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Enhancement of *Bacillus thuringiensis* Cry3Aa and Cry3Bb Toxicities to Coleopteran Larvae by a Toxin-Binding Fragment of an Insect Cadherin▽†

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The Cry3Aa and Cry3Bb insecticidal proteins of *Bacillus thuringiensis* are used in biopesticides and transgenic crops to control larvae of leaf-feeding beetles and rootworms. Cadherins localized in the midgut epithelium are identified as receptors for Cry toxins in lepidopteran and dipteran larvae. Previously, we discovered that a peptide of a toxin-binding cadherin expressed in *Escherichia coli* functions as a synergist for Cry1A toxicity against lepidopteran larvae and Cry4 toxicity against dipteran larvae. Here we report that the fragment containing the three most C-terminal cadherin repeats (CR) from the cadherin of the western corn rootworm binds toxin and enhances Cry3 toxicity to larvae of naturally susceptible species. The cadherin fragment (CR8 to CR10 [CR8-10]) of western corn rootworm *Diabrotica virgifera virgifera* was expressed in *E. coli* as an inclusion body. By an enzyme-linked immunosorbent microplate assay, we demonstrated that the CR8-10 peptide binds α-chymotrypsin-treated Cry3Aa and Cry3Bb toxins at high affinity (11.8 nM and 1.4 nM, respectively). Coleopteran larvae ingesting CR8-10 inclusions had increased susceptibility to Cry3Aa or Cry3Bb toxin. The Cry3 toxin-enhancing effect of CR8-10 was demonstrated for Colorado potato beetle *Leptinotarsa decemlineata*, southern corn rootworm *Diabrotica undecimpunctata howardi*, and western corn rootworm. The extent of Cry3 toxin enhancement, which ranged from 3- to 13-fold, may have practical applications for insect control. Cry3-containing biopesticides that include a cadherin fragment could be more efficacious. And Bt corn (i.e., corn treated with *B. thuringiensis* to make it resistant to pests) coexpressing Cry3Bb and CR8-10 could increase the functional dose level of the insect toxic activity, reducing the overall resistance risk.

The Cry3 class of *Bacillus thuringiensis* Cry proteins is known for toxicity to coleopteran larvae in the family Chrysomelidae. Cry3Aa and Cry3Bb proteins are highly toxic to Colorado potato beetle (CPB) *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae), and both were used for the development of Bt crops (crops treated with *B. thuringiensis* to make them resistant to pests) and Bt biopesticides. Due to the limited efficacy of Cry3-based biopesticides/plants and the success of competing chemical pesticides, these biopesticides have had limited usage and sales (12). Cry3Bb is toxic to corn rootworms (8, 17), and a modified version is expressed in commercialized MON863 corn hybrids (26).

Cry3 toxins have a mode of action that is similar to, yet distinct from, the action of lepidopteran-active Cry1 toxins. The Cry3A protoxin (73 kDa) lacks the large C-terminal region of the 130-kDa Cry1 protoxins, which is removed by proteases during activation to toxin. The Cry3A protoxin is activated to a 55-kDa toxin and then further cleaved within the toxin molecule (5, 18). Activated Cry3A toxin binds to brush border membrane vesicles with a K_d (dissociation constant) of ~37 nM (19) and recognizes a 144-kDa binding protein in brush border membrane vesicles prepared from the yellow mealworm *Tenebrio molitor* (Coleoptera: Tenebrionidae) (2). Recently, Ochoa-Campuzano et al. (20) identified an ADAM metalloprotease as a receptor for Cry3Aa toxin in CPB larvae.

Structural differences between Cry3Bb and Cry3Aa toxins must underlie the unique rootworm activities of Cry3Bb toxin. As noted by Galitsky et al. (11), differences in toxin solubility, oligomerization, and binding are reported for these Cry3 toxins. Recently, Cry3Aa was modified to have activity against western corn rootworm (WCRW) *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) (27). Those authors introduced a chymotrypsin/cathepsin G cleavage site into domain 1 of Cry3Aa that allowed the processing of the 65-kDa form to a 55-kDa toxin that bound rootworm midgut.

Cadherins function as receptors for Cry toxins in lepidopteran and dipteran larvae. A critical Cry1 toxin binding site is localized within the final cadherin repeat (CR), CR12, of cadherins from tobacco hornworm *Manduca sexta* (Lepidoptera: Sphingidae) and tobacco budworm *Heliothis virescens* (Lepidoptera: Noctuidae) (14, 28). Unexpectedly, a fragment of *B. thuringiensis* R_1 cadherin, the Cry1A receptor from *M. sexta*, not only bound toxin but enhanced Cry1A toxicity against lepidopteran larvae (6). If the binding residues within CR12 were removed, the resulting peptide lost the ability to bind toxin

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and lost its function as a toxin synergist. Recently, we identified a cadherin from mosquito *Anopheles gambiae* (Diptera: Culicidae) that binds Cry4Ba toxin and probably functions as a receptor. We discovered a similar effect where a fragment of a cadherin from *A. gambiae* enhanced the toxicity of the mosquitocidal toxin Cry4Ba to mosquito larvae (15). Sayed et al. (22) identified a novel cadherin-like gene in WCRW and proposed this protein as a candidate Bt toxin receptor. The cadherin-like gene is highly expressed in the midgut tissue of larval stages. The encoded protein is conserved in structure relative to that of other insect midgut cadherins.

