virescens* BBMV, but these bindings were neutralized by 500-fold unlabeled same peptide, indicative of binding specificity. (* = TAMRA-labeled; Scale bar = 10 μm; AMv = apical tip of microvilli; BL = basal lamina; BMv = base of microvilli; Nu = nucleus; PM = peritrophic matrix).

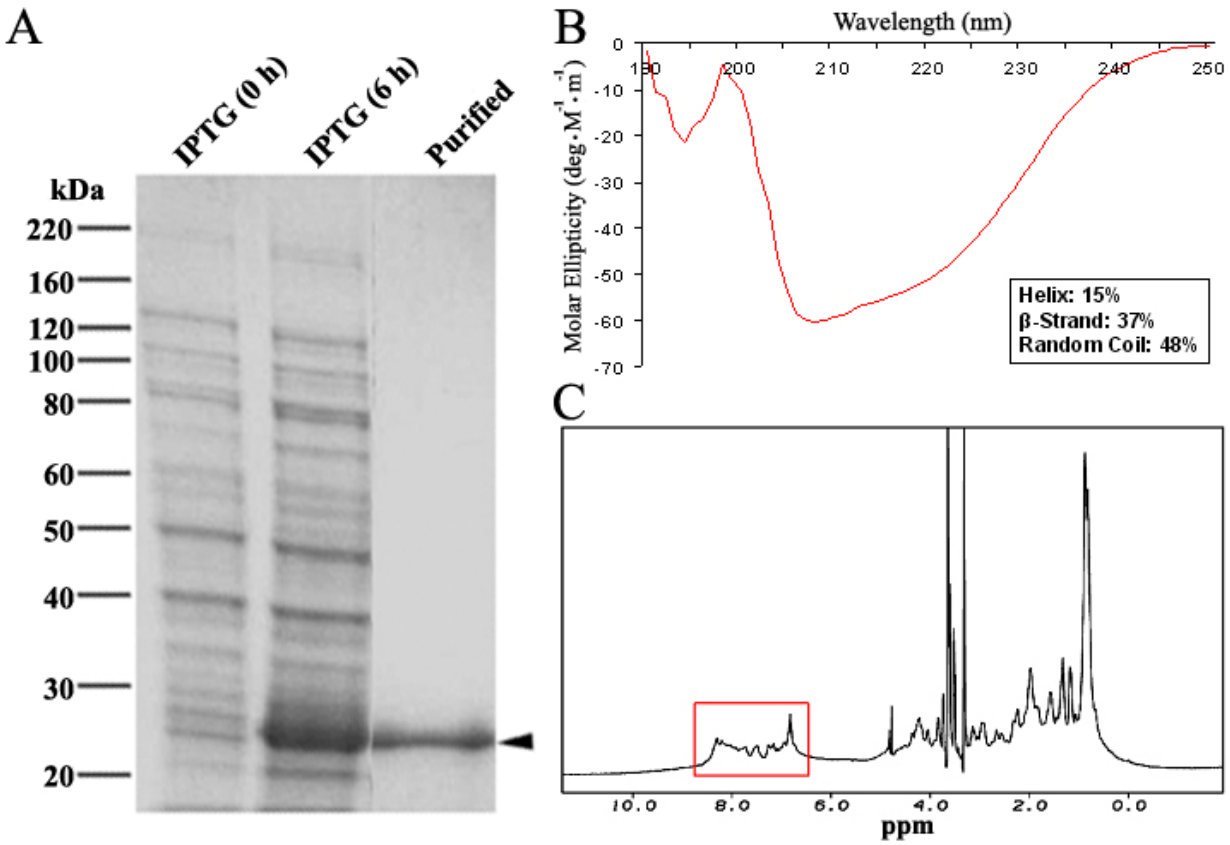

A
IPTG (0 h)
IPTG (6 h)
Purified
kDa
220
160
120
100
80
60
50
40
30
20
B
Wavelength (nm)
Molar Ellipticity (deg·M-1·m-1)
Helix: 15%
β-Strand: 37%
Random Coil: 48%
C
ppm

Fig. 1

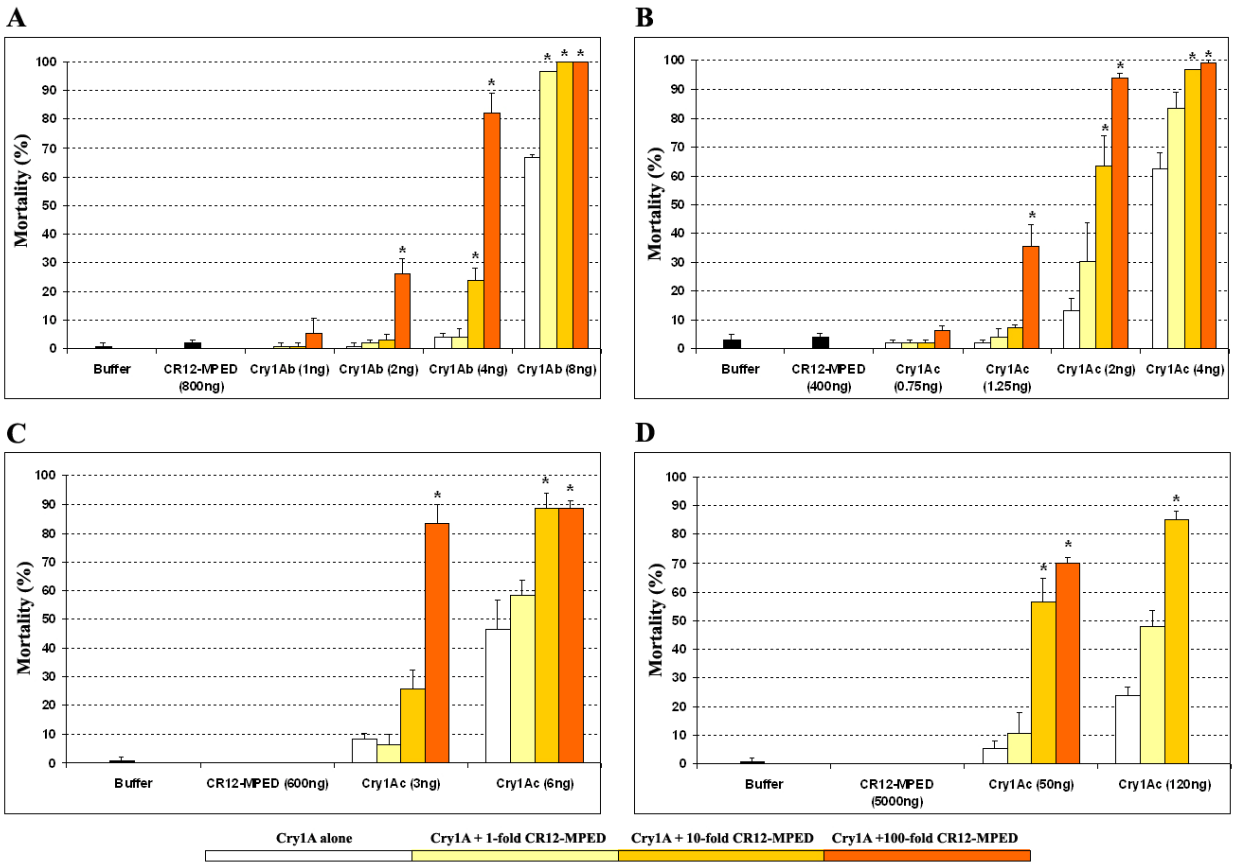

A
B
C
D
Mortality (%)
Buffer
CR12-MPED (800ng)
Cry1Ab (1ng)
Cry1Ab (2ng)
Cry1Ab (4ng)
Cry1Ab (8ng)
CR12-MPED (400ng)
Cry1Ac (0.75ng)
Cry1Ac (1.25ng)
Cry1Ac (2ng)
Cry1Ac (4ng)
CR12-MPED (600ng)
Cry1Ac (3ng)
Cry1Ac (6ng)
CR12-MPED (5000ng)
Cry1Ac (50ng)
Cry1Ac (120ng)
Cry1A alone
Cry1A + 1-fold CR12-MPED
Cry1A + 10-fold CR12-MPED
Cry1A +100-fold CR12-MPED

