

Reduction of *Bacillus thuringiensis* Cry1Ac toxicity against *Helicoverpa armigera* by a soluble toxin-binding cadherin fragment

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ABSTRACT

A cadherin-like protein has been identified as a putative receptor for *Bacillus thuringiensis* (Bt) Cry1Ac toxin in *Helicoverpa armigera* and plays a key role in Bt insecticidal action. In this study, we produced a fragment from this *H. armigera* Cry1Ac toxin-binding cadherin that included the predicted toxin-binding region. Binding of Cry1Ac toxin to this cadherin fragment facilitated the formation of a 250-kDa toxin oligomer. The cadherin fragment was evaluated for its effect on Cry1Ac toxin-binding and toxicity by ligand blotting, binding assays, and bioassays. The results of ligand blotting and binding assays revealed that the binding of Cry1Ac to *H. armigera* midgut epithelial cells was reduced under denaturing or native conditions *in vitro*. Bioassay results indicated that toxicities from Cry1Ac protoxin or activated toxin were reduced *in vivo* by the *H. armigera* cadherin fragment. The addition of the cadherin fragment had no effect on Cry2Ab toxicity.

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1. Introduction

Bt toxins are among the most important biopesticides used to control agricultural pests. The pest management tactics associated with Bt-expressing cotton have resulted in a drastic reduction in both insecticide use (Wu and Guo, 2005) and cotton bollworm suppression from host crops (Wu et al., 2008). However, populations of diamondback moth (*Plutella xylostella*) and cabbage looper (*Trichoplusia ni*) have evolved resistance to Bt sprays (Tabashnik et al., 1990; Janmaat and Myers, 2003), and decreased susceptibility to Bt toxins has been documented in field populations of three other insect species exposed to Bt crops (Tabashnik, 2008; Gassmann et al., 2009). In addition, a small increase in the frequency of resistance of *Helicoverpa armigera* to Cry1Ac toxin has been reported in specific areas of China with large areas of Bt cotton (Xu et al., 2009; Liu et al., 2008). Several strains of *H. armigera* have been selected in the lab for high levels of resistance to Cry1Ac toxin (Akhurst et al., 2003; Xu et al., 2005; Kranthi et al., 2006; Luo et al., 2006). Resistance to Bt in the laboratory in some insect species has led to predictions of a serious threat to agricultural production (Ferré and van Rie, 2002).

The proposed mode of action for Bt toxins indicates that Cry protoxins are solubilized in the insect midgut lumen and activated by midgut proteases to release a toxin fragment that crosses the peritrophic matrix and binds to specific receptors located on the brush border membrane of midgut columnar cells, eventually leading to cell death. One model proposes that the Cry toxin first binds to a cadherin-like protein, resulting in conformational change (Bravo et al., 2004). After further proteolytic activation and oligomerization, toxin oligomers display increased binding affinity for aminopeptidase N (APN), which facilitates the insertion of toxins into the membrane with a concomitant pore formation that results in cell death by osmotic shock. According to this model, the two receptors interact sequentially with different structural species of the toxin, resulting in its efficient membrane insertion. An alternative model has recently been proposed wherein Cry toxin monomers bind to the cadherin-like receptor and a protein kinase A-dependent oncotic signaling pathway is activated, leading to cell death (Zhang et al., 2005, 2006, 2008). In this model, APN binding and pore formation are irrelevant to biological activity.

Cadherin-like proteins of 175–210 kDa, belonging to the cadherin superfamily of transmembrane glycoproteins, have been identified as putative Cry1A toxin-binding receptors on midgut epithelial cells in many lepidopteran species, including *Manduca sexta* (Vadlamudi et al., 1993, 1995), *Heliothis virescens* (Gahan

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et al., 2001; Jurat-Fuentes and Adang, 2006a), *Bombyx mori* (Nagamatsu et al., 1998a,b), *H. armigera* (Wang et al., 2005), *Pectinophora gossypiella* (Morin et al. 2003) and *Ostrinia nubilalis* (Flannagan et al., 2005). Cadherin-gene changes involving resistance to Cry1A toxin were found in laboratory-selected strains of *Heliothis virescens* (Gahan et al., 2001; Jurat-Fuentes et al., 2004), field populations of *P. gossypiella* (Morin et al., 2003) and a laboratory-selected strain of *H. armigera* GYBT strain (Xu et al., 2005; Yang et al., 2007). Site-directed mutagenesis of the *Heliothis virescens* cadherin gene reduced the ability of cadherin to bind to Cry1Ac toxin (Xie et al., 2005).

