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Enhancement of insecticidal activity of *Bacillus thuringiensis* Cry1A toxins by fragments of a toxin-binding cadherin correlates with oligomer formation

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Abstract

Cry1A toxins produced by *Bacillus thuringiensis* bind a cadherin receptor that mediates toxicity in different lepidopteran insect larvae. Insect cadherin receptors are modular proteins composed of three domains, the ectodomain formed by 9 to 12 cadherin repeats (CR), the transmembrane domain and the intracellular domain. Cry1A toxins interact with three regions of the *Manduca sexta* cadherin receptor that are located in CR7, CR11 and CR12 cadherin repeats. Binding of Cry1A toxin to cadherin induces oligomerization of the toxin, which is essential for membrane insertion. Also, it has been reported that cadherin fragments containing the CR12 region enhanced the insecticidal activity of Cry1Ab toxin to *M. sexta* and other lepidopteran larvae. Here we report that cadherin fragments corresponding to CR7 and CR11 regions also enhanced the activity of Cry1Ac and Cry1Ab toxin to *M. sexta* larvae, although not as efficient as the CR12 fragment. A single point mutation in the CR12 region (I1422R) affected Cry1Ac and Cry1Ab binding to the cadherin fragments and did not enhance the activity of Cry1Ab or Cry1Ac toxin in bioassays. Analysis of Cry1Ab *in vitro* oligomer formation in the presence of wild type and mutated cadherin fragments showed a correlation between enhancement of Cry1A toxin activity in bioassays and *in vitro* Cry1Ab-oligomer formation. Our data shows that formation of Cry1A toxin oligomer is in part responsible for the enhancement of Cry1A toxicity by cadherin fragments that is observed *in vivo*.

1. Introduction

Bacillus thuringiensis produces crystalliferous proteins known as δ -endotoxins or Cry toxins with insecticidal activity against different insect orders and nematodes [4]. Different Cry toxins are used worldwide either as sprays or in transgenic plants in the control of insect pests in agriculture and insect vectors of human diseases [4].

The structure of five different Cry toxins and one Cry protoxin-with different insect specificity-belonging to the family of three-domain Cry toxins was solved and shown to share a similar structure [2,3,9,16,20,22]. Domain I is a seven α -helix bundle in which a central helix α -5 is surrounded by six outer helices involved in membrane insertion. Domain II consist of three anti-parallel β -sheets packed around a hydrophobic core in a "beta-prism" and is involved in

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receptor interaction. Finally, domain III is a β -sandwich of two antiparallel β -sheets involved in receptor binding [4].

The mode of action of Cry toxins is a multi-step process, involving sequential interaction with at least two different receptors and has been studied primarily in lepidopteran insects [5]. Cry1A toxins active against lepidopteran pests are synthesized as 130 kDa protoxins. In order to exert their toxic activity, crystal inclusions are ingested by larvae, dissolved and activated in the midgut lumen by proteases to yield a 60 kDa monomer. The activated toxin binds with high affinity (K_d 1 nM) to a cadherin receptor (Bt-R₁ in the case of *M. sexta*) that is located in the apical membrane of midgut epithelial cells [25]. Binding of Cry1A toxins to cadherin facilitates the proteolytic cleavage from helix α -1 of the N-terminal end of the toxin facilitating the formation a pre-pore oligomeric structure [11]. Engineered Cry1A toxins lacking helix α -1 form oligomers *in vitro* in the absence of cadherin receptor and kill resistant lepidopteran insects that have mutations in the cadherin structural gene [24]. The toxin-oligomer binds to secondary receptors, glycosylphosphatidylinositol-anchored proteins such as aminopeptidase-N or alkaline-phosphatase, resulting in the insertion of the pre-pore in membrane lipid rafts leading finally to pore-formation and cell lysis resulting in death of the insect [5,19,23].