In this study, we hypothesized that a fragment from a beetle cadherin that contains a putative Bt toxin binding region might enhance the insecticidal toxicities of Cry3Aa and Cry3Bb toxins. The region spanning CR8 to CR10 (CR8-10) of the WCRW cadherin (22) was cloned and expressed in *E. coli*. This cadherin fragment significantly enhanced the toxicities of Cry3Aa and Cry3Bb toxins to CPB and rootworms.

MATERIALS AND METHODS

Cloning and expression of CR8-10 of the WCRW cadherin-like protein. The location of CR domains in the WCRW (22) cadherin-like protein (GI:156144975) was predicted using the ISREC ProfileScan server (http://hits.isb-sib.ch/cgi-bin/PFSCAN). A DNA fragment encoding amino acids 961 to 1329 (corresponding to the CR8-10 sequence, the three most C-terminal repeats) of the WCRW cadherin-like protein was synthesized by GenScript Corp. (Piscataway, NJ). The native WCRW coding sequence was optimized for *E. coli* expression, and most of the cytosine-phosphate-guanine sequences in the native cadherin sequence were removed to decrease potential DNA methylation in planta. The synthetic CR8-10 peptide of 377 amino acid residues has an initiation methionine, a C-terminal six-histidine tag, and a molecular size of 42,814 Da (see Fig. S1 in the supplemental material). The synthetic gene was inserted between the NdeI and HindIII restriction enzyme sites of an expression vector, pET30a(+) (Novagen, Madison, WI). The coding sequence and clone orientation were confirmed by sequencing (Molecular Genetics Instrumentation Facility at the University of Georgia). The pET construct was transformed into *E. coli* strain BL21(DE3)/pRIL (Stratagene, La Jolla, CA), and positive clones were selected on LB plates containing kanamycin and chloramphenicol. The CR8-10 peptide was overexpressed in *E. coli* as inclusion bodies. The expression and purification protocol for the truncated cadherin fragment was as described in a previous paper (6). The inclusion body form was prepared as a suspension in sterile deionized water. The total protein was measured by a Bio-Rad protein assay using bovine serum albumin (BSA) as a standard (3). One microgram of the cadherin peptide was analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie brilliant blue R-250 staining. The specific concentration of the target protein, such as toxin or the cadherin peptide, in the total protein was determined from Coomassie-stained gel by gel image analyzer (Alpha Innotech, San Leandro, CA) using BSA as a standard.

Culture of *B. thuringiensis* subsp. *tenebrionis* and purification of Cry3Aa crystals. A *B. thuringiensis* subsp. *tenebrionis* strain harboring the *cry3Aa* gene (23) was grown in peptone glucose salts medium (4) until sporulation and cell lysis. The spore crystal mixture was harvested by centrifugation, suspended in 0.1 M NaCl, 2% Triton X-100, 20 mM Bis-Tris (pH 6.5), and sonicated. Spores and crystals were pelleted by centrifugation at 12,000 × *g* for 10 min and then washed twice with 0.5 M NaCl and once with distilled water. The pellet was diluted 10-fold with 4 M NaBr, and solubilized Cry3Aa was processed for recrystallization according to Slaney et al. (24). The final Cry3Aa protein concentration was determined based on band density on 15% SDS-PAGE gels using BSA as a standard. The crystals were stored in distilled water at 4°C until used for experimentation.

Cloning and expression of the *cry3Bb* gene. The Cry3Bb (8) coding region was cloned by PCR using total DNA extracted from the Bt biological insecticide Raven (Ecogen, Inc., Langhorne, PA) as a template with primers Cry3Bb/FWD (CAGGTCTAGAGTTATGTATTATGATAAGAATGGG) and Cry3Bb/REV (TAAACTCGAGTTACAATTGTACTGGGATAAATTC). The PCR-amplified *cry3Bb* gene was cloned into the pGEM-T Easy vector (Promega, Madison, WI) and then subcloned between the XbaI and XhoI sites of pET30a(+). The clone called Cry3Bb/pET was transformed into *E. coli* strain BL21-CodonPlus (DE3)/pRIL (Stratagene, La Jolla, CA). The coding sequence and clone orientation were confirmed by sequencing. The expression and purification protocol for the Cry3Bb protein inclusion bodies was as described above for the CR8-10 peptide.

