MODE OF ACTION OF CADHERIN-BASED *BACILLUS THURINGIENSIS* CRY TOXIN SYNERGISTS AND TOXIN INTERACTIONS WITH MIDGUT RECEPTORS IN LEPIDOPTERAN PESTS OF CORN

by

KHALIDUR RAHMAN

(Under the Direction of MICHAEL J. ADANG)

ABSTRACT

The Cry toxins produced by *Bacillus thuringiensis* (Bt) are the active component of the most widely used biopesticides effective against a range of crop pests and disease vectors. The high synergistic potential of recently discovered insect receptor (cadherin)-based Bt Cry toxin synergist provides a way to potentially surmount insecticidal activity of Cry toxins. However, a lack of understanding of cadherin synergist's mode of action in the insect midgut presents a major challenge to fully realizing its potential. In the first part of my dissertation research I have elucidated the mechanism of cadherin-based Bt synergism by utilizing the differential Cry1Fa toxin enhancing properties of *Spodoptera frugiperda* (SfCad) and *Manduca sexta* (MsCad) cadherin fragments. I have shown that cadherin fragments enhance Bt toxicity by at least two different mechanisms or a combination thereof; increasing probability of toxin-receptor interactions by prolonging toxin stability in the insect gut and increasing the rate of pore formation in the midgut epithelium.

The Cry1Ab and Cry1Fa toxins are two important Cry toxins that are widely used for lepidopteran pest control in corn and cotton. Co-expressing Cry1Ab and Cry1Fa toxins in corn is an approach being employed to broaden the insecticidal spectrum of transgenic Bt corn varieties. However, the long term utility of this Cry1Ab/Cry1Fa stacked corn variety will depend on whether these toxins share common binding sites in the targeted pests. Therefore, in the second part of my dissertation research, I have developed and used a fluorescence-based binding assay to study Cry1Fa and Cry1Ab toxin interaction with brush border membrane vesicles (BBMV) from three important pests of corn, *Ostrinia nubilalis* (European corn borer; ECB), *Diatraea grandiosella* (south western corn borer; SWCB), and *Helicoverpa zea* (corn earworm; CEW). Our results showed that Cry1Fa shares binding site(s) with Cry1Ab toxin in ECB, and SWCB for which Cry1Ab has higher affinity than Cry1Fa. Apart from the shared binding sites, Cry1Ab binds additional site(s) in ECB, and both toxins bind additional site(s) in SWCB. In CEW, Cry1Fa and Cry1Ab each have a high affinity binding site(s), which binds the heterologous toxin with low affinity.

INDEX WORDS: *Bacillus thuringiensis*, Binding, Cadherin, Cry1Fa, Cry1Ab, Corn, *Diatraea grandiosella*, *Helicoverpa zea*, *Manduca sexta*, *Ostrinia nubilalis*, Synergist

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DEDICATION

I dedicate my dissertation to my parents and my wife for their encouragement, love, and support.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 *Bacillus thuringiensis* and its insecticidal proteins

Bacillus thuringiensis (Bt) is a Gram positive, spore-forming bacterium that belongs to the *Bacillus cereus* group, which also includes *Bacillus anthracis* and *Bacillus cereus* (Helgason et al., 2000; Rasko et al., 2005). Unlike *B. anthracis*, which is highly pathogenic to humans and other mammals, *B. thuringiensis* is a highly specific insect pathogen with no known pathogenicity against mammals. *B. thuringiensis* produces three important classes of insecticidal proteins during various phases of its life cycle; Vip (vegetative insecticidal proteins) proteins during vegetative phase, and Cry (Crystal proteins) and Cyt (cytolytic toxin) proteins during the sporulation phase (Schnepf et al., 1998; Bravo et al., 2011). Apart from these insecticidal proteins, Bt also produces non-insecticidal Cry proteins that are toxic to many cancerous cells (Mizuki et al., 1999; Lee et al., 2000a). Parasporins are one such example of non-insecticidal Cry proteins that are toxic against MOLT-4 and HeLa cells and are being developed as potential cancer therapy drugs (Mizuki et al., 2000; Krishnan et al., 2010; Okumura et al., 2011).

Insecticidal proteins produced by *B. thuringiensis* exhibit high, specific toxicity against a number of coleopteran, dipteran, and lepidopteran pests of economic and medical importance. Due to their high specific toxicity, proteinaceous nature, and biodegradability, Bt toxins are used as a safe alternative to chemical pesticides and have been in use for the past 60 years as biopesticidal formulations or genetically modified plants producing Bt toxins. Since the

commercialization of transgenic plant varieties protected by Bt toxins in 1996, more than 200 million ha of transgenic crops expressing Cry toxins have been planted worldwide, making it the second most used trait after herbicide resistance (James, 2007).

1.2 *Bacillus thuringiensis* Cry toxins

Of the three most important classes of insecticidal proteins produced by *B. thuringiensis*, Cry toxins are the most extensively studied and widely used for crop pest control. Nucleotide sequences of more than 300 *cry* genes have been reported that constitute >200 unique Cry toxins with unique or overlapping insecticidal properties highly specific to a narrow range of insect species (Crickmore et al., 2011). The diversity in Cry toxins are attributed to three properties; high degree of genetic plasticity of the *cry* genes, association of *cry* genes with transposable elements which may facilitate evolution of new toxin (de Maagd et al., 2001), and association of *cry* genes with transmissible plasmids facilitating creation of new strains due to horizontal transfer by conjugation (Thomas et al., 2000; Thomas et al., 2001).

Cry toxin structure

The three-dimensional structures determined for Cry toxins Cry3Aa (Li et al., 1991) , Cry1Aa (Grochulski et al., 1995), Cry1Ac (Derbyshire et al., 2001), Cry2Aa (Morse et al., 2001), Cry3Bb (Galitsky et al., 2001), Cry4Ba (Boonserm et al., 2005), Cry4Aa (Boonserm et al., 2006) and Cry8Ea1 (Guo et al., 2009) show a great degree of similarity in their overall topology in spite of diversity in their amino acid sequences, indicated by 30-50% similarity in amino acid sequences.

The tertiary structure of Cry toxins produced by *B. thuringiensis* are formed by three conserved domains; Domains I, II, and III. Domain I is formed by five to seven amphiphilic α-helices with the hydrophobic α-helix 5 positioned in the middle surrounded by the remaining α-helices (Li et al., 1991; Grochulski et al., 1995). Domain II consists of three anti-parallel beta sheets (Li et al., 1991; Grochulski et al., 1995), and Domain III is a β sandwich with a "Jelly Roll" topology (Grochulski et al., 1995; Li and Ellar 1991).

With respect to the mode of action of Cry toxins, the long hydrophobic and amphipathic helices of Domain I mediate membrane insertion and pore formation by Cry toxins (Pigott et al., 2007; Girard et al., 2008; Girard et al., 2009; Lebel et al., 2009). The α-helices of Domain I are also shown to be necessary for triggering toxin oligomerization, an important step in the mode of action of Cry1A toxins (Jimenez-Juarez et al., 2007; Rodriguez-Almazan et al., 2009). The loops formed by the β-sheets of Domain II are involved in toxin-receptor interactions and are critical for toxicity (Rajamohan et al., 1995; Rajamohan et al., 1996; Lee et al., 2000b; Lee et al., 2001; Gomez et al., 2006; Pigott et al., 2007; Girard et al., 2009; Pacheco et al., 2009a). Domain III is shown to be involved in toxin stability and is also implicated to play a role in toxin binding to receptors (de Maagd et al., 1999a; de Maagd et al., 1999b; Lee et al., 1999; Pigott et al., 2007; Kitami et al., 2011).

Mode of action of Cry toxins

Unlike most bacterial pore forming toxins, Bt Cry toxins are produced as insoluble crystals, which upon ingestion are solubilized in the alkaline lumen of the midgut into a 70-160 kDa protoxin form (Hofmann et al., 1988; Aronson et al., 1991; Du et al., 1994). The solubilized protoxins are then activated by the gut proteinases, such as trypsins and chymotrypsins (Choma

et al., 1990a; Choma et al., 1990b; Lightwood et al., 2000), to its active form of 60-65 kDa (Choma et al., 1990b; Rukmini et al., 2000). The activated toxins traverse peritrophic matrix and interact with cadherin, a primary Bt receptor proteins present on the midgut microvilli, which causes a conformational change in the toxin leading to further proteolytic processing of the N-terminal end of the toxin. This enables toxins to form pre-pore oligomer complexes, that have increased affinity for secondary GPI-anchored receptors such as aminopeptidases (APN), or alkaline phosphatases (ALP) localized in special membrane islands called lipid rafts (Zhuang et al., 2002; Bravo et al., 2004). Oligomers insert into the membrane and disrupt membrane integrity by forming lytic-pores, which ultimately cause insect death by septicemia (Knowles et al., 1987; Bravo et al., 2004; Broderick et al., 2006).

A recent modification to the pore formation model proposes that activated toxin monomers first bind highly abundant low-affinity APN receptors before binding to high-affinity cadherin receptors which results in toxin oligomerization. Oligomers, while anchored to cadherin through loop-3 of domain II, interact with APN through domain III leading to membrane insertion and pore-formation (Pacheco et al., 2009a).

In contrast to the pore-formation model, the cell-signaling model (Zhang et al., 2005; Zhang et al., 2006; Zhang et al., 2008) proposes that binding of activated toxin monomers to cadherin activates an intercellular signaling pathway, which ultimately results in cell death. According to the cell signaling model, toxin-cadherin interaction causes an increase in cAMP production through activation of heterotrimeric G- protein (Gα) receptor and adenylyl cyclase. The cAMP activates protein kinase A, which in turn activates a cascade of events leading to programmed cell death by oncosis (Zhang et al., 2005; Zhang et al., 2006; Zhang et al., 2008).

Bt toxin receptors

Although a number of different Cry toxin binding proteins have been identified in the midgut epithelia of susceptible lepidopteran, dipteran, and coleopteran insects, the functionality of these binding proteins in the mode of action of Cry toxins may differ between insect species and toxins. Cry toxin binding proteins in Lepidoptera include cadherins (Vadlamudi et al., 1995; Nagamatsu et al., 1999), glycosylphophatidylinositol (GPI) anchored aminopeptidase N (APN; (Knight et al., 1994; Sangadala et al., 1994), GPI-anchored alkaline phosphatases (ALP; (McNall et al., 2003; Jurat-Fuentes et al., 2004a), glycoconjugates (Valaitis et al., 2001), and glycolipids (Griffitts et al., 2005; Ma et al., 2011). Of the various Cry toxin binding proteins, roles of cadherin, APN and ALP in the mode of action of Cry toxins are extensively studied in lepidopteran insects.

Cadherins belong to the super family of integral transmembrane single-chain glycoproteins, which mediate intercellular cell-cell adhesion through homotypic interactions in the presence of extracellular calcium (Gumbiner, 1996). Unlike mammals, where cadherins are localized in the basolateral cell-to-cell contact regions, intestinal cadherins in Bt susceptible lepidopterans and dipterans are localized on the apical border of the midgut microvilli (Chen et al., 2005; Hua et al., 2008). With regard to Bt toxins, there are numerous reports demonstrating the role of cadherin as a functional Cry toxin receptor in Bt susceptible species belonging to Lepidotera and Coleoptera (Pigott et al., 2007; Fabrick et al., 2009; Gao et al., 2011). Furthermore, correlation between mutations in cadherin genes and resistance to Bt toxins substantiates the importance of cadherin in Bt mode of action (Gahan et al., 2001; Morin et al., 2003; Jurat-Fuentes et al., 2004b; Xu et al., 2005; Fabrick et al., 2007; Soberon et al., 2007;

Yang et al., 2007; Xu et al., 2008a; Xu et al., 2008b; Bel et al., 2009; Khajuria et al., 2009; Yang et al., 2009; Liu et al., 2010; Zhao et al., 2010; Fabrick et al., 2011; Porta et al., 2011).

APNs are a group of exopeptidases that cleave amino acids from the N-terminus of peptides. Although Cry toxin binding to APN has been established in a number of lepidopteran and dipteran species (Pigott et al., 2007; Perera et al., 2009; Liu et al., 2010), the functional role of APN as an independent receptor of Cry toxin is not completely elucidated. Initial attempts at validating functionality of APN by heterologously expressing APN in *Drosophila melanogaster* S2 cells failed to increase cell susceptibility to Cry toxins suggesting that APN alone is not sufficient to facilitate Cry toxicity (Garner et al., 1999; Luo et al., 1999b; Banks et al., 2003). Functional role of APN in Cry1C toxicity in *Spodoptera litura* (Rajagopal et al., 2002), Cry1Ac toxicity in *Helicoverpa armigera* (Sivakumar et al., 2007), and Cry1Ab toxicity in *Ostrinia nubilalis* (Khajuria et al., 2011) and *Diatraea saccharalis* (Yang et al., 2010) was later shown by silencing midgut APNs by double-stranded RNA mediated gene silencing. Susceptibility of transgenic *D. melanogaster* expressing *Manduca sexta* APN to Cry1Ac toxin is another example demonstrating a functional role of APN in Cry toxicity (Gill et al., 2002). Recently, differential expression of APN was shown to correlate with Cry1Ac resistance in *Trichoplusi ni* (cabbage looper) (Tiewsiri et al., 2011) , *O. nubilalis* (Khajuria et al., 2009) and *H. armigera* (Zhang et al., 2009).

Alkaline phosphatases are another group of GPI-anchored Cry toxin binding proteins present in the insect midgut. Binding of Cry toxins to ALP has been reported in a number of susceptible lepidopteran insects; *M. sexta* (McNall et al., 2003; Arenas et al., 2010), *Heliothis virescens* (Jurat-Fuentes et al., 2004a), *Anthonomus grandis* (cotton boll weevil) (Martins et al., 2010), and *H. armigera* (Upadhyay et al., 2011). A low level of ALP detected in Cry1A toxin

resistant *H. virescens*, Cry1Ac toxin resistant *H. armigera*, and Cry1Fa toxin resistant *Spodoptera frugiperda* evidence that ALP does play a functional role in the mode of action of Cry toxins (Jurat-Fuentes et al., 2004a; Jurat-Fuentes et al., 2011).

Apart from the above mentioned major toxin binding proteins, Cry toxin binding to glycolipids (Griffitts et al., 2005; Ma et al., 2011) and glycoconjugates (Valaitis et al., 2001) is reported. Modification of glycolipids allows resistance to Cry5B, as *Caenorhabditis elegans* mutants lacking a glycosyltransferase gene, *bre5*, survive Cry5B intoxication (Griffitts et al., 2001). Subsequently, specific binding of Cry1Ac, Cry1Ab and Cry1Aa toxins to *M. sexta* glycolipids was shown (Griffitts et al., 2005). In contrast to toxin interaction with glycolipids through Domain III of Cry toxins that results in toxicity, Ma et al. (2011) reported Cry1Ac and Cry2Ab toxin interaction through Domain II, which results in toxin sequestration and tolerance. Cry toxin binding to glycoconjugates was reported in gypsy moth, *Lymantria dispar* where Cry1Aa and Cry1Ab toxins bind with high affinity to a 270 kDa glycoconjugate (Valaitis et al., 2001).

1.3 Role of toxin binding and receptor sharing in toxin selection for pest control

Toxin binding to receptors present on midgut microvilli is one of the most crucial steps in the mode of action of Cry toxins. Activated Cry toxins bind with high affinity and specificity to receptors present in insect midgut microvilli of susceptible insects (Ferre et al., 2002; Pigott et al., 2007). High affinity binding to midgut receptors however does not always correlate with high toxicity (Wolfersberger, 1990; Garczynski et al., 1991; Luo et al., 1999b) as disruption in any of the downstream steps in the mode of action of Cry toxins including toxin oligomerization, membrane-insertion, and pore-formation, can also affect Cry toxicity.

Toxin binding studies have shown that multiple toxins can share a single binding site(s). The high correlation between receptor sharing and cross-resistance among multiple Cry toxins suggests that toxins that share a common binding site(s) will have a high probability of cross resistance in the event that the common binding site(s) is altered (Ferre et al., 2002; Hernandez et al., 2005; Heckel et al., 2007; Pigott et al., 2007; Gouffon et al., 2011). Conversely, toxins that do not share binding site(s) or bind additional site(s) apart from the shared site(s) will have less probability of developing cross-resistance. Due to its accuracy in predicting cross-resistance development, toxin-binding models are widely used for selecting Cry toxins for toxin-stacking (Ferre et al., 1991; Tabashnik et al., 1994; Ferre et al., 2002).

The role of receptor sharing in cross-resistance development is most extensively studied in the diamondback moth (*Plutella xylostella*); the first insect reported to have evolved high levels of resistance to Bt toxins in the field (Tabashnik et al., 1990; Ferre et al., 2002). A four-site toxin-binding model was proposed to explain receptor sharing and cross-resistance development in the diamondback moth. According to this model, site one binds Cry1Aa, site two binds Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, and Cry1Ja, site three binds Cry1Ba, and site four binds Cry1Ca (Ferre et al., 2002). This model explains the cross-resistance pattern to different Cry toxins in the resistant *P. xylostella* strains studied thus far (Ferre et al., 2002; Sayyed et al., 2005b; Higuchi et al., 2007; Gong et al., 2010).

In *H. virescens*, a three-site binding model was proposed to explain Cry1A, Cry1Fa and Cry1Ja toxin interaction (Van Rie et al., 1989; Jurat-Fuentes et al., 2001; Jurat-Fuentes et al., 2006). According to this model, Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa and Cry1Ja toxins share a common binding site A. Cry1Ab and Cry1Ac share binding site B, while Cry1Ac binds to a third distinct binding site which it does not share with the other toxins. A recent modification to the

above mentioned model proposes another Cry1A specific population of binding sites not shared by Cry1Fa toxin (Jurat-Fuentes et al., 2001; Gouffon et al., 2011).

Toxin binding models are developed for Cry toxins that have utility in pest control due to their high, selective toxicity against economically important pests. Cry1Ab, Cry1Ac, Cry1Fa, and Cry1Ca are some of the important toxins used in crop pest management. In *Spodoptera exigua* and *S. frugiperda,* Cry1Fa shares one binding site(s) with Cry1Ac and a second binding site(s) with Cry1C (Luo et al., 1999a; Sena et al., 2009). In *Helicoverpa zea* and *H. armigera*, Cry1Fa, Cry1Ac, and Cry1Ja share a common binding site(s) (Hernandez et al., 2005). Gouffon et al. (2011) reported an additional Cry1A binding site(s) in *H. zea* not shared with Cry1Fa toxin. In *O. nubilalis*, Cry1Ab, Cry1Ac, and Cry1Fa share a binding site(s) for which Cry1Fa has lower affinity than the Cry1A toxins (Hua et al., 2001). Apart from the shared binding site(s), Cry1Ab has an additional binding site(s) in *O. nubilalis* that is not shared with Cry1Ac and Cry1Fa toxins (Hua et al., 2001).

1.4 Cry toxins and crop pest management

The insecticidal properties of *B. thuringiensis* were first used as biopesticides in France around 1938. The discovery of the insecticidal crystal by Angus in 1956 (Angus, 1968) and a growing awareness of the hazards of chemical pesticides reinvigorated studies of Cry toxins and their use in crop and forest pest management. Initial use of Bt biopesticides as formulations was inconsistent in controlling crop pests, due to environmental inactivation of the toxins under unfavorable weather conditions and due to poor coverage (Nester et al., 2002). This problem was solved for crop plants by incorporating *cry* genes into the genome and expressing Cry proteins

directly in plant tissue. The efficacy of Cry toxin expressing crops in suppressing targeted pests made Cry toxins a versatile tool in crop pest management.

First generation transgenic crop varieties expressing Cry toxin were commercialized in 1996 (Reviewed in de Maagd et al., 1999c). Monsanto's NewLeaf potato variety expressing Cry3A, corn varieties KnockOut by Syngenta (Basel, Switzerland) and NatureGard by Mycogen (Indianapolis, IN) expressing Cry1Ab toxin, and the cotton varieties Bollgard and Ingard by Monsanto (St. Louis, MO) expressing a modified Cry1Ac toxin were the first transgenic Bt varieties released for commercial cultivation. Subsequently, a number of singe-gene transgenic crop varieties were developed and released for commercial cultivation. Corn varieties included Agrisure CB (by Northrup King, Minneapolis, MN; expressing Cry1Ab), YieldGard (by Monsanto; expressing Cry1Ab), Herculex (jointly developed by Pioneer Hi-Bred, Johnston, IA and Dow AgroSciences, Indianapolis, IN; expressing Cry1F), and YieldGard Rootworm (by Monsanto; expressing a synthetic variant of the *cry3Bb1* gene).

Although the single-gene transgenic crop varieties expressing Cry toxin were effective in suppressing targeted pests, due to their narrow spectrum of specificity they failed to substantially reduce the need for chemical pesticides (NAS 1999, p. 114). Crop damage from secondary pests was another disadvantage of the narrow specificity of first generation transgenic crop varieties expressing Cry toxins. The limitations of the first generation transgenic crop varieties expressing single Cry protein were mitigated by second generation transgenic crop varieties expressing multiple Cry toxins with alternative modes of action and specificity. The transgenic cotton variety expressing Cry1Ac and Cry2Ab toxins, Monsanto's Bollgard II cotton, was the first transgenic variety expressing multiple toxins approved for commercial use in 2002. This was followed by the release of another cotton hybrid expressing Cry1F and Cry1Ac toxins in 2005;

Widestrike cotton (Dow Agrosciences). Bollgard II and Widestrike varieties provided better protection against bollworm and budworm compared to the first generation transgenic cotton varieties expressing single Cry protein (Gore et al., 2008).

With respect to transgenic corn expressing Cry toxins, the second-generation corn varieties broadened pest control spectrum by combining corn borer protection with root worm protection. Examples of transgenic corn varieties with dual protection against lepidopteran and coleopteran pests include YieldGard Plus expressing Cry1Ab1 and Cry3Ab1, and Herculex XTRA and Optimum AcreMax1expressing Cry1F and Cry34/35Ab1 toxins. Two transgenic corn varieties expressing vegetative insecticidal protein (Vip3A) together with Cry1Ab toxin (Agrisure Viptera 3110) or Cry1Ab and modified Cry3A toxins (Agrisure Viptera 3111) were released by Syngenta (Basel, Switzerland) to provide protection against all major lepidopteran and coleopteran pests of corn. Recently, Monsanto released Genuity corn lines that include Genuity VT Double Pro (Cry1A.105 and Cry2Ab2) resistant to earworm, borer, and armyworm; Genuity VT Triple Pro (Cry1A.105, Cry2Ab2, and Cry3Bb1) resistant to earworm, corn borer, rootworm, and armyworm; and Genuity SmartStax (Cry1A.105, Cry2Ab2, Cry1F, Cry34/35Ab1, and Cry3Bb1) resistant to corn earworm, borer, rootworm, cutworm, and armyworm. Another transgenic corn variety released in China combined Cry1Ac toxin with modified cowpea trypsin inhibitor (CpTI) to delay resistance development against Cry1Ac toxin (He et al., 2009).

1.5 Insect resistance to Cry toxins

While great strides have been made in pest control by the introduction of transgenic Bt crops, the commercial use of Bt toxins carries the threat that insects will become resistant to Bt toxins (Bravo et al., 2008; Tabashnik, 2008; Tabashnik et al., 2009). The potential to develop

resistance against Cry toxin was first shown in laboratory-selected colonies of Indian meal moth, *Plodia interpunctella (McGaughey, 1985)*, but field-evolved resistance against Bt toxins was not observed until 1988. *P. xylostella* is the first insect that developed resistance against sprayable Bt formulations in the field in the Phillipines and Hawaii (Kirsch, 1988; Tabashnik et al., 1990). Subsequently, potential to develop resistance against Cry toxins was demonstrated in laboratory-selected populations of tobacco budworm, *H. virescens* (Gould et al., 1992); diamondback moth, *P. xylostella* (Tabashnik et al., 1994*)*; beet armyworm, *S. exigua* (Moar et al., 1995); European corn borer, *O. nubilalis* (Huang et al., 1999; Siqueira et al., 2004); pink bollworm, *Pectinophora gossypiella* (Tabashnik et al., 2004b); and cotton bollworm, *H. armigera* (Akhrst et al., 2003; Gunning et al., 2005; Xu et al., 2005).

Although rare, field-evolved resistance against commercially available transgenic Bt crops has been reported in some populations of *S. frugiperda* (fall armyworm), *Busseola fusca* (African corn stem borer), *H. zea, and P. gossypiella*. The widespread resistance of fall armyworm to Cry1F corn in Puerto Rico resulting in withdrawal of the Bt corn variety from the market is the fastest documented case of field-evolved resistance to Bt crops (Storer et al., 2010). In *H. zea*, evidence of field-evolved resistance to Bt cotton expressing Cry1Ac toxin and Bt corn expressing Cry2Ab toxin was reported in the Southeastern cotton and corn growing belt of USA (Luttrell et al., 1999; Ali et al., 2006; Ali et al., 2007; Tabashnik, 2008; Tabashnik et al., 2008). Recently, field evolved resistance against YieldGard corn expressing Cry1Ab toxin was reported in African corn stem borer in South Africa that caused severe damage to Bt corn, requiring additional insecticidal sprays (van Rensburg, 2007). Reported Cry1Ac resistance in *P. gossypiella* populations in Bt cotton expressing Cry1Ac toxin in India is another case of field-evolved resistance to Bt crops (Tabashnik et al., 2010; Dhurua et al., 2011).

