APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2001, p. 323–329 Vol. 67, No. 1
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.1.323–329.2001

Importance of Cry1 δ-Endotoxin Domain II Loops for Binding Specificity in *Heliothis virescens* (L.)

JUAN LUIS JURAT-FUENTES[1] AND MICHAEL J. ADANG[1,2]*

Department of Entomology[1] and Department of Biochemistry and Molecular Biology,[2] University of Georgia, Athens, Georgia 30602-2603

Received 13 April 2000/Accepted 2 November 2000

We constructed a model for *Bacillus thuringiensis* Cry1 toxin binding to midgut membrane vesicles from *Heliothis virescens*. Brush border membrane vesicle binding assays were performed with five Cry1 toxins that share homologies in domain II loops. Cry1Ab, Cry1Ac, Cry1Ja, and Cry1Fa competed with ^{125}I-Cry1Aa, evidence that each toxin binds to the Cry1Aa binding site in *H. virescens*. Cry1Ac competed with high affinity (competition constant [K_{com}] = 1.1 nM) for ^{125}I-Cry1Ab binding sites. Cry1Aa, Cry1Fa, and Cry1Ja also competed for ^{125}I-Cry1Ab binding sites, though the K_{com} values ranged from 179 to 304 nM. Cry1Ab competed for ^{125}I-Cry1Ac binding sites (K_{com} = 73.6 nM) with higher affinity than Cry1Aa, Cry1Fa, or Cry1Ja. Neither Cry1Ea nor Cry2Aa competed with any of the ^{125}I-Cry1A toxins. Ligand blots prepared from membrane vesicles were probed with Cry1 toxins to expand the model of Cry1 receptors in *H. virescens*. Three Cry1A toxins, Cry1Fa, and Cry1Ja recognized 170- and 110-kDa proteins that are probably aminopeptidases. Cry1Ab and Cry1Ac, and to some extent Cry1Fa, also recognized a 130-kDa molecule. Our vesicle binding and ligand blotting results support a determinant role for domain II loops in Cry toxin specificity for *H. virescens*. The shared binding properties for these Cry1 toxins correlate with observed cross-resistance in *H. virescens*.

Bacillus thuringiensis produces parasporal crystals composed of Cry proteins during the sporulation phase of growth (1, 45). Most Cry proteins are toxic to insects, and Cry1 proteins are specifically toxic to larvae of Lepidoptera. Cry1 crystals are solubilized in the midgut of larvae, releasing 130- to 140-kDa protoxins that are subsequently processed to 55- to 60-kDa active toxins by midgut proteinases. Passing through the peritrophic membrane, Cry toxins bind to specific receptors on the brush border membrane of the midgut cells (53, 54) in a reversible manner. An irreversible binding phase, attributed to toxin insertion into the midgut membrane, takes place (29), followed by toxin oligomerization (3) and pore formation. The osmotic shock resulting from toxin-induced pores leads to cell lysis, gut paralysis, and insect death (24).

Cry toxins consist of three structural domains (23, 28) that are associated with different steps of the toxin mode of action. Domain I, composed of α-helices, is involved in pore formation (28, 46). Domain II, composed mainly of β-sheets, contains the primary determinants that specify binding to receptors on the midgut brush border (5, 10, 42, 43). Domain III (also composed of β-sheets) has been implicated in toxin stability and binding specificty in some insects (7, 11).

Since the commercial introduction of *B. thuringiensis* corn, cotton, and potatoes in 1996, *B. thuringiensis* toxins have become one of the most important tools for pest insect control. However, the development of resistance by insects challenges their future efficacy. Insects are capable of developing high levels of resistance to *B. thuringiensis* toxins after laboratory (20, 21, 40, 56) or field (48, 49) selection. Resistant insects are often cross resistant to *B. thuringiensis* toxins that were not in the environment of selection (20, 21, 50, 51), suggesting that the mechanism(s) of acquired resistance can be effective against toxins that have never been used against that insect. Although several mechanisms of resistance have been proposed (16, 41), the best-documented mechanism is the alteration of binding to the specific receptors in the midgut (15, 26, 55).

Toxins that share high homology in the loops of domain II (50) often share midgut binding sites (4, 14) and display cross-resistance. For example, Cry1Fa and Cry1A toxins have a common high-affinity binding site on brush border membrane vesicles (BBMV) prepared from *Plutella xylostella* (22). Cry1Ac-resistant *P. xylostella* show greatly reduced Cry1Ac binding and cross-resistance to Cry1Fa. Though direct binding studies of Cry1Fa are not reported, it appears that when *P. xylostella* adapted to Cry1Ac toxin the modification that reduced Cry1Ac binding also reduced Cry1Fa toxicity. Cry1Ja, another toxin that shares high sequence homology in the loops of domain II with Cry1A toxins, presents a similar pattern of cross-resistance in *P. xylostella* (51).