Binding affinities of Cry3Aa and Cry3Bb toxins to CR8-10. To determine the binding affinities of Cry3Aa and Cry3Bb to CR8-10, a protein-protein binding assay using coated microtiter plates and enzyme-linked immunosorbent assay (ELISA) was developed from the protocol of Craig et al. (7). The CR8-10 peptide was purified from inclusion bodies on a HiTrap Ni^{2+}-chelating HP column (GE Healthcare, Piscataway, NJ) according to Chen et al. (6). The purified CR8-10 peptide was dialyzed against phosphate-buffered saline at 4°C and quantified by a method described previously (3) with BSA as a standard. The purified CR8-10 peptide (338 μg) was biotinylated using a 50-fold molar excess of sulfo-NHS (*N*-hydroxysuccinimide)–photocleavable biotin (Pierce, Rockford, IL) according to the manufacturer's instructions. The final reaction was dialyzed against 200 mM NaCl, 20 mM Na_2CO_3 (pH 8.0) at 4°C and stored in aliquots at 4°C until needed for the binding assays. The Cry3Aa and Cry3Bb toxins were prepared from crystals and *E. coli*-derived inclusion bodies, respectively, using chymotrypsin as in a previously described method (27). The microtiter plates (high-binding, 96-well Immulon 2HB plates; Thermo Fisher Scientific, Inc., Waltham, MA) were coated with 1.3 μg Cry3Aa/well or 0.5 μg Cry3Bb/well in 50 μl coating buffer (100 mM Na_2CO_3 [pH 9.6]). Toxin-coated plates were washed with wash buffer (phosphate-buffered saline plus 0.05% Tween 20), blocked with 0.5% BSA in wash buffer, and incubated for 2 h with increasing concentrations of biotinylated CR8-10 peptide (0.01 nM to 18 nM) to determine total binding. Nonspecific binding was determined by incubating the plates with increasing concentrations of biotinylated CR8-10 peptide (0.01 nM to 18 nM) with a 1,000-fold molar excess of nonlabeled CR8-10 peptide. The plates were washed, incubated with horseradish peroxidase-conjugated streptavidin (SA-HRP; Pierce), diluted 1:10,000 in wash buffer, washed, and incubated with HRP chromogenic substrate (1-Step Ultra TMB-ELISA; Thermo Fisher Scientific, Inc.) to detect bound SA-HRP. Color development was stopped by adding 3 M sulfuric acid, and absorbance was measured at 450 nm using a microplate reader (MDS Analytical Technologies, Sunnyvale, CA). Specific binding was determined by subtracting nonspecific binding from total binding. The 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.

Insect bioassays. Adult CPBs were obtained from the New Jersey Department of Agriculture (Trenton, NJ). A CPB colony was maintained on containerized potato plants in an environmental chamber at 25°C and 70% relative humidity with a photoperiod of 16 h of light and 8 h of darkness. Neonate CPB larvae were fed fresh potato leaves for 5 h before the bioassay. Suspensions (30 ml) of Cry3Aa crystals or Cry3Bb protein inclusions (referred to subsequently as crystals) or crystals plus CR8-10 inclusions were prepared in diluent (0.12% [vol/vol] Kinetic nonionic wetting agent [Helena Chemical Co., Collierville, TN] plus 0.12% [vol/vol] commercial-grade polyethylene and octyl phenol polyethoxy ethanol spreader-sticker [Southern Agricultural Insecticides, Inc., Boon, NC] in tap water). For the concentration response bioassays with CPB, the concentrations tested for the Cry3Aa crystals were 0.76, 1.53, 3.06, 6.12, 12.5, 25, 50, and 100 μg/ml; for the Cry3Bb crystals, the concentrations tested were 0.5, 1, 2, 4, 10, 20, 50, and 100 μg/ml. For the synergistic effect bioassays against CPB, CR8-10 inclusions were added to Cry3Aa crystals at 0.76, 1.53, 3.06, 6.12, and 100 μg/ml and Cry3Bb crystals at 0.25, 0.5, 1, 2, 4, and 100 μg/ml using a 1:10 (Cry3-peptide) molar ratio. The suspensions were sprayed on potato leaves, and the leaves were air-dried in a fume hood for 15 min. Five larvae were applied to treated leaves in a 1-oz. Solo plastic cup (Solo Cup Company, Highland Park, IL). Each bioassay was conducted with 50 larvae per replicate and two replicates per concentration. To determine the specificity of the synergistic effect, control bioassays were performed using inclusion body preparation of a cadherin fragment from *A. gambiae* (15) that does not bind Cry3Aa (data not shown). The *A. gambiae* cadherin fragment consisted of the transmembrane-cytoplasmic (TM-Cyto) regions (1570D to 1735F).

The eggs of southern corn rootworm (SCRW) *Diabrotica undecimpunctata howardi* (Coleoptera: Chrysomelidae) and WCRW were obtained from French Agricultural Research, Inc. (Lamberton, MN), and incubated at 25°C for 8 days and 16 days until hatching, respectively. The molten SCRW artificial diet (Bio-Serv, Frenchtown, NJ) was adjusted to pH 7.0 (according to the manufacturer) for the SCRW bioassay and to pH 9.0 for the WCRW bioassay with KOH and then aliquoted into 128-well bioassay trays (C-D International, Pitman, NJ). Cry3 crystals or crystals with CR8-10 inclusions were serially diluted with sterile deionized water and then overlaid onto the diet surface and air-dried. One newly hatched larva was transferred into each well; the trays were sealed with perforated lids (C-D International, Pitman, NJ) and then covered with brown paper to