Cry1A toxin-binding cadherin-like proteins share a structure composed of four domains: an ectodomain, a membrane proximal extracellular domain (MPED), a transmembrane domain, and a cytoplasmic domain. The ectodomain consists of 11–12 cadherin repeats (CRs). CRs adjacent to the MPED contain the Cry1A toxin-binding region (Dorsch et al., 2002; Hua et al., 2004; Wang et al., 2005; Fabrick and Tabashnik, 2007).

The toxicity of Cry1A toxins in *M. sexta* larvae was reduced by premixing the toxin with a toxin-binding cadherin soluble peptide containing the putative toxin-binding site (Dorsch et al., 2002; Griko et al., 2004; Xie et al., 2005). Interestingly, a very similar peptide (CR12-MPED) produced in *Escherichia coli* cells as inclusion bodies enhanced the activity of Cry1A toxins in *M. sexta* (Chen et al., 2007). It was suggested that this enhancing peptide increased the toxin concentration around the gut brush border microvilli, since the peptide bound this membrane fraction with high affinity (K_d , 32 nM). It was also suggested that enhancement of Cry1A activity by CR12-MPED was due in part to enhanced oligomer formation (Pacheco et al., 2009). However, little is known regarding the effect of cadherin fragments on Cry toxicity in other insects. To shed light on the role of *H. armigera* cadherin in Cry toxin action, we evaluated the insecticidal activity of Cry1Ac and Cry2Ab toxins in

the presence of a soluble toxin-binding cadherin fragment through bioassays, and further analyzed the potential molecular mechanism via ligand blot and binding assays.

2. Materials and methods

2.1. Insect strains

H. armigera strain 96S was used in this study and was originally collected from Xinxiang County, Henan Province of China in 1996. Larvae were reared on an artificial laboratory diet as previously described (Yang et al., 2007).

2.2. Preparation of Cry1Ac and Cry2Ab protoxin and toxin

Cry1Ac and Cry2Ab protoxin were kindly supplied by the Biotechnology Research Group, Institute of Plant Protection, Chinese Academy of Agricultural Science. For activation, Cry1Ac protoxin was incubated 2 h at 37 °C with 1/25 ratio of trypsin (Sigma), and the soluble trypsinized toxin was purified by a Superdex 200 HR 10/30 column (Amersham Biosciences) on a fast protein liquid chromatography (FPLC) system.

2.3. Cloning, expression and purification of a cadherin fragment

A 1092 bp DNA fragment of *H. armigera* cadherin (GenBank accession no. AF519180, Fig. 1A) harboring the Cry1Ac toxin-binding region was cloned and expressed in *E. coli* cells as a His-tag recombinant protein. Total RNA was extracted from the midgut of the fifth instar larvae with TRIzol[®] reagent (Invitrogen) according to the manufacturer's instructions and reverse-transcribed with SuperScript III RNase H⁻ reverse transcriptase (Invitrogen). The cDNA fragments served as templates for subsequent PCR