Heliothis virescens, the subject of this study, is an important pest of cotton in the United States and the major target of transgenic cotton expressing the *B. thuringiensis cry1Ac* gene. *H. virescens* selected for Cry1Ac resistance in the laboratory is cross resistant to Cry1Aa, Cry1Ab, and Cry1Fa toxins (21). In *H. virescens*, a model of three populations of receptor molecules for Cry1A toxins is generally accepted (17, 30, 37, 53). According to this model, receptor A (previously identified as a 170-kDa aminopeptidase N [APN]) binds Cry1Aa, Cry1Ab, and Cry1Ac. Receptor B binds Cry1Ab and Cry1Ac, and receptor C only binds Cry1Ac. Alteration of receptor A is implicated as a mechanism for *H. virescens* resistance to Cry1A toxins (26). The 170-kDa APN elicits binding and pore formation by all three Cry1A toxins (30). Other authors (26, 39) have

* Corresponding author. Mailing address: Department of Entomology, University of Georgia, Biosciences Building, 125 Cedar St., Athens, GA 30602-2603. Phone: (706) 542-2436. Fax: (706) 542-2640. E-mail: adang@arches.uga.edu.

suggested that a 130-kDa aminopeptidase may also function as receptor A. The 170-kDa APN in *H. virescens* was found to be a low-affinity binding site for Cry1Ac, while the 130-kDa aminopeptidase showed high affinity (32.1 nM) for this toxin. The 170- and 130-kDa APNs may be the product of differential posttranslational glycosylation of the same protein precursor (39).

The objective of this study was to determine if toxins that share homology in the loops of domain II share binding sites on BBMV from *H. virescens*. Four of the toxins (Cry1Aa, Cry1Ab, Cry1Ac, and Cry1Fa) are highly active against *H. virescens*, while Cry1Ja has low toxicity. We then used ligand blotting to determine the molecular sizes of toxin binding proteins for each Cry1 toxin. By integrating data from vesicle binding experiments, ligand blotting, and published results, we further developed the model of Cry1A toxin binding in *H. virescens* to include Cry1Fa and Cry1Ja toxins.

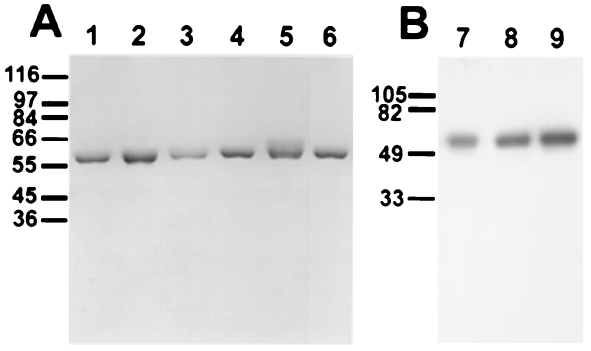

FIG. 1. SDS-PAGE (A) and autoradiography (B) analyses of purified and ^{125}I-labeled Cry1 toxins. Lane 1, Cry1Aa; lane 2, Cry1Ab; lane 3, Cry1Ac; lane 4, Cry1Fa; lane 5, Cry1Ea; lane 6, Cry1Ja; lane 7, ^{125}I-Cry1Aa; lane 8, ^{125}I-Cry1Ab; lane 9, ^{125}I-Cry1Ac. Molecular mass markers (in kilodaltons) are shown on the left.

MATERIALS AND METHODS

Insect bioassays. The *H. virescens* strain used in this work was started from insects collected in Alabama by J. Graves (Louisiana State University). The colony has been maintained on an artificial diet (Southland Products, Lake Village, Ark.) in the laboratory for 23 generations.

For insect bioassays, at least five dilutions of each toxin were prepared in 20 mM Na_2CO_3 (pH 9.6), and 50-μl aliquots were applied uniformly over the surface of artificial diet in 2-cm^2 wells (EC-International). Each toxin dilution was assayed with at least 12 neonate larvae, and the bioassay was repeated three times. Mortality was scored after 7 days. The 50% lethal concentration (LC_{50}) values and the slopes of concentration-mortality regression lines were obtained using the Polo-PC program (44).

Bacterial strains and toxin purification. *B. thuringiensis* HD-37 and HD-73 producing Cry1Aa and Cry1Ac, respectively, were obtained from the *Bacillus* Genetic Stock Center (Columbus, Ohio). *B. thuringiensis* strains producing Cry1Fa and Cry1Ja were obtained from Ecogen Inc. (Langhorne, Pa.). *B. thuringiensis* strain MR522 producing Cry1Ea was obtained from Dow Agrosciences (San Diego, Calif.). An *Escherichia coli* strain carrying the *B. thuringiensis* NRD-12 *cry1Ab* toxin gene was kindly provided by Luke Masson (National Research Council of Canada, Montreal).

Toxins were prepared and purified from *B. thuringiensis*, as described elsewhere (31). Cry1Ab inclusions were prepared from *E. coli* NRD-12, and toxin was purified as previously described (34).

Fractions containing pure toxin (as determined by gel electrophoresis) were pooled, quantified by the Bradford protein assay (6) using bovine serum albumin (BSA) as a standard, and stored at −80°C until used.

Gel electrophoresis. Purified Cry1 toxin samples were analyzed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, 10 μg of toxin (for gels stained with Coomassie brilliant blue R-250) or 10^5 cpm (for ^{125}I-Cry1A toxins) per lane was used in SDS-PAGE. Gels were stained with Coomassie brilliant blue R-250 or exposed to Kodak XAR-5 film with an intensifying screen at −80°C for 1 h.