Fig. 2

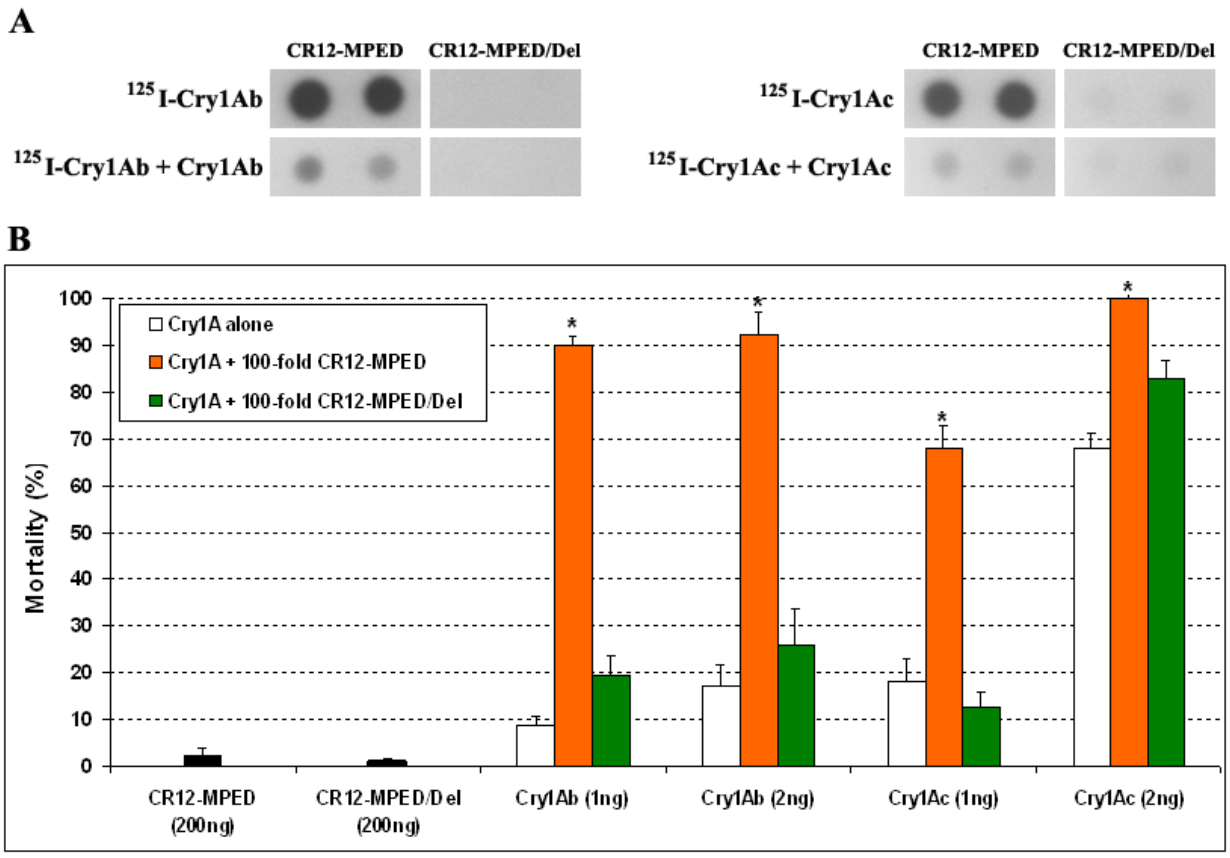

A
CR12-MPED
CR12-MPED/Del
125 I-Cry1Ab
125 I-Cry1Ab + Cry1Ab
125 I-Cry1Ac
125 I-Cry1Ac + Cry1Ac
B
Cry1A alone
Cry1A + 100-fold CR12-MPED
Cry1A + 100-fold CR12-MPED/Del
Mortality (%)
0
10
20
30
40
50
60
70
80
90
100
CR12-MPED (200ng)
CR12-MPED/Del (200ng)
Cry1Ab (1ng)
Cry1Ab (2ng)
Cry1Ac (1ng)
Cry1Ac (2ng)

Fig. 3

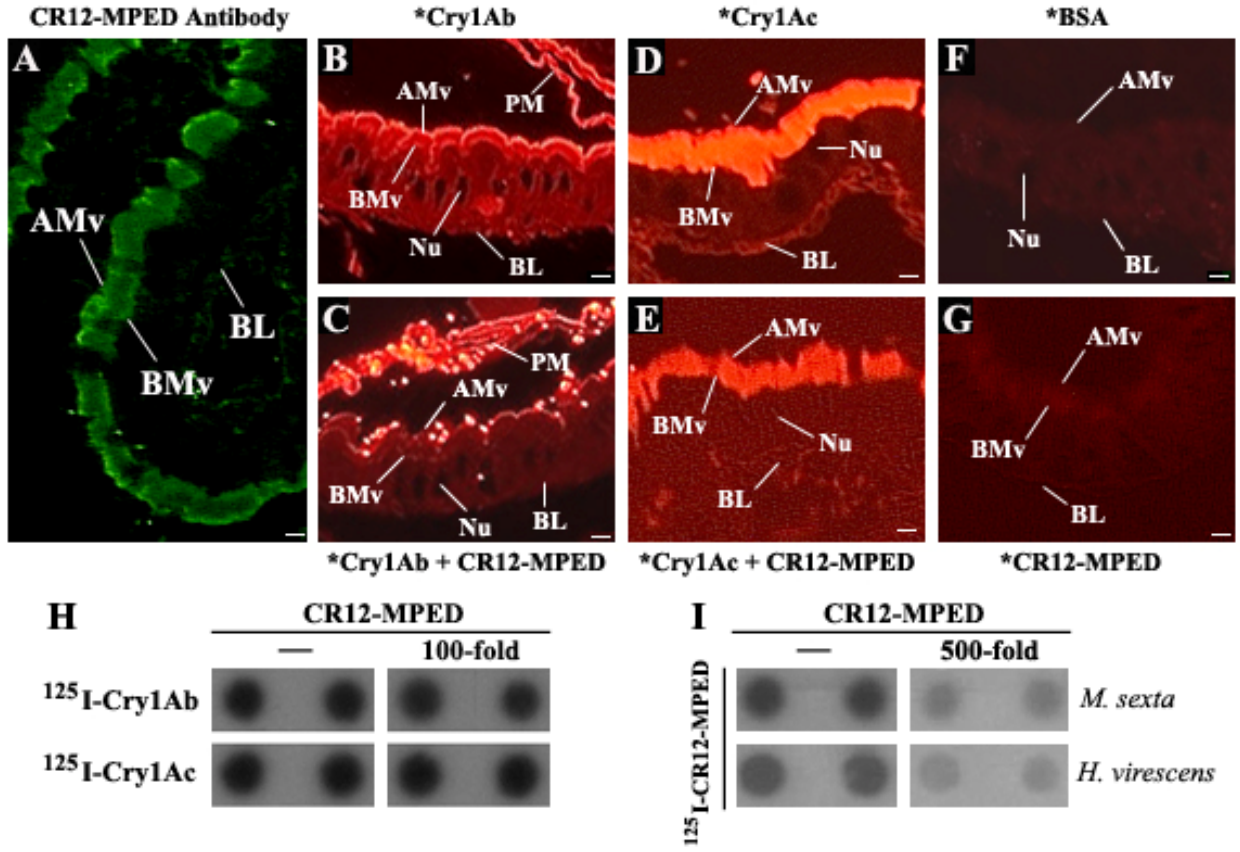
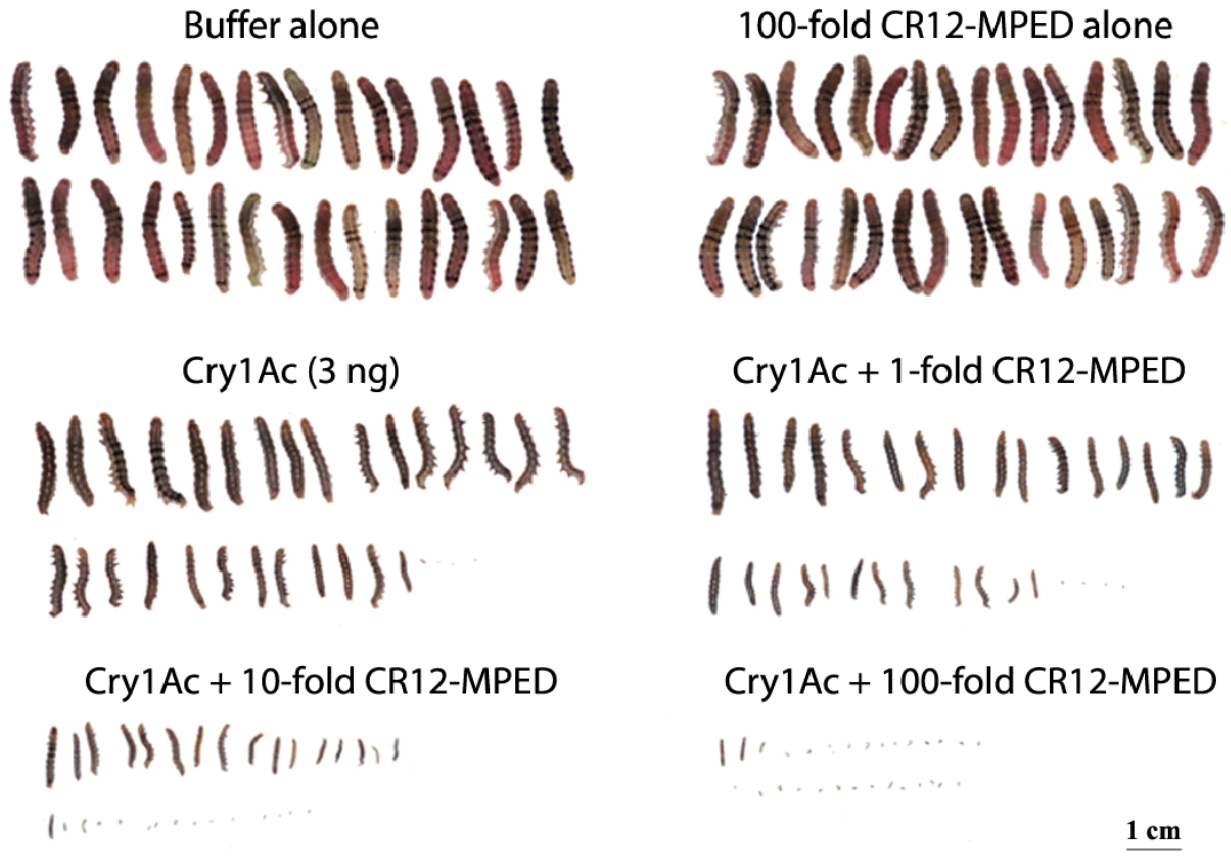