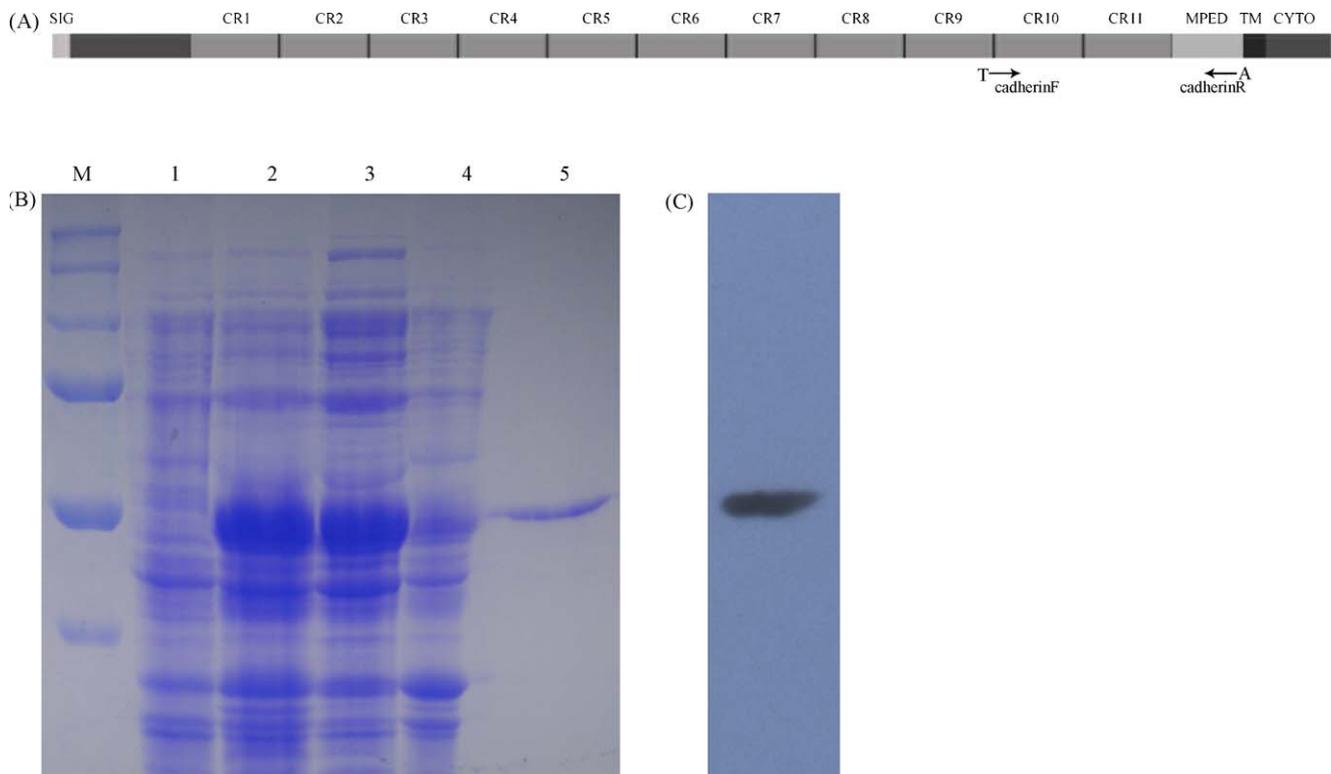


Fig. 1. SDS-PAGE and ligand blot analysis of the expression of the cadherin fragment. (A) Structure of *Helicoverpa armigera* cadherin. The arrows indicate the location of primers in the *H. armigera* cadherin sequence, used in the PCR to obtain the cadherin fragment. (B) Expressed and purified proteins were Coomassie-stained following 10% SDS-PAGE. Lane 1: IPTG-0 h; lane 2: IPTG-6 h; lane 3: supernatant; lane 4: pellet; lane 5: purified protein. (C) Purified protein transferred to a PVDF membrane was probed with activated Cry1Ac toxin and was detected by a polyclonal anti-Cry1Ac antibody.

amplification using primers cadherin-F (5'-AGTCATATGAC-GATTTCGTCTACGGAC-3') and cadherin-R (5'-ATACTC-GAGTGGCTCGCGCTGCGCGT-3'). Amplicons were cloned into the T-Easy vector (Promega) following manufacturer's instructions. The recombinant plasmid was excised with Nde I and Xho I, subcloned into the His-tagged expression vector pET28a+ (Novagen), and transfected into *E. coli* BL21 (DE3) cells. The transfected cells were cultured, and expression was induced with 0.2 mM IPTG for 6 h at 25 °C. For protein purification, the pellet cells were collected by centrifugation at 3000 × g, 4 °C for 10 min, washed with ice-cold PBS buffer, re-suspended in PBS buffer, and sonicated for 3 min on ice. After 25,000 × g centrifugation for 20 min at 4 °C, the supernatant was subjected to affinity purification using Ni-Sepharose beads (Amersham Biosciences). After washing with 40 mM imidazole in PBS buffer, the recombinant protein was eluted with 400 mM imidazole and dialyzed against PBS buffer.

