

Helicoverpa armigera cadherin fragment enhances Cry1Ac insecticidal activity by facilitating toxin-oligomer formation

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Abstract The interaction between *Bacillus thuringiensis* insecticidal crystal protein Cry1A and cadherin receptors in lepidopteran insects induces toxin oligomerization, which is essential for membrane insertion and mediates Cry1A toxicity. It has been reported that *Manduca sexta* cadherin fragment CR12-MPED and *Anopheles gambiae* cadherin fragment CR11-MPED enhance the insecticidal activity of Cry1Ab and Cry4Ba to certain lepidopteran and dipteran larvae species, respectively. This study reports that a *Helicoverpa armigera* cadherin fragment (HaCad1) containing its toxin binding region, expressed in *Escherichia coli*, enhanced Cry1Ac activity against *H. armigera* larvae. A binding assay showed that HaCad1 was able to bind to Cry1Ac in vitro and that this event did not block toxin binding to the brush border membrane microvilli prepared from *H. armigera*. When the residues ¹⁴²³GVLSLNQ¹⁴³⁰ were deleted from the fragment, the subsequent mutation peptide lost its ability to bind Cry1Ac and the toxicity enhancement was also significantly reduced. Oligomerization tests showed that HaCad1 facilitates the formation of a 250-kDa oligomer of Cry1Ac-activated toxin in the midgut fluid environment. Oligomer formation was dependent upon the toxin binding to HaCad1, which was also necessary for the HaCad1-mediated enhancement effect. Our discovery reveals a novel strategy to enhance insecticidal activity or to overcome the resistance of insects to *B. thuringiensis* toxin-based biopesticides and transgenic crops.

Keywords *Helicoverpa armigera* cadherin · Cry1Ac insecticidal protein · Toxicity enhancement · Oligomer formation

Introduction

Bacillus thuringiensis is a ubiquitous gram-positive, spore-forming bacterium that forms parasporal insecticidal crystal proteins (ICPs) which exhibit toxicity to certain insect species. These ICPs display insect-specific toxicity and are harmless to humans and nontarget animals. Consequently, they have been widely used in biopesticides and transgenic plants to control agricultural pests (Schnepf et al. 1998; Pigott and Ellar 2007).

Studies on the mode of action of Cry1A toxins to lepidopteran insects have revealed several types of receptors: cadherin-like proteins (Vadlamudi et al. 1995; Nagamatsu et al. 1994), aminopeptidase N (APN; Knight et al. 1994), alkaline phosphatase (ALP; McNall and Adang 2003; Jurat-Fuentes and Adang 2004), glycoconjugate (Valaitis et al. 2001), and glycolipids (Griffitts et al. 2005). A current model (Bravo et al. 2004) suggests that these receptors work in a stepwise fashion to mediate Cry1A toxicity to lepidopteran insects. Firstly, after ingestion by the lepidopteran larva, the 130-kDa Cry1A protoxin is solubilized and activated to a 65-kDa toxic form by the insect midgut digestive fluids. The activated monomer toxin binds to the first receptor, cadherin, on the midgut microvilli and forms toxin oligomers. The toxin oligomers then undergo a conformational change that facilitates cleavage of a single α -helix by membrane-bound proteases (Gómez et al. 2002). This modification causes the toxin oligomers to bind with high affinity to a second receptor protein attached to the cell membrane by a glycosylphosphatidylinositol anchor, such as ALP and APN

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(Pardo-López et al. 2006). Finally, the insertion of the prepore complex into the membrane leads to the formation of ion channels or pores in the brush border membrane microvilli (BBMV) of the larval gut, leading to cell death by osmotic shock.

A crucial step in this mode of action is the binding of Cry1A toxin to cadherin receptor (Gómez et al. 2002). The first Cry1A cadherin receptor protein was isolated and cloned from the midgut epithelium of *Manduca sexta* (Vadlamudi et al. 1995). Since then, additional lepidopteran cadherins have been identified (Nagamatsu et al. 1994; Gahan et al. 2001; Morin et al. 2003; Flannagan et al. 2005; Wang et al. 2005; Hua et al. 2008), and all have been shown to have a similar domain organization: an ectodomain formed by nine to 12 cadherin repeats, a membrane proximal extracellular domain, a transmembrane domain, and a cytoplasmic domain (Pigott and Ellar 2007).