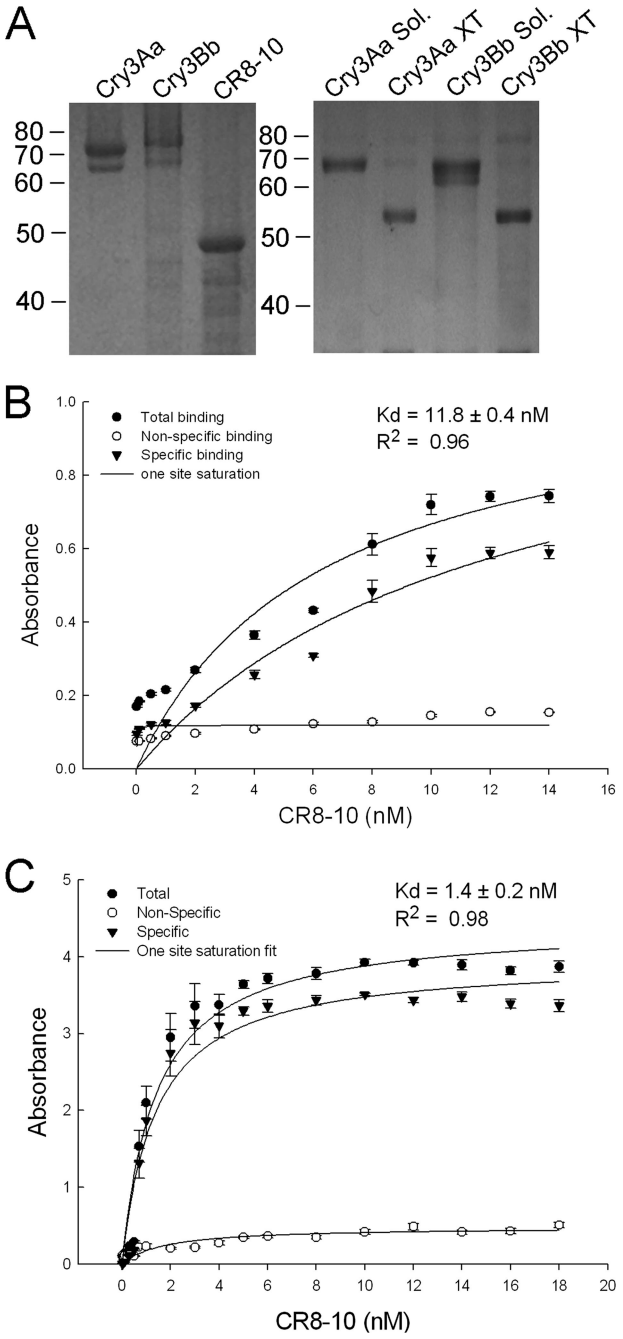

FIG. 1. SDS-PAGE of Cry3Aa, Cry3Bb, and the CR8-10 peptide (A) and the binding saturation of the biotinylated CR8-10 peptide to Cry3 toxins (B and C). (A) Right panel, lane 1, Cry3Aa (crystals); lane 2, Cry3Bb (crystal inclusions); lane 3, CR8-10 (crystal inclusions). Left panel, solubilized Cry3Aa and Cry3Bb before and after chymotrypsin treatment (designated with XT). (B and C) The binding of biotinylated CR8-10 to Cry3Aa (B) or Cry3Bb (C) protein was determined using an ELISA-based binding assay. Microtiter plates were coated with chymotrypsin-treated Cry3Aa or Cry3Bb and then incubated with increasing concentrations of biotinylated CR8-10 peptide. Bound biotinylated CR8-10 peptide was detected with an SA-HRP conjugate and substrate. Nonspecific binding was determined in the presence of 1,000-fold excess unlabeled CR8-10 peptide. Each data point is the mean of the results from two experiments done in duplicate. Error bars depict standard deviations. Binding affinities (K_d) were calculated based on specifically bound biotinylated CR8-10 peptide using a one-site saturation binding equation.

provide a dark environment. Each bioassay was conducted with 16 larvae per replicate and two replicates per concentration. The trays were incubated at 28°C for 6 days for WCRW or 7 days for SCRW before mortality counts were determined.

The optimal ratio of Cry3 to CR8-10 was determined by performing bioassays with a fixed amount of Cry3 and an increasing amount of CR8-10 (i.e., 1:0, 1:1, 1:10, 1:100 ratios of Cry3–CR8-10). From these data, we determined that a 1:10 ratio was optimal for CPB, SCRW, and WCRW; bioassays for determining 50% lethal concentrations (LC_{50}s) were then performed at a Cry3-to-CR8-10 ratio of 1:10.

Statistical analysis. Mortality values used for calculating the LC_{50}s were corrected for the background mortality using Abbott's formula (1). There was no significant difference between the rate of mortality caused by the highest concentration of CR8-10 in the tested insects and the background rate of mortality of the diluent or water control group. The LC_{50}s for each experiment were calculated by Probit analysis (9) using the EPA Probit Analysis program, version 1.5 (U.S. Environmental Protection Agency, Cincinnati, OH). The relative toxicity was determined by dividing the LC_{50} of a Cry protein alone with the LC_{50} of the Cry protein with CR8-10. The differences in the LC_{50}s were considered significant if the 95% confidence limits did not overlap. Pairwise chi-square analysis was performed to analyze the effect of the synergist in the dose-response bioassays.