CR12-MPED Antibody
*Cry1Ab
*Cry1Ac
*BSA
A
B
C
D
E
F
G
AMv
BL
BMv
PM
Nu
*Cry1Ab + CR12-MPED
*Cry1Ac + CR12-MPED
*CR12-MPED
H
CR12-MPED
—
100-fold
125I-Cry1Ab
125I-Cry1Ac
I
CR12-MPED
—
500-fold
125I-CR12-MPED
M. sexta
H. virescens

Fig. 4

Supplemental Information:

Suppl. Fig. 1 *H. virescens* larval growth after 7 d of feeding on diet containing 3 ng/cm^2 of Cry1Ac. In controls, insects feeding with buffer or 600 ng/cm^2 CR12-MPED alone (*upper left and right panels*) did not result in toxicity or reduced growth. Compared with the treatment using Cry1Ac alone (*middle left panel*), larval growth was clearly stunted by the addition of 10 or 100-fold CR12-MPED (*lower left and right panels*). Scale bar = 1 cm.


Buffer alone
100-fold CR12-MPED alone
Cry1Ac (3 ng)
Cry1Ac + 1-fold CR12-MPED
Cry1Ac + 10-fold CR12-MPED
Cry1Ac + 100-fold CR12-MPED
1 cm

Suppl. Fig. 1

CHAPTER 4

BT-R_1 EXTRACELLULAR CADHERIN REPEAT 12 ENHANCES CRY1AC TOXICITY AGAINST CORN EARWORM (*HELICOVERPA ZEA*) [1]

[1] Chen, J., G. Hua and M.J. Adang. To be submitted to *FEMS Microbiol Lett*.

Abstract:

The cadherin Bt-R_1 is a Bt Cry1A binding protein localized on the midgut epithelia of *Manduca sexta* larvae. The extracellular cadherin repeat (CR) is the minimum fragment necessary for mediating Cry1Ab binding and cytotoxicity (Hua et al. 2004). We recently discovered that a 23 kDa truncation of Bt-R_1 consisting of the CR12 and membrane proximal extracellular domain (MPED) synergizes the Cry1A toxicity toward lepidopteran larvae (Chen et al., 2006). To define the functional region of CR12-MPED with toxin enhancing properties, we constructed and expressed sub-truncations of CR12-MPED in *Eschericia coli*. In diet surface bioassays, the 12 kDa CR12 peptide at toxin:peptide ratios of 1:50 and 1:100 exhibited the same ability as the original CR12-MPED peptide to enhance Cry1Ac toxicity against corn earworm (*Helicoverpa zea*). Other peptide regions MPED, T(1362-1424) and T(1384-1444) increased insect mortality due to Cry1Ac intoxication, but the effect was over 20 % lower than CR12. In dot-blot binding assays, Cry1Ac bound CR12-MPED and CR12, bound slightly to MPED, but did not bind T(1424-1524), T(1362-1424) and T(1384-1444) sub-truncations. Thus we conclude that the CR12 region is the essential component of the CR12-MPED peptide necessary to potentiate Cry1Ac toxicity against lepidopteran larvae, and toxin-peptide binding is an important factor for this synergism. Because the CR12 peptide enhances insect mortality from about 20% to greater than 80% for the same toxin dosage, this peptide may have utility for insect control by both Bt biopesticides and transgenic plants.

Keywords: *Bacillus thuringiensis*, *Helicoverpa zea*, cadherin, synergist

Introduction:

The Gram-negative bacterium *Bacillus thuringiensis* (Bt) produces insecticidal crystal proteins (ICP) during parasporulation stage. These ICPs are toxic to insects with a narrow spectrum, which are species mostly in the order of Lepidoptera, Diptera or Coleoptera. The Cry1A family contains ICPs specifically against lepidopteran larvae. Although the functional mechanism of ICPs has not been completely understood, an accepted mode of action is that after fed by insects, ICPs are processed with solubilization, activation, binding to target molecules on midgut epithelia, oligomerization and pore-formation which leads to enterocytolysis and insect death (Aronson and Shai 2001, Whalon and Wingerd 2003). In lepidopteran, one family of Cry1A-binding molecules is the cadherin on insect midgut epithelia (Vadlamudi et al. 1995). Unlike traditional members in cadherin family, these Cry1A-binding cadherins do not localize at cell-cell contacting areas for adhesion, but distribute on epithelial microvilli and expose to the gut lumen (Aimanova et al. 2006, Chen et al. 2005).