Mechanisms of acquired insect resistance to Cry toxins

A number of different mechanisms have been reported by which insects can develop resistance to Bt toxins; these include receptor alteration resulting in decrease or loss of toxin binding to gut receptors (Van Rie et al., 1990; Gahan et al., 2001; Loseva et al., 2002; Sayyed et al., 2004; Sayyed et al., 2005a; Heckel et al., 2007; Bel et al., 2009; Caccia et al., 2010; Gahan et al., 2010), altered proteolytic activation of toxin (Oppert et al., 1997; Lightwood et al., 2000; Candas et al., 2003; Chandrashekar et al., 2004; Li et al., 2004; Rajagopal et al., 2009), increased toxin degradation (Forcada et al., 1999; Oppert et al., 2011), and toxin sequestration (Gunning et al., 2005; Ma et al., 2011). Another mechanism of resistance reported in both *M. sexta* (Spies et al., 1985) and *H. virescens* (Forcada et al., 1999) is the faster regeneration of midgut epithelial cells in resistant insects.

Despite the possibility of developing resistance through multiple mechanisms reported in laboratory-selected resistant strains, binding site alteration is the most widely reported mechanism of resistance observed in field-derived Cry toxin resistant populations (Heckel et al., 2007). Binding site alteration can occur either due to mutations or due to down regulation of receptors important for toxicity. For example, a mutation in the cadherin gene has been reported to correlate with Cry1Ac resistance in three lepidopteran species; *H. virescens*, *P. gossypiella* (pink bollworm), and *H. armigera* (cotton bollworm). In *H. virescens*, high level of resistance to Cry1Ac toxin has been linked to loss of toxin binding to cadherin due to retrotransposon-mediated insertions in the cadherin gene (Gahan et al., 2001). Cry1Ac resistance in *P. gossypiella* has been associated with reduced toxin binding to cadherin due to deletion mutations in the cadherin gene (Morin et al., 2003; Fabrick et al., 2011). In *H. armigera*, loss of binding

due to a premature stop codon or a deletion in the cadherin gene has been shown to cause Cry1Ac resistance (Xu et al., 2005; Yang et al., 2006; Zhao et al., 2010).

Apart from alterations in cadherin binding, downregulation of APN and ALP receptors are also implicated to play a role in resistance development against Cry toxins. For example, Cry1C resistance in *S. exigua* correlates with the complete absence of an APN isoform (Moar et al., 1995; Herrero et al., 2005). Reduced expression of an APN isoform in *T. ni* (Tiewsiri et al., 2011) and a deletion mutation in an APN isoform in *H. armigera* (Zhang et al., 2009) are associated with Cry1Ac resistance. Additionally, reduced level of ALP is shown to correlate with Cry1A toxin resistance in *H. virescens*, Cry1Ac toxin resistance in *H. armigera,* and Cry1Fa toxin resistance in *S. frugiperda* (Jurat-Fuentes et al., 2004a; Jurat-Fuentes et al., 2007; Jurat-Fuentes et al., 2011)**.**

1.6 Important pests of corn

Corn (*Zea mays* L.) is cultivated world-wide and is the third leading cereal crop after wheat and rice. Corn production is affected negatively by a number of lepidopteran and coleopteran insect pests, which increase cost of production and/or reduce crop yields. In North and South America, some of the important pests of corn include *O. nubilalis* (European corn borer; ECB), *D. grandiosella* (southwestern corn borer; SWCB), *H. zea* (corn earworm; CEW), *S. frugiperda* (fall armyworm; FAW), and corn rootworm complex (CRW) (Steffey, 1999).

The European corn borer (ECB) which was introduced into North America in the early 1900s, has established itself as the most destructive pest of corn in North America with damages exceeding $1 billion annually. Apart from corn, ECB is also a pest of snap beans, tomatoes, soybeans, and cotton. ECB completes one generation per year in the North, two generations in

the Midwest, and three to four generations in the South. ECB over winters as late stage larvae within corn stubble and other corn debris left in the field after harvesting. Newly hatched first generation larvae feed on the developing whorls. Late stage larvae bore into stalks and excavate tunnels, in which they pupate. The second generation larvae coincide with the tassel stage of the crop and mainly feed by boring into the ears and stalks. Tunneling in the stalk and ear interferes with the plant's ability to translocate water and nutrients resulting in reduced ear and kernel size. Stalk and ear tunneling also cause yield losses due to broken plants and dropped ears. Corn ear damage causes seed yield loss and can reduce quality of seedcorn, popcorn, and fresh market sweet corn (Steffey, 1999).

The corn rootworm (CRW) complex, common pests of corn in the Corn Belt of the United States includes three rootworm species; western (*Diabrotica virgifera virgifera*), northern (*Diabrotica barberi*), and southern (*Diabrotica undecimpunctata howardi*) corn rootworms. The CEW complex causes an estimated loss of $1 billion per annum in the United States in yield loss and money spent to control this pest (Gray et al., 2009). CRW larvae feed on the roots, which results in reduced ability of the plant to absorb water and nutrients from soil. Larval feeding on the root called root pruning also causes harvesting difficulties due to plant lodging. Root feeding and larval tunneling into the roots results in infection by plant pathogens that causes root rot; the most prevalent disease that occurs due to CRW feeding. The adults feed on leaf tissue, freshly exposed corn silks, pollen, and young corn kernels on exposed ear tips (Steffey, 1999).

The SWCB is an important pest of corn in the Southern corn belt from Arizona to Georgia. It completes two to three generations per year. The first generation larvae feed on the corn whorl giving rise to the shot-hole appearance when the whorl unfurls. Larvae also feed on the terminal bud resulting in stunted and bushy plants, and the late instar larvae bore into the

stem. The second generation larvae typically feed on cobs, husks, shanks, leaf sheaths, and ear kernels. Substantial plant injury occurs when the second and third generation larvae bore into the stem causing stunted plants and plant lodging (Steffey, 1999).

The CEW is native to the Americas and is found throughout the United States. In addition to corn, it is also a pest of cotton, tomato, and soybean. CEW completes several generations during the growing season. The larvae feed on corn leaves, tassels, silk, and ear kernels. Damage of the corn ear also provides a point of entry for secondary pests and microbes (Steffey, 1999).

FAW is an important and most difficult to control pest of corn found throughout the United States east of the Rocky Mountains and South America. FAW over-winters as larvae in Southern Florida and Southern Texas and the adults migrate northward in spring. FAW completes one to three generations in the northern states, and more than five generations in the Southern states. Larvae cause extensive defoliation, damage to whorls, corn ears and kernels (Steffey, 1999).

1.7 Cry toxin synergists

Like chemical pesticides, resistance development in targeted pests is the major threat to the long term efficacy of Bt Cry toxins. Recent reports of field-evolved resistance against transgenic Bt crops exemplifies the need for strategies to delay resistance development in the field. Of the several strategies employed to delay resistance development against Cry toxins; refuge strategy (Tabashnik, 2008) and stacking multiple Cry toxins in transgenic Bt hybrids are the most successful strategies that have proven effective in the field. However, success of these strategies depends on the expression of high-dose of Cry toxins in Bt plants (Gould, 1998;

Tabashnik et al., 2004a). Use of synergists to enhance toxicity, a strategy successfully employed to delay resistance development against chemical pesticides (Bernard et al., 1993), is a possible alternative to achieving high level expression of Cry toxins in Bt crops. The term synergist refers to materials with no or low activity when applied alone but enhance the effect of active compounds in a formulation (Walker, 2001). With respect to Bt toxins, several Cry toxin synergists of Bt and non-Bt origin have been reported that show moderate to high level of synergism of Cry toxicity.

Cry toxin synergists of non-Bt origin

A number of Bt Cry synergists of non-Bt origin have been reported that show low to moderate enhancement of Cry toxicity; zwittermicin (Broderick et al., 2000), enhancin (Granados et al. 2001), endochitinase (Regev et al., 1996), and avidin (Zhu et al., 2005). Zwittermicin is a broad spectrum antibiotic produced by *Bacillus cereus* (He et al., 1994), which enhances toxicity of Bt subsp. *kurstaki* to *L. dispar* larvae (Broderick et al., 2000). The synergism of Cry toxins by zwittermicin is attributed to the antimicrobial activity of zwittermicin, which inhibits the growth of a variety of gut microbes required for normal growth and development of insects (Broderick et al., 2000).

Avidin, a biotin-binding glycoprotein found in egg white, is another example of a synergist that synergizes Cry1Ac toxicity against *H. zea*, *S. exigua*, and *Anticarsis gemmatalis* (Zhu et al., 2005). Avidin is toxic to insects as it sequesters availability of biotin resulting in stunted growth and mortality (Kramer et al., 2000). Avidin is also expressed in transgenic avidin corn (Kramer et al., 2000), tobacco (Markwick et al., 2001), and apple (Markwick et al., 2001) to provide protection against important crop pests.

Two peritrophic membrane (PM) disrupters, enhancin and ChiAII, are also reported to synergize Cry toxicity by increasing the permeability of peritrophic matrix. Enhancin is a metalloprotease from granulosis virus (Lepore et al., 1996), while ChiAII is an endochitinase from the bacterium *Serratia marcescens(Regev et al., 1996)*. Enhancin has been shown to enhance toxicity of Dipel to *Trichoplusia ni, Pseudoplusia includens, Anticarsia gemmatalis*, *H. virescens*, *H. zea* and *S. exigua* larvae (Granados et al., 2001). ChiAII was shown to enhance Cry1C toxicity to Cry1C tolerant *S. littoralis* (Regev et al., 1996).

Cry toxin synergists of Bt origin

Cry toxin synergists of Bt origin include cytolytic toxins (Cyt) and Bt enhancin like proteins (Bel). Cyt toxins are usually produced by the mosquitocidal Bt strains and can lyse a wide variety of cell types (Wirth et al., 2000). In contrast to Cry toxins, Cyt toxins have a different mechanism of action. Cyt toxins insert into the membrane and form pores by directly interacting with membrane lipids (Li et al., 1996; Butko, 2003; Promdonkoy et al., 2003). Due to their ability to bind membrane lipids, Cyt toxins of Bt subsp. *israelensis* show high-level synergism to mosquitocidal Cry toxins (Wirth et al., 2000; Sayyed et al., 2001; Perez et al., 2007) by functioning as a membrane bound receptor for the Cry toxins (Perez et al., 2005).

Similar to viral enhancins, Bel proteins produced by Bt disrupt PM by degrading insect intestinal mucin (Fang et al., 2009). The synergistic property of Bel protein was reported in *H. armigera*, where Bel protein enhanced Cry1Ac toxicity by 6-fold when larvae were fed a mixture of Bel protein and Cry1Ac toxin (Fang et al., 2009).

Receptor-based Cry toxin synergists

The discovery of *M. sexta* cadherin as a Bt Cry toxin synergist was the first time a receptor has been reported to enhance toxicity (Chen et al., 2007) . Chen et al. (2007) showed that a *M. sexta* cadherin fragment containing cadherin repeat (CR) 12 and membrane proximal region (MPED) enhances Cry1Ab toxicity to *M. sexta* and Cry1Ac toxicity to *H. virescence* and *H. zea* larvae. Further research showed that the *M. sexta* cadherin peptide CR10-12 has increased synergy relative to the original CR12-MPED peptide (Abdullah et al., 2009). The synergistic potential of the *M. sexta* cadherin fragment has encouraged further research in this area (Chen et al., 2007). Subsequently, synergistic effect of cadherin has been demonstrated using cadherin fragments from dipterans (mosquito) with mosquitocidal Cry4Ba (Hua et al., 2008; Park et al., 2009a), from coleopterans (beetle) with beetle-toxic Cry3Aa and Cry3Bb toxins (Park et al., 2009a), and an additional example of cadherin synergy of lepidopteran-active Cry toxins (Peng et al., 2010). Additionally, the role of APN as a Cry toxin synergist was recently reported in *Anopheles gambiae* where a fragment of the APN2 receptor enhanced Cry11Ba toxicity (Zhang et al., 2010).

Although the synergistic potential of cadherin fragments generated a great deal of interest (Chen et al., 2007; Hua et al., 2008; Pacheco et al., 2009b; Park et al., 2009a; Park et al., 2009b; Peng et al., 2010; Gao et al., 2011), how cadherin fragments enhance Cry toxicity is yet to be fully understood. Two mode of action models were proposed to explain the mechanism of cadherin-based Cry toxins synergism; surrogate receptor model and oligomer formation model (Fig. 1.1). According to the surrogate receptor model, cadherin fragments enhance Cry toxicity by anchoring toxins to the membrane resulting in increased probability of toxin-membrane interactions (Chen et al., 2007; Peng et al., 2010). The high toxin binding affinity of cadherin

fragments has been shown to be essential for their synergistic properties as deletion of the toxin binding site resulting in loss of binding renders them inactive (Chen et al., 2007; Peng et al., 2010). Additionally, different cadherin fragments that do not bind Cry toxin are shown to lack synergistic ability (Park et al., 2009b); Dr. Mohd. Amir Abdullah, personal communication). However, the role of BBMV binding ability and surrogate receptor function of synergistic cadherin fragments are yet to be fully established. A cadherin fragment from *Anopheles gambiae* that inhibits toxin binding to BBMV, but enhances Cry4Ba toxicity (Park et al., 2009b) suggests that ability of synergistic cadherin fragments to bind BBMV and act as surrogate receptors may not be the only synergistic mechanism.

According to the oligomer formation model, ability of cadherin fragment to induce toxin oligomerization was proposed as a mechanism of cadherin based Cry toxin synergism (Fig. 1.1) (Pacheco et al., 2009b; Peng et al., 2010). Toxin oligomer formation is an essential step in the mode of action of Cry toxins and oligomers are shown to have higher toxicity than monomeric toxins (Bravo et al., 2004; Munoz-Garay et al., 2006). Therefore, ability of cadherin fragments to induce toxin oligomers seemed a plausible explanation of mechanism of Cry toxin synergism by cadherin fragments. However, both studies reported oligomer formation when cadherin fragments were incubated with protoxin in the presence of gut extract (Pacheco et al., 2009b; Peng et al., 2010). Since, cadherin fragments have limited ability to enhance protoxin (Amir Abdullah, personal communication), a correlation between ability of cadherin fragments to induce oligomer formation and their synergistic ability is questionable. Additionally, a *H. armigera* cadherin fragment that induces formation of pre-pore oligomers but reduces Cry1Ac toxicity (Liu et al., 2009) suggest existence of alternative or additional synergistic mechanisms. Taken together, data on the mode of action of cadherin based Cry toxin synergism suggest that

cadherin fragments may enhance Cry toxicity by acting as a surrogate receptor or by inducing toxin oligomers, however, these two are not the only mechanisms of cadherin based Cry toxin synergism.

1.8 Dissertation rationale

The Cry toxins produced by *Bacillus thuringiensis* (Bt) are the active component of the most widely used biopesticide. Since 1996, farmers have reduced use of chemical insecticides by growing crops genetically engineered to produce Bt Cry toxins. Nevertheless, like chemical insecticides, development of resistance is a primary threat to the long-term efficacy of Bt Cry toxins.

Several insect resistance management strategies have been proposed to delay resistance development against Cry toxins; high dose refuge strategy mandated by the United States Environmental Protection Agency and use of multiple Cry toxins or toxin-stacking are the two most widely used strategies effective in delaying resistance development in the field (Gould, 1998; Tabashnik, 2008). The refuge strategy is based on three assumptions; inheritance of resistance is recessive, Bt crops express high dose of toxin, and the resistant pests surviving on Bt crop will mate with susceptible pests in the nearby refuge to produce hybrid progeny that will die on the Bt crop (Tabashnik, 2008). As the name suggests, the high dose refuge strategy requires expression of high level of Cry toxins to kill all the hybrid progeny (Gould, 1998; Tabashnik et al., 2004a). Since further increase in the amount of Cry toxins expressed in Bt plants is not technologically feasible, use of synergists to enhance toxicity is a possible alternative to achieving high level expression of Cry toxins in Bt crops.

Cry1Fa is an important toxin used in commercial corn cultivars to control lepidopteran pests. Cry1Fa is highly toxic to the primary pests of corn, *O. nubilalis*, in the North American corn growing regions but is only moderately toxic against *S. frugiperda*, a secondary pest of corn in North America but primary pest of corn in South America (Sena et al., 2009). Corn cultivars expressing Cry1Fa toxins are effective in suppressing *O. nubilalis* and no incidence of field evolved resistance against Cry1Fa toxin was reported in the North American corn growing regions. Recently, Cry1Fa corn was introduced in Puerto Rico where *S. frugiperda* is a primary pest of corn. The moderate toxicity of Cry1Fa toxin against *S. frugiperda* along with other environmental factors resulted in rapid resistance development against Cry1Fa corn in Puerto Rico resulting in crop loss and withdrawal of the variety from the market (Table 1.1) (Matten, 2007; Matten et al., 2008; Storer et al., 2010). As a Cry1Fa toxin synergist will have utility in managing *S. frugiperda*, I tested the ability of *M. sexta* cadherin fragment CR10-12 (MsCad) to enhance Cry1Fa toxicity against *S. frugiperda*. Although MsCad is an excellent Cry1A toxin synergist against lepidopteran larvae (Abdullah et al., 2009), MsCad only marginally synergized Cry1Fa toxicity against *S. frugiperda* larvae. Reasoning that CR10-MPED peptide from the orthologue *S. frugiperda* cadherin may enhance Cry1Fa toxicity to *Spodoptera* larvae, a region in *S. frugiperda* cadherin most similar to MsCad was identified, synthesized, and cloned into *E. coli* for protein production. To test whether SfCad is a better Cry1Fa synergist, the synergistic effect of the peptides was quantified using a dose response bioassay. As hypothesized, SfCad caused a significant increase in Cry1Fa toxicity to *S. frugiperda* compared to MsCad. Although this solved our problem of finding a Cry1Fa synergist, it raised another question - how did MsCad and SfCad differentially enhance Cry1Fa toxicity against *S. frugiperda*? To address this question, in the first part of my dissertation research (Chapter 2), I have studied the mode of action of

cadherin based Bt cry toxins synergists by utilizing differential Cry1Fa toxin enhancing properties of *S. frugiperda* (SfCad) and *M. sexta* (MsCad) cadherin fragments.

The second part of my dissertation relates to the use of toxin stacking which is the co-expression of multiple toxins in the same plant. Toxin stacking is a resistance management strategy which can be used to not only delay resistance development but also to broaden the spectrum of insecticidal activity of Bt crops (Bates et al., 2005; Tabashnik et al., 2008; Bravo et al., 2011). One of the criteria used to select multiple toxins for co-expression is that the toxins should have the least probability of cross-resistance development in the targeted pests (Roush, 1998; Bravo et al., 2008). Since alterations in a common binding site have been shown to confer resistance to toxins that share that binding site (Lee et al., 1995; Ballester et al., 1999; Gonzalez-Cabrera et al., 2003), toxin-binding models are used to predict cross-resistance development among multiple toxins (Ferre et al., 1991; Tabashnik et al., 1994; Ferre et al., 2002).

Cry1Ab and Cry1Fa toxins are two important toxins used in corn and cotton pest control and as such are incorporated in many Bt corn or cotton hybrids alone or in combination with other toxins. Although both Cry1Ab and Cry1Fa are highly toxic to the primary pests of corn *O. nubilalis* and *D. grandiosella*, only Cry1Fa has moderate toxicity against *S. frugiperda* (Table 1.1). The toxicity range of Cry1Ab and Cry1Fa toxins make them ideal candidates for stacking to improve efficacy and target range of transgenic Bt corn varieties. To this end, a number of Bt corn varieties expressing Cry1Ab and Cry1Fa toxins along with other Cry toxins are being developed (Lang, B. A. et al. 2008. Pub. NO. US 2008/031106). However, inadequate knowledge of mode of action of Cry1Fa toxin questions the long term utility of these Bt varieties.

The mode of action of Cry1Ab toxin is well studied, but difficulties in labeling Cry1Fa without compromising its activity has limited direct study of Cry1Fa interactions with binding sites in larval brush border. The current Cry1Ab toxin binding model developed using ^{125}I-Cry1Ab as probe and unlabeled Cry1Fa as competitor shows that Cry1Ab and Cry1Fa share a binding site(s) in *O. nubilalis* for which Cry1Fa has lower affinity than the Cry1Ab toxin (Fig. 1.2) (Hua et al., 2001). Apart from the shared binding site(s), Cry1Ab has an additional binding site(s) in *O. nubilalis* that is not shared with Cry1Fa toxins (Hua et al., 2001). In *H. zea*, Hernandez et al. (2005) reported a single binding site(s) shared by both Cry1Ab and Cry1Fa, while Gouffon et al. (2011) reported an additional Cry1Ab binding site(s) not shared with Cry1Fa . Although these binding site models describe binding characteristics of Cry1Ab toxin, they do not provide information as to whether Cry1Fa binds to additional high affinity site(s) apart from the ones shared with Cry1A toxins. The correct prediction of cross-resistance development between Cry1Ab and Cry1Fa toxin will require determining binding characteristics of Cry1Fa toxin and its interaction with Cry1Ab toxin. Therefore, in the second part of my dissertation research (Chapter 3), I have developed a fluorescence-based binding assay to enable direct study of Cry1Fa toxin interactions with BBMV preparations from three important pests of corn, and determined whether Cry1Fa shares binding site(s) with Cry1Ab toxin in these species.

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Table 1.1. LC_{50} (ng/cm^2) of Cry1Fa and Cry1Ab toxins to lepidopteran corn pests.

	***O. nubilalis* (ECB)[ab]**	***H. zea* (CEW)[ab]**	***S. frugiperda* (FAW)[ab]**	***D. grandiosella* (SWCB)[ac]**
Cry1Ab	13.1	229.9	8200	0.14 (0.11-0.17)
Cry1Fa	29.3	459.8	587	

a LC_{50} values are reported as ng/cm^2

b Malvar et al., 2007 United States Patent 7618942

c Huang et al., 2006 J. Econ. Entomol. 99(1) 194-202

Figure 1.1. Schematic diagram of proposed mechanisms of cadherin-based Cry toxin synergism. According to the surrogate receptor model, cadherin fragments may increase probability of toxin membrane interactions by acting as a surrogate receptor of Cry toxin. Alternatively, cadherin peptides may enhance Cry toxicity by inducing formation of toxin oligomers in the insect midgut. Modified from Jurat-Fuentes and Adang, 2006.

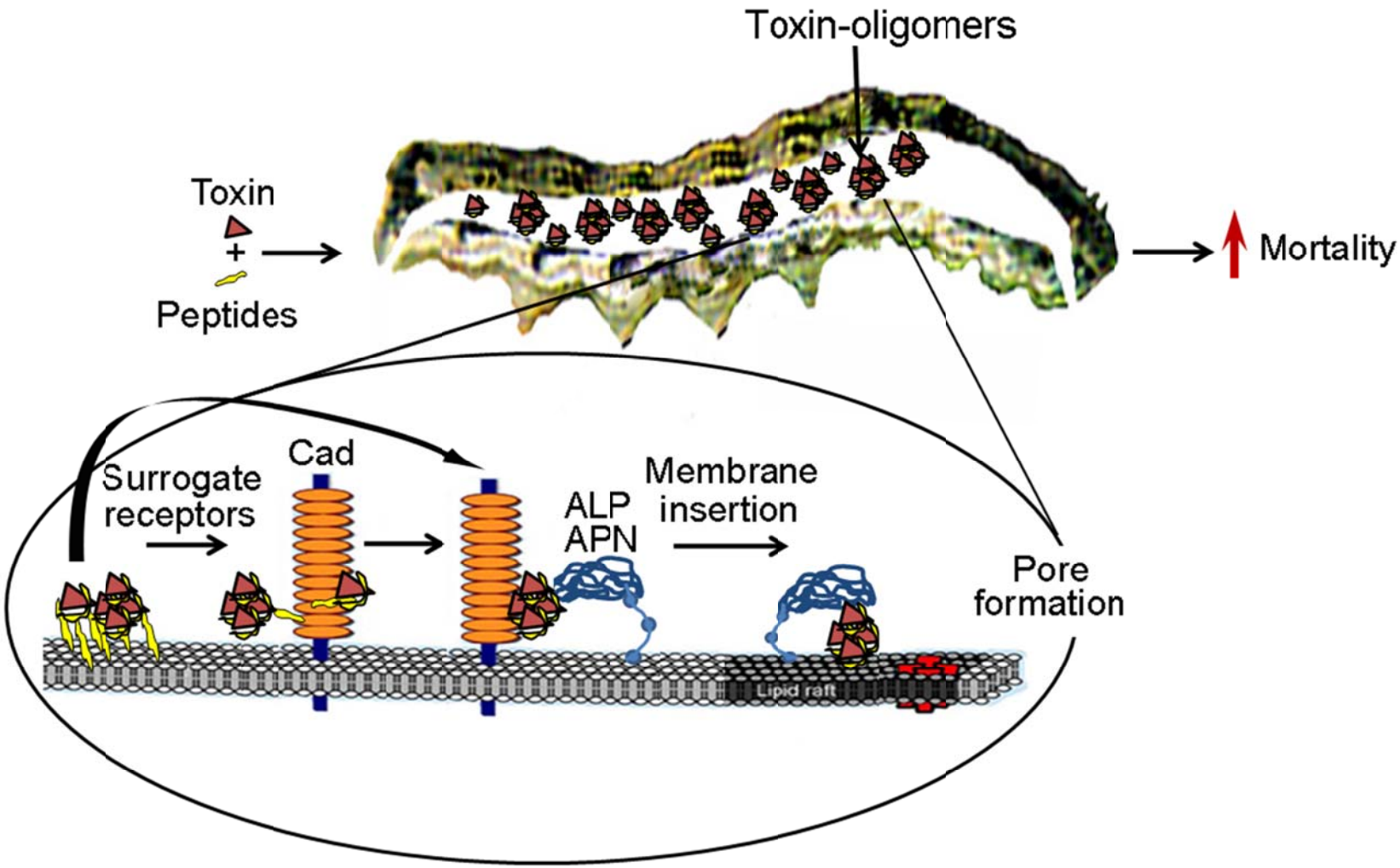
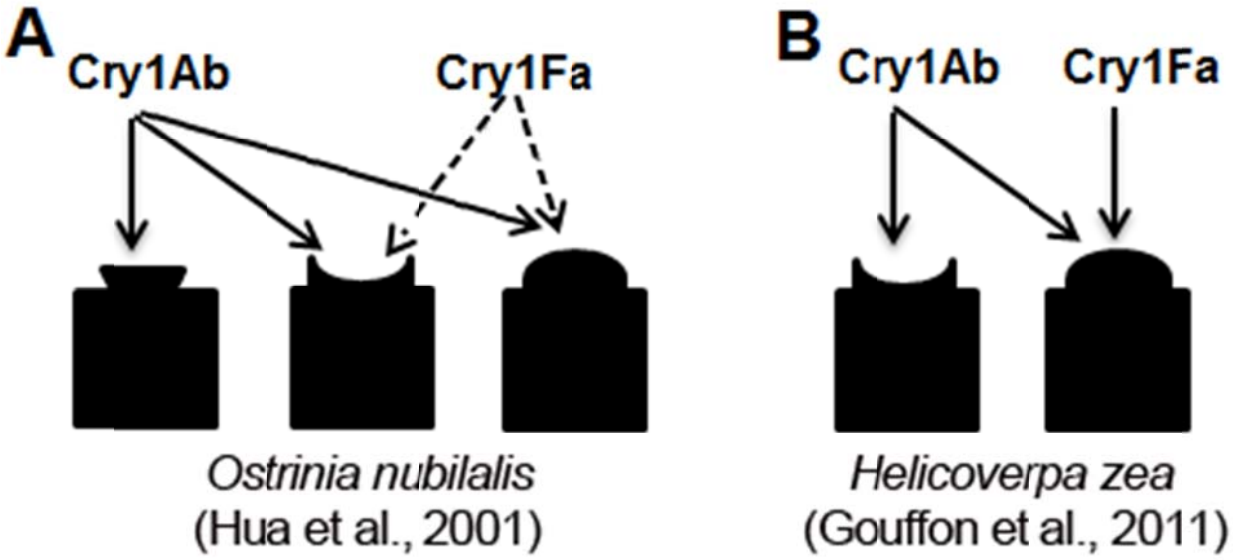

Toxin-oligomers
Toxin
+
Peptides
Mortality
Surrogate receptors
Cad
ALP
APN
Membrane insertion
Pore formation
Lipid raft

Figure 1.2. Cry1Ab and Cry1Fa toxins interaction with the midgut receptors in the *O. nubilalis* and *H. zea*. Dashed arrows indicate lower binding affinity than solid arrows.