Mortality data from the mass ratio experiments (see Fig. 2C and D, 3C, and 4D) were normalized using arcsine-square root (x) transformation and were analyzed using analysis of variance with the significance level set at an α value of 0.05. When significant F values were detected, the means were separated using a Fisher protected least significant difference test to compare the treatment means with the control and with each other. 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). All data are presented in the original scale.

RESULTS

In this study, the CR8-10 region of the WCRW cadherin (22) was overexpressed in *E. coli* and tested for the ability to bind Cry3Aa and Cry3Bb toxins and enhance toxicity to CPB and corn rootworm larvae. The CR8-10 inclusion bodies isolated from recombinant *E. coli* were composed of the expected 45-kDa protein, plus lesser amounts of smaller-sized peptides (Fig. 1A). Cry3Aa crystals prepared from *B. thuringiensis* subsp. *tenebrionis* and Cry3Bb inclusions isolated from recombinant *E. coli* were composed of the expected 73- and 75-kDa protoxin-sized proteins (Fig. 1A).

Chymotrypsinized Cry3Aa and Cry3Bb bind the CR8-10 peptide. The affinity of CR8-10 peptide binding to Cry3Aa and Cry3Bb toxins was analyzed by a protein-protein binding assay based on ELISA. Chymotrypsinized Cry3Aa and Cry3Bb toxins used in binding assays were 55 kDa (Fig. 1A). As shown in Fig. 1B and C, the biotin-labeled CR8-10 peptide bound specifically to Cry3Aa and Cry3Bb, respectively. Using a one-site saturation fit model, we calculated the CR8-10 peptide K_d of 11.8 ± 0.4 nM for Cry3Aa and 1.4 ± 0.2 nM for Cry3Bb (Fig. 1B and C).

The CR8-10 peptide enhances Cry3Aa and Cry3Bb toxicity to CPB larvae. Leaflets of potato were sprayed with suspensions of Cry3 crystals alone or crystals with CR8-10 inclusions, air dried, and fed to first-instar CPB larvae. The calculated LC_{50} mortality values were 3.56 (2.41 to 4.97) μg Cry3Aa/ml and 6.86 (5.54 to 8.27) μg Cry3Bb/ml (Table 1 and Fig. 2A and B). The addition of CR8-10 inclusions at a 1:10 mass ratio of Cry3–CR8-10 to the crystal suspensions significantly reduced the Cry3Aa and Cry3Bb LC_{50}s 3.7-fold and 6.4-fold, respectively (Table 1). To determine the extent that CR8-10 could enhance a low dose of Cry3 crystals, we added increasing

TABLE 1. Effect of CR8–10 peptide on Cry3Aa and Cry3Bb toxicity to CPB, SCRW, and WCRW larvae[a]

Treatment	Insect	No. of larvae	LC_{50} (95% confidence limit)	Slope ± SE	χ^2	Relative toxicity
Cry3Aa[b]	CPB	400	3.56 (2.41–4.97)	1.06 ± 0.11	1.43	
Cry3Aa + CR8–10[d]	CPB	200	0.96 (0.78–1.12)	1.66 ± 0.59	1.91	3.7
Cry3Bb[c]	CPB	350	6.86 (5.54–8.27)	1.33 ± 0.46	1.18	
Cry3Bb + CR8–10	CPB	250	1.08 (0.85–1.34)	1.55 ± 0.27	2.43	6.4
Cry3Aa	SCRW	192	167.84 (106.82–246.07)	1.38 ± 0.24	1.10	
Cry3Aa + CR8–10	SCRW	192	57.20 (42.92–72.55)	1.48 ± 0.31	1.92	2.9
Cry3Bb	SCRW	224	2.10 (0.65–4.38)	0.96 ± 0.17	1.29	
Cry3Bb + CR8–10	SCRW	224	0.26 (0.15–0.42)	1.06 ± 0.24	2.51	8.4
Cry3Bb	WCRW	192	9.49 (3.48–19.66)	0.95 ± 0.22	1.29	
Cry3Bb + CR8–10	WCRW	128	0.65 (0.23–1.24)	1.22 ± 0.22	2.30	13.1

[a] The results are LC_{50}s (with 95% confidence limits) and are expressed as micrograms of Cry protein per ml for the CPB bioassays and micrograms of Cry protein per square centimeter of diet surface for the SCRW and WCRW bioassays. The LC_{50}s for each experiment were calculated using the EPA Probit Analysis program version 1.5. SE, standard error.

[b] Cry3Aa crystals were purified from a sporulated *B. thuringiensis* subsp. *tenebrionis* culture.

[c] Cry3Bb crystal inclusions were prepared from recombinant *E. coli*.

[d] CR8-10 crystal inclusions were isolated from *E. coli* expressing the CR8-10 region of WCRW cadherin.

amounts of CR8-10 inclusions to a Cry3 crystal concentration predicted to cause about 20% larval mortality. As seen in Fig. 2C, the enhancement effect reached a plateau at a 1:10 (Cry protein–CR8-10) mass ratio. The CR8-10 peptide enhanced the low Cry3Bb dose to a greater extent than Cry3Aa. The control bioassay using the TM-Cyto cadherin fragment from *A. gambiae* showed no synergistic effect with Cry3Aa (Fig. 2D).