A limiting step in the mode of action of Cry1A toxins is the binding to the cadherin receptor. Insect cadherin receptors are modular proteins composed of three domains, the ectodomain formed by 9 to 12 cadherin repeats (CR) depending on the insect species, the transmembrane domain and the intracellular domain [25]. In different lepidopteran insect colonies that have evolved resistance to Cry1A toxins, the resistance is linked to mutations in the cadherin structural gene [8,21,27,28]. Cry1Ab and Cry1Ac share identical domain II amino acid regions involved in receptor interaction and bind the cadherin receptor by means of domain II loop α -8, loop 2 and loop 3 that interact with amino acid regions of Bt-R₁ receptor located in CR11, CR7 and CR12, respectively [12,13,14,26]. Of these binding regions, CR12 has been shown to mediate toxicity in cultured insect cells [17]. Interestingly, a CR12 fragment (named CR12-MPED) produced in *E. coli* cells enhanced the activity of Cry1Ab toxin in different lepidopteran insect larvae [6]. It was suggested that the CR12 fragment increases the toxin concentration in the microvillar membrane of the larvae, since this fragment binds this membrane with high affinity (K_d 32nM) and was localized in the microvilli of the larvae fed CR12. The Cry toxin would then be able to interact with GPI-anchored Cry1A secondary-receptors [6].

Since binding of Cry1A toxin to cadherin receptor facilitates the formation of the pre-pore oligomer, we hypothesized that enhancement of Cry1A activity by CR12-MPED may be in part due to enhanced oligomer formation. In this article we analyzed the insecticidal activity of Cry1Ab and Cry1Ac toxin to *M. sexta* larvae in the presence of different cadherin fragments and in mutant cadherin fragments that affected toxin binding. Also we analyzed the *in vitro* oligomer formation in the presence of the different cadherin fragments. Our data shows that the enhanced activity of Cry1A toxin by CR12 correlates with oligomer formation. These data are in agreement with the role of the cadherin receptor in facilitating Cry1A toxin oligomerization and support the hypothesis that the oligomers are essential for toxicity.

2. Methods

2.1 Growth of *Bacillus thuringiensis*, purification of Cry1A crystal inclusions and toxin activation

Crystal inclusions of Cry1Ab and Cry1Ac were produced in the acrySTALLIFEROUS Bt strain 407cry⁻ transformed with the pHT315Ab plasmid harboring the *cry1Ab* gene or with pHT3101 plasmid harboring the *cry1Ac* gene [1] (kindly supplied by Dr. A. Aronson, Purdue University). Bt strains were cultured for 3 days at 29C with shaking at 200rpm in nutrient broth sporulation

medium supplemented with 10 µg erythromycin per ml. The crystals of Cry1Ab or Cry1Ac were recovered and purified by sucrose gradients and solubilized in 10 mM carbonate buffer pH 10.5, 0.2% β-mercaptoethanol at 37°C for 2 h and activated with 1:50 (w/w) TPCK-treated bovine trypsin (Sigma-Aldrich Co.) w/w for 2 h at 25 °C. Protoxin digestion was stopped with 1 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentration was determined using the Bio-Rad Protein assay (Bio-Rad Labs. Hercules, CA).

2.2 Site directed mutagenesis of cadherin fragments

The CR7-12 and CR12 fragments were previously cloned by PCR as described elsewhere [18,24]. Mutagenesis of pET22b plasmids harboring either CR7-12 or CR12 cadherin fragments [18,24] was performed using QuikChange XL-kit (Stratagene, La Jolla CA). A mutagenic oligonucleotide (5'-CCA CTT CCT GCT TCC CGC CTT ACT GTC ACT GTT AC-3') was synthesized for I1422R mutant construction. Mutant I1442R was sequenced and transformed into BL21 *E. coli* strain for protein production.