Iodination and biotinylation of Cry1A toxins. Labeling of purified Cry1A toxins with Na^{125}I was done using the Chloramine T method (17). Toxin (1 μg) was labeled with 0.5 mCi of Na^{125}I (Amersham-Pharmacia). Cry1A toxicity to insect larvae is not reduced by iodination using the Chloramine T method (53). The specific activities were 6.2 μCi/μg for Cry1Aa, 10.5 μCi/μg for Cry1Ab, and 28.4 μCi/μg for Cry1Ac (based on input toxin).

For toxin biotinylation, 0.5 mg of purified toxin was incubated (1:30 molar ratio) with EZ-Link Sulfo-NHS-LC-Biotin (Pierce) for 30 min at room temperature. To remove excess biotin, samples were dialyzed overnight in 20 mM Na_2CO_3–200 mM NaCl (pH 9.6). Biotinylated toxins were quantified as described above and stored at −80°C until used.

Midgut isolation and BBMV preparation. Midguts were dissected from fifth instar *H. virescens* larvae, washed in ice-cold SET buffer (250 mM sucrose, 17 mM Tris [pH 7.5], 5 mM EGTA), and kept at −80°C until used.

BBMV were prepared by the differential magnesium precipitation method (57), as modified by Carroll and Ellar (8). The final BBMV pellet was suspended in ice-cold TBS (25 mM Tris [pH 7.5], 2 mM KCl, 135 mM NaCl), and the protein concentration was quantified by the method of Bradford (6), as described above. BBMV were frozen in dry ice and kept at −80°C until used. After thawing, BBMV were centrifuged for 10 min at 13,500 × *g* and resuspended in binding buffer for binding assays (see below).

Aminopeptidase-specific activity was used as an enzymatic marker of enrichment for brush border membranes (52), using leucine-ρ-nitroanilide as the substrate. Typical activity enrichment in the BBMV preparations was five to seven times the activity measured in the initial midgut homogenates.

Binding of ^{125}I-Cry1A to BBMV. For qualitative binding experiments, increasing amounts of BBMV were incubated with either 0.5 nM (^{125}I-Cry1Aa) or 0.1 nM (^{125}I-Cry1Ab and ^{125}I-Cry1Ac) labeled toxin in 0.1 ml (final volume) of binding buffer (25 mM Tris [pH 7.5], 3 mM KCl, 135 mM NaCl, 0.1% BSA) for 1 h at room temperature. After incubation, samples were centrifuged at 13,500 × *g* for 10 min, and the pellets were washed twice with 0.5 ml of cold binding buffer. Nonspecific binding was determined by adding a 1,000 nM concentration of the respective unlabeled toxin to the reaction mixtures. Radioactivity was measured in a Beckman Gamma 4000 detector.

Homologous and heterologous competition experiments were done by incubating 40 μg (for Cry1Aa), 10 μg (for Cry1Ab), or 5 μg (for Cry1Ac) of BBMV with 0.5 nM (^{125}I-Cry1Aa) or 0.1 nM (^{125}I-Cry1Ab and ^{125}I-Cry1Ac) labeled toxin for an hour at room temperature. Increasing amounts of unlabeled homologous or heterologous competitor were used to compete binding. Competition reactions were stopped by centrifugation at 13,500 × *g* for 10 min, and the pellets were washed as described before. Data were analyzed using the LIGAND program (35) to obtain a representative value of the binding affinity constant and the concentration of receptors for all toxins. The competition constant (K_{com}) is used as the binding constant instead of K_d, due to the two-step binding process (reversible plus irreversible) taking place (7, 58).

Ligand blotting. BBMV proteins (15 μg) were separated by SDS–8% PAGE and transferred to a polyvinylidene difluoride Q membrane filter (Millipore). The filter was blocked for 1 h in 3% BSA in TBST (25 mM Tris [pH 7.5], 3 mM KCl, 135 mM NaCl, 0.1% Tween 20) and then cut into strips for the different treatments after washing. Subsequent incubations and washes were done in 0.1% BSA–TBST.

Ligand blotting with radiolabeled toxins was done by incubating the filters with 10^6 cpm of ^{125}I-labeled toxin in 10 ml of 0.1% BSA–TBST for 3 h. After this, membranes were washed before exposure to film overnight at −80°C.

For biotinylated toxin ligand blots, the blocked filters were incubated with biotin-toxin (1.6 nM) for 1 h. After washing, filters were incubated with anti-biotin (Sigma) antibody (1:50,000 dilution) for an hour. Binding proteins were visualized using the ECL kit (Amersham-Pharmacia) following the manufacturer's instructions.

For the ligand blots with antibodies against the toxins, blocked filters were incubated with toxin (5 nM) for 1 h. After washing, filters were incubated with rabbit polyclonal antibodies against Cry1Ac or Cry1Fa (1:5,000 dilution) for an hour. After washing, filters were incubated with donkey anti-rabbit peroxidase-conjugated antibody (1:30,000 dilution) (Amersham-Pharmacia) for another hour. Binding proteins were visualized with an ECL kit, as described for biotinylated toxins.