The CR8-10 peptide enhances Cry3Aa and Cry3Bb toxicities to SCRW larvae. The different and expected insecticidal properties of Cry3Aa and Cry3Bb were detected in our bioassays

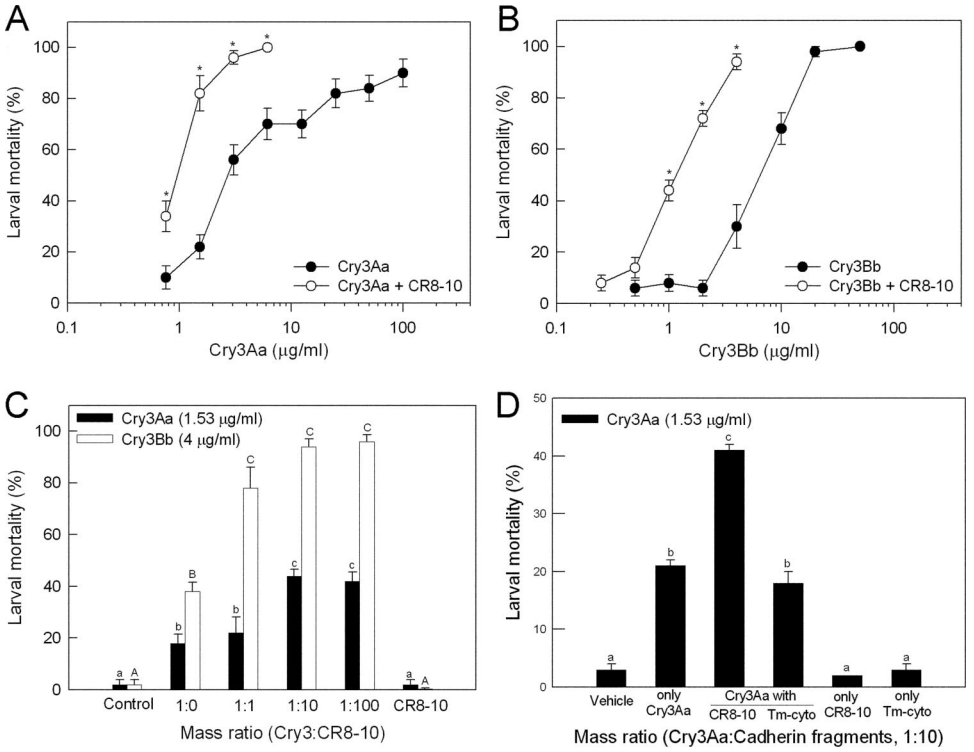

FIG. 2. The CR8-10 peptide enhances Cry3Aa and Cry3Bb toxicity to CPB larvae. Suspensions of Cry3Aa crystals (A) or Cry3Bb inclusions (B) without or with CR8-10 inclusions at a toxin/peptide mass ratio of 1:10 were sprayed onto excised potato leaves. (C) Treatments consisted of Cry3Aa or Cry3Bb alone or with various toxin/peptide mass ratios of CR8-10 peptide. Buffer and CR8-10 peptide at 400 μg/ml were not toxic to the larvae. (D) Treatments consisted of Cry3Aa alone, Cry3Aa with CR8-10 or Tm-Cyto cadherin fragment inclusions of *A. gambiae* cadherin (15), and buffer, CR8-10, or Tm-Cyto fragments alone. Each bioassay consisted of five CPB larvae per cup with 10 cups per treatment. Larval mortality was scored on day 3. Concentrations are expressed as micrograms Cry protein per ml suspension. Each data point represents the mean ± standard error of the results from a bioassay with 50 larvae per concentration. In panels A and B, an asterisk denotes a significant difference (chi-square analysis; $P < 0.05$) between larval mortality with Cry3 treatment alone and that with Cry3 plus peptide treatment at the same toxin dose. In panels C and D, lowercase letters above the error bars indicate significant differences in Cry3Aa treatments, while uppercase letters indicate significant differences in Cry3Bb treatments.