2.3 Expression and purification of cadherin fragments

CR7 and CR11 fragments were amplified by PCR using the pET22b:CR7-12 as template with the following primers which include *EcoRI* and *HindIII* restriction sites in 5' end of the forward or the reverse primers, respectively. For CR7 amplification: CR7For, 5'-TAC AGA ATT CCA TGA TCG ACT TCC TCA CGG GTC AAA TTT CC-3' and CR7Rev, 5'-TCT TAA GCT TTG CAT AAT TGA TGA CGT AGC TCA C-3'. For CR11 amplification: CR11For, 5'-TAC AGA ATT CAA TGG AAG AGT CTC ACC AAC TTC-3' and CR11Rev: TCT TAA GCT TTG CAT AAT TGA TGA CGT AGC TCA C-3'. The PCR reactions were performed with Vent-Polymerase (New England BioLabs, Beverly, MA). The PCR products (342 bp for CR7 and 429 bp for CR11) were purified with QIAquick PCR Purification Kit (QIAGEN, Valencia, CA), digested with *EcoRI* and *HindIII* (New England BioLabs, Beverly, MA) and ligated into the previously digested vector pET22b (Novagen, EMD Biosciences, Inc.). All cadherin fragments including the CR7-12 and CR12 fragments previously cloned [18,24] were purified to homogeneity from BL21 *E. coli* cultures grown at 37 °C in 2X TY (Tryptone 1.6 % w/v, Yeast extract 1% w/v and NaCl 0.5 % w/v, supplemented with 100 µg/ml ampicillin and 0.1% glucose) until they reached an OD of 0.7 at 600 nm. Induction of cadherin fragments was done by adding of 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) that triggers the *lacZ* promoter, to the culture and grown for 4 h at 25°C. All cadherin fragments were solubilized from inclusion bodies with 8M Urea in 300 mM Tris-HCl pH 8. The solubilized fragments were applied to a Ni-agarose column (Qiagen, GmbH, Germany), washed with PBS and the cadherin protein fragments eluted with 2 ml of 250 mM imidazole, 0.2% azide in PBS.

2.4 Proteolytic activation and oligomer formation assay

For oligomer formation, 1–2 µg of Cry1Ab protoxin was incubated for 1 h with cadherin fragments CR7-12, CR7, CR11 or CR12 (in a mass ratio 1:1), digested 1 h at 37 °C with 5% midgut juice from *M. sexta*. PMSF was added to a final 1 mM concentration to stop proteolysis. The reaction mixture was separated in SDS-PAGE, transferred onto a PVDF nitrocellulose membrane, blocked with skimmed milk (5 %) and detected with anti-Cry1Ab polyclonal antiserum (1/30,000; 1 h) and visualized with a goat anti-rabbit antibody coupled with horseradish peroxidase (HR) (Sigma, St. Louis, MO) (1/1000; 1 h), followed by SuperSignal chemiluminescent substrate (Pierce, Rockford, IL) as described by the manufacturers.

2.5. Toxin overlay assays

Toxin overlays to CR7-12 and CR7 (10 µg) loaded in 10% SDS-PAGE were performed as reported [12] using 10 nM biotinylated Cry1Ac or Cry1Ab, and detected with streptavidin-HRP conjugate (1:5000) and SuperSignal chemiluminescent substrate.

2.6 ELISA binding assays

ELISA plates, 96-wells, were incubated 12 h at 4 °C with 0.5 µg/ml of CR7-12 in 50 mM NaHCO₃ pH 9.6, followed by five washes with PBS, 0.2% Tween 20. The plates were then incubated with PBS, 0.5% gelatin (BioRad) 0.2%/Tween 20, for 1 h at 37 °C and washed five times with buffer A (PBS, 0.1% Tween 20). The ELISA plates were incubated with 5 nM Cry1Ac for 2 h at 37 °C and washed again with buffer A. The Cry1Ac proteins bound to CR7-12 were detected with anti-Cry1Ac antibody (1:5000) 2 h at 37 °C, followed by a secondary goat-anti-rabbit-HRP antibody (1:5000) 1 h at 37 °C. The HRP enzymatic activity was revealed with a freshly prepared substrate (40 mg of *o*-phenylenediamine, 18 ml of H₂O₂ in 100 ml of 100 mM NaH₂PO₄, pH 5.0). The enzymatic reaction was stopped with 6 N HCl and the absorbance read at 490 nm with a precision microplate reader from Molecular Devices (Sunnyvale, Cal).