Hua et al. (2004) identified the *M. sexta* cadherin Bt-R1 region CR12-MPED as the essential binding region required for Cry1Ab-mediated cytotoxicity. Then, they expressed and purified CR12-MPED from inclusion bodies in *E. coli*. They found that 4 ng/ml Cry1Ab alone induced 4.2% insect mortality against *M. sexta*, but when the same amount of toxin was mixed with 100-fold of CR12-MPED, larval mortality reached 82.3%. Further studies revealed that CR12-MPED can also enhance the activity of Cry1Ab and Cry 1Ac toxin to some other lepidopteran insect larvae (Chen et al. 2007). Later, an *Anopheles gambiae* cadherin (AgCad1) was identified as a receptor for Cry4Ba and the CR11-MPED peptide of AgCad1 potentiated the toxicity of Cry4Ba to *A. gambiae* larvae (Hua et al. 2008).

Wang et al. (2005) published the cDNA sequence of a cadherin gene in *Helicoverpa armigera* (HaCad) and mapped the Cry1Ac toxin binding region (TBR) to residues 1217 to 1461. In the present study, this region of HaCad (named HaCad1) was cloned and expressed in *E. coli*. It was found to dramatically enhance Cry1Ac activity to *H. armigera* larvae. In vitro toxin binding and oligomer formation tests showed that the HaCad1-mediated enhancement depends on HaCad1-toxin binding and toxin-oligomer formation. These discoveries indicated that HaCad1 peptide may have practical utility to enhance insecticidal activity or to overcome resistance of insects to *B. thuringiensis* products.

Materials and methods

Purification and activation of Cry1Ac toxin

Growth of *B. thuringiensis* strain BMB871 (Wu et al. 2000) and purification and activation of Cry1Ac toxin were carried out as described by Luo et al. (1999). Purified toxin samples were identified by 10% sodium dodecyl

sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then quantified (Bradford 1976), and stored at -80°C .

Cloning, expression, and purification of HaCad1

RNA was extracted from fourth-instar *H. armigera* larvae using a total RNA isolation system (Promega, Madison, WI, USA). First-strand cDNA was synthesized from total RNA with oligo(dT)17 primer, deoxyribonucleotide triphosphates, and reverse transcriptase avian myeloblastosis virus according to the manufacturer's protocols (Takara, Tokyo, Japan).

HaCad1 (amino acids T1217 to L1461; Fig. 1a) coding gene was amplified from synthesized cDNA by polymerase chain reaction (PCR) using a pair of primers designed using the sequences reported for the cadherin gene (GenBank no. DQ973295): P1, 5'-ATGAATTCACGATTCGTGCTACG GACGGT-3'; P2, 5'-ATGTCGACCAGGTACACCTT CACTTCCG-3'. The amplified fragments were purified, digested with *EcoRI* and *SalI*, and cloned into vector pGEX-6P-1 (Amersham Biosciences, Uppsala, Sweden) to create a recombinant vector pEMB1058. The constructed plasmid and coding sequence were identified further by restriction enzyme mapping and DNA sequencing, respectively. Then, plasmid pEMB1058 was transformed into *E. coli* strain BL21(DE3) (Amersham Biosciences, Uppsala, Sweden), and positive transformants were selected on an Luria-Bertani plate containing $100\ \mu\text{g ml}^{-1}$ ampicillin. The HaCad1 peptides were overexpressed in *E. coli* as inclusion bodies. The inclusion bodies were solubilized and purified on a GStrap FF column (Amersham Biosciences, Uppsala, Sweden). Purified proteins were dialyzed against 10 mM Tris-HCl (pH 8.0, 4°C), quantified, and stored at -80°C .

Site directed mutagenesis of HaCad1

A pair of primers (P3: 5'-ATAGCATGCTCAGAG TCCCAACTCCTACTTCCG-3' and P4: 5'- ATAG CATGCGGTTGGCGGGCGGTACGTGCCGTACAA-3') containing *SphI* cleavage sites were designed to delete the putative Cry1Ac-binding epitope $^{1423}\text{GVLSLNQ}^{1430}$ within the HaCad1 peptide. PCR was performed by using the Expand High Fidelity PCR System kit (Takara, Tokyo, Japan) with pEMB1058 as template. The PCR product was purified, digested with *SphI*, and cloned into the pGEX-6p-1 vector. The correct recombined plasmid was verified by restriction enzyme mapping and DNA sequencing. The expressed peptide was named HaCad1-M, and the Cry1Ac-binding epitope $^{1423}\text{GVLSLNQ}^{1430}$ in this mutation peptide was replaced with AC because of the introduced *SphI* sites in the DNA sequence. The expression and purification of HaCad1-M was as described above for wild-type HaCad1.