Ligand blot analysis was used to determine the binding of the Cry1Ac toxin to the purified cadherin fragment. Briefly, the purified protein was separated by a 10% SDS-PAGE and transferred onto a PVDF membrane that was subsequently blocked with dry skim milk (5%). The membranes were incubated in PBST buffer containing 10 nM Cry1Ac toxin for 2 h, and toxin was detected using polyclonal anti-Cry1Ac antibody (1:10,000, 1 h) and a horseradish peroxidase (HRP)-conjugated secondary antibody (ZSGB-BIO, China) (1:20,000, 1 h). The blot was developed with Super ECL Plus Detection Kit (Applygen, China).

2.4. Oligomer formation assay

For oligomer formation, 5 µg of Cry1Ac protoxin was incubated for 1 h either with or without *H. armigera* cadherin peptide, at a ratio of 1 µg protoxin:1 µg cadherin fragment, in the presence of *H. armigera* midgut fluid. To obtain midgut fluid, fifth instar *H. armigera* larvae were chilled 10 min on ice and midgut tissue was dissected. Midgut fluid was separated from solid material by centrifugation (13,350 × g, 15 min, 4 °C) and filtered through 0.22 µm filters. Small aliquots of midgut fluid were stored at -80 °C. Protoxin with or without peptide was activated by digestion with 5% *H. armigera* midgut fluid for 1 h at 37 °C. Proteolysis was stopped by the addition of PMSF to a final concentration of 1 mM. The reaction mixture was separated by a 7% SDS-PAGE, transferred onto a PVDF membrane, and detected with polyclonal anti-Cry1Ac antibody (1:10,000, 1 h) and HRP-conjugated secondary antibody (ZSGB-BIO, China) (1:20,000, 1 h). The blot was developed as described above.

2.5. Preparation of brush border membrane vesicles

Midguts from fifth instar larvae of *H. armigera* were dissected longitudinally, and were washed in ice-cold MET buffer (250 mM mannitol, 17 mM Tris-HCl, and 5 mM EGTA, pH 7.5) and stored at -80 °C until use. Brush border membrane vesicles (BBMV) were prepared from midguts with the differential centrifugation method (Wolfersberger et al., 1987). Briefly, frozen midguts were mechanically homogenized in MET buffer. One volume of 24 mM MgCl₂ was added and the mixture was incubated for 15 min. Following centrifugation of the mixture (15 min, 2500 × g at 4 °C), the supernatant was further centrifuged (30 min, 30,000 × g at 4 °C), and the final pellet was suspended in resuspension buffer (300 mM mannitol, 1 mM DTT, 10 mM HEPES-Tris, pH 7.4), frozen, and stored at -80 °C until use. The concentration of proteins in BBMV preparation was determined using bovine serum albumin as a standard (Bradford, 1976).

2.6. Toxin labeling and direct binding assay

Trypsin-activated Cry1Ac toxin was labeled with the fluorescent dye fluorescein isothiocyanate (FITC) according to the manufacturer's protocol (Sigma). The labeled Cry1Ac toxin was separated from unconjugated dye by dialysis. A calibration curve was constructed using fluorescence intensities measured at 490 (excitation) and 528 (emission) nm by a multi-mode microplate reader (Synergy HT, BioTek).

The FITC-labeled Cry1Ac toxin was diluted with resuspension buffer, and then incubated for 30 min in the dark at 25 °C either with or without a ratio of 1 µg FITC-labeled Cry1Ac toxin:1 µg cadherin fragment, and then mixed with *H. armigera* BBMV (corresponding to 15 µg proteins) for 1 h in 100 µl of resuspension buffer in the dark at 25 °C. BBMV were pelleted by 30,000 × g centrifugation for 30 min at 4 °C and rinsed twice with 500 µl of ice-cold resuspension buffer to remove the unbound labeled toxins. The final BBMV pelleted was suspended with 300 µl of resuspension buffer and sonicated for 25 s on ice, and the fluorescence of FITC bound to BBMV was determined as described above.