2.7 Insect bioassays

M. sexta larvae were reared on an artificial diet in 24-well trays (Cell Wells, Corning Glass Works, Corning New York 14831). Cry1Ac or Cry1Ab activated toxins either at 1 ng or 0.05 ng/cm² in the presence of 5 ng/cm² of cadherin fragments was applied on the surface of diet and one neonate larvae was placed in each well. Mortality was recorded after five days at 28° C, 65±5% relative humidity and a photoperiod of 16:8 h light:dark. This entire experiment was replicated twice, yielding a total sample size of 48 larvae reared on Cry1Ab or Cry1Ac-treated diet.

3. Results

3.1 Different cadherin fragments enhance Cry1Ac toxicity

Cry1A toxins interact with cadherin through three binding epitopes located in cadherin repeats CR7, CR11 and CR12 [10,12,13,26]. Previously it was demonstrated that a cadherin fragment corresponding to CR12 (CR12-MPED) enhanced the activity of Cry1Ab toxin in different lepidopteran insect larvae [6]. In order to determine if CR7 or CR11 also enhanced Cry1A toxin activity, a bioassay that used sub-lethal doses of Cry1Ab or Cry1Ac (10% mortality) in the presence of three peptides corresponding to these cadherin regions was performed against *M. sexta* larvae. Figure 1 shows the cadherin protein structure and the cadherin fragments that were characterized in this work. Previously two cadherin fragments, one include from CR7 to CR12 (M810-A1485) and fragment CR12 (G1370-A1485), were cloned into pET22b vector and produced in *E. coli* [18,24]. Using appropriate primers and CR7-12 clone as template, a CR7 (M810-A926) and a CR11 (M1257-S1400) fragments were amplified by PCR and cloned also into pET22b. The four protein fragments, CR7-CR12, CR7, CR11 and CR12, were purified from *E.coli* transformant cells. Figure 2 show that all fragments enhanced the activity of Cry1Ab and Cry1Ac toxins. The CR7-12 fragment showed 8-fold enhancement of Cry1Ab or Cry1Ac activity. From the three individual fragments we observed that CR12 was the most effective since it enhanced the activity 7-fold followed by CR11 that increased 4-fold and CR7 that showed 3-fold higher activity in response to a sub-lethal dose of both Cry1A toxins.

3.2 A single amino acid change I1422R affects Cry1Ac binding to cadherin fragments

In order to determine the role of toxin binding in the enhanced activity of Cry1A toxins by cadherin fragments a single point mutation in the CR12 fragment, which affected its interaction with Cry1A was constructed. Previously, extensive mutagenesis of a CR12 amino acid region in the *Heliothis virescens* cadherin protein revealed a Cry1Ac binding region (¹⁴²³GVLTLN¹⁴³⁰) [26]. Single arginine changes of L1425 and F1429 affected binding of Cry1Ac to a *H. virescens* cadherin fragment containing these mutations [26]. Furthermore a deletion mutant in the *M. sexta* homologous region (¹⁴¹⁶GVL¹⁴²³TNIQ¹⁴²³) affected binding of Cry1Ab and the synergistic effect of CR12-MPED fragment [6]. We therefore replaced I1422

to arginine in the *M. sexta* CR7-12 and CR12 fragments (see Methods). Binding analysis of the two cadherin fragments and the corresponding mutant fragments (I1422R) by toxin overlay assays showed that this single amino acid change affected Cry1Ac binding (Fig. 3A and 3B). ELISA binding assays confirmed that the I1422R mutation affected Cry1Ab and Cry1Ac binding to both cadherin fragments (Fig. 3C).

Bioassays of Cry1Ac toxin in the presence of the mutated cadherin fragments revealed that the I1422R mutant did not enhance Cry1Ab or Cry1Ac toxicity as the wild type cadherin fragments, CR7-12 (8-fold) or CR12 (7 fold) (Fig. 2).