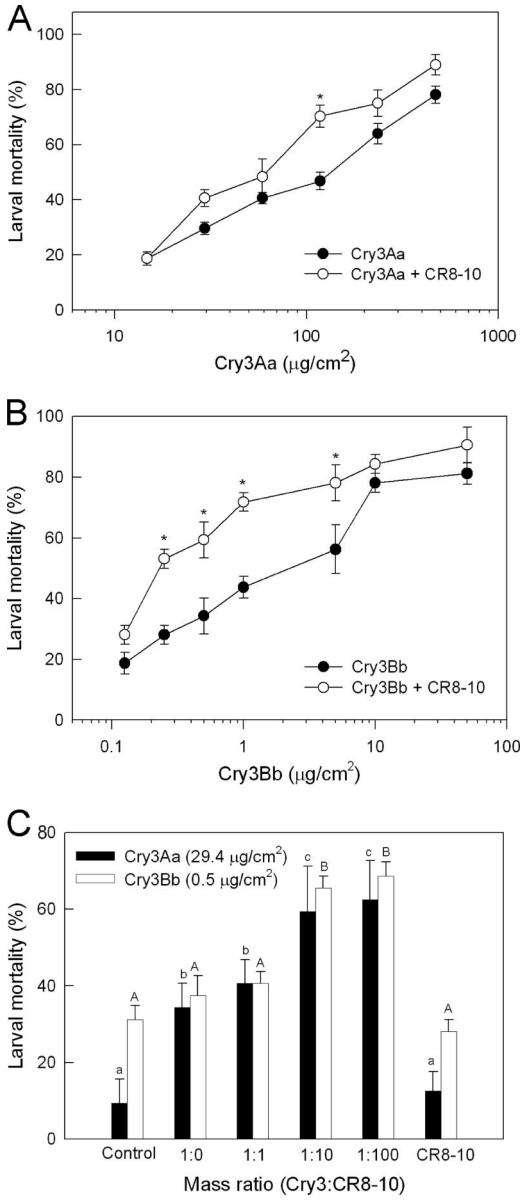

FIG. 3. Comparison of Cry3Aa and Cry3Bb toxicities to SCRW larvae without and with CR8-10 peptide. Suspensions of Cry3Aa crystals (A) or Cry3Ba inclusions (B) without or with CR8-10 inclusions at a toxin/peptide mass ratio of 1:10 were fed to first-instar SCRW larvae in a surface overlay bioassay. Panel C shows the enhancement effects of increasing ratios of CR8-10 peptide in Cry3Aa or Cry3Bb suspensions. No toxicity was observed when treated with CR8-10 peptide alone. Mortality was scored on day 7. Each data point represents the mean ± standard error of the results from a bioassay with 32 larvae per concentration. In panels A and B, an asterisk denotes a significant difference (chi-square analysis; $P < 0.05$) between larval mortality with Cry3 treatment and that with Cry3 plus peptide treatment at the same toxin dose. In panel C, different letters above the error bars indicate significant differences between means. Lowercase letters compare Cry3Aa treatments, while uppercase letters compare Cry3Bb treatments.

against SCRW. Whereas Cry3Bb is toxic to SCRW, Cry3Aa has extremely low toxicity to this pest (Fig. 3A and Table 1) (8, 24). The ability of the CR8-10 peptide to enhance Cry3Aa and Cry3Bb toxicity to SCRW was tested using a 1:10 (Cry3–CR8-10) mass ratio. The addition of the CR8-10 peptide to Cry3Aa and Cry3Bb significantly reduced the LC_{50}s about 2.9- and 8.4-fold, respectively (Table 1). As seen in Fig. 3A, the highest dose of Cry3Aa and Cry3Aa plus the CR8-10 peptide killed less than 100% of the larvae. Cry3Bb was about 80-fold more toxic than Cry3Aa to SCRW larvae in diet overlay bioassays (Table 1 and Fig. 3A and B). The addition of CR8-10 inclusions increased Cry3Aa and Cry3Bb toxicity to SCRW larvae (Table 1) and the mass ratio 1:10 (Cry3–CR8-10) was optimal for toxicity enhancement (Fig. 3C).

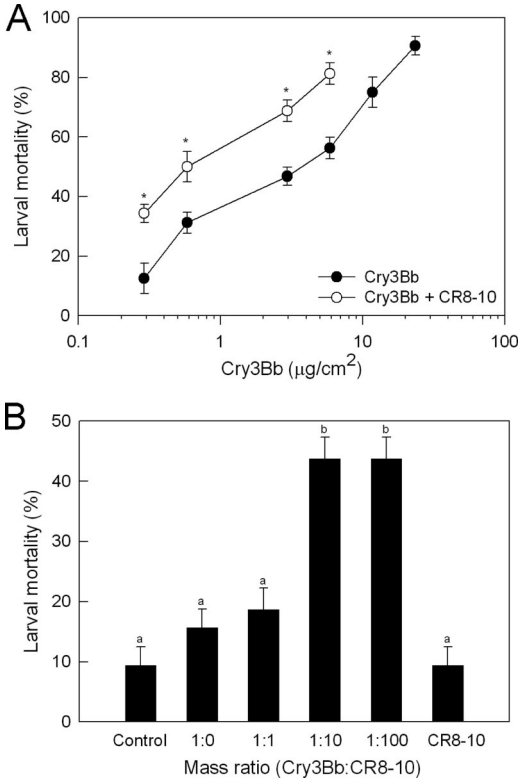

FIG. 4. CR8-10 peptide enhancement of Cry3Bb toxicity to WCRW larvae. Larvae were exposed to diet treated with Cry3Bb crystals alone or Cry3Bb plus CR8-10 inclusions at fixed 1:10 (A) or various toxin/peptide ratios (B). In panel B, the Cry3Bb concentration was 1.0 μg/cm^2. Mortality was scored on day 6. Each data point represents the mean ± standard error of the results from a bioassay with 32 larvae per concentration. In panel A, an asterisk denotes a significant difference (chi-square analysis; $P < 0.05$) between larval mortality with Cry3 treatment and that with Cry3 plus peptide treatment at the same toxin dose. In panel B, different letters above the error bars indicate significant differences between means.