Ostrinia nubilalis
(Hua et al., 2001)

Helicoverpa zea
(Gouffon et al., 2011)

CHAPTER 2

DIFFERENTIAL PROTECTION OF CRY1FA TOXIN AGAINST *SPODOPTERA FRUGIPERDA* LARVAL GUT PROTEASES BY CADHERIN ORTHOLOGS CORRELATES WITH INCREASED SYNERGISM

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Abstract

The Cry proteins produced by *Bacillus thuringiensis* (Bt) are the most widely used biopesticides effective against a range of crop pests and disease vectors. Like chemical pesticides, development of resistance is the primary threat to the long-term efficacy of Bt toxins. Recently discovered cadherin based Bt Cry synergists showed the potential to augment resistance management by improving efficacy of Cry toxins. However, the mode of action of Bt Cry synergists is thus far unclear. Here we elucidate the mechanism of cadherin-based Cry toxin synergism utilizing two cadherin peptides, *Spodoptera frugiperda* cadherin (SfCad) and *Manduca sexta* cadherin (MsCad), which differentially enhance Cry1Fa toxicity to *Spodoptera frugiperda* neonates. We show that differential SfCad and MsCad mediated protection of Cry1Fa toxin in the *S. frugiperda* midgut correlates with differential Cry1Fa toxicity enhancement. Both peptides exhibited high affinity for Cry1Fa toxin and an increased rate of Cry1Fa induced pore-formation in *S. frugiperda*. However, only SfCad bound *S. frugiperda* brush border membrane vesicles and more effectively prolonged the stability of Cry1Fa toxin in the gut explaining higher Cry1Fa enhancement by this peptide. This study shows that cadherin fragments may enhance *B. thuringiensis* toxicity by at least two different mechanisms or a combination thereof: (i) protection of Cry toxin from protease degradation in the insect midgut, and (ii) enhancement of pore-forming ability of Cry toxin.

2.1 Introduction

Bacillus thuringiensis (Bt) Cry toxins are a family of bacterial pore-forming proteins that are highly toxic to a range of crop pests and disease vectors. Cry proteins are produced as crystals during the sporulation phase. Crystals are ingested, solubilized in the gut lumen to protoxin, and activated by host gut proteases. Activated toxin crosses the peritrophic matrix (PM) and binds cadherin, which is the primary high-affinity receptor for Cry1 toxins on the apical border of midgut microvilli. A current model postulates that toxin interaction with cadherin causes a conformational change in toxin allowing a specific proteolytic cleavage and formation of a prepore toxin oligomer. Evidence suggests that the prepore oligomer has increased affinity for secondary glycosylphosphatidylinositol (GPI)-anchored receptors such as aminopeptidases (APN), or alkaline phosphatases (ALP) localized in lipid rafts. Oligomers insert into the membrane and disrupt membrane integrity by forming lytic-pores, which lead directly to insect mortality or indirectly to mortality due to septicemia (Knowles et al., 1987; Schnepf et al., 1998; Bravo et al., 2004; Broderick et al., 2006). A recent modification to the pore formation model described above proposes that activated toxin monomers first bind to abundant low-affinity APN receptors before binding to high-affinity cadherin receptors which results in toxin oligomerization (Pacheco et al., 2009a). In contrast to the pore-formation model, the cell-signaling model (Zhang et al., 2005) proposes that binding of activated toxin monomers to cadherin activates an intracellular signaling pathway, which ultimately results in cell death. However, toxin activation by gut proteases and toxin-receptor interactions are the most essential steps of Bt mode of action and are common to all models and alterations in these steps have been linked with resistance development (Oppert et al., 1994; Oppert et al., 1997; Gahan et al., 2001; Xu et al., 2005; Yang et al., 2006).

A strategy successfully employed to delay development of resistance against chemical pesticides is the use of synergists to enhance toxicity (Bernard et al., 1993). Several synergists of Bt Cry have been reported that show low to moderate synergism of Cry toxicity. Bt Cry synergists of non-Bt origin include zwittermicin (Broderick et al., 2000), endochitinase (Regev et al., 1996), and avidin (Zhu et al., 2005). Cyt toxin of Bt subsp. *israelensis* that shows high-level synergism of mosquitocidal Cry toxins (Wirth et al., 2000; Sayyed et al., 2001; Perez et al., 2007) and Bel protein that enhanced Cry1Ac toxin are examples of synergists of Bt origin (Fang et al., 2009). However, the discovery of *M. sexta* cadherin as a Bt Cry synergist is the first time a receptor has been reported to enhance toxicity (Chen et al., 2007). The synergistic potential of the *M. sexta* cadherin fragment has encouraged further research in this area (Chen et al., 2007). Subsequently, the *M. sexta* cadherin peptide CR10-12 (called MsCad in this study) was found to have increased synergy relative to the original CR12-MPED peptide (Abdullah et al., 2009). Additionally, a synergistic effect has been demonstrated using cadherin fragments from Dipterans (mosquito) with mosquitocidal Cry4Ba (Hua et al., 2008; Park et al., 2009b), from Coleopterans (beetle) with beetle-toxic Cry3Aa and Cry3Bb (Park et al., 2009a; Gao et al., 2011), and an additional example of cadherin synergy of lepidopteran-active Cry toxins (Peng et al., 2009). Due to the potential of cadherin as a Bt synergist, research is underway to enable commercial use of cadherin fragments either as an additive in Bt formulations or expressed in transgenic Bt plants.

Although several papers have been published on the use of cadherin fragments as Bt synergists (Chen et al., 2007; Hua et al., 2008; Pacheco et al., 2009b; Park et al., 2009a; Park et al., 2009b; Peng et al., 2009; Peng et al., 2010; Gao et al., 2011), the mode of action of insect cadherin-based synergists remains to be fully understood. Chen et al. (2007) proposed that

cadherin fragments increase the probability of toxin-receptor interaction by anchoring Bt toxins to the membrane. Formation of cadherin-induced toxin oligomers is another mechanism proposed to explain cadherin-based Bt synergism (Pacheco et al., 2009b; Peng et al., 2009). Nevertheless, Cry4Ba toxicity enhancement by an *Anopheles gambiae* cadherin fragment that inhibits toxin binding to BBMV (Park et al., 2009b), and a *Helicoverpa armigera* cadherin fragment that induces formation of prepore oligomers but reduces Cry1Ac toxicity (Liu et al., 2009) suggest existence of alternative or additional synergistic mechanisms.

Cry1Fa, the subject Bt toxin of this paper, is used in genetically modified corn cultivars to control lepidopteran pests. Cry1Fa is highly toxic to *Ostrinia nubilalis*, European corn borer, and has significant but not high level toxicity to *S. frugiperda*, fall armyworm (Sena et al., 2009). Unfortunately, in Puerto Rico where *S. frugiperda* is a major corn pest, *S. frugiperda* larvae have acquired resistance to Cry1Fa corn and the product was withdrawn from the market (Matten et al., 2008; Storer et al., 2010).

Our initial hypothesis was that CR10-12 peptide of *M. sexta* cadherin (MsCad) (Abdullah et al., 2009) would significantly synergize Cry1Fa toxicity to *S. frugiperda*. When data did not support this hypothesis, we hypothesized that the corresponding region of the ortholog cadherin from *S. frugiperda* (called SfCad in this study) would synergize Cry1Fa toxicity to *S. frugiperda*. The MsCad and SfCad peptides were then used in a comparative approach to investigate mechanisms of cadherin-based Cry toxin synergism.

2.2 Materials and methods

Bacterial strains and toxin purification

The Bt strain harboring *cry1Fa* gene was obtained from Ecogen Inc. (Langhorne, PA). The conditions for Bt cultures, toxin purification, and activation were as described previously by Luo et al. (1999). All protein concentrations, unless otherwise specified, were measured by BioRad protein assay using bovine serum albumin (BSA) as standard (Bradford, 1976).

BBMV preparation

Insects for all experiments were purchased from Benzon Research Inc. (Carlisle, PA) and larvae were fed multiple species artificial diet (Southland Products, Lake Village, AK). Midguts were excised from fourth-instar *S. frugiperda* larvae and BBMV were prepared by the $MgCl_2$ precipitation method (Wolfersberger et al., 1987), with modifications according to Carroll and Ellar (1993). The final BBMV pellet was suspended in SET buffer (250 mM sucrose, 5 mM EGTA, 20 mM Tris, pH 7.5), and frozen at -80ºC until used.

Preparation of gut extract

To prepare gut extract (GE), midguts from ten late 4^{th} instar *S. frugiperda* larvae, reared on artificial diet (Southland Products Inc., Lake Village, AR), were dissected, pooled, vortexed, and centrifuged at 13,000 xg for 15 min at 4°C to separate gut extract from the solid materials (Oppert et al., 1994). Supernatant was collected, filtered through a 0.22 µm filter, aliquoted, and stored at -80°C.

Cloning, expression and purification of SfCad and MsCad peptides

The sequence of *S. frugiperda* cadherin, an orthologue of *M. sexta* BtR1 cadherin, was published by Flannagan and Meyer (US patent number 7,572,889; GenBank accession number: CAC41167). The amino acid sequence and putative domains of *S. frugiperda* cadherin are shown in Appendix Fig. 2.S1. The cadherin *M. sexta* BtR1 repeat CR10 to 12 (MsCad, 35,148 Da) was previously identified as a Cry synergist (Abdullah et al., 2009). A region most similar to MsCad was identified on the *S. frugiperda* cadherin by protein alignment using ClustalW (version 1.83, from EMBL-EBI). The corresponding region of *S. frugiperda* cadherin (amino acids 1168 to 1479), SfCad, consists of 311 amino acids that have 51.8% identity with the aligned MsCad (Appendix Fig. 2.S2). For expression in *E. coli*, we optimized the SfCad coding region based on *E. coli* codon bias (Carbone et al., 2003), modified the peptide by adding MK at the N-terminus and six histidines at the C-terminus resulting in a 35,068 Da peptide. Both SfCad and MsCad encoding DNA were synthesized (Genscript, Piscataway, NJ) and then subcloned into an expression vector, pET30a(+) (Novagen, Madison, WI) for high expression in *E. coli*. The expression, solubilization, purification, and quantification methods are described elsewhere (Chen et al., 2007).

Insect bioassays

The toxicity of Cry1Fa toxin and the synergistic effect of cadherin fragments were evaluated using diet surface bioassays with *S. frugiperda* neonates as described previously by Abdullah et al. (2009). Insects were purchased from Benzon Research Inc. (Carlisle, PA). The maximum doses of SfCad and MsCad peptides applied in combination with toxin were tested alone in deionized water as negative controls. Toxin alone and buffer controls were also included

with each larval bioassay. Bioassays were scored 7 days after treatment (DAT). Different batches of larvae were used for dose response and the single point toxicity enhancement bioassays. Bioassays were repeated three times and the data were pooled. Probit regression analysis (Finney, 1971) using EPA Probit Analysis Program v.1.5 was used to calculate LC_{50} values. The enhancement in toxicity is expressed as fold decrease in the LC_{50} value of a Cry protein when the protein was used in combination with the cadherin fragments.

Labeling of SfCad and MsCad peptides

SfCad and MsCad peptides were biotinylated using a 50-fold molar excess of sulfo-NHS-PC-biotin according to the manufacturer's (Pierce, Rockford, IL) instructions. The final reaction was dialyzed against 20 mM Na_2CO_3, 150 mM NaCl, pH 9.6 and stored in aliquots at -20^{o}C until needed for binding assays.

For the dot blots experiments with Cry1Fa toxin, SfCad (6 µg) and MsCad (12 µg) were radiolabeled with 0.5 mCi of $Na^{125}I$ using the chloramine-T method (Garczynski et al., 1991) and stored at 4°C. Specific activities of the labeled peptides were 9.0 µCi/µg or 7.7 µCi/µg for SfCad and MsCad, respectively. For the dot blot experiments with BBMV, SfCad (5 µg) and MsCad (6 µg) were radiolabeled as above and the specific activities of the radioligands were 27 µCi/µg and 7 µCi/µg, respectively.

Labeling Cry1Fa toxin with Alexa-488

Trypsin activated Cry1Fa toxin was labeled with Alexa Fluor® 488 according to the manufacturer's (Invitrogen, Molecular Probes, Inc, Eugene, OR) instructions. The final reaction

was dialyzed against 20 mM Na_2CO_3, 200 mM NaCl, pH 9.6 at 4°C, quantified according to the manufacturer's instructions, and stored in aliquots at -20°C until needed for binding assays.

Toxin-synergist binding assays

The binding affinities of the cadherin fragments and Cry1Fa toxin was calculated using a microplate-based protein-protein interaction binding assay described previously (Park et al., 2009a). For both saturation and competition binding assays, microtiter plates were coated with 0.5 µg toxin/well. Plates were incubated with increasing concentrations of biotinylated SfCad or MsCad (0.01 nM to 18 nM) alone or in the presence of 1000-fold molar excess of unlabeled MsCad or SfCad to determine total and non-specific binding. For the competition binding assays, plates were probed with 2 nM biotinylated SfCad in the presence of increasing molar concentrations of non-labeled SfCad or MsCad to determine homologous and heterologous binding, respectively. Bound biotinylated SfCad or MsCad was detected with horseradish peroxidase (HRP)-conjugated streptavidin and HRP chromogenic substrate according to (Park et al., 2009a). Binding assays were replicated three times. Data were analyzed using SigmaPlot software (Version 9; Systat Software Inc., San Jose, CA) and the curves were fitted based on a best fit of the data to a one site saturation binding equation or one site competition equation with the maximum set at 100%.

Dot–blot binding assays

For the dot blot experiments, toxin or BBMV (5 µg) were dotted, in duplicates, onto a polyvinylidene difluoride (PVDF) filter (Millipore, Bedford, MA). For the toxin binding experiments, filters were probed with 0.5 nM ^{125}I-SfCad or ^{125}I-MsCad alone or in the presence

of 1,000-fold molar excess of non-labeled SfCad or MsCad. For the BBMV binding experiments, filters were probed with 0.5 nM ^{125}I-SfCad or ^{125}I-MsCad alone or in the presence of 1,000-fold molar excess of non-labeled SfCad, MsCad, or Cry1Fa. Bound ^{125}I-labeled peptides were detected by autoradiography at - 80°C. Binding assays were replicated four times and representative blots are shown.

^{Alexa}Cry1Fa toxin binding assays

To determine the effect of SfCad and MsCad in Cry1Fa toxin binding to *S. frugiperda* BBMV, 200 µg/ml BBMV were incubated with 2.5 nM ^{Alexa}Cry1Fa toxin with or without 10-fold mass ratio of SfCad or MsCad in binding buffer (20mM Na_2CO_3, 0.1% Tween20, 0.15M NaCl, pH 9.6). The reactions were incubated at room temperature for 2 h. At the end of incubation, BBMV were pelleted, washed three times with wash buffer (20mM Na_2CO_3, 0.1% Tween20, 0.15M NaCl, 0.1% BSA, pH 9.6), and solubilized in SDS (sodium dodecyl sulfate) sample buffer (Laemmli, 1970) by heating the samples for 10 min at 100° C. Proteins were separated using a Criterion 4-20% Tris-HCl gradient gel (Bio-Rad, Hercules, CA) and the bound Alexa-488 conjugated toxins were detected by scanning the gels using a Typhoon Imager with 488 nm excitation and 520 nm BP40 emission filters (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Assays were replicated three times and representative data are shown.

Toxin oligomerization and protease K protection assays

The experiments were based on the methodology of Aronson et al. (1999) with some modifications. To determine toxin oligomer formation, 60 ng of activated Cry1Fa toxin was incubated with or without 10-fold mass of SfCad or MsCad in $NaHCO_3$, 0.25 M NaCl, pH 9.5 at

37°C for 1 h. After 1h, 20 µg of *S. frugiperda* BBMV was added followed by further incubation at 30°C for 1 h. For protease K digestion, BBMV plus toxin (preincubated with or without SfCad or MsCad) was incubated as described above; then protease K (Sigma) was added to 0.17 mg/ml final concentration, and the suspensions were further incubated at 37°C for 30 min. Protease K treated Cry1Fa toxin (60 ng) preincubated with or without the peptides and protease K untreated BBMV plus toxin served as experimental controls. Ten millimolar AEBSF (4-(2-Aminoethyl)-benzenesulfonyl fluoride) (final concentration) was added to the samples that contained BBMV to stop serine protease activity (including protease K) and incubated at 37°C for 12 min. Samples were mixed with Tris-SDS as in (Aronson et al., 1999) to yield a final SDS concentration of 0.1% and incubated at 65°C for 5 min. Samples were separated on a Criterion 4-15% Tris-HCl gel (Bio-Rad, Hercules, CA) and electroblotted onto a PVDF filter. Cry1Fa was detected using rabbit antisera (produced at the Animal Resources Facility, University of Georgia) against Cry1Fa toxin. Goat anti-rabbit IgG horse radish peroxidase, ECL chemiluminescence substrate, and a FluorChem 8900 digital imaging system (AlphaInnotech, San Leandro, CA) were used to visualize Cry1Fa bands.

In vitro protease protection assays

The effect of SfCad or MsCad on Cry1Fa toxin stability in *S. frugiperda* GE was evaluated by incubating Cry1Fa toxin (100 ng) alone or pre-incubated with 1 µg cadherin fragments for 1 h with 1 µl GE in a final volume of 20 µl carbonate buffer (0.1 M Na_2CO_3, 0.25 M NaCl, pH 9.6) for 5, 10, or 20 min at 30°C. At the end of each time point, toxin proteolysis was terminated by heating the samples for 10 min, at 100°C (Oppert et al., 1994). Samples in SDS sample buffer were heated for 10 min at 100° C, separated by gel electrophoresis as above,

electroblotted onto a PVDF membrane, and residual Cry1Fa toxin was detected using anti-Cry1Fa serum as described above. Experiments were replicated three times and representative blots are shown.

In vivo protease protection assays

In vivo protease protection assays were performed on fourth instar *S. frugiperda* larvae starved for 1 h prior to force-feeding. The larvae were force-fed 2 µL treatment solution containing 0.2 µg Cry1Fa toxin alone or pre-incubated for 1 h with 1 µg SfCad or MsCad in 20 mM Na_2CO_3, pH 10, using a Gilmont syringe (Cole-Parmer, Vernon Hills, IL) fitted with a 26 gauge blunt-end needle. The larvae were then placed on artificial diet until dissected. Midguts from 8 larvae per treatment per time point were dissected and the midgut tissue was separated from gut content including the PM. The tissue and the gut content were pooled separately in 80 µl SET buffer, 10 µl 100 mM PMSF (phenylmethanesulfonylfluoride), and 20 µl 50x protease inhibitor cocktail (COMPLETE mini EDTA-free tablet; Roche, Indianapolis, IN) and homogenized on ice using a 2 ml Potter-Elvehjem glass tissue grinder for 30-60 seconds.

A microplate based BCA protein assay kit compatible with reducing agents (Pierce Biotechnology, Rockford, IL) was used to quantify total protein content of the samples as per the manufacturer's instructions. Based on the total protein concentration, 600 µg protein was solubilized in SDS-sample buffer (Laemmli, 1970) by boiling. Samples were centrifuged and the supernatant was separated using a Criterion 12.5 % gel (Bio-Rad, Hercules, CA), electroblotted onto a PVDF membrane and the residual Cry1Fa toxin was detected using antisera raised against Cry1Fa toxin as described above. The Cry1Fa bands were quantified by densitometry using a FluorChem 8900 imager (AlphaInnotech, San Leandro, CA). Imager software was used to

subtract background density values from regions containing a toxin band. Experiments were replicated three times and representative blots are shown.

Membrane permeability assays

Light scattering assays were performed with a stop flow spectrofluorimeter (model RSM 1000; On-line Instrument Systems, Bogart, GA) essentially as described previously with slight modifications (Luo et al., 1999). BBMV from *S. frugiperda* were prepared one day before the assay as described above except that the final BBMV pellet was suspended in HEPES buffer (25 mM sucrose, 2.4 mM $MgCl_2$, 9 mM HEPES/KOH, pH 7.5) and stored on ice. All treatment mixtures were prepared fresh in HEPES/KCl buffer (25 mM sucrose, 2.4 mM $MgCl_2$, 0.27 M KCl, and 9 mM HEPES/KOH, pH 7.5). Assays were initiated by simultaneously injecting 0.25 ml of BBMV (75 µg BBMV) and 0.25 ml of a treatment mixture into the cuvette in the spectrofluorimeter sample compartment. The treatments mixtures consisted of various concentrations of Cry1Fa alone or mixed with either SfCad or MsCad in KCl buffer. Different concentrations of SfCad and MsCad used with Cry1Fa were tested alone as negative controls. Treatments were replicated five-times per experiment and data from two experiments were pooled to calculate the means and standard errors.

The shrinkage response signal, measured by determining the increase in the scattered light signal, was determined by comparing the light scattered from BBMV coinjected with buffer having the same osmotic strength (HEPES buffer) and the light scattered from BBMV coinjected with KCl buffer. The mean scattered light signal was converted to percentage of volume recovered using the formula:

$$\text{Volume Recovered (\%)} = (KCl_t\text{-}Treat_t)/(KCl_t\text{-}NS_t) \times 100$$

where KCl_t was the mean signal (at time *t*) obtained for BBMV mixed with the KCl buffer, $Treat_t$ was the mean signal (at time *t*) obtained for BBMV mixed with various treatments, and NS_t was the mean signal (at time *t*) obtained for BBMV mixed with the HEPES buffer. SigmaPlot, Version 9, was used to plot data and fit curves (Systat Software, Inc, San Jose, CA).

Statistical analysis

Mortality data from the bioassays were normalized using arcsine-square root (x) transformation and subjected to analysis of variance (ANOVA) with the significance level set at 0.05. When significant, means were separated using a Fisher protected least significant difference test (LSD) to compare the treatment means with the toxin alone treatment. BBMV binding data were analyzed using ANOVA and when significant, means were compared with the peptide alone treatment using LSD. All calculations were performed using PROC GLM and PROC UNIVARIATE of the Statistical Analysis System (SAS 2002–2003, version 9.1; SAS Institute, Cary, NC). Data are presented in the original scale.