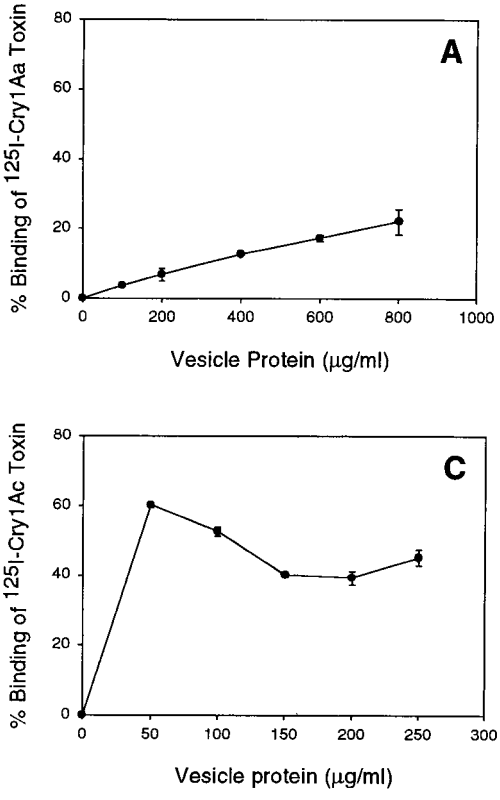

FIG. 2. Specific binding of ^{125}I-Cry1Aa (A), ^{125}I-Cry1Ab (B), and ^{125}I-Cry1Ac (C) toxins to *H. virescens* BBMV. Vesicles at the concentrations indicated were incubated with ^{125}I-Cry1A toxins. Binding is expressed as a percentage of input ^{125}I-Cry1A. Binding in the presence of 1,000 nM unlabeled homologous toxin was subtracted from total binding. Maximum nonspecific binding was 20% of total binding for ^{125}I-Cry1Aa and 40% for both ^{125}I-Cry1Ab and ^{125}I-Cry1Ac at the highest vesicle concentrations tested. Each data point is the average of the means based on independent trials with duplicate samples. Standard deviations of the mean values are depicted by error bars.

RESULTS

Toxicity of Cry1 toxins to *H. virescens*. As shown in Fig. 1, each purified toxin appeared as a single band on SDS-PAGE after staining or autoradiography for ^{125}I-labeled Cry1A toxins.

Table 1 shows the results of bioassays conducted with *H. virescens*. As previously reported, Cry1Aa, Cry1Ab, Cry1Ac, and Cry1Fa, but not Cry1Ea and Cry1Ja, are highly toxic to *H. virescens* (18, 26, 37, 53, 54). Cry1Aa was more toxic for this study than for previous reports (53, 54). Bioassays with an independent Cry1Aa preparation (kindly provided by L. Potvin, National Research Council of Canada, Montreal) gave the same toxicity results. Peptide mapping analyses confirmed the identity and purity of our Cry1Aa preparation (data not shown).

As the activities of biotinylated Cry1Fa and Cry1Ja toxins are unknown, we performed bioassays with these modified toxins. Activities of these biotinylated toxins against *H. virescens* were the same as those of unmodified Cry1Fa and Cry1Ja. The toxicity of Cry1Ab was previously reported to be unchanged by biotinylation (12).

Binding of ^{125}I-Cry1A toxins to BBMV. Total binding experiments done with ^{125}I-labeled toxins and various concentrations of BBMV provided the basis for selecting BBMV concentrations appropriate for subsequent competition experiments. The levels of ^{125}I-Cry1Aa, ^{125}I-Cry1Ab, and ^{125}I-Cry1Ac binding shown in Fig. 2 were similar to those found in previous studies (26, 53). The amounts of BBMV needed to reach maximum binding were different for the three toxins, and they correlated directly with their in vivo activities against *H. virescens*. Thus, the most toxic toxin, Cry1Ac, reached maximum specific binding at 50 μg of BBMV per ml, while Cry1Aa (the least toxic of the Cry1A toxins tested) maximum binding increased, up to 1 mg/ml. These binding data identified the lowest BBMV concentration that gave maximal specific binding. According to the binding observed, 400 μg (for Cry1Aa), 100 μg (for Cry1Ab), and 50 μg (for Cry1Ac) per ml were selected as the BBMV concentrations for binding competition experiments.

Competitive binding with ^{125}I-Cry1A toxins. Using unlabeled Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, and Cry1Ja as competitors, we performed heterologous binding competition experiments. For negative controls we also tested Cry2Aa and Cry1Ea toxins, and neither toxin competed with labeled Cry1A toxins (Cry2Aa data not shown).

When using ^{125}I-Cry1Aa, high-affinity competitive binding was observed with Cry1Aa, Cry1Ab, and Cry1Ac (Fig. 3). These toxins competed up to 90% of ^{125}I-Cry1Aa binding at low concentrations. Cry1Fa and Cry1Ja both competed with ^{125}I-Cry1Aa, although the levels of affinity were lower, as reflected in the K_{com}s (Table 2). Cry1Ja and Cry1Fa competed 90% of the ^{125}I-Cry1Aa binding, although 10 times more toxin was

TABLE 1. Toxicity of purified Cry1 toxins to neonate larvae of *H. virescens*

Toxin	LC_{50} (95% fiducial limits)[a]	Slope ± SE
Cry1Aa	5.0 (2.9–7.4)	1.8 ± 0.3
Cry1Ab	1.5 (0.8–2.3)	1.7 ± 0.2
Cry1Ac	0.5 (0.1–1.1)	1.6 ± 0.2
Cry1Fa	1.9 (1.2–2.5)	3.2 ± 0.7
Cry1Ja	660 (464.3–921.4)	3.2 ± 0.9
Cry1Ea	>9,000	2.8 ± 0.6
Cry1Fa-biotin	1.9 (0.5–5.1)	1.4 ± 0.2
Cry1Ja-biotin	793 (246–5,989)	3.2 ± 1.2

[a] LC_{50} is expressed in nanograms of protein per square centimeter of artificial diet.