In tobacco hornworm (*Manduca sexta*), the cadherin Bt-R_1 is a 210 kDa transmembrane protein for Cry1A-binding, which contains twelve cadherin repeats (CR1-12) and one membrane proximal extracellular domain (MPED) for the extracellular portion (Dorsch et al. 2002, Vadlamudi et al. 1993, Vadlamudi et al. 1995). When Bt-R_1 and its truncations were expressed on *Drosophila* S2 cell surface, the CR12 domain was identified as a critical region for Cry1Ab binding and Cry1Ab mediated cytotoxicity (Hua et al. 2004). Among all Bt-R_1 truncations, CR12-MPED was the minimum fragment for doing so (Hua et al. 2004). To further study the role of CR12-MPED involved Bt toxicity, we purified it through *Escherichia coli* expression and fed this peptide to *M. sexta* larvae for bioassays. However, the toxicity of Cry1Ab and Cry1Ac toward *M. sexta* was not inhibited, but dramatically potentiated, by the addition of CR12-MPED.

Similar results were also observed when other lepidopteran species were tested (Chen et al. 2006), indicating that CR12-MPED is a synergist of Cry1A toxins. However, our observations were contrary to other reports with similar truncations of Bt-R_1 (Dorsch et al. 2002) or its homologue HvCaLP from tobacco budworm (*Heliothis virescens*) (Xie et al. 2005). Far-UV circular dichroism (CD) and ^{1}H-NMR data indicated that this discrepancy was possible due to the unfolded state of CR12-MPED in our experiments, while folded ones in Dorsch et al. (2002) and Xie et al. (2005).

CR12-MPED is a two-domain fragment with 206 residues. In current research, we attempted to identify the critical region in this peptide for Cry1A toxicity enhancement. An agricultural important insect, corn earworm (*Helicoverpa zea*), was used in bioassays to test the synergistic ability of a series of CR12-MPED sub-truncations.

Materials and Methods:

Insect and bacterial strain

Eggs of *H. zea* were purchased from Benzon Research Inc. (Carlisle, PA). Bt strain HD-73 producing Cry1Ac was obtained from the *Bacillus* Genetic Stock Center (Columbus, OH).

Purification and radiolabeling of Cry1Ac

Growth of Bt HD73 and the preparation of Cry1Ac were conducted according to (Luo et al. 1999). Purified toxin fractions (as determined by SDS-10 % PAGE) were pooled, quantified (Bradford 1976) and stored at -80 ^{o}C.

Cloning, expression, and purification of CR12-MPED sub-truncations

The original synergist CR12-MPED is a Bt-R_1 truncation (G1362 to P1567) (Chen et al. 2006). The constructed sub-truncations of CR12-MPED were CR12, MPED, T(1424-1524), T(1362-1424) and T(1382-1444). The numerals in brackets are residues at N and C-termini of each peptide according to the amino acid sequence of Bt-R_1 in Dorsch et al. (2002). Primers, as listed in Table 1, were manufactured by Integrated DNA Technologies, Inc. (Coralville, IA). The plasmid for coding CR12-MPED (pET-CR12-MPED) had been constructed (Chen et al. 2006). Fragments for coding CR12-MPED sub-truncations were amplified by PCR with pET-CR12-MPED as the template. Amplicons were digested by Nde I and Xho I endonucleases (Promega Co., Madison, WI), and inserted to pET-30a(+) vector (Novagen, Inc., Madison, WI). The coding regions of all clones were confirmed by DNA sequencing at the Molecular Genetics Instrumentation Facility (University of Georgia, Athens, GA). Peptides were expressed in *E. coli* strain BL21(DE3)/pRIL and purified via their C-terminal 6× His-tags according to our previous descriptions (Chen et al. 2006). Briefly, bacterial cultures grew at 37^{o}C and were induced by 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) when OD_{600} reached 0.5-0.6. The bacteria were harvested by centrifugation, and expressed proteins were released to soluble form by sonication in buffer A (6 MGuHCl, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.0) on ice. Soluble extracts were loaded through a HiTrap™ Ni^{2+}-chelating HP column (GE Biosciences Co., Piscataway, NJ), washed with buffer B (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.0), and eluted with buffer C (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.0) containing imidazole concentrations ranging from 0.01 M to 1.0 M. Purity of eluted peptides was ascertained by separation on a 4-20 % Tris-HCl/Criterion Precast Gel (Bio-Rad Laboratories, Hercules, CA), followed by staining with Coomassie G-250 (ProtoBlue Safe, Atlanta, GA). The highest purity

fractions were pooled and dialyzed against TSC buffer (10 mM Tris, 20 mM Na_2CO_3, pH 9.8). Peptide concentrations were quantified by Bradford (1976) method with bovine serum albumin (BSA) as a standard. Purified peptides were stored at -20 °C until needed for experimentation.

Insect bioassay

Diet surface bioassays were conducted according to Chen et al. (2006). In preliminary bioassays with newly hatched *H. zea* larvae, a dosage of 170 ng Cry1Ac/cm^2 caused 20-30 % mortality in a 7-day bioassay period. This Cry1Ac dosage was selected for all toxin:peptide bioassays. CR12-MPED and its truncated derivatives were mixed with Cry1Ac at molar ratios of 1:50 and 1:100 (Cry1Ac:peptide), and applied uniformly to the diet surface. Controls included each peptide alone at the 100-fold dosage or TSC buffer alone. The synergistic effects of CR12-MPED and CR12 were compared at 1:0, 1:10, 1:20 and 1:40 (Cry1Ac : peptide) molar ratios. Each experimental treatment contained 16 larvae, and was repeated at least 10 times. Data were analyzed by the GLM procedure and Tukey's test of the statistical software SAS 9.1 (SAS Institute Inc., Cary, NC).

Labeling of Cry1Ac and dot-blot binding assay

Purified Cry1Ac (5 µg) was radiolabeled with 0.5 mCi $Na^{125}I$ (GE Biosciences Co., Piscataway, NJ) by the chloramine-T method according to Garczynski et al. (1991). The 7.4 kDa T(1384-144) was dotted as two spots containing 1 and 5 µg peptides, respectively, onto a polyvinylidene difluoride (PVDF) filter (Millipore Co., Bedford, MA). Other peptides, CR12-MPED (23.3 kDa), CR12 (12.1 kDa), MPED (12.3 kDa), T(1424-1524) (12.2 kDa) and T(1362-1424) (7.7 kDa) were dotted with same molar amounts according to 1 and 5 µg T(1384-1444),

respectively. After blocking in PBS-T buffer (0.1 % Tween-20 in phosphate-buffered saline, pH 7.4) with 3 % BSA, filters were bathed in ^{125}I-Cry1Ac (15 ng/ml) or the same concentration of ^{125}I-Cry1Ac in the presence of 1000-fold excess of unlabeled Cry1Ac for 1 h. After washing, the peptide-bound ^{125}I-Cry1Ac on PVDF filter was exposed to X-ray film for autoradiography at -80 ºC overnight.

Results:

Production of CR12-MPED and its sub-truncations

The CR12-MPED truncation of Bt-R_1 has the unexpected property of enhancing Cry1A toxicity when combined with toxin and fed to lepidopteran larvae (Chen et al. 2006). Sub-truncations of CR12-MPED were prepared to probe the functional region in this peptide for toxicity enhancement. Peptides were purified from a crude *E. coli* lysate using an immobilized Ni^{2+}-column and analyzed for purity on SDS-4-20 % PAGE. The apparent molecular weights of peptides in gel (Fig. 1B) were close to their theoretical sizes (Table 1). The purity of each peptide was presented according to gel.

Synergistic effect of CR12-MPED and its sub-truncations on Cry1Ac toxicity against H. zea

In diet surface bioassays, *H. zea* larvae were fed Cry1Ac with mixed CR12-MPED or its sub-truncations. The Cry1Ac-induced insect mortality was significantly increased from 23.5 ± 2.89 % to 77.5 ± 10.60 % ($P < 0.0001$) and 81.9 ± 7.07 % ($P < 0.0001$) with the addition of 50 and 100-fold CR12-MPED, respectively (fig. 2A). To test the synergistic ability of CR12-MPED sub-truncation, we first did bioassays with CR12, MPED and T(1424-1524), whose molecular weights were approximately half of CR12-MPED (Table 1, Fig. 1A). Three sub-

truncations exhibited discrepancy on synergizing Cry1Ac toxicity. The mortality enhanced by CR12 was about same as that by CR12-MPED at two toxin:peptide ratios (Fig. 2A). Insect mortality was significantly increased by 100-fold excess of MPED (59.5 ± 3.69 %, $P < 0.0001$), but not by the 50-fold one (41.3 ± 6.19 %, $P > 0.05$). However, the mixture of Cry1Ac and T(1424-1524) did not show significant synergism at either toxin:peptide ratio ($P > 0.05$). Because the synergistic ability of CR12 was about same as CR12-MPED, we further tested two 7 kDa sub-truncations, T(1362-1424) and T(1384-1444), within CR12. Although both short peptides significantly potentiated Cry1Ac toxicity, their synergistic effects were about 20 % lower than CR12 at two tested toxin:peptide ratios in bioassay (Fig. 2A). In controls, no toxicity was observed with insects fed TSC buffer (Fig. 2A) or above peptides (data not shown) alone.