The CR8-10 peptide enhances Cry3Bb toxicity to WCRW larvae. Since Cry3Bb, but not Cry3Aa, is active against WCRW, we tested the ability of the CR8-10 peptide to enhance Cry3Bb toxicity. As seen in Fig. 4A, the CR8-10 peptide significantly enhanced Cry3Bb toxicity at each toxin concentration tested. The LC_{50} for Cry3Bb was 9.49 (3.48 to 19.66) μg/cm^2 and the LC_{50} for Cry3Bb plus a 1:10 toxin-to-cadherin-fragment ratio was 0.65 (0.23 to 1.24) μg/cm^2, representing a 13.1-fold significant enhancement by the CR8-10 peptide (Table 1 and Fig. 4A). The maximal enhancement of Cry3Bb toxicity to WCRW larvae occurred with a 1:10 Cry3Bb–CR8-10 mass ratio (Fig. 4B).

DISCUSSION

Based on our reports that toxin-binding fragments of cadherins enhance Cry toxicity to target insects (6, 15), we tested the possibility that a homologous cadherin fragment from a beetle would enhance Cry3Aa and Cry3Bb toxicity to coleopteran larvae in the family Chrysomelidae.

The WCRW midgut cadherin (22) was used as a template to design a cadherin fragment that includes a potential toxin binding site. The designed CR8-10 peptide includes the predicted binding site ^{1311}SSLNVTVN^{1318} (22), which has similarity to Cry1A toxin binding region 2 (TBR 2) of *M. sexta* cadherin (13). The WCRW cadherin and the CR8-10 cadherin peptide do not contain an obvious match to TBR 3 (GVLTL NIQ, residues 1416 to 1423) of *M. sexta* cadherin (6). We show that the CR8-10 cadherin fragment binds Cry3Aa (K_d = 11.8 nM) and Cry3Bb (K_d = 1.4 nM) with nM affinity (Fig. 1B and C), a value comparable to the high-affinity (K_d = 9.17 nM) Cry1Ab binding site in the CR12–membrane-proximal extracellular domain *M. sexta* cadherin peptide (6). Since the high-affinity TBR 3 site of *M. sexta* cadherin is necessary for toxin binding and toxicity enhancement, it is quite possible that the high-affinity-binding site in the CR8-10 from WCRW is also a critical component of toxicity enhancement. The high-affinity interaction between Cry3Bb and the CR8-10 and the toxicity enhancement properties of the CR8-10 are at least suggestive that this rootworm cadherin may serve as a Cry3Bb receptor in vivo.

The CR8-10 cadherin fragment from WCRW increased the potency of Cry3Aa and Cry3Bb proteins between 2.9- and 13.1-fold against CPB and two rootworm species. In general, this level of potentiation is comparable to the 2.7-fold enhancement of Cry1Ab toxicity to *M. sexta* by a cadherin fragment (6). In Chen et al. (6), the cadherin fragment consisting of the terminal CR and membrane-proximal extracellular domain also bound toxin. Soberon et al. (25) provide an explanation for how cadherin fragments are involved in Cry1Ab toxicity. The CR12 fragment induces the formation of a prepore Cry1Ab oligomer, a critical step in the intoxication process (16), and the enhancement of Cry1A toxicity by the CR12 fragment is correlated with oligomer formation (21). We do not know if the Cry3A toxins function in a similar manner, but our data suggest this possibility.

The practical importance of a 3- to 13-fold level of synergy on the field performance of biopesticides is difficult to predict. In the case of susceptible CPB larvae and "high-dose" Cry3Aa expression in transgenic Bt Newleaf potato, a threefold enhancement of Cry3Aa toxicity may not significantly impact control. In contrast, where weekly sprays of a microbial biopesticide are required due to short field half-life and tolerance in older larvae (29), a threefold enhancement of Cry3Aa toxicity may lead to more efficacious control of CPB.

Diabrotica species are major pests of corn in the United States, significantly impacting corn grown for food and ethanol production, and adult SCRW is also an important insect pest of cucurbits. The LC_{50} (2.10 $\mu g/cm^2$) we determined for the Cry3Bb inclusions with SCRW larvae is less than the 15.9 $\mu g/cm^2$ previously reported (17). This could be due to differences in bioassay conditions, such as the Cry3Bb preparation, composition of the artificial diet, or insect strain. The Cry3Bb LC_{50} (9.49 $\mu g/cm^2$) against WCRW is within the 0.7- to 13.0-$\mu g/cm^2$ range of values calculated for laboratory and field populations of WCRW (10). The MON863 cultivar of Bt maize (also called YieldGard Rootworm) expresses a modified Cry3Bb that has eightfold increased toxicity relative to that of Cry3Bb, yet some WCRW and SCRW survive on MON863 corn. Resistance management is a critical component for Bt crops, and lower-dose products, such as MON863 corn, may have an increased resistance risk. While we were unable to test the modified Cry3Bb expressed in MON863, we see no reason why CR8-10 should not enhance this modified toxin. Once the synergistic activity is confirmed, the coexpression of CR8-10 and modified Cry3Bb in planta could reduce the resistance risk. Although factors such as feeding behavior and environmental conditions can impact rootworm survival, a peptide synergist that increases Cry3Bb potency could lead to improved rootworm control.

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