3.3 Oligomerization correlates with synergistic effect of cadherin fragments

As mentioned previously, binding of Cry1A toxins to cadherin receptors facilitates the formation of a 250 kDa toxin oligomer that is membrane-insertion competent [11]. Cry1Ab toxin oligomerizes *in vitro* when the protoxin is incubated with proteases in the presence of cadherin fragments corresponding to CR7 to CR12, or CR12, while Cry1Ac oligomerization has been observed when activated with midgut proteases in the presence of CR12 only [12, 13,14,23,24]. To determine if oligomer formation correlates with the enhancement of Cry1A toxicity by cadherin fragments, first we compare the *in vitro* oligomerization of Cry1Ab in the presence of each CR7, CR11 and CR12 cadherin protein fragments. Cry1Ab was incubated with *M. sexta* midgut juice as source of proteases and with each of the three cadherin fragments and oligomer formation was observed by Western blot after SDS-PAGE of the activation reactions. Figure 4A shows that the three cadherin fragments facilitate the formation of the 250 kDa oligomer, although oligomer formation was more efficient in the presence of CR12 than with either CR11 or CR7. Finally we determine if the mutated CR12-I1422R fragment could induce Cry1Ab oligomerization. Figure 4B shows that a 250 kDa oligomer is observed in the protein sample incubated with the CR12 fragment but not in the sample incubated with the CR12-I1422R protein.

4. Discussion

Previously we characterized the Cry1A toxin binding sites in the *M. sexta* cadherin receptor and found that cadherin fragments corresponding to CR7 (TBR1) and CR11 (TBR2) inhibited Cry1Ab toxin activity in bioassays [13]. Inhibition of Cry1Ab toxicity by TBR1 and TBR2 peptides was achieved using a concentration of the toxin corresponding to a LC₉₀, and 1000-3000 molar excess of cadherin peptides that were purified from soluble fractions [13]. Also, a cadherin peptide containing CR10 to CR12 of the *H. virescens* cadherin inhibited Cry1Ac toxicity in bioassays [26]. Moreover, a *M. sexta* cadherin fragment containing CR11-12 (TBR) was reported to inhibit Cry1Ab activity in bioassays with *M. sexta* [7]. Nevertheless, it was reported that peptide CR12-MPED extracted from *E. coli* inclusion bodies enhanced the activity of Cry1Ab in the same insect species [6]. It was argued that the differences observed in the effect of the TBR peptide could be due to an unfolded nature of proteins that enhance Cry1Ab activity since the proteins were extracted from inclusion bodies and shown to be unfolded in contrast to fragments that are purified from soluble fractions [6]. In the case of the cadherin fragments characterized in this work, all were extracted from inclusion bodies and circular dichroism spectra analysis showed that the CR12 peptides were mostly unfolded (data not shown). Another possibility is that different protein ratios of Cry1A toxins and cadherin protein competitors could have different effects, either inhibiting or enhancing toxicity. In this regard, we found that the CR7-12 peptide did not enhance Cry1Ac toxicity when used at 50 ng/cm² (ten-fold higher peptide concentration than normal bioassays) was used in the bioassay (data not shown).

Cadherin repeat 12 has been recognized as an important region of cadherin mediating Cry1A toxicity since S2 cultured insect cells expressing cadherin receptor proteins containing CR12