2.7. Ligand blot

The activated Cry1Ac toxin was incubated for 30 min at 25 °C either with or without a ratio of 1 µg activated Cry1Ac toxin:1 µg cadherin fragment, and was analyzed by ligand blot. The BBMV proteins (corresponding to 10 µg proteins) were separated by 10% SDS-PAGE, transferred onto PVDF membranes, and blocked with dry skim milk (5%). The membranes were incubated in PBST buffer containing 10 nM Cry1Ac toxin or another aliquot of the mixture described above for 2 h, and then detected as described previously.

2.8. Insect bioassays

To evaluate the effect of the cadherin fragment on Cry1Ac and Cry2Ab toxicity, either the Cry1Ac protoxin commercial formulation of 19.7% MVP II (Dow AgroSciences) or Cry2Ab protoxin were thoroughly mixed with a defined amount of artificial diet at the LC₅₀ for *H. armigera* (0.31 µg/g for MVP II and 10 µg/g for Cry2Ab, respectively). After the artificial diet solidified, one g portions of diet were transferred to a 24-well plate. Two concentrations of the cadherin fragment in each assay and PBS (used as a control) were applied to the diet surface and allowed to air-dry. One first instar larva of *H. armigera* strain 96S was placed in each well of the plate. Each treatment had three replicates, and a total of 24 larvae were used for each replicate. The environment for the bioassay was maintained at 28 °C and 75 ± 10% relative humidity (RH) with a photoperiod of 14:10 h (L:D). The survival rates of the larvae were measured after 7 days.

For a trypsin-activated Cry1Ac toxin toxicity assay, trypsin-activated Cry1Ac toxin was diluted with PBS buffer at a concentration of 0.1 µg/cm² (LC₅₀) and incubated with two concentrations of the cadherin fragment in each assay and PBS buffer (used as a control) at 25 °C for 30 min. A disc of artificial diet (1.6-cm diameter) was placed into a 24-well plate and made to fit into the inner wall and bottom of the plate through gentle pressure. The toxin solution was applied to the diet surface and allowed to air-dry. One second-instar larva of the GY strain, starved for 4 h, was placed in each well of the plate. Each treatment had three replicates, and a total of 24 larvae were used for each replicate. The environment for bioassay was maintained as described above. The survival rates were measured after 5 days.

2.9. Statistical analysis

Mortality data were transformed using an arcsine transformation and were subjected to analysis of variance (ANOVA) (SAS Institute, 1998). Treatment differences were determined using Duncan's multiple range test. Statistical significance was assumed at $P < 0.05$.

3. Results

3.1. Expression and purification of the cadherin fragment

The cDNA fragment for *H. armigera* cadherin encoding the Cry1Ac toxin-binding region was cloned (Fig. 1A). This fragment was expressed in *E. coli* cells, and the peptide was evaluated by SDS-PAGE (Fig. 1B). The results demonstrated that the cadherin fragment expressed in *E. coli* was a soluble form, had the expected size of 47.5 kDa, and was purified to homogeneity. A ligand blot analysis indicated that the fragment bound to Cry1Ac toxin (Fig. 1C).

3.2. Oligomerization of Cry1Ac toxin by binding to the soluble cadherin fragment

Previously, binding of Cry1A toxins to *M. sexta* cadherin receptors facilitated the formation of a 250-kDa toxin oligomer (Gómez et al., 2002). Therefore, the oligomerization of Cry1Ac toxin by the soluble toxin-binding cadherin fragment of *H. armigera* was analyzed. The results indicated that Cry1Ac toxin oligomerized in the presence of the cadherin fragment when activated with *H. armigera* midgut fluids (Fig. 2A). Oligomers were not observed when protoxin was incubated with midgut fluids alone (Fig. 2B).