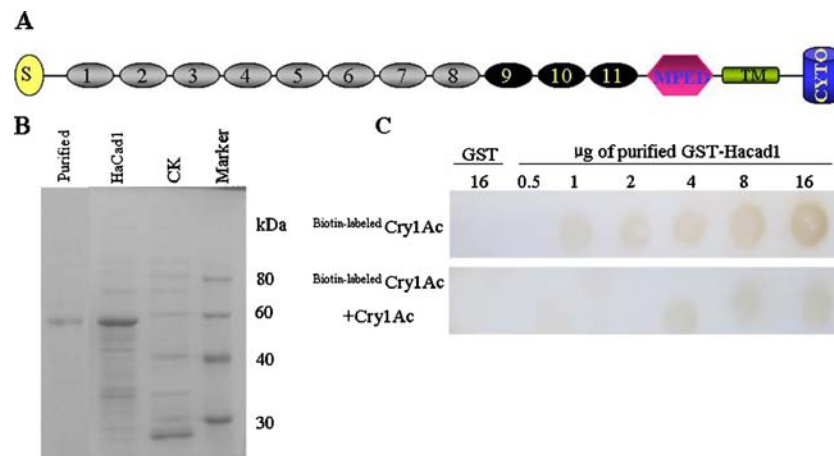


Fig. 1 HaCad1 peptide binds to Cry1Ac toxin. **a** Structure of HaCad with ectodomains reported to contain Cry1Ac binding site in black (29). **b** Overexpression and purification of HaCad1. SDS-PAGE of lysates from *E. coli* transformed with pGEX-6p-1 (CK) and pEMB0158 (*HaCad1*) after 4 h induction at 1 mM isopropyl-β-D-thiogalactopyranoside and purified HaCad1 peptides obtained from a

GSTrap FF column. **c** Analysis of the interaction of Cry1Ac and GST-HaCad1 by dot blot. The ability of GST-HaCad1 to bind Cry1Ac toxin was shown on a nitrocellulose membrane by biotinylated Cry1Ac and biotinylated Cry1Ac plus unlabeled Cry1Ac (500-fold excess) protein as a competitor

Insect bioassays

Susceptible *H. armigera* strain SS1 used in this study was provided by Hubei Academy Institute of Agricultural Sciences.

H. armigera larva bioassays were performed on an artificial diet in 24-well trays (Zhu et al. 2006). Cry1Ac toxins at 2 ng/ml in the presence of 0, 5, and 25 ng/ml of HaCad1 and HaCad1-M were applied on the surface of diet. The number of neonate larvae used per dilution was 72, and two replicates were conducted with each dilution. After a 3-day exposure at 28°C, mortalities for each treatment were scored.

BBMVs preparation and protein labeling

H. armigera BBMVs were prepared from fifth-instar larvae according to methods described by Wolfersberger et al. (1987) and stored at -80°C. Proteins were biotinylated with EZ-Link Sulfo-NHS-LC-biotin (Pierce, FL, USA) as described by Atsumi et al. (2005).

Binding assays

HaCad1 and HaCad1-M peptide (1 µg) were dotted onto a nitrocellulose membrane (Millipore, Bedford, MA, USA). After blocking with 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS)-T buffer (0.1% Tween 20 in PBS, pH 7.4), nitrocellulose membranes were bathed in 5 ng/ml biotinylated-Cry1Ac for 2 h at room temperature. Unlabeled Cry1Ac (500-fold excess) was used in competition assays. To study the effect of HaCad1 on Cry1Ac toxin binding to *H. armigera* BBMV, different quantities of BBMV proteins were dotted onto a polyvinylidene difluoride membrane. After blocking, the membranes were probed

with 5 ng/ml biotinylated-Cry1Ac alone or in the presence of 100-fold mass excess of HaCad1. All of the above binding assay results were visualized with streptavidin-horseradish peroxidase (HRP) followed by SuperSignal chemiluminescent substrate (Pierce, FL, USA) as described by the manufacturers.

Toxin-oligomer formation assay

Cry1Ac protoxin (1 µg) was incubated with HaCad1 (mass ratio 1:1) for 1 h at room temperature and then digested with 5% midgut juice from *H. armigera* for 4 h at 37°C as described by Gómez et al. 2003. The reaction mixture was then separated by SDS-PAGE, transferred onto a nitrocellulose membrane, blocked with BSA (3%), and detected with anti-Cry1Ac antibody (1:5,000) for 2 h at 37°C, followed by a secondary goat anti-rabbit HRP antibody (1:5,000) for 1 h at 37°C. Finally, the signal was visualized as described above for binding assay.