Comparisons of CR12 and CR12-MPED synergistic ability at various toxin:peptide ratios

CR12 was compared with CR12-MPED in *H. zea* bioassays across a range of toxin:peptide ratios. At the dosage of 170 ng Cry1Ac /cm^2, both peptides significantly enhanced Cry1Ac toxicity at 1:10 ($P < 0.001$), 1:20 ($P < 0.0001$) and 1:40 ($P < 0.0001$) toxin-to-peptide ratios (Fig. 2B). There was no measurable difference in the extent of mortality enhancement between CR12 and CR12-MPED (Fig. 2B). The mortality values for the lower toxin:peptide ratios were consistent with the properties of the two peptides at higher additions (50 and 100-fold). Increased amounts of CR12 and CR12-MPED in the toxin preparation directly correlated with increased larval mortality (Fig. 2A, Fig. 2B).

Binding property of CR12-MPED sub-truncations to Cry1Ac

^{125}I-Cry1Ac bound CR12-MPED and CR12 on dot blots, and the bindings could be neutralized by the addition of 1000-fold non-labeled Cry1Ac (Fig. 3). However, it seemed that CR12-MPED bound less Cry1Ac than CR12 with equal molar amount. MPED peptide bound some Cry1Ac, relative to the other sub-truncations, T(1424-1524), T(1362-1424) and T(1384-1444) which did not bind ^{125}I-Cry1Ac on dot blots (Fig. 3).

Discussion:

The first generation of Bt cotton (Bollgard I) effectively controls *H. virescens* larvae, yet *H. zea* larvae occasionally survive on the crop (Gore et al. 2001). This species-based difference is accounted for the higher Cry1Ac dosage that is necessary to kill *H. zea* as compared to *H . virescens* (Ali et al. 2006, Luttrell et al. 1999, Macintosh et al. 1990). The discovery that CR12-MPED increased Cry1Ac toxicity to *H. zea* suggested a practical application for lepidopteran pest control by using a combination of Cry1Ac with CR12-MPED (Chen et al. 2006). However, a concern is that 10 and 100-fold mass excess of CR12-MPED was necessary to effectively potentiate Cry1Ac toxicity (Chen et al. 2006). Since the molecular weight ratio of Cry1Ac (65 kDa) vs. CR12-MPED (23.3 kDa) is about 3:1, the mass excess above for CR12-MPED is switched to 30 and 300-fold in molar. Thus, any decrease in synergist size would mean that less synergist in mass would attain same molar excess.

The goal of this study is to identify the critical region in CR12-MPED with maximal toxicity-enhancing property. Since the unfolded structure is a key factor determining the synergistic property of CR12-MPED (Chen et al. 2006), no efforts are necessary to maintain a specific three-dimensional structure of the peptide during preparations. Thus, the key fragment

in CR12-MPED for enhancing Cry1Ac toxicity could be probed through constructing and expressing different sub-truncations of CR12-MPED. In insect bioassays using Cry1Ac, the 12 kDa CR12 region displayed a synergistic ability which was equivalent to the original 23 kDa CR12-MPED at a series of toxin-to-peptide ratios (1:10 to 1:100). Because the molecular weight of synergist was reduced by about 50 %, CR12 with only half mass had equal synergistic ability as CR12-MPED to enhance Cry1Ac toxicity against *H. zea*. CR12 was further truncated to 7 kDa peptide. Although still synergized Cry1Ac toxicity, both T(1362-1424) and T(1384-1444) exhibited over 20 % reduction in toxicity when compared with CR12 and CR12-MPED at two tested toxin:peptide ratios. Thus, based on our current bioassay results, CR12 in full-length was regarded as the critical fragment in CR12-MPED to enhance Cry1Ac toxicity against *H. zea*.

Xie et al. (2005) reported a Cry1Ac-binding epitope in HevCaLP from *H. virescens* larvae. A homolog of this epitope also exists as ^{1416}GVLTLNIQ^{1423} in CR12-MPED. Previously we found that the CR12-MPED mediated Cry1A toxicity enhancement was correlated with this peptide binding to toxins (Chen et al. 2006). When the Cry1A-binding epitope ^{1416}GVLTLNIQ^{1423} was removed from CR12-MPED, the derivative, called CR12-MPED/Del, lost binding to Cry1Ab and Cry1Ac, and did not demonstrate synergistic ability in bioassays with *M. sexta* (Chen et al. 2006). These indicated that ^{1416}GVLTLNIQ^{1423} was an important region for toxin-binding and synergism in CR12-MPED. This toxin-binding epitope locates in CR12, which alone was enough to bind Cry1Ac, and displayed equivalent synergism as CR12-MPED. However, the T(1362-1424) and T(1384-1444) sub-truncations of CR12, which contained ^{1416}GVLTLNIQ^{1423} fragment, did not bind to Cry1Ac in dot-blot assays, but still potentiated Cry1Ac toxicity against *H. zea* in bioassays. This suggests that ^{1416}GVLTLNIQ^{1423} is not the only critical factor for Cry1Ac-binding and synergism. However, since we did not test

T(1362-1424) and T(1384-1444) in bioassays with *M. sexta*, we are currently unable to rule out the possibility that the observed synergistic abilities in these two peptides were due to different insect species. This could be supported by the case of CR12-MPED/Del. This derivative of CR12-MPED did not enhance Cry1Ac toxicity to *M. sexta*, yet its MPED region potentiated same toxin against *H. zea*, although this synergistic effect was not as strong as CR12-MPED or CR12.

In summary, our current study indicates that CR12 is a critical region in CR12-MPED necessary for enhancing Cry1Ac toxicity against *H. zea*. Compared with CR12-MPED, this short peptide enables less amount of synergist to be used in practice. Thus, CR12 can be a surrogate of CR12-MPED. On another aspect, because the gene coding a synergist was shortened, it will be helpful to probe hot spots in CR12 for enhancing Cry1A toxicity. Although some sub-truncations of CR12-MPED lost binding to toxin, they still showed ability to potentiate Cry1Ac toxicity toward *H. zea*. It seemed that toxin-peptide binding only partially contributed to synergistic effect. Further experiments are necessary to elucidate any mechanism of CR12 synergized Cry1A toxicity enhancement beyond toxin-peptide binding.

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Figure Legends:

Fig. 1 The sub-truncations of synergist CR12-MPED peptide. (A) The cadherin Bt-R_1 for Cry1A toxin binding is diagramed. Bt-R_1 contains twelve cadherin repeats (CR) and one membrane proximal extracellular domain (MPED) for its extracellular portion, a single transmembrane fragment (TM) and one cytoplasmic domain (CYTO). The CR12-MPED truncation (residues G1362 to P1567 according to Dorsch et al. (2002)) of Bt-R_1 was identified as a synergist for Cry1A toxicity against lepidopteran larvae (Chen et al. 2006). The sub-truncations of CR12-MPED are diagramed. For each sub-truncation peptide, the residual numbers at N and C-termini are marked. The fragment ^{1416}GVLTLNIQ^{1423} within CR12 domain is a Cry1Ac binding epitope according to Xie et al. (2005). (B) The peptides CR12, MPED, T(1424-1524), T(1362-1424) and T(1384-1444) are five sub-truncations of CR12-MPED. Each peptide contained a C-terminal 6x His-tag for purification through a Ni^{2+} column. Purified peptides were checked by running a 4-20 % gradient SDS-PAGE.