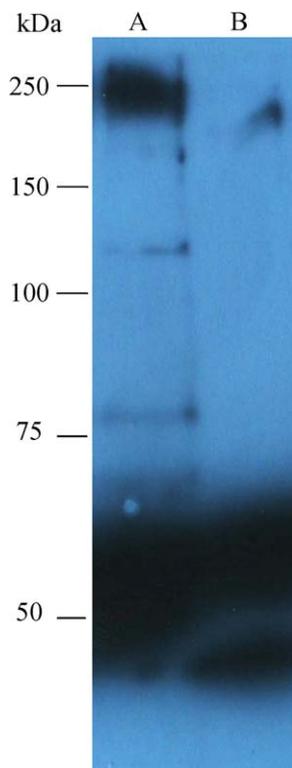


Fig. 2. Oligomer formation of Cry1Ac protoxin in the presence of the *H. armigera* cadherin fragment, detected with a polyclonal anti-Cry1Ac antibody. Lane A: Cry1Ac protoxin activated with midgut fluids in the presence of the cadherin fragment. Lane B: Cry1Ac protoxin activated only with midgut fluids.

3.3. Reduction of Cry1Ac and Cry2Ab toxin insecticidal action by a cadherin fragment

In insect bioassays, the *H. armigera* cadherin fragment containing the Cry1Ac-binding region was evaluated as a Cry1Ac toxin antagonist to demonstrate that toxin-binding and insecticidal activity are directly correlated at the organismal level. Specifically, bioassays were designed to test whether the toxicity of Cry1Ac protoxin and its activated form against *H. armigera* was reduced by the cadherin fragment. When the peptide fragment was added to Cry1Ac protoxin preincorporated into the diet, the survival of *H. armigera* larvae was increased 28% (34-fold excess fragment added Cry1Ac protoxin) to 33% (56-fold mass excess fragment) ($F = 65.46$; d.f. = 2, 8; $P = 0.001$) (Fig. 3A). The addition of the cadherin fragment to 0.1 $\mu\text{g}/\text{cm}^2$ (LC₅₀) of trypsin-activated Cry1Ac toxin resulted in a significant increase in larval survival, from 55% to 75% (18–36-fold, respectively, mass excess fragment) ($F = 51.88$; d.f. = 2, 8; $P = 0.002$) (Fig. 3B). In contrast, the addition of 10–20-fold cadherin fragment to Cry2A protoxin premixed in the diet had no effect on toxicity ($F = 1.94$; d.f. = 2, 8; $P = 0.224$) (Fig. 3C). The results of the bioassays suggested that the binding of Cry1Ac toxin to cadherin in the *H. armigera* midgut was essential for toxicity, and that cadherin (or at least the cadherin fragment) may be irrelevant to Cry2Ab protoxin toxicity. Presumably, in the presence of the cadherin fragment, the binding of both Cry1Ac protoxin and its activated form to the gut receptor was blocked and the insecticidal activities of the toxins were prevented.

3.4. Detection of Cry1Ac toxin-binding by ligand blot

BBMV proteins were prepared from the midgut of *H. armigera* and were analyzed by SDS-PAGE. The BBMV proteins transferred to PVDF membranes were probed with activated Cry1Ac toxin mixed with or without the *H. armigera* cadherin fragment. When the membranes were exposed under the same conditions, the intensities of the binding signals were quite different (Fig. 4). A much weaker binding signal was observed in the membrane probed with activated Cry1Ac toxin mixed with the cadherin fragment (Fig. 4B) than in the membrane without a cadherin fragment (Fig. 4A). The results suggested that the cadherin fragment acted as an antagonist to Cry1Ac toxin by blocking the binding of the toxin to the denatured BBMV receptor proteins. The binding of Cry1Ac toxin to a protein around 120 kDa, possibly aminopeptidase N, was also attenuated.

3.5. Reduction of the binding of Cry1Ac toxin to BBMV under native conditions

BBMV were prepared without any protein extraction procedure to maintain receptor native conformation. The toxin-receptor reaction was performed in solution to simulate true binding action *in vitro*. As shown in Fig. 5, in Cry1Ac toxin mixed with the cadherin fragment, the amount of binding was lower than that of toxin alone, and the binding capacity was attenuated significantly. These results suggested that interactions between Cry1Ac toxin and the cadherin peptide resulted in decreased aggregation of toxin on the surface of the BBMV.