Results

HaCad1 peptide binds to Cry1Ac toxin

HaCad1 (Fig. 1a) formed inclusion bodies during expression in *E. coli*. After solubilization and purification, the target peptide had the expected size of 55.8 kDa on SDS-PAGE (Fig. 1b). Circular dichroism spectral analysis showed that the purified HaCad1 peptides were mostly unfolded (data not shown) as mentioned by Chen et al. (2007).

In order to test the ability of the HaCad1 fragment to bind Cry1Ac toxin, the eluted glutathione *S*-transferase

(GST)-HaCad1 was dotted in increasing amounts (0.5 to 16 μg) directly onto a nitrocellulose membrane filter and the filter was probed with biotin-labeled Cry1Ac. The highest dose of GST (16 μg) was used as a control (Fig. 1c). No signal was detected for purified GST protein but as the quantity of dotted GST-HaCad1 peptide increased, more biotin-labeled Cry1Ac was bound. Furthermore, unlabeled Cry1Ac (500-fold excess) competed for biotin-labeled Cry1Ac binding (Fig. 1c). This result demonstrated that HaCad1 contains an epitope that can specifically bind to Cry1Ac toxin.

HaCad1 peptide enhances Cry1Ac toxicity to *H. armigera* larvae

A comparison among HaCad, Bt-R1, and AgCad1 was conducted at amino acid level (data not show), and the HaCad showed 74% and 61% identity to Bt-R1 and AgCad1, respectively. Further sequence alignment showed that the toxin binding region of these cadherins are highly conserved (Fig. 2a). Based on data of Chen et al. (2007) and Hua et al. (2008) and on evidence that Bt-R1, AgCad1, and HaCad are homologs and that the toxin binding region of these cadherins are highly conserved (Fig. 2a), we investigated whether HaCad1 was able to

enhance Cry1Ac activity. Bioassays were performed against *H. armigera* larvae using sublethal doses of Cry1Ac (2 ng/ml) in the presence and absence of HaCad1 (Fig. 2b). We observed a significant enhancement of Cry1Ac toxicity to *H. armigera* larvae when HaCad1 was included in these bioassays. The magnitude of this enhancement depended on the ratio of toxin to HaCad1 used. In contrast, insecticidal activity was not observed when insects were fed with even the highest HaCad1 concentration (25 ng/ml) used alone. This suggests that the Cry1Ac toxicity enhancement was due to a synergistic rather than direct effect of HaCad1.

Toxicity enhancement depends on Cry1Ac binding to a region of HaCad1

Xie et al. (2005) reported a critical Cry1A-binding region ($^{1423}\text{GVLTLLNFQ}^{1430}$) in the cadherin from *Heliothis virescens*. Chen et al. (2007) found that a homolog peptide ($^{1416}\text{GVLTLLNIQ}^{1423}$) of Bt-R1 was essential for Cry1Ab toxin binding and correlated to CR12-MPED mediated synergism. Interestingly, a very similar homolog to the above peptide ($^{1423}\text{GVLSLNFQ}^{1430}$) is contained within the HaCad1 region of HaCad (Fig. 3a). To confirm the function of this region during toxin binding and toxicity