2.3 Results

We tested the ability of MsCad (Fig. 2.1A) to enhance Cry1Fa toxicity to *S. frugiperda* using varying concentrations of Cry1Fa applied to diet surface while maintaining a constant 1:5 Cry1Fa:MsCad mass ratio. The LC_{50} of Cry1Fa alone was 491 (400 – 637) ng/cm^2, while Cry1Fa:MsCad was 275 (229 – 331) ng/cm^2 (a 1.8-fold increase in Cry1Fa toxicity against *S. frugiperda* larvae). In view of the possibility that a fragment of *S. frugiperda* cadherin may enhance Cry1Fa toxicity to *S. frugiperda* better than MsCad, we identified the most similar region in *S. frugiperda* cadherin. The MsCad region (311 aa) aligns to *S. frugiperda* cadherin

residues 1168 to 1479 (311 aa) with 51.6% sequence similarity (Appendix Fig. 2.S2). The SfCad region was cloned into *E. coli* for protein production and SfCad peptide was purified from inclusion bodies (Appendix Fig. 2.S3). The synergistic effect of the SfCad peptide was quantified as for MsCad peptide. SfCad lowered the Cry1Fa LC_{50} to 94 (83 – 105) ng/cm^2; a 5.2-fold significant increase in toxicity compared to Cry1Fa toxin alone treatment (Fig. 2.1B). The values in LC_{50} are considered significantly different if the 95% fiducial limits (in parentheses) do not overlap (Rosner, 1995). These results showed that SfCad was a significantly better Cry1Fa toxicity enhancer than MsCad against *S. frugiperda*.

The extent of MsCad and SfCad mediated enhancement of Cry1Fa toxicity was further tested using 100 ng/cm^2 Cry1Fa toxin alone or with 1:5 to 1:40 mass ratios of MsCad or SfCad peptides. As shown in Fig. 2.1C, the level of MsCad mediated enhancement of Cry1Fa toxicity was not significantly different from the toxin alone treatment. In contrast to the MsCad results, SfCad significantly enhanced Cry1Fa at 1:5 Cry1Fa:SfCad ratio (increased mortality from 31% to 64%), and mortality was further increased (about 91% mortality) when the Cry1Fa toxin:cadherin ratio was increased from 1:5 to 1:10 (Fig. 2.1C). As a control, BSA did not enhance Cry1Fa toxicity (data not shown). The highest concentration of the cadherin peptides was not toxic to larvae.

SfCad and MsCad bind Cry1Fa saturably and with high affinity

Since cadherin fragment binding to toxin usually correlates with synergism of insecticidal activity (Chen et al., 2007; Pacheco et al., 2009b), we tested MsCad and SfCad for binding to Cry1Fa toxin. While both cadherin fragments specifically bound Cry1Fa toxin in ELISA binding assays, SfCad had more non-specific binding to Cry1Fa toxin (Fig. 2.2B). Binding affinities

were calculated from ELISA data using a one-site saturation binding equation. MsCad and SfCad bound Cry1Fa with similar affinities, K_d = 5.8 ± 0.7 and 2.2 ± 0.5 nM, respectively (Fig. 2.2A and B). Homologous and heterologous competition of biotin-SfCad binding to Cry1Fa was tested by the addition of increasing amounts of unlabeled cadherin fragment to the binding reactions. As seen in Fig. 2.2C, MsCad and SfCad competed biotin-SfCad binding to Cry1Fa toxin, evidence that MsCad and SfCad share a common population of sites on Cry1Fa toxin. However, the shared SfCad and MsCad population of binding sites appear to be low affinity sites as 50% competition was not attained until about 1 □M unlabeled peptide was in the binding reaction. Whether or not SfCad and MsCad share a common high affinity binding site on Cry1Fa cannot be concluded from the data.

Binding to toxin was also tested by incubating ^{125}I-labeled MsCad or SfCad peptide with PVDF filters dotted with Cry1Fa toxin, visualizing bound toxin by autoradiography, and quantifying bound toxin by counting radioactivity. Specificity of binding was assessed by adding 1000-fold excess unlabeled peptide to the binding reaction. As seen in Fig. 2.2 (insets) ^{125}I-SfCad and ^{125}I-MsCad bound Cry1Fa toxin with some specificity and according to the radioactivity counts, Cry1Fa bound more ^{125}I-SfCad (14 ± 0.1 nmol) than ^{125}I-MsCad (7 ± 1.0 nmol).

^{125}I-SfCad, but not ^{125}I-MsCad, specifically binds to S. frugiperda BBMV

We tested the ability of ^{125}I-labeled cadherin fragments to bind BBMV prepared from larvae. BBMV were spotted to PVDF membrane and probed with labeled cadherin peptides alone or in the presence of 1000-fold non-labeled cadherin peptide (Fig. 2.3). ^{125}I-SfCad, but not ^{125}I-MsCad, bound BBMV and binding was competed by unlabeled SfCad (Fig. 2.3A). Binding

was not competed by Cry1Fa toxin suggesting that SfCad interacts with BBMV using different binding sites than the Cry1Fa toxin. Spots were excised, counted for radioactivity and data are presented in Fig. 2.3B as nmol peptide bound per µg of BBMV protein. This analysis of bound peptides clearly showed the specific affinity of ^{125}I-SfCad for BBMV prepared from *S. frugiperda* larvae. The ability of SfCad to bind both toxin and BBMV suggests suggested that SfCad may function as a surrogate receptor for the Cry1Fa toxin. This may occur either by SfCad binding first to toxin and then the complex to BBMV, or alternatively it is possible SfCad binds first to BBMV and then to Cry1Fa.

Effect of SfCad and MsCad on ^{Alexa}Cry1Fa toxin binding to BBMV

We analyzed whether SfCad increases Cry1Fa toxin binding to *S. frugiperda* BBMV. As shown in Fig. 2.4A (lanes 2-3), although ^{Alexa}Cry1Fa toxin bound BBMV there was limited competition by unlabeled Cry1Fa toxin. Pre-incubation of toxin with SfCad did not increase ^{Alexa}Cry1Fa binding to BBMV compared to untreated toxin (Fig. 2.4A).

SfCad and MsCad increase Cry1Fa oligomerization but not toxin membrane insertion

We assessed the ability of SfCad and MsCad to induce Cry1Fa oligomer complexes without and with *S. frugiperda* BBMV. As shown in Fig. 2.4B (lanes 1-3), pre-incubation of Cry1Fa toxin with a 10-fold mass ratio of either MsCad or SfCad increased formation of toxin aggregates, which presumably represent prepore toxin oligomer complexes (≥180 kDa). There was no difference in the amount of prepore oligomer induced by the two peptides. Interestingly, the peptide-induced oligomers observed in the absence of BBMV were not detected when BBMV were added to the mixture (Fig. 2.4B, lanes 4-6). To possibly clarify this issue we used a

protease K protection assay (Aronson et al., 1999; Nair et al., 2008) to reveal membrane-inserted toxin monomers and oligomers. As shown in Fig. 2.4B (lanes 7-9), protease K treatment resulted in the degradation of Cry1Fa toxin except for a ~37kDa fragment and oligomers were not detected. Total degradation of Cry1Fa toxin by protease K in the absence of BBMV (Fig. 2.4B; lanes 10-12) suggested that the ~37kDa fragment observed in the presence of BBMV is the membrane inserted part of Cry1Fa toxin. Pre-incubation with cadherin fragments did not affect membrane insertion suggesting that cadherin-based synergism of Cry toxin is not mediated through an increase in membrane insertion. The insertion of a smaller ~37kDa fraction of Cry1Fa toxin in membrane is in agreement with a recent analysis of Cry1Ab membrane insertion where domain I is inserted in membrane and domains II and III remain exposed to the solvent (Zavala et al., 2011).

SfCad and MsCad prolong stability of Cry1Fa toxin in S. frugiperda digestive fluid

Insect midgut proteases are essential for the activation of Bt protoxins (Gill et al., 1992), have a role in toxin specificity and are sometimes involved in resistance (Oppert et al., 1997; Miranda et al., 2001). Therefore the effect of the cadherin fragments on Cry1Fa toxin stability in *S. frugiperda* gut extract was tested *in vitro*. Both SfCad and MsCad peptides showed stabilization of Cry1Fa at the 5 min time point compared to toxin alone treatment (Fig. 2.5A and 5C). At the 10 min time point, however, more residual toxin was observed in the SfCad pre-treated lane compared to MsCad pre-treated or toxin alone lane suggestive of prolonged toxin stabilization by SfCad in the *S. frugiperda* gut extract (Fig. 2.5A and C).

SfCad and MsCad prolong stability of Cry1Fa toxin in S. frugiperda midgut

To further demonstrate the protective effect of cadherin fragments against gut proteases, *S. frugiperda* larvae were force-fed Cry1Fa toxin alone or toxin pre-incubated with MsCad or SfCad, and residual Cry1Fa toxin was evaluated at different time points after force-feeding. As seen in the Fig. 2.5B and D, peptide pre-incubated Cry1Fa was relatively more stable in the gut compared to the toxin alone at the 2 and 4 hours post-feeding times tested here. While both peptides protected Cry1Fa in the midgut, SfCad provided better Cry1Fa protection compared to MsCad as evidenced by the higher residual Cry1Fa in the SfCad treated animals. The relatively higher amount of Cry1Fa in the peptide-Cry1Fa treated larvae may explain enhancement of Cry1Fa toxicity by the peptides, while higher amount of Cry1Fa in SfCad treated larvae may explain why SfCad is a better Cry1Fa enhancer than MsCad. These results provided additional support that one of the modes of action of the synergistic peptides involved stabilization of Cry toxin in the presence of cadherin peptides in the gut. The prolonged stability of toxin could increase probability of the toxin inflicting more damage to the gut membrane via increased binding, insertion, and pore-formation.

SfCad and MsCad enhance pore formation by Cry1Fa

The effect of cadherin fragments on the pore forming ability of Cry1Fa toxin was analyzed. Pore forming ability was indirectly quantified by measuring membrane permeability using the vesicle light scattering assay previously described by Carroll and Ellar (1993). The principle behind the vesicle light scattering assay is that in the presence of external KCl, the amount of scattered light rapidly increases due to vesicle shrinkage while membrane permeabilization causes a decrease in the scattered light due to vesicle swelling (Fig. 2.6A). In

agreement with Luo et al. (1999), when *S. frugiperda* BBMV were made permeable by increasing doses of Cry1Fa, the scattered light signal decreased (Fig. 2.6A). The percentage of volume recovered for increasing concentrations of Cry1Fa is shown in Fig. 2.6B. A linear regression equation was used to calculate osmotic swelling rate at various concentrations of Cry1Fa used (% volume recovered per minute). A dose-response standard curve was obtained based on the best fit ($R^2 = 0.99$) of the osmotic swelling rates (Y) of increasing concentrations of Cry1Fa (X) to a logarithmic regression equation; $Y= -10.35 + 5.90(\ln X)$ (Fig. 2.6C). Since cadherin fragments alone slightly lowered the swelling rate of vesicles relative to the HEPES/KCl treatment (Fig. 2.6C), the swelling rate for cadherin peptide alone treatments (Table S1) were added to the osmotic swelling rates (Y) of the treatments containing Cry1Fa with cadherin peptides; this addition was based on the assumption that the dampening effect on vesicle swelling also occurs when cadherin peptides are mixed with Cry1Fa. The sum values of Cry1Fa-cadherin peptide rates plus cadherin alone rates were plotted in the equation to calculate equivalent Cry1Fa concentrations. The ratios of the calculated equivalent Cry1Fa concentration over the actual Cry1Fa concentration in the treatment represent the synergistic factor. As shown in Fig. 2.6D, a two to three-fold increase in osmotic swelling rate with two graded concentrations of Cry1Fa and peptides suggested that the peptides enhanced toxicity by increasing pore-forming ability of the toxin (Fig. 2.6D). Nevertheless, the similar increase in osmotic swelling rates by both peptides suggested that pore formation was not a factor that contributed to the differential Cry1Fa toxicity enhancement by the peptides.

2.4 Discussion

In this paper we demonstrate the greater ability of SfCad relative to MsCad to synergize Cry1Fa toxicity to *S. frugiperda* larvae and investigated the mechanisms underlying the observed synergy.

High affinity Cry binding to cadherin peptide is a prerequisite for the peptide's synergistic effect (Chen et al., 2007; Peng et al., 2009). SfCad has a predicted toxin binding region (TBR) located in CR12 that is 62% identical and similar in physico-chemical properties to the MsCad high-affinity Cry1Ab TBR (Dorsch et al., 2002; Hua et al., 2004) (Supplementary Figures 2.S1 and S2). Both cadherin fragments bound Cry1Fa with high affinity (2 nM for SfCad and 8 nM for MsCad) and shared a common binding site on Cry1Fa toxin. High affinity Cry1Fa recognition of SfCad is consistent with cadherin having a Cry1 receptor role in *S. frugiperda* as it does in *Manduca* and other lepidopteran species (reviewed in Pigott and Ellar, 2007 (Pigott et al., 2007)). However, high affinity binding to cadherin peptide did not lead to high MsCad synergism of Cry1Fa toxicity suggesting that other synergistic mechanisms may influence synergistic ability of cadherin peptides.

A model for the synergistic effect of cadherin fragments was suggested by the ability of some cadherin fragments to bind Cry toxin and BBMV (Chen et al., 2007; Peng et al., 2009). This model would be analogous to Cyt toxin of Bt subsp. *israelensis* where Cyt binds midgut membrane and functions as a surrogate Cry11Aa receptor (Perez et al., 2005). ^{125}I-labeled SfCad bound *S. frugiperda* BBMV and was competed by unlabeled SfCad unlike labeled MsCad, which did not bind BBMV. The ability of SfCad to bind BBMV was consistent with SfCad enhancing Cry1Fa toxicity and the surrogate receptor model. However, the lack of peptide mediated increase in Cry1Fa toxin binding to *S. frugiperda* BBMV suggest that the ability to increase

binding to BBMV is not an important determinant of synergistic ability of a cadherin fragment. A cadherin fragment from *Anopheles gambiae* that synergizes Cry4Ba toxicity inhibited Cry4Ba binding to BBMV (Park et al., 2009b) further questions the role of cadherin-BBMV bindability in toxin synergism by cadherin fragments. An alternative explanation is that it may not be necessary that SfCad increase the amount of toxin binding to have a positive effect on pore formation and Cry1Fa toxicity. Further research, such as 'knocking out' SfCad binding to BBMV by mutational analyses could test the relevance of the cadherin-BBMV binding property to the synergistic effect.

Another possibility is that SfCad and not MsCad induces prepore oligomer formation. Studies show that the *M. sexta* cadherin synergist containing CR12 region, a high affinity binder (Chen et al., 2007; Pacheco et al., 2009b), induces Cry1Ab oligomerization. A *Helicoverpa armigera* cadherin fragment was also shown to facilitate oligomerization of Cry1Ac toxin in the presence of midgut proteinases (Peng et al., 2009). The observed increased Cry1Fa oligomer formation in the presence of SfCad and MsCad agrees with the reported correlation between increased formation of toxin oligomers and enhancement activity of toxin-binding cadherin fragments (Pacheco et al., 2009b; Peng et al., 2009). However, unlike the Cry1Ab oligomers that are stable in *M. sexta* BBMV (Jimenez-Juarez et al., 2008), the Cry1Fa oligomers observed here were transient and disappeared in the presence of *S. frugiperda* BBMV. It is possible that the Cry1Fa oligomers observed without BBMV were present in BBMV but were unstable under our BBMV solubilization conditions. It is also possible that the formation of prepore toxin oligomer may have detrimental effect to toxicity as was reported in *Helicoverpa armigera*, where Cry1Ac toxin oligomer formation induced by *H. armigera* cadherin fragments reduced Cry1Ac toxicity

(Liu et al., 2009). Consequently, the induction of prepore toxin oligomers by cadherin fragments or other synergists cannot be used as a consistent predictor of synergistic effect on Bt toxicity.

Gut proteases activate Cry protoxin to its active toxin form and can conversely deactivate toxin by proteolytic degradation in naturally tolerant insects and in insects that have evolved resistance to Bt Cry proteins (Oppert et al., 1997; Miranda et al., 2001). A better protective, i.e. stabilization effect, by SfCad would account for why SfCad is a better Cry1Fa toxicity enhancer than MsCad. Data from our *in vitro* gut extract assay showed that both SfCad and MsCad protected Cry1Fa from proteolytic degradation and SfCad protected Cry1Fa for a longer duration compared to MsCad. In agreement with the *in vitro* experiment, results from the *in vivo* experiment further confirmed the protective role played by the peptides. Higher amount of Cry1Fa detected in the membrane and the gut content of the peptide-Cry1Fa treated larvae correlated with the enhancement of toxicity by the peptides, while higher residual Cry1Fa in SfCad treated larvae explained why SfCad is a better Cry1Fa enhancer than MsCad. A similar observation where MsCad stabilizes Cry1Ab toxicity in *S. exigua* midgut (unpublished data) suggests that cadherin-mediated stabilization of toxin may well be a general mode of action for cadherin-based Bt Cry synergists. Although the exact mechanism of protection is unknown, high affinity interaction between the peptides and the toxins points to the possibility that the peptides physically protect critical proteolytic sites on the toxin limiting digestion in the insect gut.

According to the pore-formation model, Cry toxin-induced pore-formation is an important step in the mode of Bt Cry toxin action (Carroll et al., 1993; Lorence et al., 1995; Wolfersberger et al., 1996; Peyronnet et al., 1997). Although both monomeric and prepore oligomeric complexes of Cry toxin form pores, prepore oligomeric complexes have higher pore forming ability than monomeric Cry toxins (Munoz-Garay et al., 2006). In agreement with

previous reports (Luo et al., 1999; Munoz-Garay et al., 2006), our light-scattering assay showed that Cry1Fa toxin increased the permeability of *S. frugiperda* BBMV in a dose-dependent function with toxin concentration. The observed two to- three-fold increase in the rate of pore formation by the cadherin-treated Cry1Fa toxin correlated with higher Cry1Fa enhancement by SfCad but not MsCad. Since the cadherin fragments did not increase the amount Cry1Fa toxin binding or membrane insertion, the increase in the rate of pore-formation by the cadherin treated toxin suggests that the cadherin fragments promote more efficient pore-formation by the membrane-inserted toxins. Similar to the oligomeric form of Cry toxin that forms better pores (Munoz-Garay et al., 2006) high affinity toxin-cadherin interaction may modify toxins to form more efficient or stable pores in the membrane. Although both SfCad and MsCad can increase rate of pore-formation *in vitro*, cadherin mediated protection of Cry1Fa toxin in the midgut will affect the amount of toxin available for membrane insertion and pore-formation. Therefore, the greater SfCad mediated protection of Cry1Fa toxin in the midgut along with the general ability of cadherin fragments to increase rate of pore-formation make SfCad a better Cry1Fa toxicity enhancer in *S. frugiperda*.

In conclusion, our results showed that like most cadherin-based Bt synergists, *S. frugiperda* cadherin was also a Cry1Fa toxin binding protein and may be a receptor. With regard to the modes of action of cadherin-based Bt synergists; cadherin mediated protection of Cry toxin in the midgut, and enhancement of pore-forming ability of Cry toxin by cadherin fragments likely act in concert to enhance Cry toxicity. The knowledge gained through this study may lead to better design of Bt and its synergists to prolong the utility of Bt as a valuable resource in crop pest management.

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Abbreviations used

ALP, alkaline phosphatase; APN, aminopeptidase-N; BBMV, brush border membrane vesicle(s); BSA, bovine serum albumin; Bt, *Bacillus thuringiensis*, CR, cadherin repeat; CYTO, cytoplasmic domain; GE, gut extract; LC_{50}, the lethal concentration that kills 50% of the test insects; MPED, membrane proximal extracellular domain; MsCad, *Manduca sexta* cadherin fragment consisting of the CR10-12 region; PM, peritrophic matrix; PMSF, phenylmethanesulfonylfluoride; SfCad, *Spodoptera frugiperda* cadherin fragment consisting of the CR10-MPED region; SP, signal peptide; TBR, toxin binding region; TM, transmembrane domain.

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Figure legends

Figure 2.1. Comparison of Cry1Fa and Cry1Ab toxicity enhancement by SfCad and MsCad. (A) Schematic diagram of *M. sexta* and *S. frugiperda* cadherins and the SfCad and MsCad peptides. Cadherin domains: SP, signal peptide; CR, cadherin repeat; MPED, membrane proximal extracellular domain; TM, transmembrane domain; CYTO, cytoplasmic domain. (B, C) *S. frugiperda* neonates were treated with (B) increasing concentrations of Cry1Fa toxin alone or in combination with five-fold mass excess of SfCad or MsCad; or (C) 100 µg/cm^2 Cry1Fa toxin alone or in combination with five to 40-fold mass excess of either SfCad or MsCad. Peptides alone treatments were included as controls. Experiments were replicated three times and the means ± SEM are reported. Vertical lines indicate SEM. In panels C letters above the histograms indicate significant differences between toxin control and toxin plus SfCad or MsCad treatments as determined by ANOVA (P = 0.05) and Fisher protected LSD.

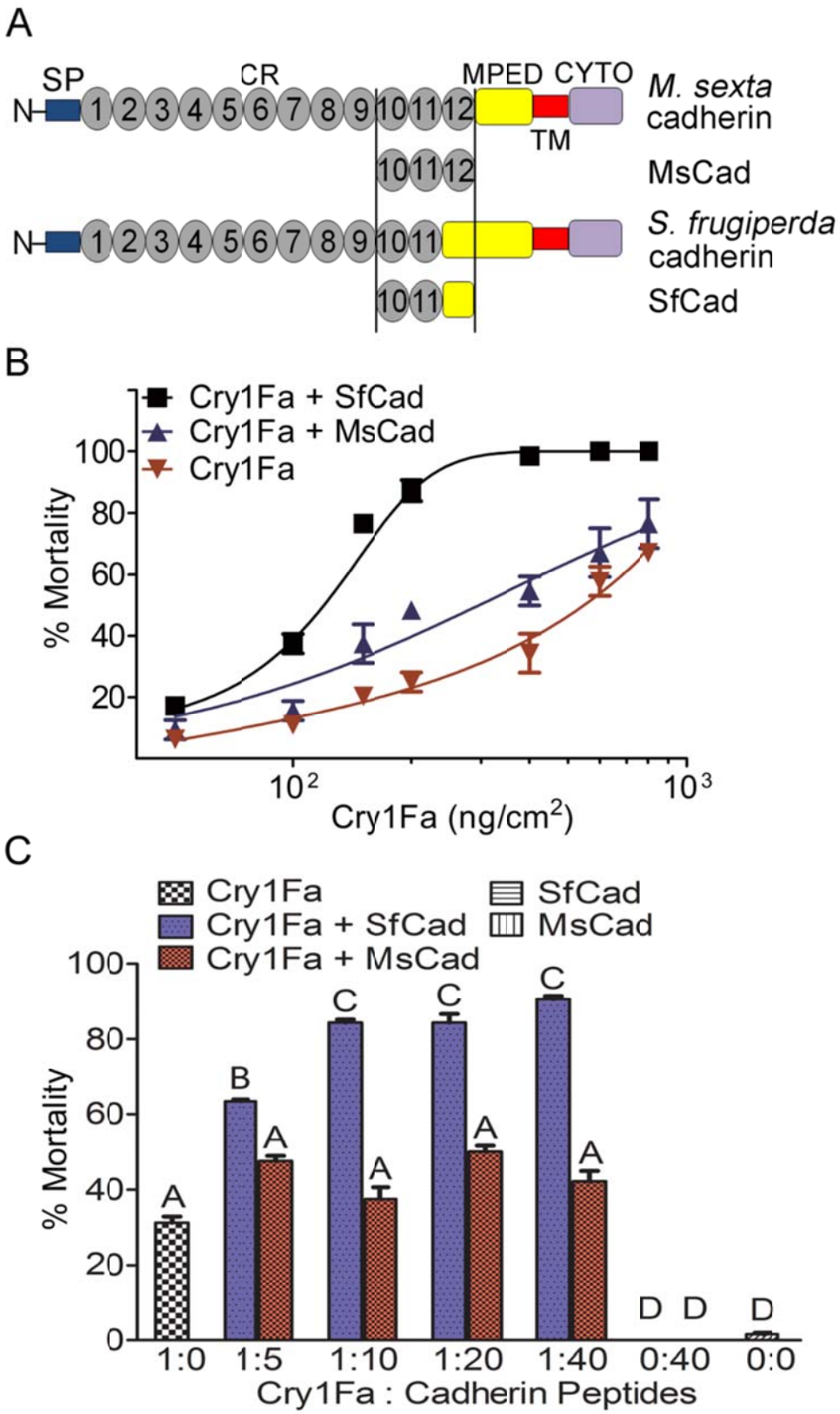

A
SP
CR
MPED
CYTO
N
1 2 3 4 5 6 7 8 9 10 11 12
TM
M. sexta cadherin
10 11 12
MsCad
S. frugiperda cadherin
10 11
SfCad
B
Cry1Fa + SfCad
Cry1Fa + MsCad
Cry1Fa
% Mortality
100
80
60
40
20
10^2
10^3
Cry1Fa (ng/cm^2)
C
Cry1Fa
Cry1Fa + SfCad
Cry1Fa + MsCad
SfCad
MsCad
% Mortality
100
80
60
40
20
0
A
B
A
C
A
C
A
C
A
D D D
1:0 1:5 1:10 1:20 1:40 0:40 0:0
Cry1Fa : Cadherin Peptides

Figure 2.2. Saturation and competitive binding characteristics of MsCad and SfCad to Cry1Fa toxins. (A, B) Ninety six-well microtiter plates coated with Cry1Fa toxin were probed with increasing molar concentrations of biotinylated MsCad (A) or SfCad (B) to determine total binding. Bound biotinylated peptide was detected with an SA-HRP conjugate and substrate. Non-specific binding was determined by probing the plates with increasing molar concentrations of biotinylated MsCad or SfCad plus 1,000-fold molar excess of non-labeled MsCad or SfCad, respectively. Specific binding was calculated by subtracting non-specific binding from total binding. (Insert) Binding of equal concentrations of MsCad and SfCad to Cry1Fa (A, B) toxin spotted in duplicate on a PVDF membrane and probed with 0.5 nM ^{125}I-MsCad (A) or ^{125}I-SfCad (B) alone or in the presence of a 1,000-fold excess of unlabeled SfCad (B) or MsCad (A), respectively. (C) Microtiter plates coated with Cry1Fa toxin were probed with 2 nM biotinylated SfCad in the presence of increasing molar concentrations of non-labeled SfCad or MsCad to determine homologous and heterologous binding. Experiments were replicated three times and the means ± SEM are reported. Vertical lines indicate SEM.