Fig. 2 Insect bioassay results. (A) The synergistic abilities of CR12-MPED, CR12, MPED, T(1424-1524), T(1362-1424) and T(1384-1444) on Cry1Ac (170 ng/cm^2) toxicity were examined with *H. zea* neonates. The molar ratios of Cry1Ac:peptide were set as 1:50 and 1:100. Solutions with buffer alone, Cry1Ac alone, or Cry1Ac plus peptide were overlaid on diet surface. After they were dry, insects were placed on diet. Each replicate contained sixteen larvae, and each experimental setup was repeated at least ten times. Insect mortality was counted on the seventh day after treatment. The synergistic ability of CR12 was approximately same as CR12-MPED, and was higher than other sub-truncations. (B) The synergistic abilities of CR12 and CR12-MPED were compared at lower molar ratios of Cry1Ac:peptide (1:10, 1:20 and 1:40).

The dosage of Cry1Ac was 170 ng/cm^2. The Cry1Ac toxicity to *H. zea* larvae was significantly enhanced even with 10-fold mixed CR12 or CR12-MPED peptides. In all bioassays, no larval mortality was observed when insects fed CR12-MPED or its sub-truncations alone (data not presented). Insect mortalities of Cry1Ac plus peptide were compared with that of Cry1Ac alone by Tukey's test. (**: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$)

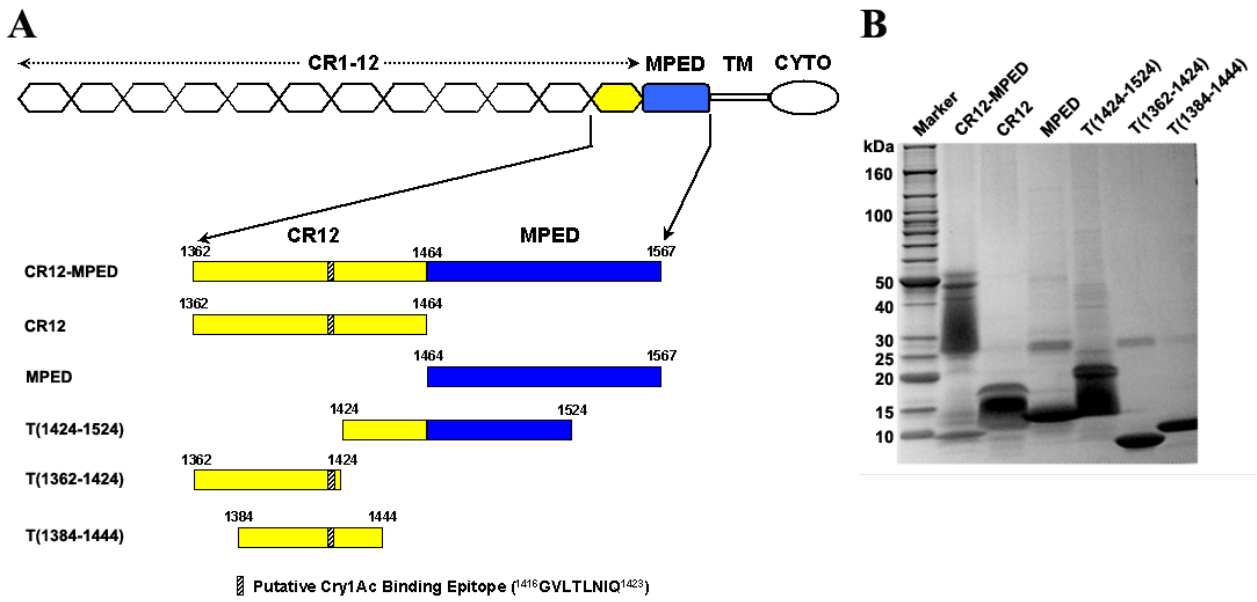
Fig. 3 Binding properties of CR12-MPED and its sub-truncations with Cry1Ac. T(1384-1444) was dotted as two spots containing 1 μg and 5 μg peptides, respectively, on PVDF filter. CR12-MPED, CR12, MPED, T(1424-1524) and T(1362-1424) were dotted with same molar amounts which were equivalent to 1 μg and 5 μg T(1384-1444), respectively. Peptides on PVDF filter were probed by 15 ng/ml ^{125}I-Cry1Ac or 15 ng/ml ^{125}I-Cry1Ac plus 1000-fold unlabeled Cry1Ac. ^{125}I-Cry1Ac bound to CR12 and CR12-MPED clearly, but to MPED faintly. All these bindings could be competed by 1000-fold unlabeled toxins. T(1424-1524), T(1362-1424) and T(1384-1444) did not show binding with ^{125}I-Cry1Ac.

Table 1 CR12-MPED and its sub-truncations

Peptide Name	Forward and Reverse Primers for PCR Cloning †	Size (kDa)	Sequence ‡
CR12-MPED	5'-GTACcatatgGGGATATCCACAGCGGACTCCATCG-3'	23.3	G1362 to P1567
	5'-GGCTctcgagAGGCGCCGAGTCCGGGCTGGAGTTG-3'		
CR12	5'-GTACcatatgGGGATATCCACAGCGGACTCCATCG-3'	12.1	G1362 to V1464
	5'-AAAActcgagCACGAAGTAGACGCGGTTCTGCGAGGAT-3'		
MPED	5'-AAAAcatatgGTGTTCGTCAACACGCTGCAACAGGTCG-3'	12.3	V1464 to P1567
	5'-GGCTctcgagAGGCGCCGAGTCCGGGCTGGAGTTG-3'		
T(1424-1524)	5'-AAAAcatatgCCCACGGCCACGATGCATGGACTGTTCA-3'	12.2	P1424 to P1524
	5'-AAAActcgagGGGTACGTTGTCCCGTATGAAGTGGCCG-3'		
T(1362-1424)	5'-GTACcatatgGGGATATCCACAGCGGACTCCATCG-3'	7.7	G1362 to P1424
	5'-AAAActcgagGGGCTGGATATTAAGCGTCAGCACTCCG-3'		
T(1384-1444)	5'-AAAAcatatgGCGGCCATTACTTATGCTATAGACTACG-3'	7.4	A1384 to A1444
	5'-AAAActcgagAGCGCCGGCCGTGTCAGTAGCTGTGACT-3'		

†: Underlined neuclotides indicate restriction site for Nde I (catatg) or Xho I (ctcgag).

‡: Residue numbers are according to the sequence of the cadherin Bt-R_1 in *M. sexta* larval midgut (GenBank AY094541).


A
CR1-12
MPED
TM
CYTO
CR12
MPED
CR12-MPED
1362
1464
1567
CR12
1362
1464
MPED
1464
1567
T(1424-1524)
1424
1524
T(1362-1424)
1362
1424
T(1384-1444)
1384
1444
Putative Cry1Ac Binding Epitope (1416GVLTLNIQ1423)
B
Marker
CR12-MPED
CR12
MPED
T(1424-1524)
T(1362-1424)
T(1384-1444)
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100
50
40
30
25
20
15
10