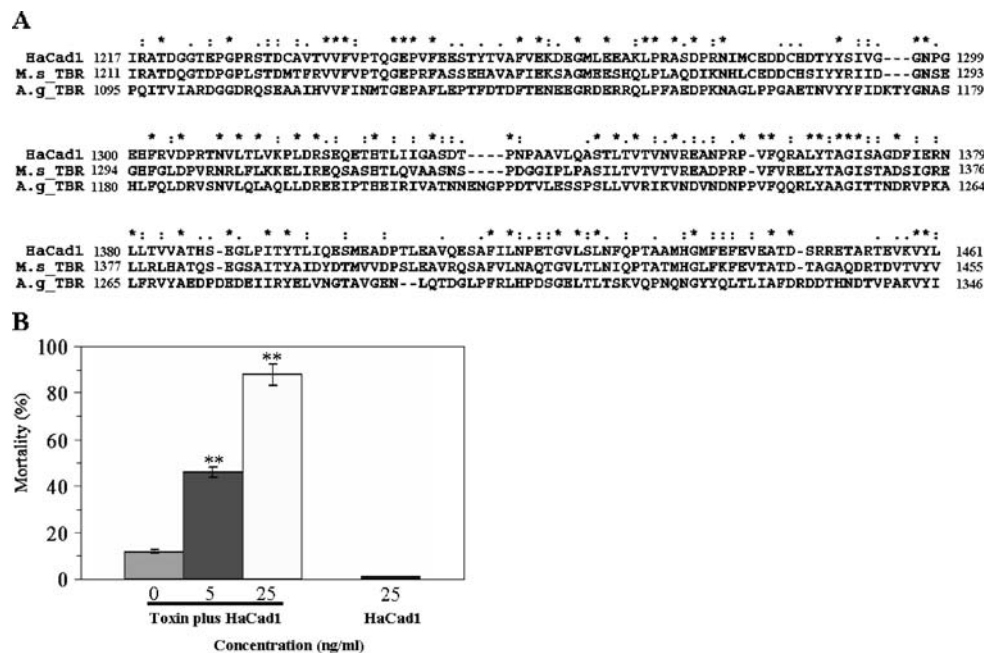


Fig. 2 HaCad1 enhances Cry1Ac toxins when fed to neonate larvae of *H. armigera*. **a** Comparison of the toxin binding regions in HaCad, Bt_R1, and AgCad1 was analyzed by Clustal X software (ver 1.83). Amino acids conserved in three peptides are indicated by asterisk, while amino acids conserved in either two peptides are shown as colon or dot. **b** Bioassay of Cry1Ac toxins to neonate larvae of *H. armigera* with HaCad1. Diets of 2 ng/ml Cry1Ac toxin were mixed with 0, 5, and 25 ng/ml of HaCad1, respectively. Twenty-five

nanograms per milliliter of HaCad1 alone was used as negative control. Mortalities of larvae after 3 days on the diet were analyzed. Each column presents data for the mean \pm standard errors from three replicate bioassays with 72 larvae per treatment. An asterisk above the column indicates that the mortality of Cry1Ac plus HaCad1 treatment was significantly different from the Cry1Ac alone treatment with the same Cry1Ac dosage (Tukey's test, $\alpha=0.05$)