in their surface become sensitive to Cry1Ab toxin [17]. Moreover, a protein fragment corresponding CR12 (CR12-MPED) enhanced activity of Cry1Ab toxin when fed to different insect larvae [6]. Nevertheless, the specific binding site of CR12 that interacts with the Cry1A toxin is in debate since two different regions of this cadherin repeat have been reported. Griko *et al.* 2007 identified a structural binding site in *M. sexta* cadherin formed by the linker region between CR11 and CR12 (amino acids 1349–1374) and the carboxi-terminal part of CR12 (residues 1349–1447) by expressing different protein fragments and showing that both regions are important in binding the Cry1Ab toxin [15]. However, Xie *et al.* 2005 using different cadherin fragments of the *H. virescens* cadherin and single point mutagenesis identified ¹⁴²³GVLTLNFQ¹⁴³⁰ as an important Cry1Ac binding epitope [26]. In agreement with Xie *et al.* 2005, a deletion mutant of the corresponding region in the *M. sexta* CR12-MPED fragment (¹⁴¹⁶GVLTLNIQ¹⁴²³) did not bind Cry1Ab toxin [6], suggesting that this site is the binding site for Cry1A toxins. In addition, in this work we describe a single point mutation (I1422R) in the *M. sexta* cadherin fragments CR7-12 and CR12 that affects Cry1Ac binding and toxicity, since it did not enhance mortality in response to a sub-lethal dose of Cry1Ac toxin, supporting the ¹⁴¹⁶GVLTLNIQ¹⁴²³ epitope as a key region in cadherin receptor involved in Cry1Ac binding and toxicity. The CR12 fragment characterized in this study, that binds the Cry1Ab and Cry1Ac toxins and enhanced toxicity, comprised only amino acids G1370 to A1485 showing that residues 1349 to 1369 are not important for Cry1A toxin interaction nor for toxicity. The protein fragments characterized by Griko *et al.* 2007 were fused to the maltose binding protein and this could affect the interaction with Cry1Ab toxin [15]. Although binding to the cadherin receptor is a limiting step in the mode of action of Cry1A toxins in lepidopteran insects, its role in the mode of action of these toxins is still controversial. Two opposing models propose a different role for the cadherin binding in mediating the toxicity of Cry1A toxins. In the pore-forming model, cadherin binding facilitates the proteolytic cleavage of helix α -1 in domain I resulting in the formation of a pre-pore oligomer structure that subsequently binds to GPI-anchored receptors and inserts into membrane causing cell death [5]. In the signaling model, binding of Cry1A to cadherin triggers a signal transduction pathway that leads to oncotic-like cell death [29]. However, recently we demonstrated that engineered Cry1A toxins lacking helix α -1 form oligomers *in vitro* in the absence of cadherin binding and kill resistant insects with either mutations in the cadherin gene or that do not express cadherin protein showing that the main role of cadherin binding is to facilitate oligomer formation [24]. In this article we show that the enhanced activity of Cry1A toxins in the presence of toxin-binding cadherin fragments correlates with oligomer formation. These data are also consistent with the pore-forming model and argue against the signal transduction model.

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Figure 1.

Organization of *M. sexta* cadherin Bt-R₁ receptor. Sig is the signal peptide, in numbers are depicted the cadherin repeats (CR) and cadherin repeats 7, 11, 12 show the toxin binding regions. MPED is the extracellular membrane proximal region, TMR the transmembrane region and IR the intracellular region. The hatched areas represent the toxin binding regions. Below the peptides characterized in this work with the molecular weight are shown.

Figure 2.

Cadherin peptides enhance Cry1Ac (black bars) and Cry1Ab (empty bars) toxins when fed to *M. sexta* larvae. LC10 concentration of Cry1Ac or Cry1Ab toxins (0.05 ng/cm² of diet) were mixed with 5ng/cm² of different cadherin peptides corresponding to CR7-12, CR12, CR11, CR7, CR7-12-I1422R and CR12-I1422R. Mortality % of 48 larvae after five days in diet. Representative results of two experiments are shown.

Figure 3.

Mutation of I1422R in the cadherin fragments affects binding to Cry1Ab and Cry1Ac toxin. A. Ligand blot of CR7-12 and CR7-12-I1422R peptides with Cry1Ac toxin and revealed with anti-Cry1Ac antibody. B. Ligand blot of CR12 and CR12-I1423R peptides with Cry1Ac toxin and revealed with anti-Cry1Ac antibody. Similar results were obtained with Cry1Ab toxin (not shown) C. ELISA binding assay of CR7-12, CR7-12-I1422R and CR12-I1422R with Cry1Ac (black bars) and Cry1Ab (empty bars) toxins revealed with anti-Cry1Ac or anti-Cry1Ab antibodies..

Figure 4.

Oligomer formation of Cry1Ab protoxin in the presence of different cadherin fragments. A. Cry1Ab protoxin was incubated with 5% midgut juice in the presence of CR7, CR11 or CR12 and oligomer formation was revealed after SDS-PAGE electrophoresis and Western blot using anti-Cry1Ab antibody. NR is without any CR peptide. B. Cry1Ab protoxin was incubated with 5% midgut juice in the presence of CR12 and CR12-I1422R and oligomer were revealed as described above.