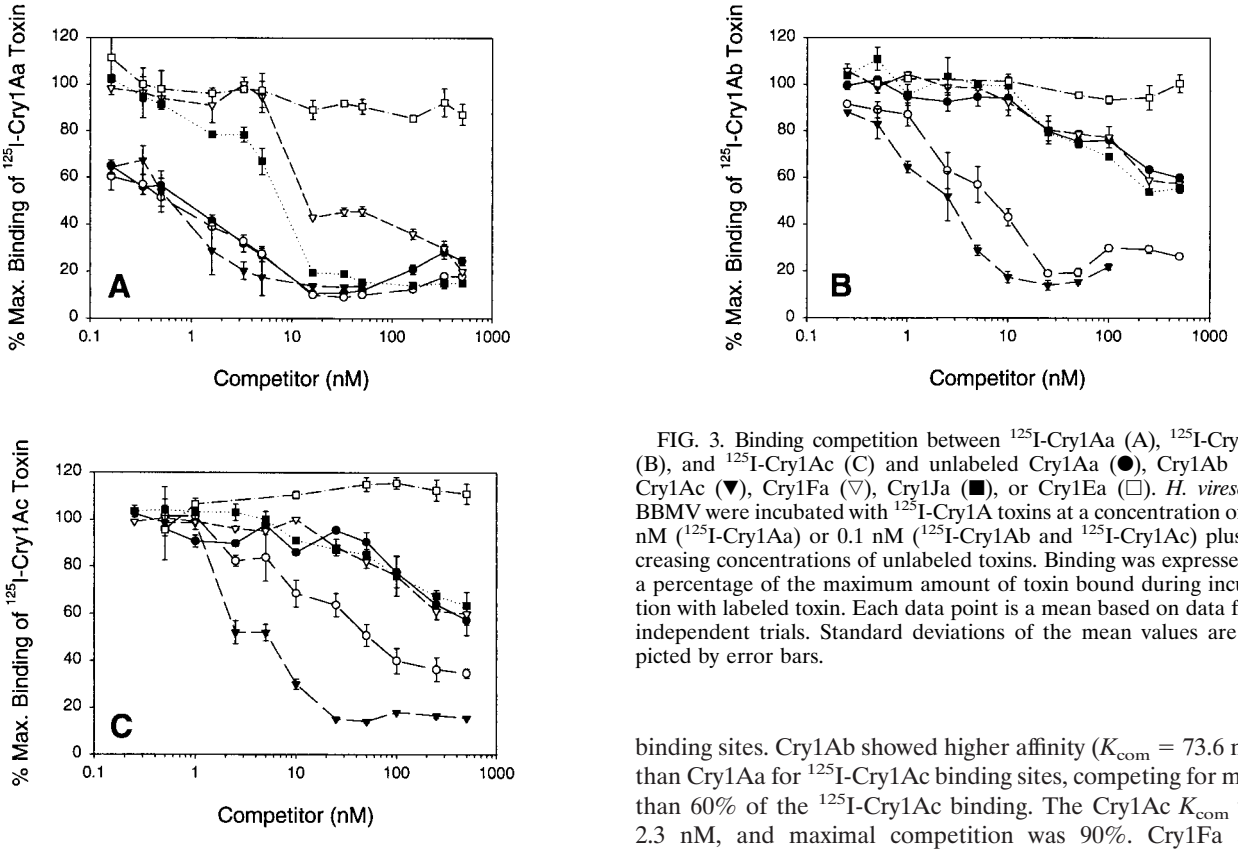

FIG. 3. Binding competition between ^{125}I-Cry1Aa (A), ^{125}I-Cry1Ab (B), and ^{125}I-Cry1Ac (C) and unlabeled Cry1Aa (●), Cry1Ab (○), Cry1Ac (▼), Cry1Fa (▽), Cry1Ja (■), or Cry1Ea (□). *H. virescens* BBMV were incubated with ^{125}I-Cry1A toxins at a concentration of 0.5 nM (^{125}I-Cry1Aa) or 0.1 nM (^{125}I-Cry1Ab and ^{125}I-Cry1Ac) plus increasing concentrations of unlabeled toxins. Binding was expressed as a percentage of the maximum amount of toxin bound during incubation with labeled toxin. Each data point is a mean based on data from independent trials. Standard deviations of the mean values are depicted by error bars.

required to reach the 90% level of competition in the case of Cry1Fa. The fact that these unlabeled toxins competed up to 90% of the ^{125}I-Cry1Aa binding is evidence that these toxins all recognize the single population of Cry1Aa binding sites present in the BBMV.