4. Discussion

In susceptible lepidopteran species, the interactions between Cry toxin and midgut epithelial cell receptors play key roles in toxin insecticidal action, and studies of Cry toxin-receptor interactions have provided insight into the toxin mode of action. Using recombinant and synthetic peptides incubated with toxins, and specific antibodies against toxin receptors, the Cry toxin

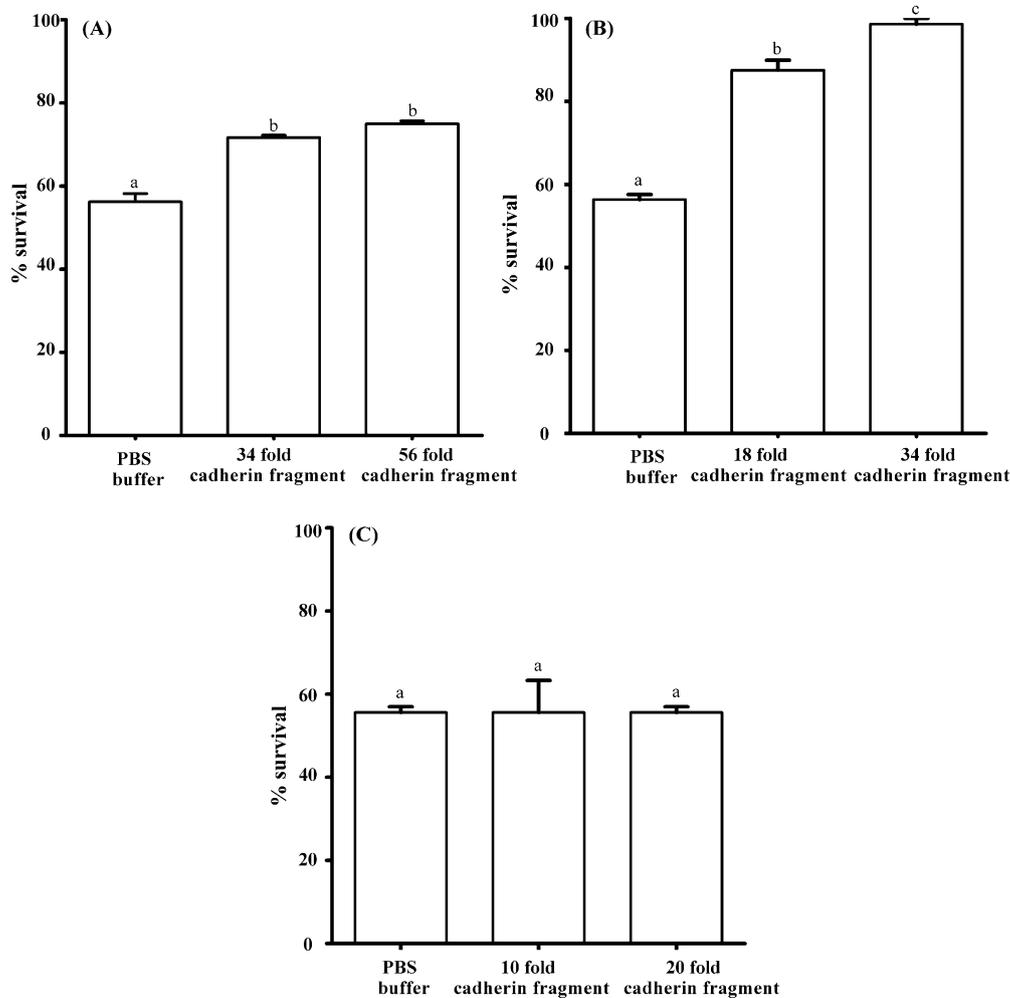


Fig. 3. Reduction of the toxicity of Cry1Ac by the *H. armigera* cadherin fragment. Larvae of *H. armigera* were fed a diet containing Cry1Ac protoxin (A), trypsin-activated toxin (B), or Cry2Ab protoxin (C) at an LC₅₀ for the toxin and two concentrations of the cadherin fragment in each assay. PBS buffer was used as a control. Survival was assessed after 5 or 7 days. Mean with different letters were statistically different ($P < 0.05$).