Fig. 1

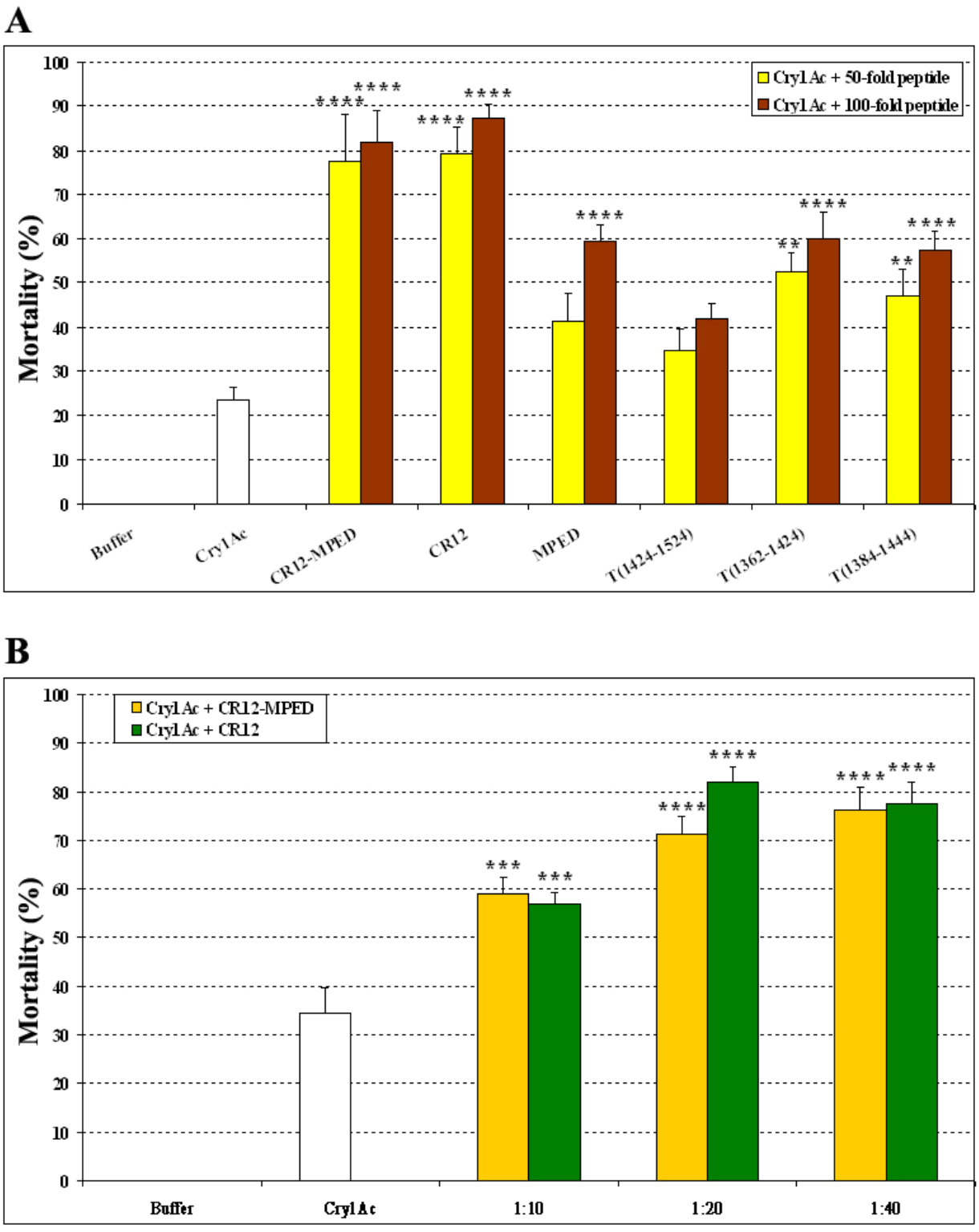
A
Cry1Ac + 50-fold peptide
Cry1Ac + 100-fold peptide
Mortality (%)
Buffer
Cry1Ac
CR12-MPED
CR12
MPED
T(1424-1524)
T(1362-1424)
T(1384-1444)
B
Cry1Ac + CR12-MPED
Cry1Ac + CR12
Mortality (%)
Buffer
Cry1Ac
1:10
1:20
1:40

Fig. 2

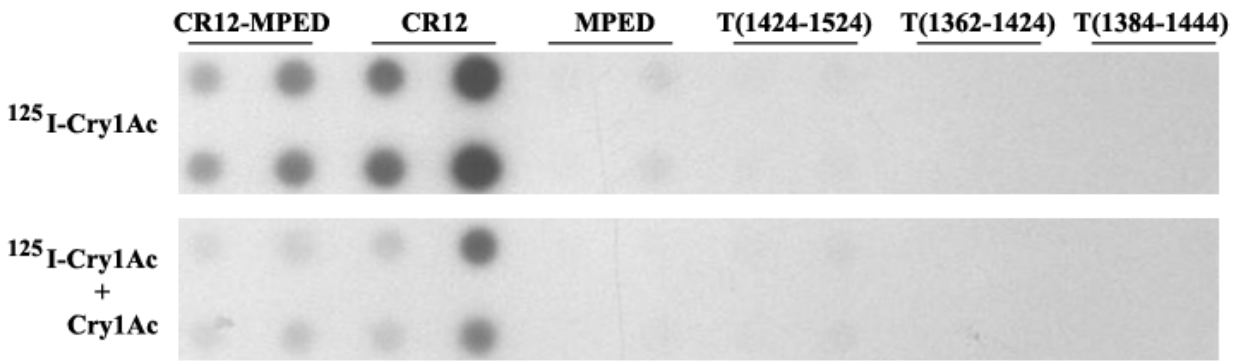
CR12-MPED
CR12
MPED
T(1424-1524)
T(1362-1424)
T(1384-1444)
125I-Cry1Ac
125I-Cry1Ac
+
Cry1Ac

Fig. 3

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

A critical step in Bt-mediated toxicity is the binding of Bt toxins to their target molecules on insect midgut epithelia. This dissertation described (1) the immunolocalization of three Cry1A-binding proteins (MsAPN1, Bt-R_1 and m-ALP) on the midgut of *M. sexta* larva, and (2) the synergist effect of CR12-MPED on Cry1A toxicity.

1. Immunolocalization of Cry1A-binding Proteins on *M. sexta* Larval Midgut

M. sexta larva is a model insect for Bt toxin mode-of-action research. Two major Cry1A-binding proteins, MsAPN1 and Bt-R_1, were recognized in BBMV prepared from *M. sexta* larval midgut more than 10 years ago (Knight et al. 1994, Sangadala et al. 1994, Vadlamudi et al. 1993). Although BBMV mainly contained cell membranes of midgut epithelial microvilli, the distributions of MsAPN1 and Bt-R_1 in midgut tissue had never been investigated. In Chapter 2 of this dissertation, the localization of MsAPN1 and Bt-R_1, as well as mALP, a recently identified Cry1A-binding protein (McNall and Adang 2003), on the midgut of *M. sexta* larvae were determined through the use of immunohistochemistry. The three Cry1A-binding proteins had different immunolocalization patterns on *M. sexta* larval midgut. MsAPN1 mainly localized on the entire length of microvilli from the posterior midgut, and was only slightly detected at the apical tip of microvilli in the anterior and middle regions. The immunostaining of m-ALP was observed at the apex of microvilli consistantly along the midgut. It is interesting that unlike other members in the cadherin family, Bt-R_1 localized at the apical tip of microvilli and the hammock areas of adjacent microvilli. These distributions allow Bt-R_1 exposure to the midgut lumen, so it serves as a binding protein for Cry1A toxins. To my knowledge, this was the first report showing a cadherin for non-adhesion purpose although its real physiological function in insect midgut has not been determined yet. Very recently, D'Alterio et al. (2005) found that

another cadherin, Cad99C, also localized on the apical microvilli of ovarian follicle cells in *Drosophila*, and regulated the length of microvilli. I deduce that similar to Cad99C, the cadherin Bt-R_1 possibly functions in controlling the length of epithelial microvilli in *M. sexta* larval midgut.

In Chapter 2, I also studied the binding localization of three Cry1A toxins (Cry1Aa, Cry1Ab and Cry1Ac) on *M. sexta* midgut. This allowed me to make a direct comparison between toxin binding localization and the distribution of toxin-binding protein on midgut epithelial microvilli. Like binding proteins, each Cry1A toxin had a distinct binding localization on insect microvilli. Through out the midgut, Cry1Aa only bound to the apical microvilli, yet Cry1Ab bound to both the apex and the base of microvilli. The binding patterns of Cry1Aa and Cry1Ab are like the immunolocalization of mALP and Bt-R_1 in *M. sexta* midgut, respectively. However, the distribution of Cry1Ac binding varied in different regions of the midgut. This toxin only slightly bound to the apex of microvilli in anterior and middle midgut regions, but accumulated on the entire length of microvilli in the posterior portion. The pattern of Cry1Ac binding is very similar to the immunostaining of MsAPN1 along the whole midgut. Overall, my research clearly demonstrated that Cry1A toxins and their binding proteins co-localized on the microvilli of *M. sexta* larval midgut.