In competition experiments, only the heterologous Cry1Ac and the homologous Cry1Ab competed with high affinity for ^{125}I-Cry1Ab binding sites. While Cry1Aa, Cry1Fa, and Cry1Ja reduced the amount of ^{125}I-Cry1Ab bound, relatively higher concentrations of competitor toxins were needed, and lower total competition was observed. These results support a two-binding-site model, whereby Cry1Aa, Cry1Fa, and Cry1Ja have low affinity and Cry1Ac has high affinity for one Cry1Ab site. Only the homologous toxin and Cry1Ac recognize this Cry1Ab binding site.

In heterologous competition assays Cry1Aa competed with a low affinity (K_{com} = 292.7 nM) for 40% of the ^{125}I-Cry1Ac binding sites. Cry1Ab showed higher affinity (K_{com} = 73.6 nM) than Cry1Aa for ^{125}I-Cry1Ac binding sites, competing for more than 60% of the ^{125}I-Cry1Ac binding. The Cry1Ac K_{com} was 2.3 nM, and maximal competition was 90%. Cry1Fa and Cry1Ja competed with low affinity for a Cry1Ac binding site. These binding results are evidence for a population of receptors recognized exclusively by Cry1Ac, a second population recognized by Cry1Ac and Cry1Ab, and a third population recognized by Cry1Ac, Cry1Aa, Cry1Ab, Cry1Fa, and Cry1Ja.

Ligand blot analyses. Ligand blotting with BBMV was performed to relate the *H. virescens* Cry1 receptor model constructed from BBMV binding studies to Cry1 toxin binding proteins. Ligand blotting with ^{125}I-labeled Cry toxins has inherent challenges due to differences in toxin labeling efficiencies and, as with Cry1Fa (31), loss of toxicity attributed to modification of critical amino acids. We employed ^{125}I-labeled toxins, biotinylated toxins, and anti-Cry protein antibodies to ensure that toxin binding proteins visualized on blots were a consequence of toxin recognition and not the labeling technique.

Binding molecules detected with ^{125}I-Cry1A toxins are shown in Fig. 4 (lanes 2 to 4). Each ^{125}I-Cry1A toxin bound to 170- and 110-kDa proteins. Apart from these common mole-

TABLE 2. K_{com}s and concentrations of binding sites (R_ts) of Cry1 toxins on BBMV from *H. virescens*

Toxin	^{125}I-Cry1Aa		^{125}I-Cry1Ab		^{125}I-Cry1Ac	
	K_{com} (nM) ± SE	R_t (pmol/mg of protein) ± SE	K_{com} (nM) ± SE	R_t (pmol/mg of protein) ± SE	K_{com} (nM) ± SE	R_t (pmol/mg of protein) ± SE
Cry1Aa	1.1 ± 0.3	0.7 ± 0.0	304.4 ± 100.0	69.5 ± 13.0	292.7 ± 100.0	142.0 ± 15.6
Cry1Ab	1.0 ± 0.9	0.5 ± 0.1	3.5 ± 1.0	1.1 ± 0.1	73.6 ± 44.0	16.6 ± 3.7
Cry1Ac	1.5 ± 2.2	0.4 ± 0.1	1.1 ± 0.1	0.3 ± 0.0	2.3 ± 2.0	0.9 ± 0.1
Cry1Fa	12.6 ± 0.4	5.0 ± 0.6	284.5 ± 100.0	64.2 ± 11.4	233.0 ± 60.0	97.2 ± 10.2
Cry1Ja	2.4 ± 1.1	1.3 ± 0.1	179.0 ± 100.0	6.5 ± 2.1	326.0 ± 138.0	150.0 ± 98.5