interactions with BBMV were studied to develop models for Cry1A toxin insecticidal action in *M. sexta*, *Helicoverpa zea*, and *Bombyx mori* (Gómez et al., 2001, 2003; Dorsch et al., 2002; Griko et al., 2004; Xie et al., 2005; Chen et al., 2007; Ibiza-Palacios et al., 2008; Pacheco et al., 2009). In these studies, synthetic peptides and soluble expressed toxin-binding peptides derived from cadherins reduced Cry1Ac toxicity against *M. sexta* and *Bombyx mori* (Fig. 6). In contrast, an unfolded peptide derived from a *M. sexta* cadherin (EC12-MPED) was expressed in an inclusion body and combined at up to 100-fold excess with Cry1A to enhance toxicity to *M. sexta* (Chen et al., 2007). Similarly, 100-fold excess of cadherin inclusion body to Cry1Ac resulted in enhanced toxicity in *H. zea* (Pacheco et al., 2009). In our study, we designed a soluble *H. armigera* toxin-binding cadherin fragment that included CR10 and -11, which are predicted to contain the toxin-binding region by homology to other lepidopteran Cry1A toxin receptors (Wang et al., 2005). The *H. armigera* cadherin fragment reduced Cry1Ac toxicity, almost completely disabling activity with 36-fold excess peptide, and blocked the binding of Cry1Ac to *H. armigera* BBMV under denaturing and native conditions. We suspected that the reduction of toxicity was due to the native conformation of the soluble toxin-binding cadherin fragment blocking amino acid residues of Cry1A toxin that bind to receptors. It is possible that an unfolded toxin-binding cadherin fragment with more exposed amino acid residues can modify interactions with Cry1A toxins and other molecules on the insect midgut epithelium and may be responsible for the

synergism of toxins. Alternatively, differences in the cadherin sequences used to design these fragments may account for the differences that have been observed (Fig. 6). However, the sequences of synergistic and competing cadherin fragments overlap, and the reason for the different activities of these peptides is not obvious. With the exception of *Heliothis virescens* CAD0, all peptides demonstrating toxin reduction activity have a complete CR10 region, and this may suggest the importance of this region toxin-binding to the cadherin. To clarify, more studies are needed on homologous cadherin peptides in different species and their effect on Bt toxicity.

Binding of Cry1A toxin to cadherin-like receptor facilitated oligomer pre-pore formation (Gómez et al., 2002). Oligomeric structures were correlated with higher *in vitro* pore formation activity than monomeric toxin and slightly enhanced Cry1A toxin insecticidal activity (Muñoz-Garay et al., 2006; Gómez et al., 2002). The enhancement of insecticidal activity in Cry1A toxins through unfolded toxin-binding cadherin fragments extracted from inclusion bodies was correlated with oligomer formation (Pacheco et al., 2009). Although we observed the formation of a 250-kDa toxin oligomer obtained by binding of a Cry1Ac toxin to our soluble toxin-binding cadherin fragment, Cry1Ac toxicity was reduced by the soluble cadherin fragment. These results indicate that the soluble cadherin fragment, which was analyzed under conditions that would maintain native conformation, blocked the binding epitopes of Cry1Ac toxin oligomer and monomer, and reduced the

selected strain was cross-resistant to Cry2Aa, and Cry2Aa-selected *Heliothis virescens* was cross-resistant to Cry1Ac (Jurat-Fuentes et al., 2003). However, cross-resistance to Cry2Ab by other Cry1Ac-resistant lepidopteran species has not been documented (Tabashnik et al., 2002; Akhurst et al., 2003; Luo et al., 2007). It was proposed that Cry1A and Cry2Ab have different binding sites in *H. armigera* (Luo et al., 2007; Hernández-Rodríguez et al., 2008). In our study, the addition of the cadherin fragment had no effect on Cry2Ab toxicity, suggesting that cadherin was irrelevant to the insecticidal activity of Cry2Ab toxin and therefore not a receptor protein of Cry2Ab toxin. These results support the hypothesis that second-generation transgenic cotton expressing Cry2Ab could be deployed to manage Cry1Ac-resistant *H. armigera* (Akhurst et al., 2003; Xu et al., 2005; Luo et al., 2007; Mahon et al., 2007).

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