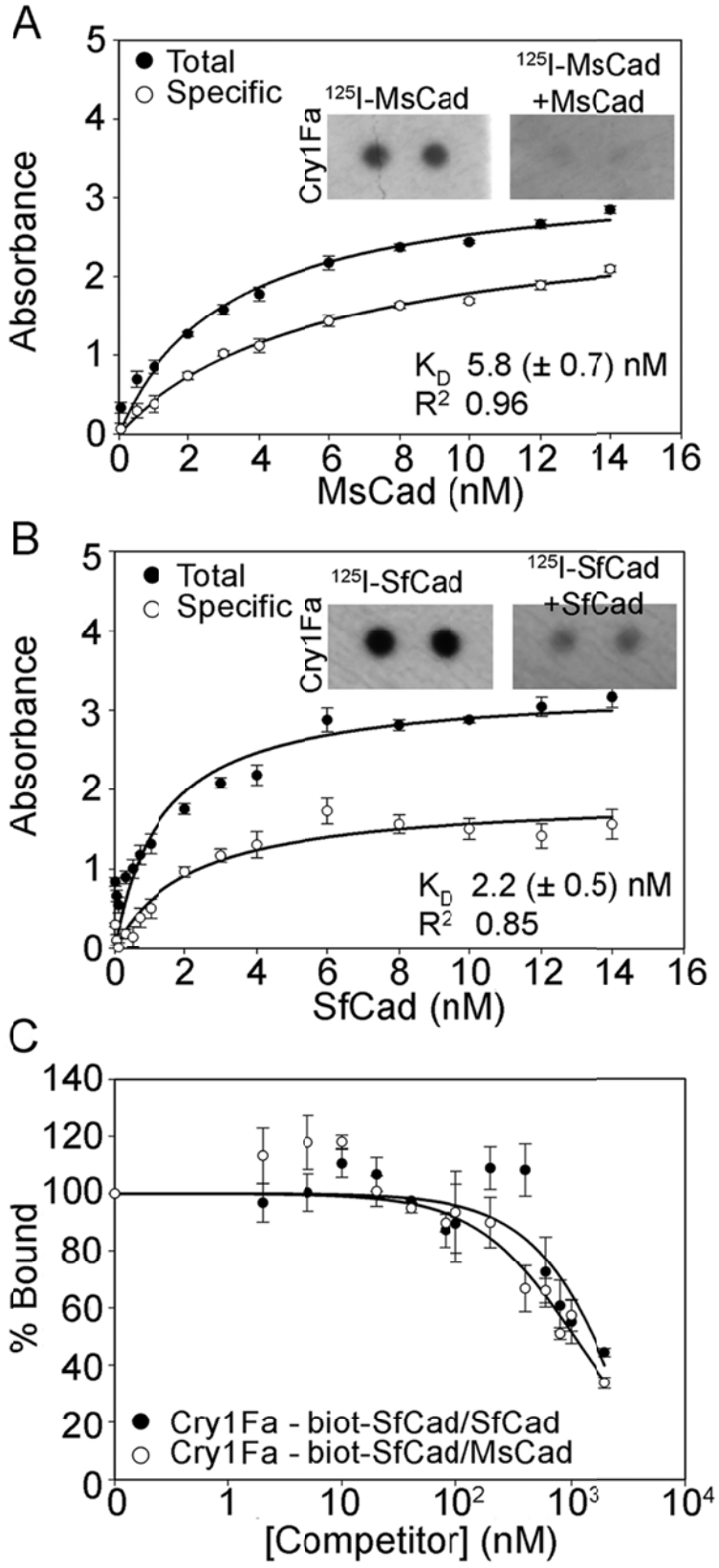

A
Total
Specific
125I-MsCad
125I-MsCad
+MsCad
Cry1Fa
Absorbance
KD 5.8 (± 0.7) nM
R2 0.96
MsCad (nM)
B
Total
Specific
125I-SfCad
125I-SfCad
+SfCad
Cry1Fa
Absorbance
KD 2.2 (± 0.5) nM
R2 0.85
SfCad (nM)
C
% Bound
Cry1Fa - biot-SfCad/SfCad
Cry1Fa - biot-SfCad/MsCad
[Competitor] (nM)

Figure 2.3. Binding of ^{125}I-labeled SfCad & MsCad to *S frugiperda* BBMV. (A) *S. frugiperda* BBMV (5µg) spotted on a PVDF membrane was probed with either ^{125}I-SfCad or ^{125}I-MsCad (0.5 nM) alone or in the presence of a 1,000-fold excess of unlabeled SfCad, MsCad, or Cry1Fa. (B) Quantitative representation of the amount of ^{125}I-labeled peptides bound to the BBMV spots in panel A. In panel B, uppercase or lowercase letters to the right of the bars indicate significant differences as determined by ANOVA (P =0.05) and Fisher protected LSD.

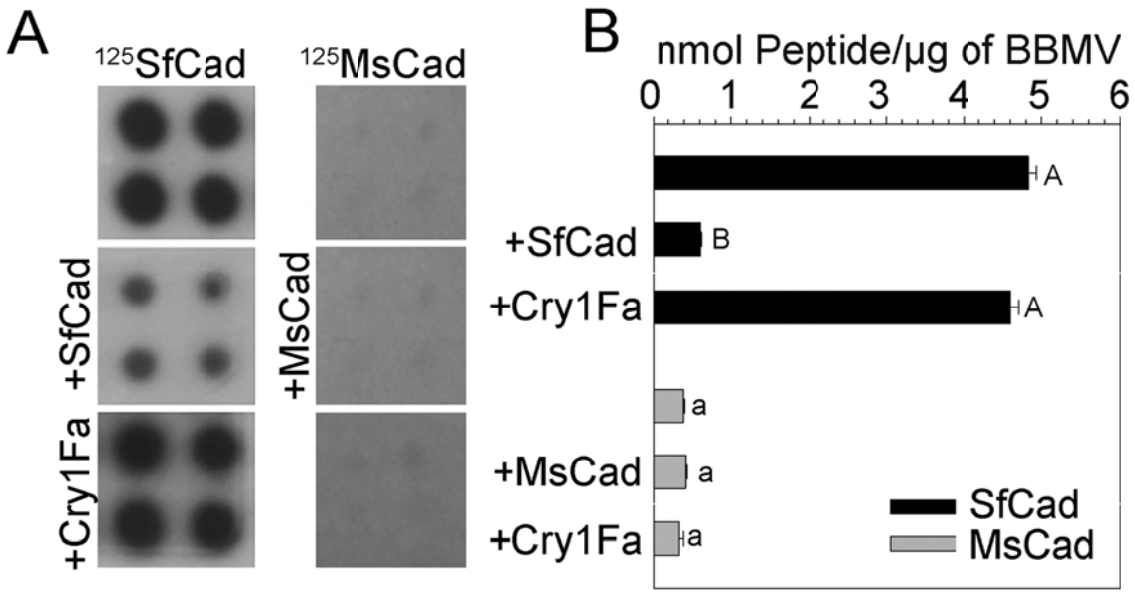

A
125SfCad
125MsCad
+SfCad
+MsCad
+Cry1Fa
B
nmol Peptide/µg of BBMV
0 1 2 3 4 5 6
+SfCad
+Cry1Fa
+MsCad
+Cry1Fa
A
B
A
a
a
a
SfCad
MsCad

Figure 2.4. Comparison of SfCad and MsCad induced Cry1Fa toxin binding, oligomerization, and membrane insertion. (A) The binding of Cry1Fa toxin to *S. frugiperda* BBMV was tested by.incubating ^{Alexa}Cry1Fa toxin alone or with 1000-fold molar excess of unlabeled Cry1Fa toxin with BBMV (A; lanes 2-3). The effect of SfCad and MsCad on ^{Alexa}Cry1Fa binding to BBMV was tested by incubating SfCad or MSCad pre-incubated ^{Alexa}Cry1Fa toxin with BBMV (A; lanes 4-5). (B) The ability of the cadherin fragments to facilitate Cry1Fa pre-pore oligomeric complex is tested in lanes 1-3. The ability of the cadherin fragments to facilitate oligomeric structure in BBMV is tested in lanes 4-6. The ability of the cadherin fragments to facilitate Cry1Fa toxin membrane-insertion in BBMV in a protease K protection assay is tested in lanes 7-9. Lanes 10-12 show the complete degradation of Cry1Fa toxin by protease K in the absence of BBMV. Reactants are indicated above the blot. Residual toxin was detected using Cry1Fa antibody. Reactants are indicated above the blot.

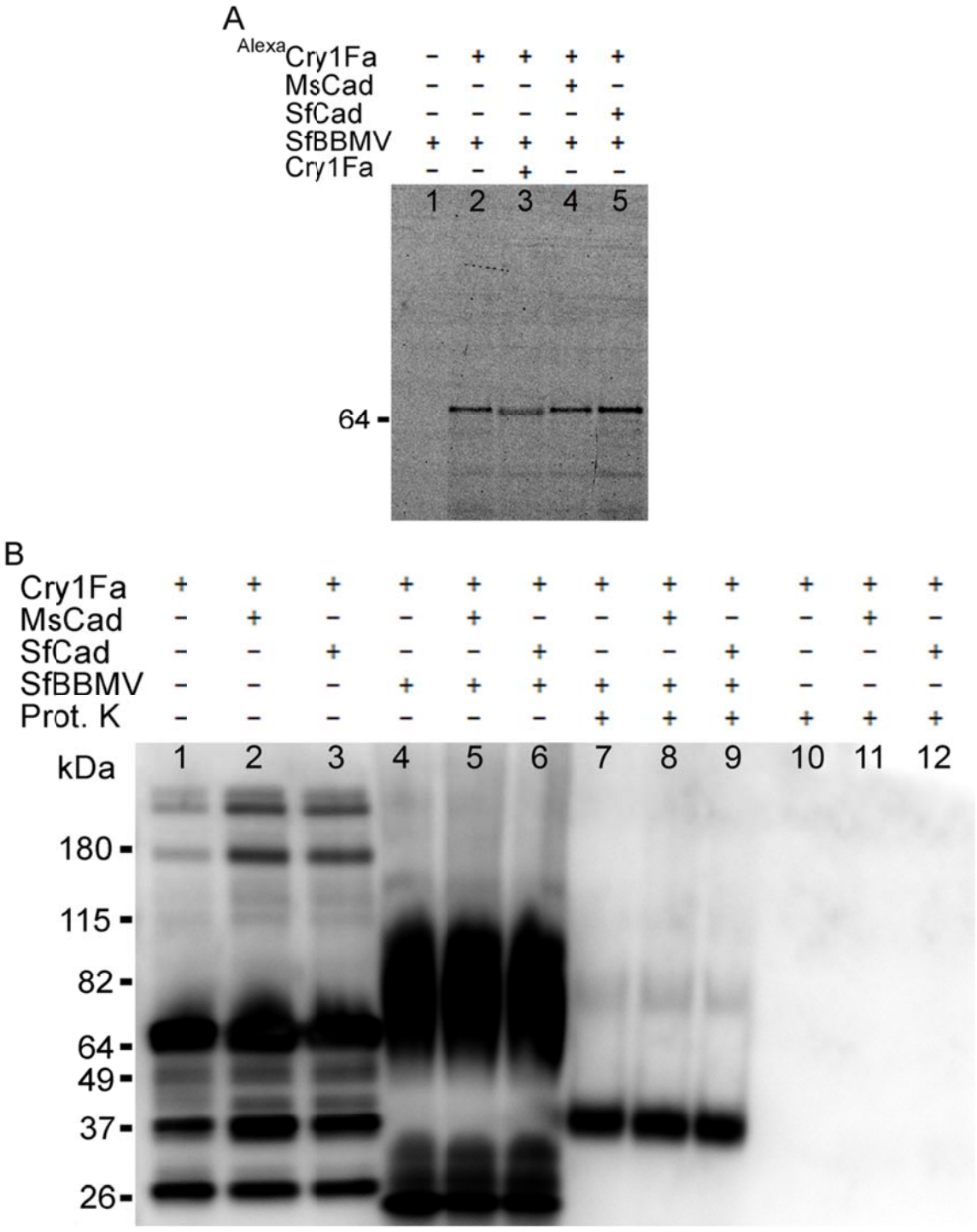

A
Alexa Cry1Fa - + + + +
MsCad - - - + -
SfCad - - - - +
SfBBMV + + + + +
Cry1Fa - - + - -
1 2 3 4 5
64
B
Cry1Fa + + + + + + + + + + + +
MsCad - + - - + - - + - - + -
SfCad - - + - - + - - + - - +
SfBBMV - - - + + + + + + - - -
Prot. K - - - - - - + + + + + +
kDa 1 2 3 4 5 6 7 8 9 10 11 12
180
115
82
64
49
37
26

Figure 2.5. Cadherin mediated protection of Cry1Fa toxin in *S. frugiperda*. (A) *In vitro* analysis of Cry1Fa toxin degradation in *S. frugiperda* gut extract in the presence or absence of cadherin peptides. Cry1Fa toxin alone or pre-treated with SfCad or MsCad was incubated with 5% (v/v) gut extract at 30°C for 5, 10, or 20 min. At the end of each time point, toxin proteolysis was terminated by heating the samples for 10 min at 100°C. (B) *In vivo* analysis of residual Cry1Fa in the tissue and gut content of *S. frugiperda* in the presence or absence of SfCad and MsCad. Midguts were extracted at various time points after treatment, and the tissue was separated from the gut content along with the PM. (A, B) Samples were homogenized, separated by SDS-PAGE, electroblotted to PVDF membrane, and the residual Cry1Fa was detected using anti-sera against Cry1Fa. (C, D) The band intensities of the residual Cry1Fa toxin in Panels A and B, respectively, were used to calculate fold differences in band intensities compared to toxin alone treatment.

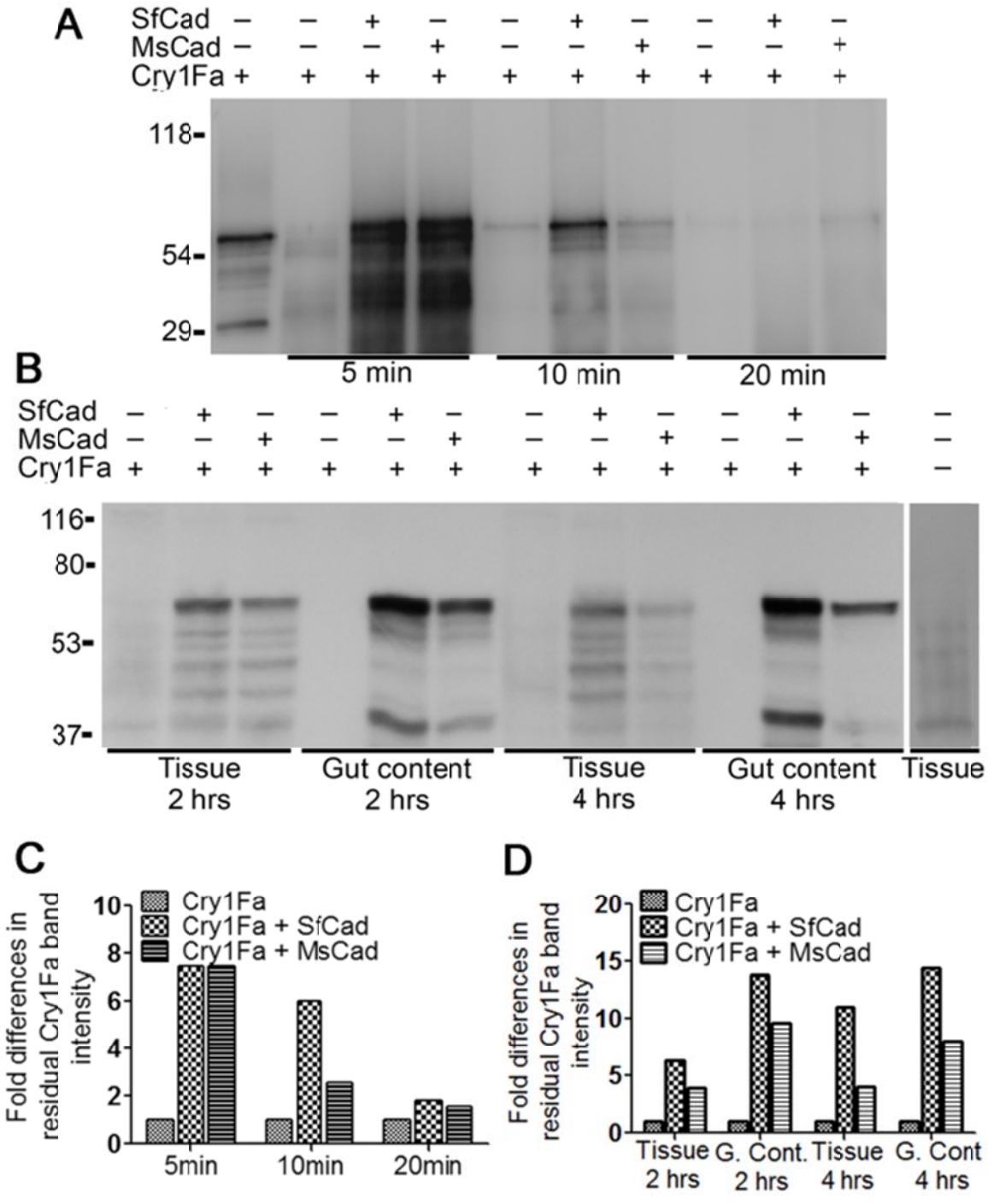
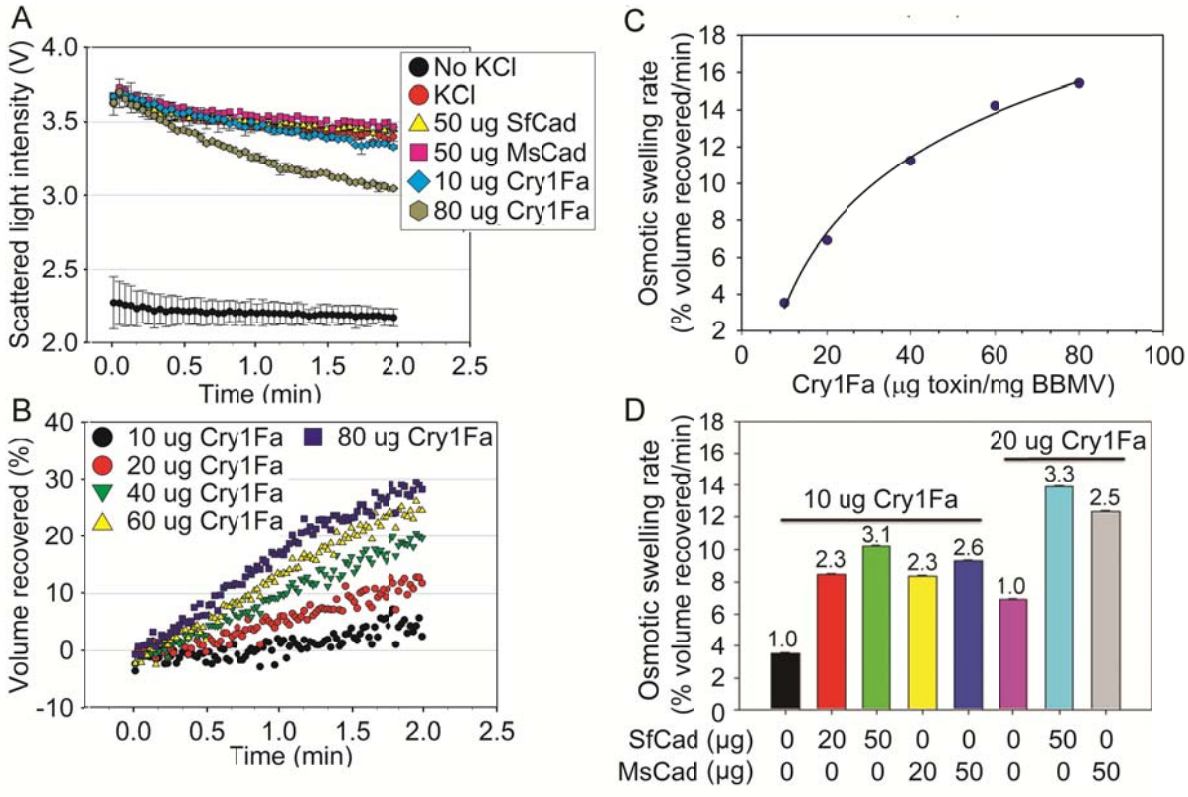

A
SfCad
MsCad
Cry1Fa
118
54
29
5 min
10 min
20 min
B
SfCad
MsCad
Cry1Fa
116
80
53
37
Tissue 2 hrs
Gut content 2 hrs
Tissue 4 hrs
Gut content 4 hrs
Tissue
C
Fold differences in residual Cry1Fa band intensity
Cry1Fa
Cry1Fa + SfCad
Cry1Fa + MsCad
5min
10min
20min
D
Fold differences in residual Cry1Fa band intensity
Cry1Fa
Cry1Fa + SfCad
Cry1Fa + MsCad
Tissue 2 hrs
G. Cont. 2 hrs
Tissue 4 hrs
G. Cont 4 hrs

Figure 2.6. SfCad and MsCad increase Cry1Fa-induced permeabilization of *S. frugiperda* BBMV. (A) Direct traces of scattered light from HEPES buffer treated, KCl buffer treated, toxin-treated, and cadherin-treated BBMV. Scattered light traces of only highest concentration of cadherin-treated BBMV, and two representative concentrations of toxin-treated BBMV were shown here. For clarity, the traces obtained during initial 0.012 min after the stopped-flow shots were omitted. This removed the initial traces that showed the initial very rapid increase in scattered light intensity as the vesicles shrank in the presence of KCl. V, volts. (B) The effect of treatment was determined by converting the scattered light signal into percentage of volume recovered. For clarity, only Cry1Fa treatments at various concentrations are shown. (C) The osmotic swelling rates (slopes of the curves from (B)) plotted against the concentration of Cry1Fa to show a dose-response standard curve. (D) The osmotic swelling rates for Cry1Fa alone and Cry1Fa with cadherin fragments. The ratios of the calculated equivalent Cry1Fa concentration over the actual Cry1Fa concentration are shown above each bar. The ratios indicate how much more toxin would have been needed to obtain same level of membrane permeability.


A
Scattered light intensity (V)
Time (min)
No KCl
KCl
50 ug SfCad
50 ug MsCad
10 ug Cry1Fa
80 ug Cry1Fa
B
Volume recovered (%)
Time (min)
10 ug Cry1Fa
20 ug Cry1Fa
40 ug Cry1Fa
60 ug Cry1Fa
80 ug Cry1Fa
C
Osmotic swelling rate
(% volume recovered/min)
Cry1Fa (µg toxin/mg BBMV)
D
Osmotic swelling rate
(% volume recovered/min)
10 ug Cry1Fa
20 ug Cry1Fa
1.0
2.3
3.1
2.3
2.6
1.0
3.3
2.5
SfCad (µg) 0 20 50 0 0 0 50 0
MsCad (µg) 0 0 0 20 50 0 0 50

Supplementary figure legends

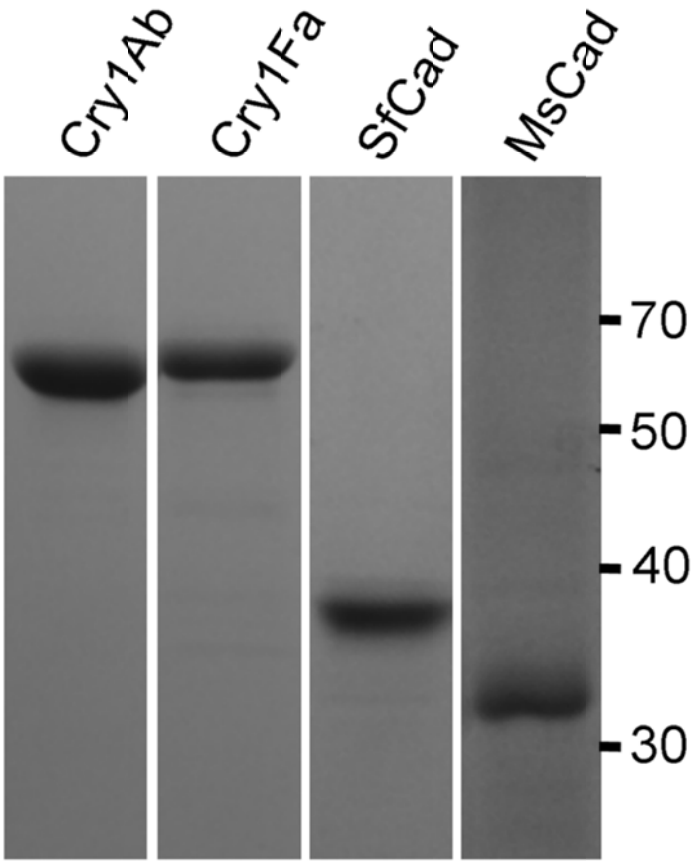
Figure 2.S1. Amino acid sequence of *S. frugiperda* cadherin. Amino acids constituting putative extracellular cadherin repeats (CR), and the putative transmembrane domain (TM) are in bold. The putative membrane proximal extracellular domain (MPED) is italicized. Arrows indicate beginning and end of each domain. The putative Cry1A toxin binding region homolog of *Manduca sexta* is in blue. Amino acids constituting putative TM are in bold, putative calcium binding sites are in green, and integrin recognition sequences are indicated in red. Protein sequence analysis was done using the ISREC ProfileScan server (http://hits.isb-sib.ch/cgi-bin/PFSCAN).