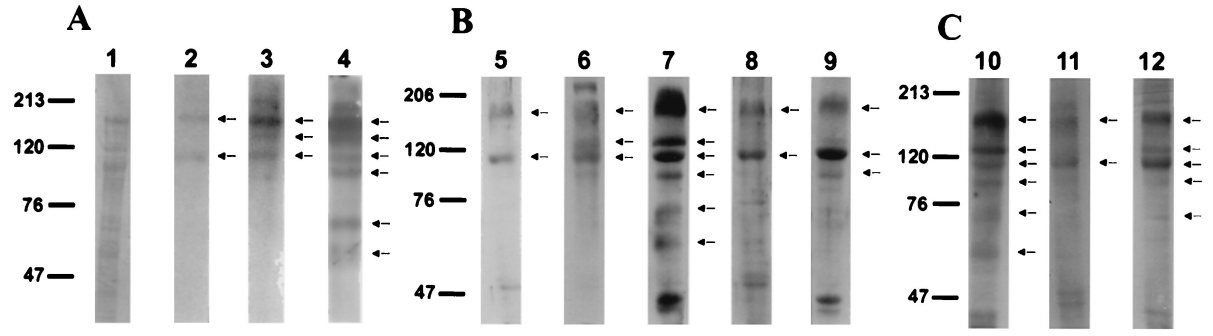

FIG. 4. Ligand blot analyses of SDS-PAGE-separated *H. virescens* BBMV proteins. (A) Lane 1, BBMV proteins transferred to a polyvinylidene difluoride filter detected by Coomassie blue staining; lanes 2 to 4, autoradiography of blots incubated with ^{125}I-Cry1Aa, ^{125}I-Cry1Ab, and ^{125}I-Cry1Ac, respectively. (B) Blots were incubated with biotinylated toxins and then antibiotin antibody-peroxidase conjugate, and detection was enhanced by chemiluminescence. Lane 5, Cry1Aa; lane 6, Cry1Ab; lane 7, Cry1Ac; lane 8, Cry1Fa; lane 9, Cry1Ja. (C) Blots were incubated with 5 nM Cry1Ac (lane 10), 5 nM Cry1Fa (lane 11), or 10 nM Cry1Fa (lane 12). The primary antibody was anti-Cry1Ac or -Cry1Fa sera, the secondary antibody was anti-rabbit peroxidase, and detection was enhanced by chemiluminescence. Molecular mass markers (in kilodaltons) are shown on the left of each panel.

cules, ^{125}I-Cry1Ab and ^{125}I-Cry1Ac also bound to a 130-kDa protein. ^{125}I-Cry1Ac also bound to 100-kDa and smaller molecules.

We used biotinylated Cry1Fa and Cry1Ja to detect binding molecules on blots (Fig. 4B). As a comparison, ligand blots with biotinylated Cry1A toxins are also shown (lanes 5, 6, and 7). Biotinylated Cry1A toxins recognized the same molecules as radiolabeled Cry1A toxins. Although both Cry1Fa and Cry1Ja bound to the 170- and 110-kDa molecules, binding to the 130-kDa protein was almost absent, unlike for Cry1Ab and Cry1Ac.

Direct detection of bound toxin on blots with antitoxin antibodies avoids the covalent modification of the toxin but requires antibodies against each toxin studied. Immunoblots with Cry1Ac and Cry1Fa revealed the same patterns of binding molecules as seen with biotinylated toxins (Fig. 4, lanes 10 to 12). In these experiments, Cry1Fa bound to the 130-kDa protein when using higher amounts of toxin (lane 12).

DISCUSSION

The objective of this study was to construct a model for the binding sites in *H. virescens* recognized by five Cry1 toxins sharing high homology in domain II loops.

We determined the in vivo potencies of the selected toxins. Cry1Aa, Cry1Ab, Cry1Ac, and Cry1Fa were highly toxic to *H. virescens*, while Cry1Ja only killed larvae at high concentrations. Cry1Aa was about 10-fold more toxic than reported by Van Rie et al. (53), but this was in agrreement with Ge et al. (18). Since the LC_{50} values for Cry1Ab and Cry1Ac are similar to reported values, there may be differences in Cry1Aa susceptibility between populations of *H. virescens*. We also tested the activity of biotinylated Cry1Fa and Cry1Ja toxins, since the toxicities of these modified toxins were unknown. Bioassay results are evidence that biotinylated Cry1Fa and Cry1Ja toxins retain their activity against *H. virescens*.

Cry1A binding to *H. virescens* fits a three-site model (53) (Fig. 5). As expected from this model (26, 53), Cry1Aa, Cry1Ab, and Cry1Ac competed with high affinity (1 nM range) for ^{125}I-Cry1Aa binding sites. Based on Cry1Fa and Cry1Ja competition with ^{125}I-Cry1Aa and ^{125}I-Cry1Ab, we conclude that those toxins recognize receptor A. Cry1Fa and Cry1Ja had high affinity (12.6 and 2.4 nM, respectively) for receptor A in ^{125}I-Cry1Aa binding assays. The Cry1Fa and Cry1Ja K_{com} values for ^{125}I-Cry1Ab and ^{125}I-Cry1Ac were of considerably lower affinity (179 to 326 nM), indicating that these toxins have low affinity for the receptor shared with these toxins (receptor A). The ability of the Cry1Ja toxin to bind with high affinity but not to kill is analogous to Cry1Ac binding to BBMV from *Spodoptera frugiperda* (17).

Receptor B is a high-affinity binding site for Cry1Ab and Cry1Ac (Table 2) (53). Cry1Aa, Cry1Fa, and Cry1Ja do not recognize receptor B. The competition of Cry1Ab for ^{125}I-Cry1Ac binding (K_{com} = 73.6 nM) is possibly the composite of competition for receptors A and B.

Cry1Ac was the only toxin tested to recognize receptor C (Fig. 5). Van Rie et al. (53) proposed the existence of receptor C. An argument for receptor C follows. Since Cry1Ac competes all ^{125}I-Cry1Aa (Fig. 3A) and ^{125}ICry1Ab (Fig. 3B), but the reciprocal heterologous competition was not detected (Fig. 3C), there must exist a population of receptors unique to Cry1Ac, and this population is termed receptor C.