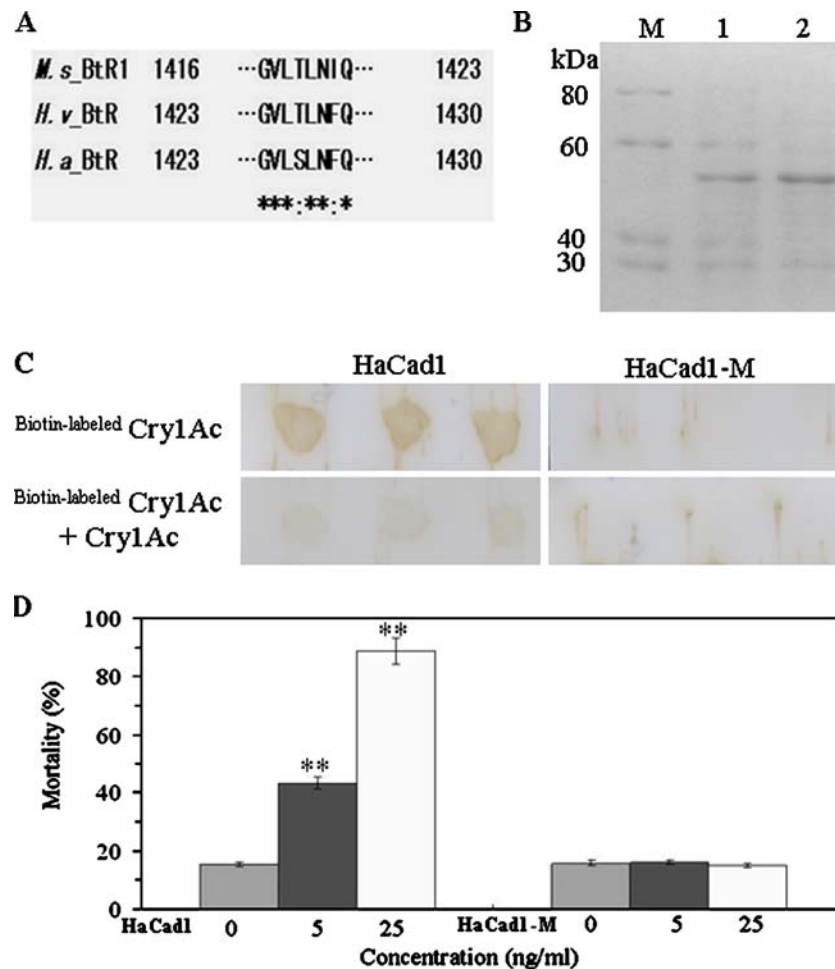


Fig. 3 HaCad1 toxicity enhancement depends on Cry1Ac binding to HaCad1. **a** Alignment of reported toxin binding regions of cadherin receptors from *M. sexta* (20), *H. virescens* (27), and *H. armigera*. **b** Overexpression and purification of HaCad1-M from *E. coli*. SDS-PAGE of lysates from *E. coli* transformed with pEMB0159 (HaCad1-M) after 4 h induction at 1 mM isopropyl-beta-D-thiogalactopyranoside and purified HaCad1-M peptides obtained from a GStrap FF column. **c** Analysis of the ability of Cry1Ac to binds to HaCad1 and HaCad1-M by dot blot. Binding abilities were shown on a nitrocellulose membrane using biotinylated Cry1Ac and biotinylated Cry1Ac plus unlabeled

Cry1Ac (500-fold excess) protein as a competitor. **d** HaCad1-mediated Cry1Ac enhancement disappeared when the toxin binding region was removed. Two nanograms per milliliter of a diet containing Cry1Ac toxin was mixed with 0, 5, and 25 ng/ml of HaCad1 and HaCad1-M, respectively. Mortalities of larvae after 3 days on the diet were analyzed. Each column presents data for the mean \pm standard errors from three replicate bioassays with 72 larvae per treatment. An asterisk above the column indicates that the mortality in the Cry1Ac plus HaCad1 treatment was significantly different from that in the treatment with Cry1Ac alone at the same Cry1Ac dosage (Tukey's test, $\alpha=0.05$)

enhancement, a mutation peptide, HaCad1-M, was constructed in which this region was removed from HaCad1. As described for the wild type, HaCad1-M was overexpressed and purified from *E. coli* BL21(DE3) cells (Fig. 3b). As shown in Fig. 3c, biotin-labeled Cry1Ac bound specifically to HaCad1 on dot blots. In contrast, HaCad1-M did not bind to the toxin, thus confirming that the deleted residues in this peptide were essential determinants of Cry1Ac toxin binding.

In order to determine whether HaCad1-M is able to enhance Cry1Ac toxicity to the same degree as its wild type, bioassays of Cry1Ac toxin were conducted in the presence of HaCad1 and HaCad1-M. In agreement with its observed inability to bind to Cry1Ac, HaCad1-M

peptide did not exhibit the enhancement shown by its wild-type peptide (Fig. 3d). These results demonstrated that HaCad1-mediated enhancement was dependent upon Cry1Ac bind to a region of HaCad1, and the residues ¹⁴²³GVLTLNFQ¹⁴³⁰ were necessary for this binding.

HaCad1 did not block Cry1Ac binding to BBMVs prepared from *H. armigera*

Previous studies reported that peptides containing the TBR competitively blocked Cry1A toxin binding to cadherin receptors in the midgut, thus neutralizing Cry1A toxicity (Gómez et al. 2003; Xie et al. 2005). In order to determine whether the binding of HaCad1 to

Cry1Ac toxin in vitro would block Cry1Ac toxin binding to the midgut epithelium of *H. armigera*, the ability of BBMV to bind toxins was tested using biotinylated-Cry1Ac alone and in the presence of 100-fold mass excess of HaCad1. BBMV extract was dotted in increasing quantities (0 to 16 μg) directly onto a nitrocellulose membrane filter and then the filter was probed with biotin-labeled Cry1Ac (Fig. 4). As the quantity of dotted BBMVs increased, more biotin-labeled Cry1Ac was bound to the groups with both the toxin alone and treated with HaCad1. Significantly, the binding ability of the competition group containing as much as 100-fold mass excess of HaCad1 was similar to that of the group containing Cry1Ac alone. These results indicated that the binding of Cry1Ac and HaCad1 in vitro did not affect the toxin-binding ability of *H. armigera* BBMVs.

Toxin-oligomer formation is responsible for HaCad1-mediated toxicity enhancement

Since the binding of Cry1A toxin to cadherin receptor facilitates the formation of a 250-kDa toxin oligomer (Pardo-López et al. 2006) and there is evidence that Cry1Ac binds specifically to HaCad1 (Fig. 1c, d), we determined whether in vitro purified HaCad1 would have the same function as the cadherin receptor. In the HaCad1 treatment (Fig. 4), we detected a strong signal corresponding to the 250-kDa oligomer of Cry1Ac toxin in the midgut juice environment but in the HaCad1-M and GST control groups, the oligomer could not be detected. These observations demonstrated that HaCad1 facilitated the formation of an oligomer of Cry1Ac toxin and that oligomer formation was dependent on the putative Cry1Ac toxin binding region of HaCad1 ($^{1423}\text{GVLSLN}^{\text{FQ}}^{1430}$). Taking these results together with the earlier bioassay results (Fig. 3d), it is clear that toxin-oligomer formation is responsible for the HaCad1-mediated enhancement effect.