→Signal Peptide 22 a.a.
MAVDVRILTATLLVLTTATAQRDRCGYMVEIPRPDRPDFPPQNFDGLTWAQQPLLPAEDR 60
EEVCLNDYEPDPWSNNHGDQRIYMEEEIEGPVVIAKINYQGNTPPQIRLPFRVGAAHMLG 120
→CR1 ←
AEIREYPDATGDWYLVITQRQDYETPDMQRYTFDVSVEGQSLVVTVRLDIVNIDDNAPII 180
→CR2
EMLEPCNLPELVEPHVTECKYIVSDADGLISTSVMSYHIDSERGDEKVFELIRKDYPGDW 240
←
TKVYMVLELKKSLDYEENPLHIFRVTASDSLPNNRTVVMMVEVENVEHRNPRWMEIFAVQ 300
→CR3
QFDEKQAKSFTVRAIDGDTGINKPIFYRIETEDEDKEFFSIENIGEGRDGARFHVAPIDR 360
← →CR4
DYLKRDMFHIRIIAYKQGDNDKEGESSFETSANVTIIINDINDQRPEPFHKEYTISIMEE 420
TAMTLDLQEFGFHDRDIGPHAQYDVHLESIQPEGAHTAFYIAPEEGYQAQSFTIGTRIHN 480
← →CR5
MLDYEDDDYRPGIKLKAVAIDRHDNNHIGEAIININLINWNDELPIFDEDAYNVTFEETV 540
GDGFHIGKYRAKDRDIGDIVEHSILGNAANFLRIDIDTGDVYVSRDDYFDYQRQNEIIVQ 600
← →CR6
ILAVDTLGLPQNRATTQLTIFLEDINNTPPILRLPRSSPSVEENVEVGHPITEGLTATDP 660
DTTADLHFEIDWDNSYATKQGTNGPNTADYHGCVEILTVYPDPDNHGRAEGHLVAREVSD 720
← →CR7
GVTIDYEKFEVLYLVVRVIDRNTVIGPDYDEAMLTVTIIDMNDNWPIWADNTLQQTLRVR 780
EMADEGVIVGTLLATDLDGPLYNRVRYTMVPIKDTPDDLIAINYVTGQLTVNKGQAIDAD 840
←
DPPRFYLYYKVTASDKCSLDEFFPVCPPDPTYWNTEGEIAIAITDTNNKIPRAETDMFPS 900
→CR8
ERIYENTPNGTKITTIIASDQDRDRPNNALTYRINYAFNHRLENFFAVDPDTGELFVHFT 960
←
TSEVLDRDGEEPEHRIIFTIVDNLEGAGDGNQNTISTEVRVILLDINDNKPELPIPDGEF 1020
→CR9
WTVSEGEVEGKRIPPEIHAHDRDEPFNDNSRVGYEIRSIKLINRDIELPQDPFKIITIDD 1080
←
LDTWKFVGELETTMDLRGYWGTYDVEIRAFDHGFPMLDSFETYQLTVRPYNFHSPVFVFP 1140
→CR10
TPGSTIRLSRERAIVNGMLALANIASGEFLDRLSATDEDGLHAGRVTFSIAGNDEAAEYF 1200
←
NVLNDGDNSAMLTLKQAIPAGVQQFELVIRATDGGTEPGPRSTDCSVTVVFVMTQGDPVF 1260
→CR11
DDNAASVRFVEKEAGMSEKFQLPQADDPKNYRCMDDCHTIYYSIVDGNDGDHFAVEPETN 1320
←→MPED
VIYLLKPLDRSQQEQYRVVVAASNTPGGTSTLSSSLLTVTIGVREANPRPIFESEFYTAG 1380
VLHTDSIHKELVYLAAKHSEGLPIVYSIDQETMKIDESLQTVVEDAFDINSATGVISLNF 1440
QPTSVMHGSFDFEVVASDTRGASDRAKVSIYMISTRVRVAFLFYNTEAEVNERRNFIAQT 1500
FANAFGMTCNIDSVLPATDANGVIREGYTELQAHFIRDDQPVPADYIEGLFTELNTLRDI 1560
←→TM 19 a.a. ←
REVLSTQQLTLLDFAAGGSAVLPGGEYALAVYILAGIAALLAVICLALLIAFFIRNRTLN 1620
RRIEALTIKDVPTDIEPNHASVAVLNINKHTEPGSNPFYNPDVKTPNFDTISEVSDDLLD 1680
VEDLEQFGKDYFPPENEIESLNFARNPIATHGNNFGVNSSPSNPEFSNSQFRS 1733

Figure 2.S2. Comparison of the deduced amino acid sequence of SfCad and MsCad. An asterisk (*) indicates identity in both sequences; a colon (:) indicates a conserved substitution, and a period (.) indicates a semi-conserved substitution. Sequence comparison was done using ClustalW (Larkin et al. 2007 Bioinformatics 21:2947-8).

```
SfCad   FLDRLSATDEDGLHAGRVTFSIAGNDEAAEYFNVLNDGDNSAMLTLKQALPAGVQQFELV 60
MsCad   HLERISATDPDGLHAGVVTFQVVGDEESQRYFQVVNDGANLGSLRLLQAVPEEIREFRIT 60
        .*:*:**** ****** ***.:.*::*: .**:*:*** * . * * **:*  :::*.:.

SfCad   IRATDGGTEPGPRSTDCSVTVVFVMTQGDPVFDDNAASVRFVEKEAGMSEKFQLPQADDP 120
MsCad   IRATDQGTDPGPLSTDMTFRVVFVPTQGEPRFASSEHAVAFIEKSAGMEESHQLPLAQDI 120
        ***** **:*** *** :. **** ***:* * ..  :* *:**.***.*..*** *:*

SfCad   KNYRCMDDCHTIYYSIVDGNDGDHFAVEPETNVIYLLKPLDRSQQEQYRVVVAASNTPGG 180
MsCad   KNHLCEDDCHSIYYRIIDGNSEGHFGLDPVRNRLFLKKELIREQSASHTLQVAASNSPDG 180
        **: * ****:*** *:***. .**.::*  * ::* * * *.*. .: : *****:*.*

SfCad   TSTLSSSLLTVTIGVEEANPRPIFESEFYTAGVLHTDSIHKELVYLAAKHSEGLPIVYSI 240
MsCad   GIPLPASILTVTVTVEEADPRPVFMRELYTAGISTADSIGRELLRLHATQSEGAAITYAI 240
          .*.:*:****: ****:***:*  *:****:  :*** :**: * *.:*** .*.*:*

SfCad   DQETMKIDESLQTVVEDAFDINSATGVISLNFQPTSVMHGSFDFEVVASDTRGASDRAKV 300
MsCad   DYDTMVVDPSLEAVRQSAFVLNAQTGVLTLNIQPTATMHGLFKFEVTATDTAGAQDRTDV 300
        * :** :* **::* :.** :*: ***::**:***:.*** *.***.*:** **.**:.*

SfCad   SIYMISTRVRV- 311
MsCad   TVYVVSSQNRLE 312
        ::*::*:: *:
```

Figure 2.S3. SDS page of trypsin activated Cry1Fa and Cry1Ab toxins, and nickel column purified MsCad and SfCad peptides. Toxin and peptides were separated using a 10% SDS-PAGE gel.


Cry1Ab
Cry1Fa
SfCad
MsCad
70
50
40
30

CHAPTER 3

BACILLUS THURINGIENSIS CRY1FA AND CRY1AB TOXINS SHARE BINDING SITES ON BRUSH BORDER MEMBRANE VESICLES OF *OSTRINIA NUBILIALIS* (HÜBNER), *DIATRAEA GRANDIOSELLA* (DYAR), AND *HELICOVERPA ZEA* (BODDIE) LARVAE

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Abstract

Bacillus thuringiensis (Bt) Cry1Fa and Cry1Ab proteins are important Cry toxins due to their high, selective toxicity against a number of lepidopteran species, including important pests of corn and cotton. The mode of action of Cry1Ab toxin is well studied, but difficulties in labeling Cry1Fa without compromising its activity has limited direct study of Cry1Fa interactions with binding sites in larval brush border. To address this issue, we developed and used a fluorescence based binding assay to study Cry1Fa and Cry1Ab toxin interaction with BBMV proteins of *Ostrinia nubilalis* (European corn borer, ECB), *Diatraea grandiosella* (south western corn borer, SWCB), and *Helicoverpa zea* (corn earworm, CEW). Homologous and heterologous binding assays with fluorophore-(Alexa488)-labeled Cry1Fa toxin showed that Cry1Fa shares binding site(s) with Cry1Ab toxin in ECB, and SWCB for which Cry1Ab has higher affinity than Cry1Fa. Apart from the shared binding sites, Cry1Ab and Cry1Fa bind an additional site(s) in ECB and SWCB. In CEW, Cry1Fa and Cry1Ab each, has a high affinity binding site(s), which binds the heterologous toxin with low affinity. The Cry1Ab-Cry1Fa toxin binding models based on these results will be helpful in predicting evolution of cross-resistance against Cry1Ab and Cry1Fa toxins in ECB, SWCB, and CEW.

3.1 Introduction

The *Bacillus thuringiensis* (Bt) proteins Cry1Fa and Cry1Ab are highly toxic to major pests of corn *Ostrinia nubilalis* (European corn borer, ECB) and *Diatraea grandiosella* Dyar (southwestern corn borer, SWCB), and cotton *Heliothis virescens* (tobacco budworm) and *Pectinophora gossypiella* (pink bollworm)**.** Cry1Fa is also moderate to highly toxic against Cry1A tolerant *Spodoptera spp.*; major pests of corn and cotton in Southern USA and South and Central America (http://www.glfc.cfs.nrcan.gc.ca/bacillus). Due to their utility in corn and cotton pest control, Cry1Fa and Cry1Ab toxins have been extensively used in different Bt formulations and are also incorporated in transgenic Bt corn and cotton varieties (Bravo et al., 2008).

One of the biggest threats to the long-term utility of Bt toxins is the development of resistance in the targeted pests (Tabashnik, 2008). Stacking multiple Bt genes in a single transgenic crop is one of several strategies employed to delay resistance development against Cry toxins (Tabashnik, 2008). The overlapping yet distinct range of insecticidal activities of Cry1Fa and Cry1Ab toxins against major pests of corn and cotton make them candidates for stacking with each other or with other Cry toxins. In the case of corn, a Cry1Ab plus Cry1Fa co-expressed stack has been suggested in the literature (Gonzalez-Cabrera et al., 2003) and in a patent application (Lang, B. A. et al. 2008. Pub. NO. US 2008/031106). A novel approach nearing commercialization, called 'refuge in a bag,' combines two varieties of corn seed, one plant variety will be protected by Cry1Ab and the other by Cry1Fa. Although, Cry1Ab-Cry1Fa combination is successful in the short term, long term efficacy of crops that combine Cry1Fa and Cry1Ab may be affected by the probability of cross-resistance between these toxins.

Receptor alteration leading to reduced binding of Cry toxins to midgut receptors is the most documented mode of resistance to Bt Cry toxins (Ferre et al., 2002). Studies have shown

that alterations in a common binding site can confer resistance to toxins that share that binding site (Lee et al., 1995; Ballester et al., 1999; Gonzalez-Cabrera et al., 2003). Alternatively, toxins that do not share binding sites or bind additional sites apart from the shared site will have less probability of developing cross-resistance. Due to their accuracy in predicting cross-resistance development, toxin-binding models are widely used for selecting Cry toxins for toxin stacking (Ferre et al., 1991; Tabashnik et al., 1994; Ferre et al., 2002). Consequently, toxin-binding data showing lack of cross-binding is part of the process of registering Bt plants co-expressing multiple Bt toxins.

Cry1Fa and Cry1Ab have high homology in Domain II loops and frequently share binding sites in larval midgut. The following Cry1Ab binding models were constructed using data obtained primarily using ^{125}I-Cry1Ab as probe and unlabeled Cry1Fa as competitor. Cry1Fa shares binding site(s) with Cry1Ab in *O. nubilalis* (Hua et al., 2001), *Plutella xylostella* (diamondback moth), *H. virescens* (Ballester et al., 1999), and *Spodoptera frugiperda* (fall armyworm) (Sena et al., 2009). In *Spodoptera exigua* (beet armyworm) and *S. frugiperda*, Cry1Fa shares one binding site with Cry1Ac and a second site with Cry1C (Luo et al., 1999). In *Helicoverpa zea* (corn earworm, CEW) and *Helicoverpa armigera* (tobacco budworm), Cry1Fa, Cry1Ac, and Cry1Ja share common binding sites (Hernandez et al., 2005). Cry1Fa shows low affinity for the binding sites it shares with Cry1A toxins except in *P. xylostella* and *S. frugiperda*, where both Cry1Ab and Cry1Fa have comparable affinities for the shared binding site(s) (Ballester et al., 1999; Sena et al., 2009). The low affinity Cry1Fa competition for the shared binding sites contradicts high Cry1Fa toxicity against these species. Since the direct binding assays using ^{125}I-Cry1Fa have been precluded due to the inability to iodinate Cry1Fa without compromising its activity (Luo et al., 1999), current Cry1Fa binding site models do not provide

information as to whether Cry1Fa binds to additional high affinity site(s) apart from the ones shared with Cry1A toxins.

The goals of the present study were to develop a fluorescence-based binding assay that enables direct study of Cry1Fa toxin interactions with BBMV preparations from three important pests of corn and cotton, and to determine if Cry1Fa shares binding site(s) with Cry1Ab toxin in these species.

3.2 Materials and methods

Bacterial strains and toxin purification

Escherichia coli strain harboring Cry1Ab toxin was provided by Dr. Donald H. Dean (Ohio State University, Columbus, OH). The conditions for the expression of Cry1Ab inclusions and toxin purification were as described previously by Lee et al. (1992). Bt strain harboring Cry1Fa was obtained from Ecogen Inc. (Langhorne, PA). The conditions for Bt cultures, toxin purification, and activation were as described previously by Luo et al. (1999).

BBMV preparation

Frozen midguts of *Ostrinia nubilalis*, *Diatraea grandiosella*, and *Helicoverpa zea* were provided by Pioneer Hi-bred International Inc., Johnston, IA. BBMV were prepared using a $MgCl_2$ precipitation method (Wolfersberger et al., 1987), with modifications according to Carroll and Ellar (1993). The final BBMV pellet was suspended in 250 mM sucrose, 5 mM EGTA, 20 mM Tris, pH 7.5 and frozen at -80ºC until used.

Iodination of Cry1Ab

Trypsin activated Cry1Ab (1 µg) was radiolabeled with 0.5 mCi of $Na^{125}I$ (PerkinElmer) using the chloramine-T method as described previously (Garczynski et al., 1991). The specific activity of ^{125}I-Cry1Ab was 34.5 µCi/µg of toxin.

Labeling of Cry1Ab and Cry1Fa with Alexa-488

Trypsin activated Cry1Ab and Cry1Fa toxins were labeled with Alexa Fluor 488 (^{Alexa}Cry1Ab and ^{Alexa}Cry1Fa) according to the manufacturer's (Invitrogen, Molecular Probes, Inc, Eugene, OR) instructions. The final reaction was dialyzed against 20 mM Na_2CO_3, 200 mM NaCl, pH 9.6 at 4^{o}C, quantified according to the manufacturer's instructions, and stored in aliquots at -20^{o}C until needed for binding assays. The moles of dye per moles of ^{Alexa}Cry1Ab and ^{Alexa}Cry1Fa were 2.7 and 2.8 moles, respectively.

Saturation and competition binding assays

Saturation and competition binding assays using iodinated toxin were as described previously by Garczynski et al. (1991). For saturation binding assays, 150 µg/ml of BBMV were incubated for 1 h at room temperature with increasing molar concentrations of iodinated toxin in the absence or presence of 1000-fold molar excess of unlabeled toxin in 100 µl binding buffer (20 mM Na_2CO_3, 0.1% Tween20, 0.15 M NaCl, 0.1% BSA, pH 9.6) to determine total and non-specific binding, respectively. For competition binding assays, 150 µg/ml BBMV were incubated with 0.22 nM ^{125}I-Cry1Ab toxin plus increasing molar concentrations of unlabeled Cry1Ab or Cry1Fa toxin in binding buffer for homologous and heterologous competition, respectively. BBMV bound ^{125}I-Cry1Ab was pelleted using a micro centrifuge, washed two times with 1 ml

binding buffer and the radioactivity of the final pellets was measured with a Beckman model Gamma 4000 detector. Each binding assay was repeated.

Saturation and competition binding assays using Alexa-488 conjugated toxin were performed as described for the iodinated toxin except for the following modifications. All assays were performed using 200 µg/ml BBMV and the reactions were incubated for 2 h at room temp. For the competition binding assays, 0.5 nM ^{Alexa}Cry1Ab or ^{Alexa}Cry1Fa toxin was used as input. At the end of the incubation, BBMV were pelleted using a micro centrifuge, washed three times with binding buffer, and solubilized in SDS sample buffer by boiling the samples for 10 min. Proteins were separated using a Criterion 4-20%Tris-HCl gradient gel (Bio-Rad, Hercules, CA) and the bound Alexa-488 conjugated toxins were detected by scanning the gels using a Typhoon Imager with 488 nm excitation and 520 nm emission filters (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Intensity of the toxin bands was quantified by densitometry with a FluorChem 8900 imager and software (AlphaInnotech, San Leandro, CA). Data were analyzed using SigmaPlot software (Version 9; Systat Software Inc., San Jose, CA) and the curves were fitted based on a best fit of the data to a one site saturation binding equation or a one site competition equation with maximum binding set at 100%. Experiments were repeated at least two times and data were pooled.

3.3 Results

Scans of SDS-gel separated ECB, SWCB and CEW brush border proteins at Alexa-488 excitation/emission wavelengths showed low endogenous fluorescence (data not shown) , enabling the use of Alexa-488 as a fluorophore for measuring Cry1Fa binding to BBMV. The relationship between fluorescence intensity and toxin quantity was established as follows.

Increasing amounts of ^{Alexa}Cry1Ab or ^{Alexa}Cry1Fa toxins were separated by SDS-PAGE, the gels were scanned for fluorescence and toxin band density was plotted against toxin quantity. The band images of ^{Alexa}Cry1Ab and ^{Alexa}Cry1Fa are shown in Fig. 3.1A and C and plots of density versus Cry amount are shown in Fig. 3.1B and D. A linear correlation between band density and toxin quantity was observed for both ^{Alexa}Cry1Ab and ^{Alexa}Cry1Fa over the range of toxins tested. The correlation coefficients for Cry1Ab ($R^2 = 0.79$) and Cry1Fa ($R^2 = 0.70$) indicated that an accurate inference of the amount of protein detected can be made over the entire toxin range tested. Conjugation of Alexa-488 to Cry1Fa did not affect toxicity, as ^{Alexa}Cry1Fa and unlabeled Cry1Fa were equally toxic to ECB neonates (Dr. Mark Nelson, personal communication).

Comparison of iodine and fluorescence based saturation and competition binding of Cry1Ab to ECB BBMV

The fluorescence-based binding assay was validated by comparing ^{125}Iodine and Alexa-488 labeled Cry1Ab binding to ECB BBMV in saturation and competition binding formats. Figures 3.2A and B shows the results of saturation binding experiments where increasing amounts of ^{125}I-Cry1Ab and ^{Alexa}Cry1Ab were incubated with a constant amount of ECB BBMV. Both labeled Cry1Ab preparations bound saturably yielding a dissociation constant (K_d) = 2.8 ± 0.2 nM for ^{125}I-Cry1Ab, a value in agreement with (Hua et al., 2001) and a K_d = 1.1 ± 0.5 nM for ^{Alexa}Cry1Ab (Table 3.1).

Homologous and heterologous competition assays conducted using ^{125}I-Cry1Ab and ^{Alexa}Cry1Ab resulted in similar competition binding curves (Fig. 3.2C and D) and calculated EC_{50}s of 0.6 ± 1.1 nm and 4.5 ± 1.1 nm for the two assay formats (Table 3.1). In both the iodine and the fluorescence-based assays, 90-100% of bound Cry1Ab was competed by non-labeled

Cry1Ab whereas the highest concentration of Cry1Fa competitor inhibited Cry1Ab binding by about 25%. The partial competition of Cry1Fa for Cry1Ab binding is evidence that Cry1Ab has a binding site not shared with Cry1Fa. Furthermore, 25% inhibition of Cry1Ab binding by Cry1Fa and the shift of the heterologous competition curve toward higher concentrations of Cry1Fa is evidence that Cry1Ab has a higher affinity for the shared binding site. The higher EC_{50} values obtained in fluorescence based assays may be due, in part, to the higher concentration of input ^{Alexa}Cry1Ab (0.5 nM) as compared to ^{125}I-Cry1Ab (0.22 nM) since EC_{50} is the sum of K_d of the competitor and the input labeled toxin concentration.

Saturation and competition binding characteristics of Cry1Fa toxin to ECB BBMV

In the saturation binding assay, ^{Alexa}Cry1Fa bound ECB BBMV saturably and with high affinity, EC_{50} of 0.4 (± 0.2) nM (Fig. 3.3A). In the homologous competition binding assay, ^{Alexa}Cry1Fa specifically bound ECB BBMV as indicated by 85% competition by unlabeled Cry1Fa (Fig. 3.3B). In the heterologous competition binding assay, unlabeled Cry1Ab competed 70% of ^{Alexa}Cry1Fa binding indicating that Cry1Ab shares most of the Cry1Fa site(s) on ECB BBMV (Fig. 3.3B). However, Cry1Ab could not completely compete off ^{Alexa}Cry1Fa binding as indicated by a 15% difference between the homologous and heterologous competition curves. This difference between Cry1Fa and Cry1Ab competition suggested that Cry1Fa has a small population of binding site(s) not shared with Cry1Ab. For a constant input of ^{Alexa}Cry1Fa, Cry1Fa homologous competition yielded a higher EC_{50} (24.6 ± 1.2 nm) value compared to Cry1Fa/Cry1Ab heterologous competition (6.8 ± 1.2 nM) suggesting a higher Cry1Ab affinity for the shared binding site(s).

Binding characteristics of Cry1Ab and Cry1Fa to SWCB BBMV

Cry1Ab bound SWCB BBMV with high affinity and specificity as indicated by 100% inhibition of ^{Alexa}Cry1Ab binding to BBMV by non-labeled Cry1Ab with EC_{50} = 2.6 ± 1.2 nM (Fig. 3.4A). In contrast, Cry1Fa inhibited ^{Alexa}Cry1Ab binding by about 30% indicating the presence of a Cry1Ab binding site(s) not shared with Cry1Fa (Fig. 3.4A). The shift of the heterologous competition curve towards higher concentrations of competitor with a relatively high EC_{50} (67 ± 1 nM) is evidence of low Cry1Fa affinity for the shared binding site(s). In competition binding assays, ^{Alexa}Cry1Fa bound saturably to SWCB BBMV as there was a 95% inhibition of ^{Alexa}Cry1Fa binding in the presence of excess unlabeled Cry1Fa with an EC_{50} of 21 (± 1 nM) (Fig. 3.4B). The lesser inhibition of bound ^{Alexa}Cry1Fa by unlabeled Cry1Ab (45% inhibition) is evidence of a Cry1Fa binding site(s) on SWCB not shared with Cry1Ab. The ^{Alexa}Cry1Fa/Cry1Ab competition curve shown in Fig. 3.4B has a calculated EC_{50} = 0.9 (± 1.3 nM) meaning that Cry1Ab has a higher affinity for the shared binding site(s) than does Cry1Fa (Table 3.1).

Binding characteristics of Cry1Ab and Cry1Fa to CEW BBMV

As shown in Fig. 3.5A, Cry1Ab bound CEW BBMV with high affinity, EC_{50} = 3.8 ± 1.2 nM (Table 3.1). However, the partial homologous inhibition of ^{Alexa}Cry1Ab binding indicated that 40% of the Cry1Ab bound to CEW BBMV was non-specific. A similar inhibition (30%) of ^{Alexa}Cry1Ab binding by Cry1Fa suggested that Cry1Ab did not have an additional binding site(s) other than the site(s) shared with Cry1Fa. However, the shift of the ^{Alexa}Cry1Ab competition curve toward high concentration of competitor and high EC_{50} with unlabeled Cry1Fa indicated that Cry1Ab has much higher affinity for the shared binding site(s).

In Cry1Fa competition assays (Fig. 3.5B), unlabeled Cry1Fa inhibited binding of about 40% of ^{Alexa}Cry1Fa (EC_{50} = 8.7 ± 1.2 nM) and unlabeled Cry1Ab inhibited binding of about 35% of the ^{Alexa}Cry1Fa. The shift of the heterologous Cry1Ab/Cry1Fa competition curve towards higher competitor concentrations is evidence of low affinity Cry1Ab binding to the shared Cry1Fa binding site(s) in CEW BBMV (Fig. 3.5B).