2. Synergistic Effect of CR12-MPED Peptide on Cry1A Toxicity to Lepidopteran Larvae

In Chapters 3 and 4, I studied the enhancement of Cry1A toxicity by CR12-MPED against lepidopteran larvae. CR12-MPED is a membrane proximal fragment of the extracellular portion of Bt-R_1. After the cadherin Bt-R_1 was recognized as a Cry1A-binding protein (Vadlamudi et al. 1993, Vadlamudi et al. 1995), researchers focused on identifying the toxin-

binding region (TBR) on this protein (Dorsch et al. 2002, Gomez et al. 2001, Hua et al. 2004). To date, three TBRs have been recognized in CR7 (Gomez et al. 2001), CR11 (Dorsch et al. 2002) and CR12-MPED (Hua et al. 2004), respectively. However, according to the methodology used in the reports, the TBR3 in CR12-MPED was regarded to be the only critical site for Cry1A binding (Hua et al. 2004, Xie et al. 2005). To investigate the role of this region in Bt-R_1 involved Cry1A toxicity *in vivo*, CR12-MPED was cloned, expressed, and purified from *E. coli*. Initially we assumed that in bioassay, more *M. sexta* larvae would survive after fed Cry1Ab with the addition of CR12-MPED peptide. However, the toxicity of Cry1Ab was not blocked, but was significantly potentiated. Such a synergistic effect was also observed in the bioassay with Cry1Ac. Thus, CR12-MPED was recognized as a synergist of Cry1A toxins against *M. sexta* larvae.

In Chapter 3, the enhanced Cry1Ac toxicity by CR12-MPED was also tested on two other lepidopterans, *H. virescens* and *H. zea*, which are severe agricultural pests on high value vegetables and field crops. Like the bioassays with *M. sexta*, the toxicity of Cry1Ac against *H. virescens* and *H. zea* was significantly enhanced by the addition of CR12-MPED peptide. Although the mechanism of CR12-MPED synergized Cry1A toxicity enhancement is still not completely understood, some aspects have been studied through a series of experiments in Chapter 3.

First, I investigated the correlation between CR12-MPED enhanced Cry1Ac toxicity and toxin-peptide binding. In other reports (Gomez et al. 2001, Dorsch et al. 2002), the addition of TBR1 or TBR2 of Bt-R_1 inhibited Cry1A toxicity to *M. sexta* larvae because the formation of TBR-Cry1A complex blocked Cry1A toxin binding to Bt-R_1 in insect midgut. Soon after CR12 was recognized to be a critical domain in Bt-R_1 for Cry1A binding (Hua et al. 2004), an 8-

residue region was found to be the Cry1Ac-binding epitope in the corresponding domain of HevCaLP, which is a homologue of Bt-R_1 in *H. virescens* (Xie et al. 2005). This Cry1Ac-binding epitope also conserved as ^{1416}GVLTLNIQ^{1423} with only one amino acid discrepancy in the CR12 domain of Bt-R_1. To investigate the effect of toxin-peptide binding on toxicity enhancement, a CR12-MPED derivative (CR12-MPED/Del) was constructed with the removal of ^{1416}GVLTLNIQ^{1423} region. In dot-blot binding assays, CR12-MPED bound to both Cry1Ab and Cry1Ac, but CR12-MPED/Del did not. In bioassays with *M. sexta* larvae, the toxicity of either Cry1Ab or Cry1Ac was significantly increased by the addition of CR12-MPED, but not by CR12-MPED/Del. Hence, we illustrated that the CR12-MPED enhanced Cry1A toxicity was associated with toxin-peptide binding.

Second, I defined the secondary structure and folding state of CR12-MPED because the function of a protein or peptide is determined not only by its primary sequence, but also by its three-dimensional structure. Since the over-expressed CR12-MPED peptides formed inclusion bodies in *E. coli*, denaturants (6 M GuHCl and 8 M urea) were used to dissolve them during purification. Although these peptides were soluble in 10 mM Tris (pH 8.0) finally, they might not refold to native structure, which was supposed to contain extensive β-sheets (Candas et al. 2002). The far-UV CD spectrum and ^{1}H-NMR spectroscopy indicated that our purified CR12-MPED peptide consisted of 15 % helix, 37 % β-strand and 48 % random coils in unfolded structure. Dorsch et al. (2002) reported a 25 kDa Bt-R_1 truncation (TBR) which inhibited Cry1Ab toxicity to *M. sexta* larvae. Although the structure of TBR was not determined, we expected that it was in folded state because this peptide was expressed as soluble form in *E. coli*. However, when TBR was expressed and purified according to our method, it exhibited the same

property as CR12-MPED by enhancing Cry1A toxicity against lepidopterans. These results suggested that unfolding may be a critical factor in CR12-MPED synergized Cry1A toxicity.

Third, we investigated the effect of CR12-MPED on Cry1A binding to *M. sexta* midgut. In histochemistry, the addition of CR12-MPED did not change Cry1Ab and Cry1Ac binding localization on *M. sexta* midgut. However, CR12-MPED alone was able to bind BBMV and tissue sections of *M. sexta* midgut.

Based on these results, I proposed a possible mechanism of CR12-MPED mediated Cry1A toxicity enhancement against lepidopteran larvae. More hydrophobic residues in CR12-MPED are exposed due to the unfolded state of this peptide. These exposed hydrophobic residues could bind some membrane molecules (protein, saccharide or lipid) on insect midgut epithelial microvilli via hydrophobic interactions. Since CR12-MPED also binds to Cry1A toxins, the CR12-MPED accumulated on microvilli could attract toxins to effectively attack these places and/or accelerate the swith of Cry1A toxin from monomer to oligomer which has pore forming ability. Hence Cry1A toxicity was increased.

3. Minimal Region in CR12-MPED for Enhancing Cry1A Toxicity

The CR12-MPED peptide was composed of 206 residues with 23.3 kDa. To probe the minimal region in this synergist for enhancing Cry1A toxicity, I constructed a series of sub-truncations of CR12-MPED and tested their synergistic abilities on Cry1Ac toxicity against *H. zea* larvae. The 12 kDa CR12 sub-truncation had equivalent synergistic ability as full-length CR12-MPED. However, when CR12 was further truncated to T(1362-1424) and T(1384-1444) with 7 kDa, neither peptide exhibited comparable synergistic ability as CR12 or CR12-MPED in bioassays. In dot-blot assays, CR12 was the only sub-truncation binding Cry1Ac. Although

T(1362-1424) and T(1384-1444) contained ^{1416}GVLTLNIQ^{1423} toxin-binding epitope, neither of them bound Cry1Ac. These results indicated that (1) CR12 was the minimal fragment with the maximal toxicity-enhancing property in CR12-MPED, (2) ^{1416}GVLTLNIQ^{1423} was not the only epitope for Cry1Ac-binding and toxicity enhancement, and (3) the enhanced Cry1Ac toxicity beyond toxin-peptide binding need to be further studied.

Overall, the first part of my dissertation illustrates that each Cry1A-binding protein has a distinct distribution in *M. sexta* larval midgut, and three binding proteins did co-localize with Cry1A toxins on insect midgut epithelial microvilli. This may help people better understand the interaction of Cry1A toxins and their binding proteins. The second part of this dissertation demonstrates that the toxicity of Cry1A toxin can be potentiated by the addition of CR12-MPED peptide, and the CR12 fragment is the minimal region in CR12-MPED for Cry1A toxicity enhancement. This will bring some benefits to Bt microbial control because Cry1A toxins are still able to effectively control lepidopteran larvae even at low dosages.

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