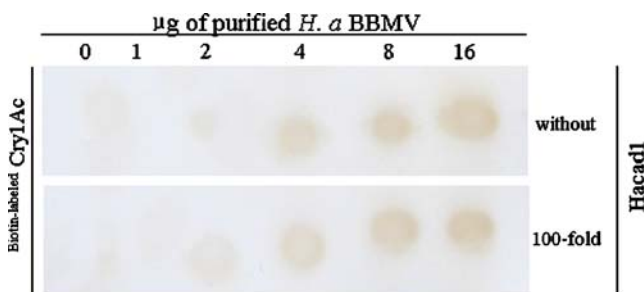


Fig. 4 Analysis of the effect of HaCad1 on Cry1Ac binding to BBMVs prepared from *H. armigera*. The ability of Cry1Ac to bind to BBMVs prepared from *H. armigera* was shown on a nitrocellulose membrane by dot blot. Biotinylated Cry1Ac alone (*upper line*) and incubated with unlabeled HaCad1 (100-fold excess) protein (*lower line*) were both detected

Discussion

The Cry1Ac toxin binding sites of HaCad have been mapped to residues 1217 to 1461 by Wang et al. (2005). The results reported in the present study support their finding (Fig. 1c). Additionally, we found that HaCad1 enhanced the insecticidal activity of *B. thuringiensis* Cry1Ac toxins to *H. armigera* larvae. These phenomena have been previously reported in *M. sexta* (Chen et al. 2007) and *A. gambiae* (Hua et al. 2008). By contrast, several studies have reported that TBR peptides block Cry1A toxin binding to cadherin receptors in the midgut, consequently neutralizing Cry1A toxicity (Gómez et al. 2003; Xie et al. 2005; Liu et al. 2009). It was argued that these differences observed in the effects of the same TBR fragments could be due to differences in the state of the proteins used. All of the TBR peptides used in the studies that showed enhancement were extracted from inclusion bodies and were shown to be in an unfolded state (Chen et al. 2007; Hua et al. 2008). In contrast, TBR fragments that neutralized Cry1A toxicity were purified from soluble fractions (Gómez et al. 2003; Xie et al. 2005; Liu et al. 2009). It has also been suggested that the unfolded conformations of TBR fragments possess more exposed amino acid residues and could modify interactions with Cry1A toxins and other molecules on insect

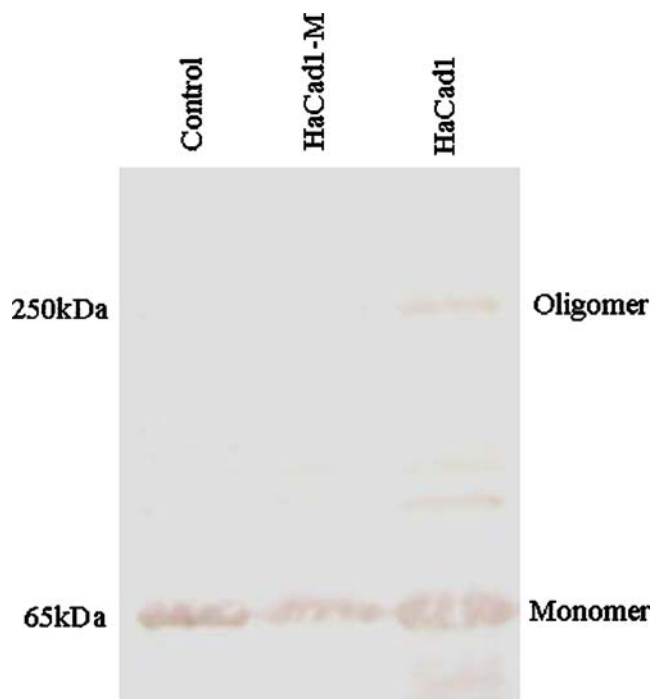


Fig. 5 Oligomer formation of Cry1Ac protoxin in the presence of HaCad1 and HaCad1-M. Biotin-labeled Cry1Ac protein was incubated with 5% midgut juice in the presence of HaCad1 and HaCad1-M; Cry1Ac treated with GST was used as a control. Oligomer formation was revealed after SDS-PAGE electrophoresis and visualized

midgut epithelium (Chen et al. 2007). In the present study, the HaCad1 fragments were all extracted from inclusion bodies and were mostly unfolded. Our bioassay results are also consistent with the finding of Chen et al. (2007) and Hua et al. (2008).