3.4 Discussion

The fluorescence-based Cry1 toxin binding assay utilized in this study allowed the construction of models for Cry1Fa and Cry1Ab binding to BBMV from ECB, SWCB and CEW (Fig. 3.6).

Figure 3.6 shows the three-site model constructed according to analyses of Cry1Ab and Cry1Fa binding to ECB BBMV. In agreement with our previous report based on ^{125}I-Cry1Ab binding experiments (Hua et al., 2001), ^{125}I-Cry1Ab and ^{Alexa}Cry1Ab bound ECB BBMV with high affinity and Cry1Fa showed low affinity for one of the two Cry1Ab binding sites. The limited Cry1Fa sharing of total Cry1Ab binding to ECB BBMV is also in agreement with low level Cry1Fa cross-resistance in a Cry1Ab-resistant ECB strain (Li et al., 2004; Siqueira et al., 2004; Crespo et al., 2011). Results of the reciprocal heterologous competition assay using ^{Alexa}Cry1Fa as probe and Cry1Ab as competitor (Fig. 3.3B) showed the ability of Cry1Ab to inhibit 70% of ^{Alexa}Cry1Fa binding supporting the conclusion that Cry1Fa shares most of its binding site(s) with Cry1Ab. Moreover, Cry1Ab inhibited Cry1Fa binding with a ~3-fold lower EC_{50} than Cry1Fa, a result supporting the reciprocal experiment where Cry1Ab showed higher affinity than Cry1Fa for the shared binding site. The existence of a Cry1Fa binding site not shared with Cry1Ab is suggested by our binding data and a previous Cry1Fa binding analysis

using surface plasmon resonance (SPR) (Hua et al., 2001). In our competition assays Cry1Ab did not compete 15% of ^{Alexa}Cry1Fa bound to ECB BBMV. Similarly in SPR experiments Cry1Ab did not compete 12% of bound Cry1Fa (Cry1Fa-Cry1Fa gave 75% inhibition versus 63% for Cry1Ab-Cry1Fa) (Hua et al., 2001). The presence of a Cry1Fa binding site(s) not recognized by Cry1Ab agrees with the toxicity analyses of Cry1Fa-resistant ECB strains where no resistance to Cry1Ab is reported (Lang, B.A. Publication No. US 2008/0311096; Pereira et al., 2010). A caveat is that Cry1Ab and Cry1Fa resistance in laboratory selected ECB strains involves a resistance mechanism other than loss of toxin binding to midgut receptors (Li et al., 2004; Siqueira et al., 2006; Pereira et al., 2010; Crespo et al., 2011). Based on binding analyses and studies of Cry1Ab or Cry1Fa resistant ECB strains, there are unique components to Cry1Fa action that support stacking Cry1Ab and Cry1Fa toxins in corn. Further research is needed to conclusively identify a unique Cry1Fa binding site and to determine the frequencies of resistance mechanisms involving loss of Cry1Ab or Cry1Fa toxin binding.

High affinity Cry1Ab and Cry1Fa binding to SWCB BBMV correlated with high Cry1Ab and Cry1Fa toxicity to SWCB (Huang et al., 2006) and in agreement with a prior analysis of Cry1Ab binding affinity (Rang et al., 2004). Similar to the competition binding pattern of Cry1Ab in ECB, Cry1Ab showed higher affinity for the binding site(s) shared with Cry1Fa and Cry1Ab bound an additional high affinity site(s) not shared with Cry1Fa. The low affinity of Cry1Fa for the shared site(s) was confirmed by the results of the reciprocal heterologous experiments with Cry1Ab as competitor. Similar to Cry1Ab binding to SWCB BBMV, Cry1Fa also bound an additional site(s) not shared with Cry1Ab. The Cry1Fa and Cry1Ab interaction in SWCB can be explained by a three-site binding model where Cry1Ab and Cry1Fa share binding site(s) for which Cry1Ab has higher affinity than Cry1Fa (Fig. 3.6). Apart from the shared

binding site(s), both toxins bind additional high affinity site(s) not shared with the other toxin. The high binding affinity of Cry1Ab toxin for the shared binding site(s) raises the issue of whether or not shared toxin binding increases the likelihood of Cry1Ab cross-resistance in Cry1Fa resistant SWCB. Since both Cry1Ab and Cry1Fa are highly toxic to SWCB, transgenic corn co-expressing Cry1Fa and Cry1Ab toxins will be under high selection pressure to alter the common binding site(s). However, the possibility that the alteration of the shared binding site(s) will confer resistance to both toxins will depend on the functionality of the shared site(s) in Cry1Fa and Cry1Ab toxicity. Further studies with, Cry1Ab or Cry1Fa resistant SWCB are needed to rule out the possibility of cross-resistance development against these toxins.

CEW is moderately susceptible to Cry1Ab but tolerant to Cry1Fa toxin. In agreement with previous reports (Wolfersberger, 1990; Chambers et al., 1991), susceptibility of CEW to Cry1Ab and Cry1Fa did not correlate with the high affinity of these toxins to CEW BBMV. Cry1Ab and Cry1Fa bound CEW BBMV with high affinity exhibited by an EC_{50} comparable to Cry1Ab and Cry1Fa binding to ECB and SWCB. The Cry1Ab binding affinity calculated from our assay is comparable to previously reported Cry1Ab affinity for CEW (Karim et al., 2000; Gouffon et al., 2011). The high non-specific Cry1Ab and Cry1Fa binding to CEW BBMV suggesting the presence of fewer toxin binding sites correlate with low susceptibility of CEW to these toxins. Furthermore, higher non-specific Cry1Fa binding to CEW BBMV compared to Cry1Ab also correlate with higher tolerance of CEW to Cry1Fa toxin. Similar to our findings, Karim et al. (2000) also observed high non-specific Cry1Ab binding to CEW BBMV, up to 25% non-specific binding. Gouffon et al. (2011), however, observed up to 90% specific binding of Cry1Ab to CEW BBMV. It is possible that the differences in susceptibility of CEW strains used in these assays and the assay conditions may have contributed to the differences in non-specific

binding observed. Taken together, results of the competition binding assays indicate that Cry1Fa and Cry1Ab share two binding site(s) in CEW. Cry1Ab has higher affinity for one while Cry1Fa has higher affinity for the second site(s) (Fig. 3.6). Cry1Fa and Cry1Ab receptor sharing in CEW is not of concern, as CEW is tolerant to Cry1Fa toxin. Nevertheless, our binding data are suggestive of a low probability of cross-resistance between Cry1Ab and Cry1Fa toxins in CEW.

The Cry1Fa binding site models developed here will be helpful in predicting evolution of cross-resistance to Cry1Fa and Cry1Ab toxins in the species tested. However, further research is needed to determine the identity of the shared binding site(s) and its importance in Cry1Fa and Cry1Ab toxicity.

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Table 3.1. Binding affinity constants (K_D) and 50% effective concentrations (EC_{50}) calculated from saturation and competition binding assays.

	ECB				SWCB	CEW
	^{125}Toxin		^{Alexa}Toxin		^{Alexa}Toxin	^{Alexa}Toxin
Toxin	K_D[1]	EC_{50}[2]	K_D[1]	EC_{50}[2]	EC_{50}[2]	EC_{50}[2]
Cry1Ab	2.8 + 0.23	0.6 ±1.1	1.1 ± 0.5	4.5 ± 1.1	2.6 ± 1.2	3.8 ± 1.2
Cry1Fa	N	N	0.4 ± 0.2	24.6 ± 1.2	21 ± 1.2	8.7 ± 1.2

[1] K_D (nM) ± SEM

[2] EC_{50} (nM) ± SEM

Figure legends

Figure 3.1. Relationship between ^{Alexa}Cry1Fa and ^{Alexa}Cry1Ab concentration and fluorescence intensity. (A, C) Various concentrations of ^{Alexa}Cry1Ab and ^{Alexa}Cry1Fa were separated on gradient gels and fluorescence was detected using a Typhoon imager. (B, D) Fluorescence signal intensities represented by the densities of the toxin bands were plotted against corresponding toxin concentrations and a linear regression line was fitted.

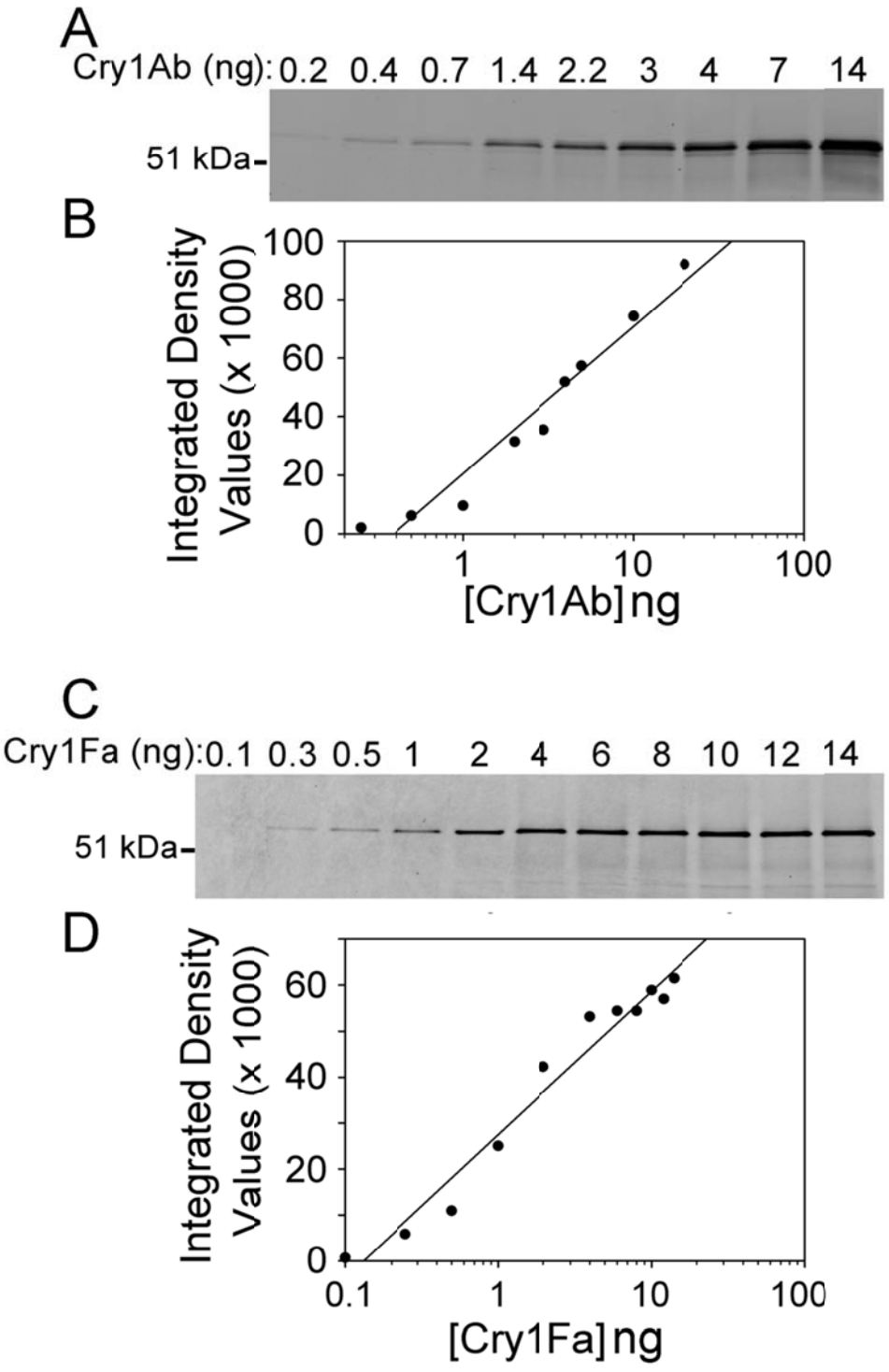

A
Cry1Ab (ng): 0.2 0.4 0.7 1.4 2.2 3 4 7 14
51 kDa
B
Integrated Density Values (x 1000)
100
80
60
40
20
0
1
10
100
[Cry1Ab] ng
C
Cry1Fa (ng): 0.1 0.3 0.5 1 2 4 6 8 10 12 14
51 kDa
D
Integrated Density Values (x 1000)
60
40
20
0
0.1
1
10
100
[Cry1Fa] ng

Figure 3.2. Saturation and competition binding of ^{125}I-Cry1Ab and ^{Alexa}Cry1Ab to *Ostrinia nubilalis* BBMV. For saturation binding assays, BBMV were incubated with increasing molar concentrations of ^{125}I-Cry1Ab (A) or ^{Alexa}Cry1Ab (B) to determine total binding. Non-specific binding was determined by incubating BBMV with increasing molar concentrations of labeled Cry1Ab plus 1,000-fold molar excess unlabeled Cry1Ab. Specific binding was calculated by subtracting non-specific binding from total binding. For the competition binding assays, BBMV were incubated with ^{125}I-Cry1Ab (C) or ^{Alexa}Cry1Ab (D) in the presence of increasing molar concentrations of unlabeled Cry1Ab or Cry1Fa to determine homologous and heterologous binding, respectively. Binding was expressed as percentage of the maximum amount of labeled toxin bound during incubation in the absence of competitors. Each data point is a mean of two independent experiments. Vertical lines indicate SEM.

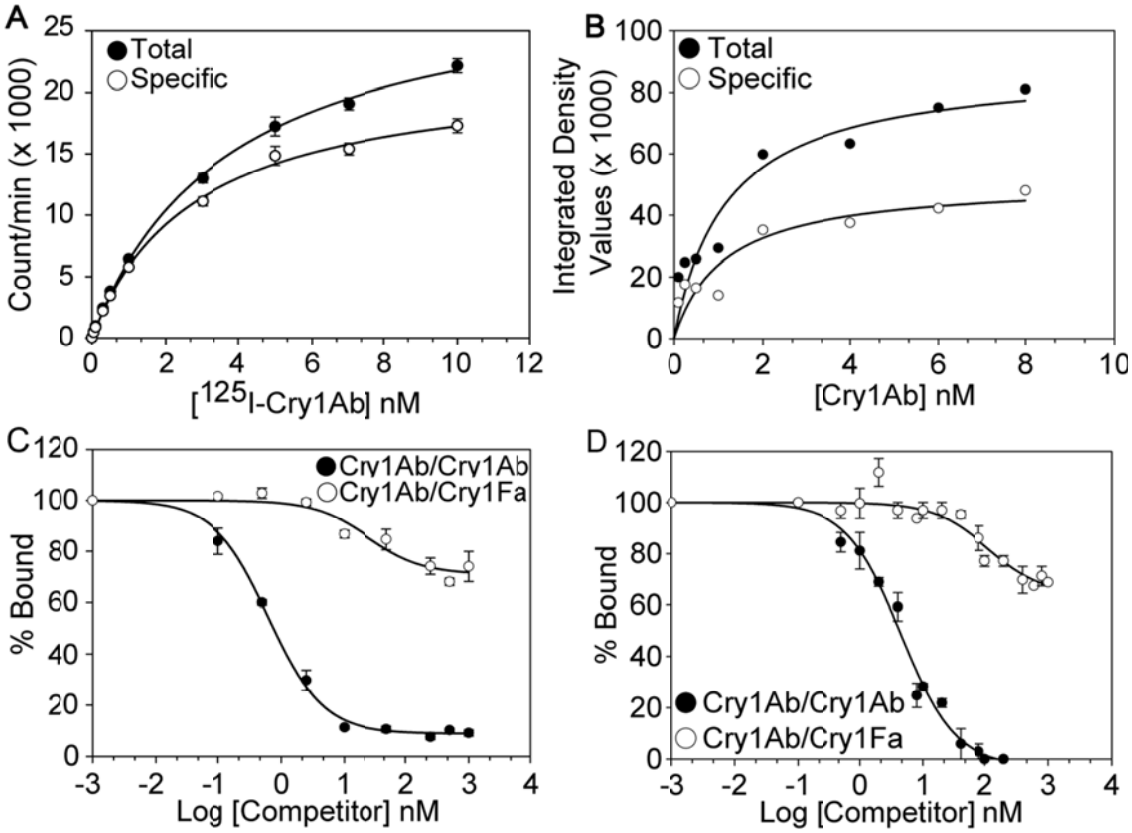

A
Total
Specific
Count/min (x 1000)
[125I-Cry1Ab] nM
B
Total
Specific
Integrated Density Values (x 1000)
[Cry1Ab] nM
C
Cry1Ab/Cry1Ab
Cry1Ab/Cry1Fa
% Bound
Log [Competitor] nM
D
Cry1Ab/Cry1Ab
Cry1Ab/Cry1Fa
% Bound
Log [Competitor] nM

Figure 3.3. Saturation and competition binding of ^{Alexa}Cry1Fa to *Ostrinia nubilalis* BBMV. (A) Saturation ^{Alexa}Cry1Fa binding to BBMV showing total and specific binding. (B) Homologous and heterologous competition binding of ^{Alexa}Cry1Fa to BBMV in the presence of increasing molar concentrations of unlabeled Cry1Ab or Cry1Fa toxin, respectively. Each data point is a mean of two independent experiments. Vertical lines indicate SEM.

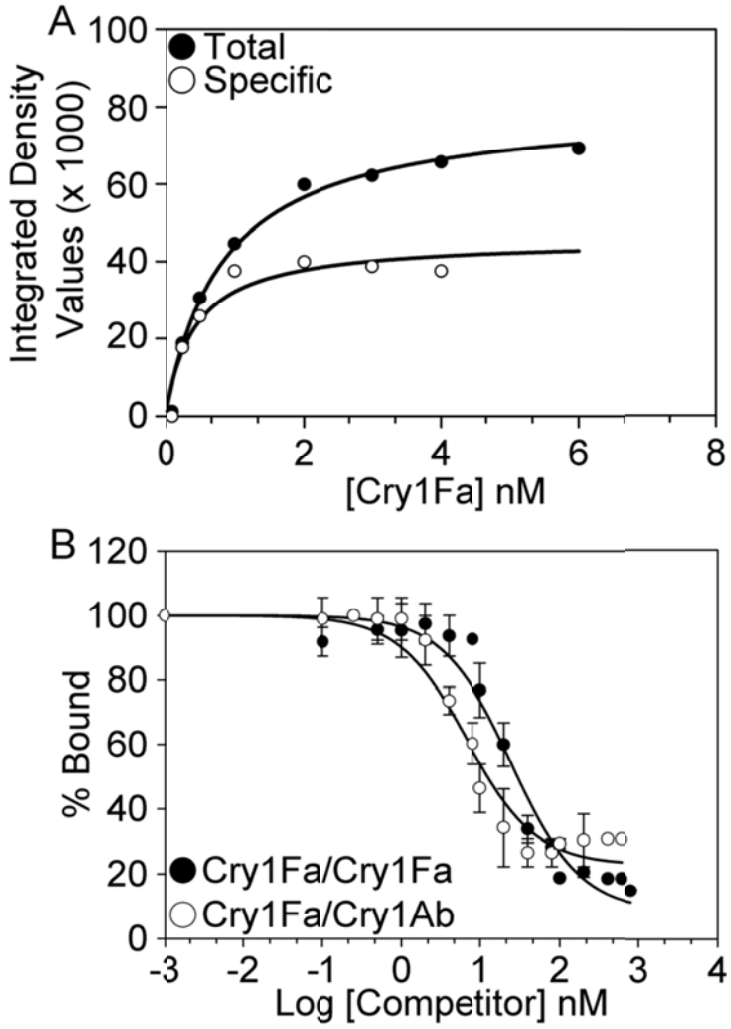

A
Total
Specific
Integrated Density Values (x 1000)
[Cry1Fa] nM
B
% Bound
Cry1Fa/Cry1Fa
Cry1Fa/Cry1Ab
Log [Competitor] nM

Figure 3.4. Competition binding characteristics of ^{Alexa}Cry1Ab and ^{Alexa}Cry1Fa to *Diatraea grandiosella* BBMV. For competition binding assays, BBMV were incubated with ^{Alexa}Cry1Ab (A) or ^{Alexa}Cry1Fa (B) toxin in the presence of increasing molar concentrations of unlabeled Cry1Ab or Cry1Fa toxin. Each data point is a mean of two independent experiments. Vertical lines indicate SEM.

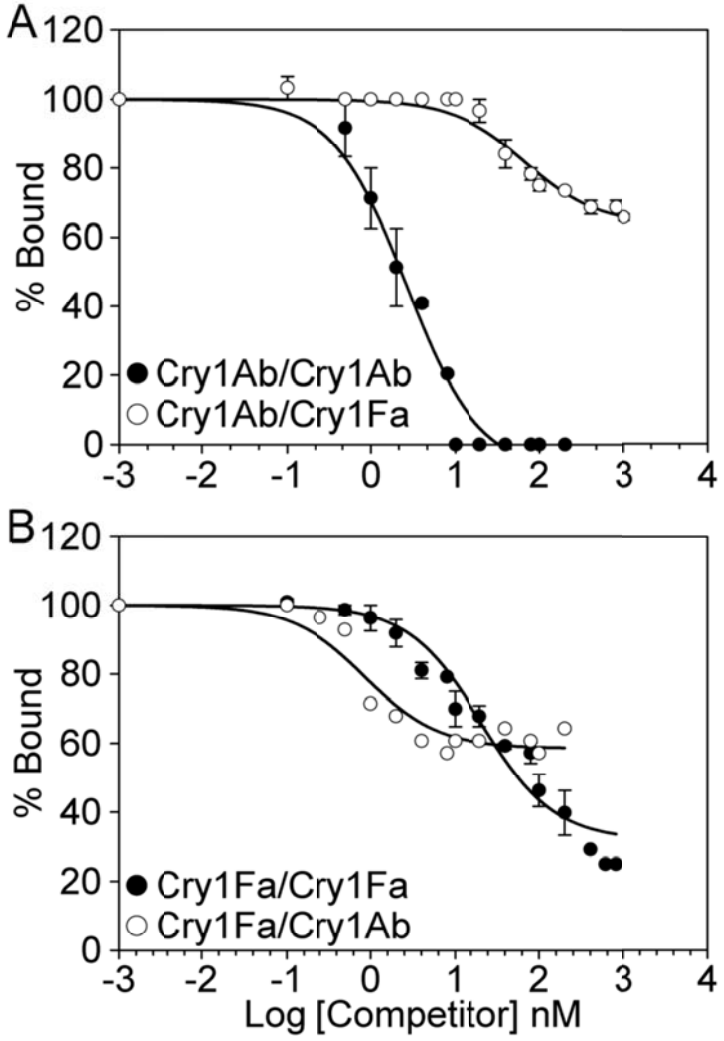

A
B
% Bound
Cry1Ab/Cry1Ab
Cry1Ab/Cry1Fa
Cry1Fa/Cry1Fa
Cry1Fa/Cry1Ab
Log [Competitor] nM

Figure 3.5. Competition binding of ^{Alexa}Cry1Ab and ^{Alexa}Cry1Fa toxins to *Helicoverpa zea* BBMV. BBMV were incubated with ^{Alexa}Cry1Ab (A) or ^{Alexa}Cry1Fa (B) alone or in the presence of increasing molar concentrations of unlabeled Cry1Ab or Cry1Fa toxin. Each data point is a mean of two independent experiments. Vertical lines indicate SEM.