Our ligand blotting results were internally consistent for detection of similar-sized binding proteins by three techniques (^{125}I-labeled Cry1A toxins, antibodies against biotinylated Cry

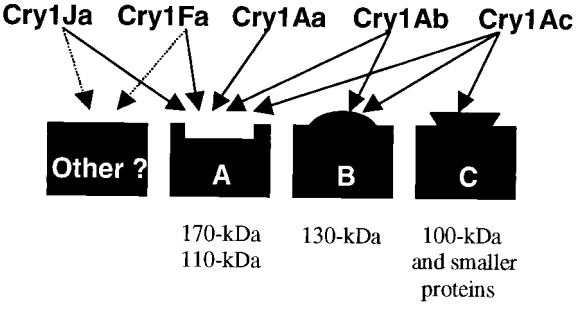

FIG. 5. Model proposed for binding of *B. thuringiensis* Cry1 toxins to sites in the *H. virescens* midgut membrane. Binding proteins correlated with specific sites are listed. Dashed arrows indicate predicted, but not determined, sites.

toxins, or antibodies against Cry1Ac and Cry1Fa). Similar patterns have been previously reported for ^{125}I-Cry1A toxins (9, 17, 30). ^{125}I-labeled Cry1A toxins bound to 170- and 110-kDa proteins on ligand blots. Cry1Fa and Cry1Ja bound the 170- and 110-kDa proteins. The 170-kDa protein is an isoform of APN. Both the 170-kDa and the 110-kDa proteins reacted with anti-APN serum (data not shown).

Although ligand blotting data have to be interpreted with caution (27), in our model of Cry1 binding proteins (Fig. 5), binding competition experiments and ligand blot observations agree. Based on the definition of receptor A being recognized by Cry1Aa, Cry1Ab, and Cry1Ac, receptor A would be comprised of 170- and 110-kDa proteins. How do we reconcile this model with the previous designation of the 170-kDa APN as a low-affinity binding site for Cry1Ac and receptor A (39)? It is not surprising that multiple molecules bind related Cry1A toxins, since this has been shown previously in *Manduca sexta* (47). Schwartz et al. (47) reported that multiple binding proteins were associated in a complex containing glycosylphosphatidyl inositol-anchored proteins. Also, it is possible that the high-affinity Cry1Ac binding site is a consequence of combined affinity for the 170- and 110-kDa molecules.

Modification of receptor A in a Cry1Ac-selected strain (YHD2) of *H. virescens* is implicated in high levels of resistance against Cry1Ac and cross-resistance to Cry1Aa and Cry1Ab (26). Apparently, the observed loss of Cry1Aa binding in strain YHD2 was due to a modification of receptor A (26). Our vesicle binding and ligand blotting results may explain the *H. virescens* cross-resistance to Cry1Fa (21) and Cry1Ja (F. Gould, unpublished personal communication) observed in strain YHD2: the modification of the shared receptor A also affects Cry1Fa and Cry1Ja binding and toxicity. This highlights the important role of the shared receptor A for toxicity in *H. virescens*, as suggested previously (26).

Cry1Ab and Cry1Ac also recognized a 130-kDa molecule. The 130-kDa molecule was recognized slightly by Cry1Aa but not by biotinylated Cry1Fa and Cry1Ja. Cry1Fa binding to the 130-kDa molecule was visualized only when high amounts of toxin (10 nM) were used, suggesting that the 130-kDa protein is a low-affinity binding molecule for Cry1Fa. The 130-kDa protein recognized by Cry1Ac and Cry1Ab on ligand blots is a candidate for receptor B.

Molecules of 100 kDa and smaller were recognized only by ^{125}I-Cry1Ac. These molecules may constitute receptor C, although some of these proteins seem to also bind Cry1Ja and Cry1Fa. We occasionally detected a 205-kDa molecule that bound Cry1 toxins, although this observation seemed to depend on the efficacy of the transfer, due to the high molecular size of this molecule. Cry1A binding molecules of this size in ligand blots have been previously described for *M. sexta* (33).

The 170- and 110-kDa proteins bind Cry1A toxins and the toxins with the highest homology in domain II loops: Cry1Fa and Cry1Ja. This domain II homology suggests that binding to receptor A is specified within the loops of domain II. In agreement with this notion, highly decreased toxicity and reduced binding to the 170-kDa protein have been reported for a Cry1Ab with mutated domain II loop 2 amino acids (42). Specificity of binding to receptors B and C seems not to be related to this homology, since high homology does not relate to competition for these sites.

Our data also imply that resistance in *H. virescens* strain YHD2 is directed against the homologous domain II loops in these toxins. This conclusion is especially relevant when considering strategies to decrease the development of *H. virescens* resistance to *B. thuringiensis* toxins. Thus, toxins with low homology to Cry1A toxins in domain II loops are reasonable alternative toxins to Cry1A toxins in *B. thuringiensis* plant and biopesticide formulations. In support of this strategy, high levels of toxicity against the resistant YHD2 strain are reported for transgenic tobacco plants producing Cry2Aa2 (25). Cry2Aa2 clusters in a group distant from Cry1A toxins in a domain II loop on a sequence similarity dendrogram (51).

The availability of receptor models for Cry toxins provides a framework for exploring mechanisms of resistance and reduces the chance of selecting toxin combinations that promote cross-resistance. Our results encourage further investigations into the relationship between domain II loops and toxin binding in *H. virescens*.

ACKNOWLEDGMENTS

We thank D. Banks, S. F. Garczynski, and F. Granero for helpful comments on the binding assays, J. Ferré for critically reading the manuscript, and L. Potvin for a gift of purified Cry1Aa toxin.

This research was supported by the NRI Competitive Grants Program of the U.S. Department of Agriculture.

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