Based on previous studies (Gómez et al. 2003; Xie et al. 2005), it was expected that the HaCad1 peptide would inhibit Cry1Ac binding to BBMV. However, we did not find competition in this study (Fig. 4). A plausible explanation is that Cry1Ac binding to HaCad1 peptide is reversible, or of low affinity, and that it has alternative high affinity binding sites in the BBMV. It is interesting that, like the TBR-induced toxicity inhibition (Gómez et al. 2003), TBR fragment-synergized Cry1A toxicity was also related to specific toxin-peptide binding residues. Chen et al. (2007) reported that when the TBR residues ¹⁴¹⁶GVLTLNIQ¹⁴²³ were removed, CR12-MPED lost its Cry1A binding ability and also its capacity for toxicity enhancement. In agreement with these reports, HaCad1 also lost its ability to bind to Cry1Ac toxin and its synergistic effect on toxicity when the homologous epitope ¹⁴²³GVLTLNFQ¹⁴³⁰ was replaced by the amino acids AC in this study (Fig. 3).

Previously, it was reported that binding of cadherin receptors to Cry1A toxins facilitates the formation of a 250-kDa toxin oligomer (Gómez et al. 2002). In this study, we found that purified HaCad1 has a function similar to that of cadherin (Fig. 5). Likewise, this toxin-oligomer formation was dependent on toxin–HaCad1 interaction (Fig. 5) and was correlated with HaCad1-mediated toxicity enhancement (Fig. 3). Combining previous reports with our own results, we suggest that the HaCad1-mediated toxicity enhancement may be due to the fact that the additional HaCad1 provided more toxin oligomer after the toxin was solubilized and activated in insect midgut fluid. The additional toxin oligomer perhaps improved toxin docking and membrane insertion. These data and this explanation of the observed synergism are in agreement with the role of native cadherin receptors in facilitating Cry1A toxin oligomerization, and they support the hypothesis that the oligomers are essential for toxicity in the pore-forming model (Bravo et al. 2004). Because homologs of cadherin are present in other lepidopteran species (Pigott and Ellar 2007; Fabrick and Tabashnik 2007), it is likely that HaCad1 would also enhance the toxicity to other lepidopterans of Cry1Ac, or of other toxins which have similar receptors.

The development of resistance to *B. thuringiensis* toxin is now considered the major threat to the long-term effectiveness of *B. thuringiensis* products (Bravo and Soberón 2008). So far, several cadherin mutations have been reported to be linked to Cry1Ac resistance, including some with stop codons (*Pg* r2, *Ha* r1, *Hv* r1) that lack toxin binding regions and some deletions upstream of the toxin binding regions (*Pg* r1 and r3) that alter the toxin binding

regions (Gahan et al. 2001; Morin et al. 2003; Xu et al. 2005; Yang et al. 2006, 2007). All of the mutant cadherins are predicted to be shortened proteins, which interrupt the interaction between cadherin and Cry1Ac toxin and result in resistance of the insects to *B. thuringiensis* toxin. Another example of Cry1Ac resistance in *H. armigera* was associated with decreased accumulation of cadherin in the midgut (Wang et al. 2005). In this study, we found that the additional HaCad1 can function in the same way as native cadherin receptors to facilitate toxin-oligomer formation in the insect midgut environment (Fig. 5). Thus, the use of additional HaCad1 could replace the role of native cadherin in insects whose resistance is linked to cadherin mutations, in order to recover the function of facilitating toxin-oligomer formation, or to provide more toxin oligomers. Based on this HaCad1 mechanism and models of cadherin-linked insect resistance, HaCad1 might potentially restore Cry1A toxicity to resistant insects resulting from cadherin receptor mutation.

In summary, the present study reported a truncated TBR fragment HaCad1 that enhanced Cry1Ac toxicity to *H. armigera* larvae by facilitating the formation of Cry1Ac oligomer in vitro. These discoveries are consistent with the pore-forming model (Bravo et al. 2004) and provide a novel strategy to enhance insecticidal activity or to overcome the resistance of insects to *B. thuringiensis* toxin-based biopesticides or transgenic crops.

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