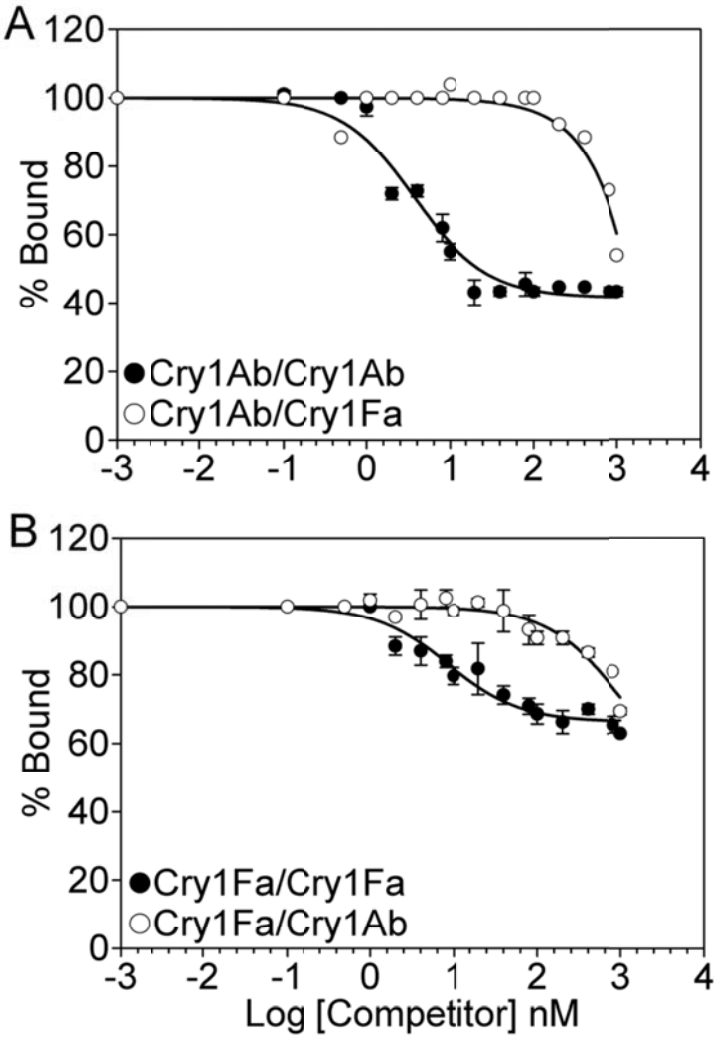
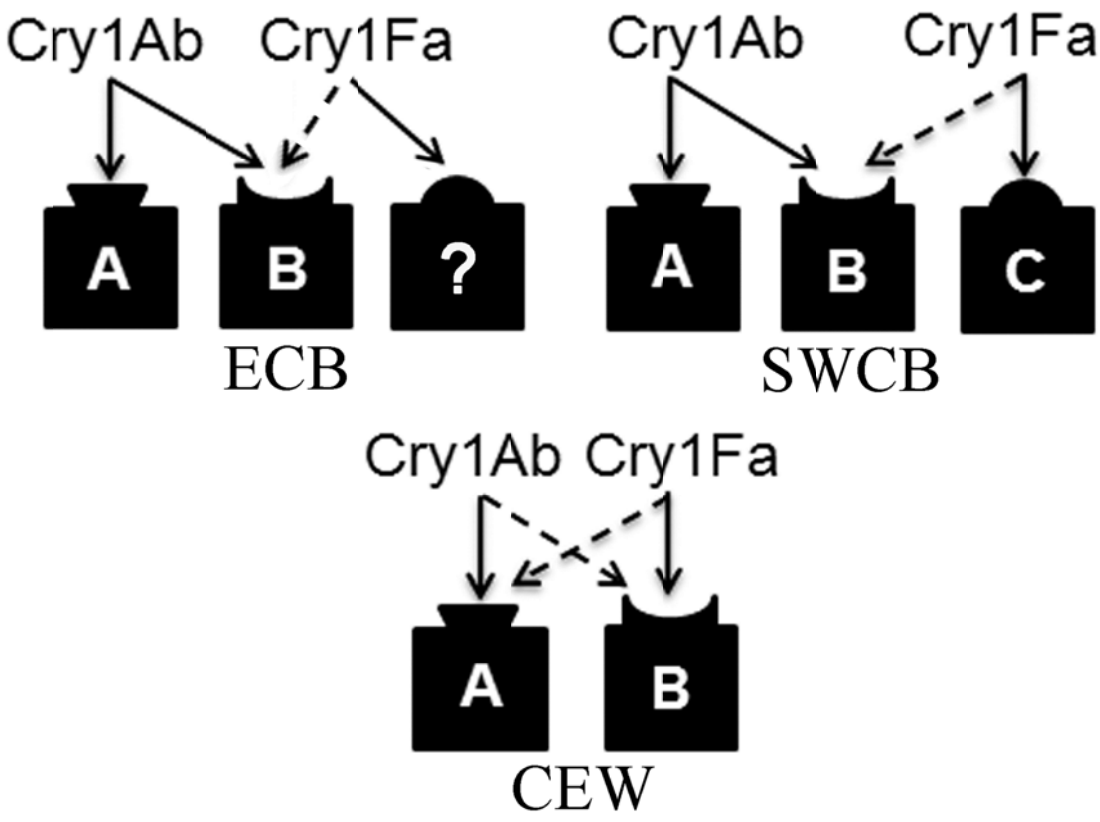

A
120
100
80
60
40
20
0
% Bound
Cry1Ab/Cry1Ab
Cry1Ab/Cry1Fa
-3
-2
-1
0
1
2
3
4
B
120
100
80
60
40
20
0
% Bound
Cry1Fa/Cry1Fa
Cry1Fa/Cry1Ab
-3
-2
-1
0
1
2
3
4
Log [Competitor] nM

Figure 3.6. Toxin binding models describing Cry1Fa and Cry1Ab toxins interactions with the midgut receptors in *Ostrinia nubilalis* (ECB), *Diatraea grandiosella* (SWCB), and *Helicoverpa zea* (CEW). Dashed arrows indicate lower binding affinity than solid arrows.


Cry1Ab
Cry1Fa
A
B
?
ECB
Cry1Ab
Cry1Fa
A
B
C
SWCB
Cry1Ab
Cry1Fa
A
B
CEW

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

The success of Bt corn and cotton in suppressing important lepidopteran and coleopteran pests has revolutionized pest management in these crop systems. However, the challenge of avoiding and managing insect resistance to Bt toxins threatens long term utility of Bt technology. The problem also remains of controlling relatively Cry toxin tolerant pests, such as *Spodoptera frugiperda* (Fall Armyworm). For example, even the highest attainable dose of Cry1Fa in corn provides marginal control of *S. frugiperda*. Recently discovered cadherin-based Cry synergists have the potential to increase the efficacy of Cry toxins to the high-dose status that is preferred for Bt crop insect resistance management (Chen et al., 2007; Tabashnik, 2008).

A number of cadherin-based Cry toxin synergists with efficacy against lepidopteran, dipteran and coleopteran larvae are reported (Chen et al., 2007; Hua et al., 2008; Abdullah et al., 2009; Pacheco et al., 2009; Park et al., 2009a; Park et al., 2009b). However, without understanding their synergistic mechanisms it is difficult to design improved synergists. The differential enhancement of Cry1Fa toxicity by MsCad and SfCad in *S. frugiperda* provided me the means to dissect the mechanism of cadherin-based Cry toxin synergism. The results of this mechanistic study of differential SfCad and MsCad synergism of Cry1Fa is presented in Chapter 2.

Use of multiple Bt toxins in a single crop, a practice referred to as toxin-stacking, is another approach used to improve the efficacy of transgenic Bt crops by extending the range of targeted insect species and possibly ameliorating the threat of insect. The success of Bt crop

varieties expressing multiple Cry toxins depends on the probability of cross-resistance development against the stacked toxins (Bates et al., 2005; Tabashnik et al., 2008; Bravo et al., 2011). Cry1Fa toxin, the subject of the second part of my dissertation research (Chapter 3), is used alone in Bt corn and cotton and has now been stacked with Cry1Ab in corn. The technical problem of labeling Cry1Fa toxin without destroying its toxicity has precluded measuring Cry1Fa binding to brush border membrane (BBMV). The first goal of the second part of my dissertation research was to develop a Cry1Fa labeling and binding assay that could be applied to the direct study of Cry1Fa toxin interactions with BBMV preparations from three important lepidopteran pests of corn. The second goal was to determine whether Cry1Fa shares binding site(s) with Cry1Ab toxin in these species. The results of this Cry1Fa binding research is presented in Chapter 3.

4.1. Mode of action of cadherin-based Cry toxin synergism.

The mode of Cry toxin action involves sequential steps of crystal solubilization, proteolytic activation of protoxin, toxin binding to receptors on the brush border of the midgut epithelium, toxin pre-pore oligomer formation, membrane insertion, and formation of transmembrane pores; which ultimately cause lysis of the midgut epithelial cells leading to septicemia and insect death (Bravo et al., 2007). Alterations in these steps, most importantly loss of toxin binding to midgut epithelium, and changes in gut proteases that either hinder activation or cause rapid degradation of toxin results in development of insect resistance to Bt Cry toxins (Tabashnik, 2008). Reasoning that cadherin peptides may enhance Cry toxicity by modulating any or all of the steps in the Cry intoxication process, my experimental approach examined critical steps in Cry1Fa toxin action that may be differentially effected by SfCad or MsCad.

Binding of Cry toxin to receptors on midgut brush border membrane is one of the most important steps of Cry toxin action (Bravo et al., 2011). Relative to toxin binding, the ability of cadherin fragments to bind both toxin and BBMV has suggested that cadherin fragments enhance Cry toxicity by acting as surrogate receptors by anchoring toxin to midgut membrane (Chen et al., 2007; Peng et al., 2009). Using an ELISA-based protein binding assay I showed that both SfCad and MsCad bind Cry1Fa toxin with high affinity and share a common binding site on toxin. In contrast, SfCad, but not MsCad, binds to *S. frugiperda* BBMV. These results support the surrogate receptor model proposed by Chen et al. (2007) whereby a cadherin peptide binds toxin and BBMV and anchors the toxin to the brush border membrane. However, the ability of SfCad to bind Cry1Fa toxin and *S. frugiperda* BBMV did not necessarily mean that SfCad would enhance Cry1Fa toxin binding to BBMV so this effect was tested. Alexa-488 labeled Cry1Fa toxin pre-incubated with or without the cadherin peptides was incubated with BBMV and the fluorescence intensity of bound Cry1Fa toxin was detected. The results of the binding experiment showed that the cadherin peptides did not enhance Cry1Fa toxin binding to *S. frugiperda* BBMV (Chapter 2; Fig. 2.4); evidence that the surrogate receptor function of SfCad may not be an important determinant of its synergistic ability (Chapter 2, Fig 2.4).

Although cadherin peptides did not increase Cry1Fa binding to BBMV, peptides may enhance Cry1Fa toxin insertion into membrane. Toxin insertion was tested using a proteinase K protection assay (Chapter 2; Fig. 2.4). The principle of the proteinase K protection assay is that toxins inserted into BBMV are protected from degradation by proteinase K. The results showed that the peptides did not increase the amount of BBMV-inserted Cry1Fa toxin. The inability of SfCad to enhance Cry1Fa toxin insertion in BBMV reaffirms our conclusion that the potential surrogate receptor function of SfCad may not be an important determinant of its synergistic

ability (Chapter 2, Fig 2.4). In a similar observation Park et al (Park et al., 2009b) reported a cadherin fragment from *Anopheles gambiae* that synergizes Cry4Ba toxicity but inhibits Cry4Ba binding to BBMV (Park et al., 2009b).

Gut proteases activate Cry protoxins (Gill et al., 1992), and can also deactivate toxin by proteolytically degrading toxin in naturally tolerant insects and in insects that have evolved resistance to Bt Cry toxins (Oppert et al., 1997; Forcada et al., 1999; Miranda et al., 2001; Oppert et al., 2011). Reasoning that a cadherin mediated increase in toxin protection in insect midgut may increase toxicity, the ability of cadherin peptides to protect Cry1Fa toxin in *S. frugiperda* midgut was tested both *in vitro* and *in vivo*. In the *in vitro* experiment, Cry1Fa toxin pre-incubated with or without the cadherin peptides was treated with *S. frugiperda* gut extract for various durations and residual Cry1Fa toxin was examined by western blotting. The *in vitro* experiment showed that pre-incubation of toxin with the peptides stabilizes toxin in *S. frugiperda* gut extract and SfCad stabilizes toxin for a longer duration than MsCad (Chapter 2; Fig. 2.5).

I next tested whether the protective effect of cadherin fragments against gut proteases observed *in vitro* would also be observed *in vivo*. *S. frugiperda* larvae were force-fed Cry1Fa toxin pre-incubated with or without the cadherin peptides, and residual Cry1Fa toxin present in the midgut was evaluated at different time points after feeding. In agreement with the *in vitro* results, *in vivo* experiments showed that the peptides stabilize Cry1Fa toxin in the midgut evidenced by the higher amount of Cry1Fa toxin detected in the membrane and gut content of peptide-Cry1Fa treated larvae (Chapter 2; Fig. 2.5). Additionally, higher amount of Cry1Fa toxin in the SfCad-Cry1Fa treated larvae compared to MsCad-Cry1Fa treated larvae confirms that SfCad is better at stabilizing Cry1Fa toxin in *S. frugiperda* midgut. A similar observation where MsCad stabilizes Cry1Ab toxicity in *S. exigua* midgut (unpublished data) suggests that cadherin-

mediated stabilization of toxin may well be a general mode of action for cadherin-based Bt Cry toxin synergists.

Since Cry toxin-induced pore-formation is another important step in the mode of Bt Cry toxin action (Carroll et al., 1993; Lorence et al., 1995; Wolfersberger et al., 1996; Peyronnet et al., 1997), the ability of the cadherin peptides to enhance Cry1Fa toxin mediated pore-formation was indirectly tested using a membrane permeability assay. Results from our membrane permeability assays showed that peptide treated Cry1Fa toxins are better pore -formers than the untreated toxins (Chapter 2; Fig. 2.6). A similar rate of pore formation by Cry1Fa toxin in the presence of both peptides suggests that the ability to increase pore-formation may not be sufficient to enhance Cry toxicity unless aided by the ability to protect toxin in the midgut. Since the stability of Cry toxin in the midgut affects the amount of toxin available for membrane insertion and pore-formation, higher Cry1Fa toxicity enhancement by SfCad can be explained by the better stabilization of Cry1Fa toxin by SfCad in the midgut.

Taken together, the first part of my dissertation research shows that like most cadherin-based Bt synergists, *S. frugiperda* cadherin is also a Cry1Fa and Cry1Ab toxin binding protein and may be a putative receptor shared by both Cry1Ab and Cry1Fa toxins. With respect to the mode of action of cadherin-based Cry toxin synergists, cadherin-mediated enhancement of Cry toxicity involves decreased degradation of Cry toxin in the insect midgut and enhancement of pore-forming ability of toxins (Table 4.1). The better protective effects of SfCad against gut proteases suggest that protease protection of Cry1Fa by SfCad is the factor that allows SfCad to be a better synergist. How this protective effect works is unknown. Considering that cadherin peptides bind loops of toxin domain II with high affinity, it is possible that the critical loops are susceptible to protease cleavage and the presence of cadherin peptides blocks this degradative

cleavage event. Further studies are needed to determine the exact mechanism of cadherin mediated Cry toxin stability in the midgut. Fig. 4.2 shows an integrative model based on my data of how cadherin-based Bt synergists function to synergize Bt Cry toxin.

The knowledge gained from this study will lead to the design of improved cadherin synergists for better insect control. The goal is to utilize cadherin synergists with Bt Cry toxins in biopesticides and transgenic plants. The addition of cadherin synergists to currently used Bt biopesticides and transgenic plants has the potential to broaden the target range of insects controlled, and delay the onset of insect resistance. This will lead to better pest management, reduce the use of harmful chemical pesticides, and consequently reduce associated costs of pest management

4.2. Characterization of Cry1Fa and Cry1Ab toxin interaction with BBMV proteins of three economically important corn pests.

The toxicity spectrum of the lepidopteran-active Cry1Fa includes high toxicity to corn borers, and good toxicity to corn earworm and armyworms made this toxin valuable for engineering into corn and cotton. Based on data that Cry1Fa does not compete for all Cry1Ab binding sites and data of non-cross resistance in Bt-resistant ECB colonies, these toxins were stacked together in Bt corn (Lang, B. A. et al. 2008. Pub. NO. US 2008/031106). However, partial information on Cry1Fa toxin binding and receptor sharing characteristics makes the broad prediction of cross-resistance between Cry1Fa and other Cry toxins somewhat speculative. The inability to label Cry1Fa toxin without compromising its activity precluded direct study of Cry1Fa toxin interactions with midgut brush border proteins, which would strengthen the binding models used in predicting cross-resistance. Therefore, in the second part of my

dissertation research, (Chapter 3) I have developed a fluorescence based Cry1Fa toxin binding assay to study Cry1Fa toxin interaction with midgut proteins from three important corn pests. Alexa-488 was used to label Cry1Fa toxin as Alexa-488 labeled Cry1Fa toxin retained its toxicity and bindability.

The fluorescence based binding assay was validated by comparing ^{125}I and Alexa-488 labeled Cry1Ab toxin binding to ECB BBMV in saturation and competition binding formats (Chapter 3; Fig. 2A & 2B). Cry1Ab toxin was chosen because Cry1Ab toxin binding to ECB BBMV has been previously characterized (Hua et al., 2001). The results of the saturation binding assays showed that both ^{125}I and Alexa-488 labeled toxins yield similar binding affinities indicated by comparable dissociation constants (Chapter 3; Fig. 3.2). The binding affinities calculated from our binding assays are comparable to previously reported Cry1Ab toxin binding affinity to ECB BBMV (Hua et al., 2001). The competition binding assays also showed similar binding characteristics of ^{125}I and Alexa-488 labeled toxins. These results demonstrate the accuracy of the fluorescence-based Cry1 toxin binding assay relative to previously established ^{125}I-Cry1Ab binding assays. Using results from fluorescence-based toxin binding assay with Cry1Fa and Cry1Ab toxins, I constructed models for Cry1Fa and Cry1Ab toxin binding to BBMV from ECB, SWCB and CEW. Briefly, my binding results show that Cry1Fa and Cry1Ab share binding site(s) in ECB (Fig. 3.2 & 3.3), SWCB (Fig. 3.4), and CEW (Fig. 3.5) with different affinities for the shared binding site(s).

In ECB, the interaction between Cry1Fa and Cry1Ab fits a two-site binding model where Cry1Ab and Cry1Fa share one binding site(s) for which Cry1Ab has higher affinity than Cry1Fa (Fig. 3.6). Cry1Ab also binds an additional site(s) in ECB not shared with Cry1Fa. The Cry1Ab and Cry1Fa toxin binding model in ECB suggests a possibility of cross-resistance against these

toxins. However, lack of cross-resistance between Cry1Ab and Cry1Fa toxins in the laboratory selected Cry1Ab (Siqueira et al., 2004) and Cry1Fa (US Patent Application 20070006340; Lang et al.; Mar. 5, 2004(Pereira et al., 2008) resistant ECB strains suggests that Cry1Ab and Cry1Fa resistance mechanism in ECB may not involve alterations in toxin binding site(s). This hypothesis is supported by the lack of correlation between toxin binding and Cry1Fa resistance in a Cry1Fa resistant ECB strain reported by Pereira et al., (Pereira et al., 2010). Furthermore, involvement of a resistance mechanism other than loss of toxin binding observed in Cry1Ab and Cry1Ac resistant ECB strains that developed very low level of cross-resistance to Cry1Fa toxin (Li et al., 2004; Siqueira et al., 2006; Crespo et al., 2011) suggest that Cry1Fa resistance in ECB may occur through a mechanism which is yet to be characterized.

The Cry1Fa and Cry1Ab interaction in SWCB can be explained by a three-site binding model where Cry1Ab and Cry1Fa share binding site(s) for which Cry1Ab has higher affinity than Cry1Fa (Fig. 3.6). Apart from the shared binding site(s), both toxins bind additional high affinity site(s) not shared with the other toxin. The presence of unshared binding sites reduces the probability of cross-resistance development between these toxins. However, high susceptibility of SWCB to both Cry1Fa and Cry1Ab toxins, transgenic corn co-expressing these toxins will be under high selection pressure to alter the common binding site(s). Therefore further studies with Cry1Ab or Cry1Fa resistant SWCB are needed to rule out the possibility of cross-resistance development against these toxins.

In CEW, Cry1Fa and Cry1Ab share two binding site(s) (Fig. 3.6). Cry1Ab has higher affinity for one while Cry1Fa has higher affinity for the second site(s). Cry1Fa and Cry1Ab receptor sharing in CEW is not of concern, as CEW is tolerant to Cry1Fa toxin. Nevertheless, our binding data are suggestive of a low probability of cross-resistance between Cry1Ab and

Cry1Fa toxins in CEW.

The Cry1Fa binding site models developed here will be helpful in predicting evolution of cross-resistance between Cry1Fa and Cry1Ab toxins in the species tested. In ECB, although the data on cross-resistance development support stacking Cry1Ab and Cry1Fa toxins (Li et al., 2004; Siqueira et al., 2006; Crespo et al., 2011), high Cry1Ab affinity for the shared binding site(s) suggests that alterations in this site(s) may lead to cross-resistance against both toxins. In case of SWCB, presence of unshared binding site(s) suggests that possibility of cross-resistance development is negligent in this species. Since Cry1Fa and Cry1Ab have overlapping, yet distinct toxicity against important pests of corn, stacking them together are likely to be beneficial to management of corn pests. Further research correlating specific Cry1Fa and Cryt1Ab binding sites with receptor proteins in midgut of ECB, CEW and SWCB would strengthen the binding models. Characterizing resistance mechanisms of Cry1Fa and Cry1Ab resistance in ECB and other Bt resistant insect species would further enhance the ability to accurately predict possibility of cross-resistance development against these toxins.

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Table 4.1. Summary table.

Synergist	Enhancement		Toxin binding affinity		Binding to *S. frugiperda* BBMV	Pre-pore oligomer formation in the absence of BBMV	Pre-pore oligomer formation in the presence of BBMV	Membrane insertion of Cry1Fa	Cry1Fa induced membrane permeability	*In vitro* protection of Cry1Fa from proteolysis	*In vivo* protection of Cry1Fa from proteolysis
	Cry1Fa[1]	Cry1Ab[3]	Cry1Fa[4]	Cry1Ab[4]							
SfCad	5-fold[2]	13.5-fold[2]	2.2 ± 0.5 nM 0.85	7.8 ± 2.5 nM 0.87	Yes	Yes[2]	No	No	Yes[2]	Yes[2]	Yes[2]
MsCad	1.8-fold[2]	14-fold[2]	5.8 ± 0.7 nM 0.96	0.8 ± 0.2 nM 0.78	No	Yes[2]	No	No	Yes[2]	Yes[2]	Yes[2]
Difference between SfCad and MsCad	3-fold	No	3-fold SfCad>MsCad	10-fold SfCad<MsCad	Yes	No	No	No	No	Yes SfCad>MsCad	Yes SfCad>MsCad

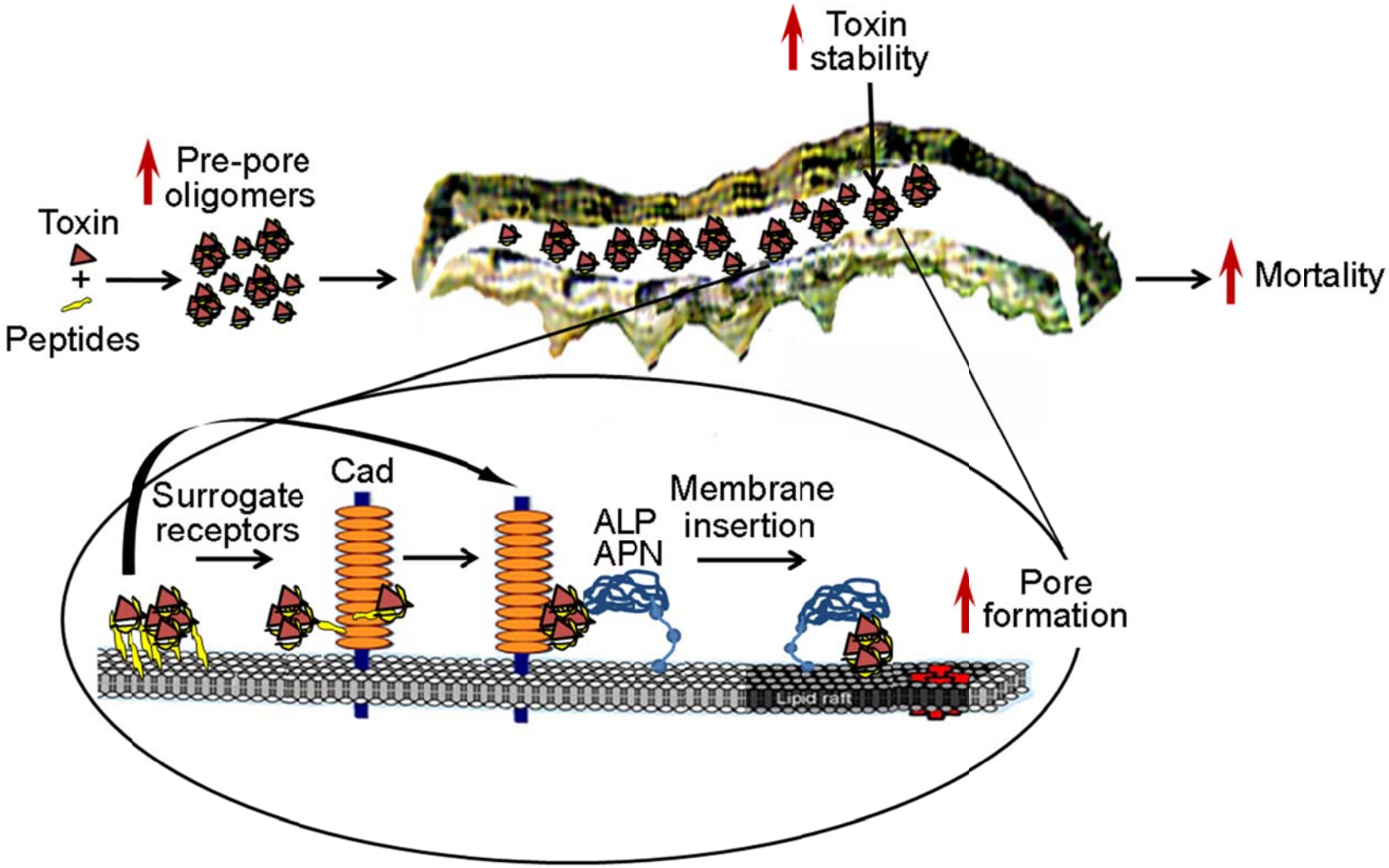
1 Fold decrease in LC_{50} compared to toxin alone

2 Compared to toxin alone treatment

3 Single point bioassay on *S. frugiperda* neonates

4 $K_D \pm SEM$ and R^2

Figure 4.1. Modes of action of cadherin-based Cry toxin synergists . Incubation of toxin with cadherin peptides induces pre-pore toxin oligomers, which upon ingestion bind to as yet unknown receptors on the membrane, inserts into the membrane and form lytic pores. It is also possible for the cadherin peptide induced pre-pore oligomers to directly interact with cadherin and secondary Bt receptors such as ALP and APN leading to membrane insertion and pore formation. Cadherin peptides also increase stability of toxin in the midgut and increase rate of transmembrane pore formation. The red upward arrows indicate observed increase in pre-pore toxin oligomer formation, toxin stability in the midgut, or rate of toxin mediated pore formation induced by cadherin peptides.


Toxin
stability
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oligomers
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+
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Mortality
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Surrogate
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ALP
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Membrane
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Pore
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