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# Identification of Putative Insect Brush Border Membrane-Binding Molecules Specific to *Bacillus thuringiensis* δ-Endotoxin by Protein Blot Analysis

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**Binding sites for insecticidal toxins of *Bacillus thuringiensis* are located in the brush border membranes of insect midguts. Two approaches were used to investigate the interactions of *B. thuringiensis* subsp. *kurstaki* HD-73 CryIA(c) toxin with brush border membrane vesicles from sensitive and naturally resistant insects: $^{125}$I-toxin–vesicle binding assays and protein blots probed with $^{125}$I-CryIA(c) toxin. In bioassays, *Manduca sexta* and *Heliothis virescens* larvae were highly sensitive, *Helicoverpa zea* larvae were moderately sensitive, and *Spodoptera frugiperda* larvae were resistant to CryIA(c) toxin. Studies of binding of $^{125}$I-CryIA(c) toxin to brush border membrane vesicles from the larval midguts revealed that all insects tested had high-affinity, saturable binding sites. Significantly, *S. frugiperda* larvae bind but are not killed by CryIA(c) toxin. Labeled CryIA(c) toxin incubated with protein blots identifies a major binding molecule of 120 kDa for *M. sexta* and 148 kDa for *S. frugiperda*. *H. virescens* and *H. zea* are more complex, containing 155-, 120-, 103-, 90-, and 63-kDa proteins as putative toxin-binding molecules. *H. virescens* also contains a minor toxin-binding protein of 81 kDa. These experiments provide information that can be applied toward a more detailed characterization of *B. thuringiensis* toxin-binding proteins.**

The bacterium *Bacillus thuringiensis* produces insecticidal crystals during sporulation. These crystals consist of protein subunits called δ-endotoxins, or Cry proteins. Genes encoding Cry proteins have been classified into four groups on the basis of relatedness and insect specificity (10). CryI proteins are toxic to insects of the order Lepidoptera. The genetics, specificity, and protein engineering of the CryI δ-endotoxins have recently been reviewed (7).

When susceptible larvae ingest CryI crystals, 130- to 140-kDa protoxins are released in the alkaline midgut. Midgut proteases process the protoxin to form a stable 55- to 70-kDa toxin core. Activated toxin subsequently binds to epithelial cells lining the midgut. Bound toxin disrupts the midgut epithelial cells, causing lysis. To study early events in the action of *B. thuringiensis* toxins, investigators have developed various in vitro assays. For studying binding phenomena, the most useful of these assays is one that utilizes brush border membrane vesicles (22). It has been shown that *B. thuringiensis* toxins bind with high affinity to specific binding molecules present in the brush border membranes of susceptible insects. Recent works have correlated the presence of high-affinity binding sites to insecticidal activity (6, 18, 20, 21).

We have used CryIA(c)-type δ-endotoxin from *Bacillus thuringiensis* subsp. *kurstaki* HD-73 to examine toxin-binding molecule interactions with brush border membrane vesicles from *Manduca sexta*, *Heliothis virescens*, *Helicoverpa zea*, and *Spodoptera frugiperda* midguts. These insects represent highly susceptible (*M. sexta* and *H. virescens*), moderately susceptible (*H. zea*), and tolerant (*S. frugiperda*) species. We report the presence of CryIA(c) toxin-binding sites in both susceptible and naturally resistant insects. Secondly, we have identified possible binding proteins by bathing the labeled toxin with proteins extracted from brush border membrane vesicles and transferred to nitrocellulose filters.

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## MATERIALS AND METHODS

**Materials.** *B. thuringiensis* subsp. *kurstaki* HD-73 was obtained from the *Bacillus* Genetic Stock Culture Collection (Columbus, Ohio). *H. virescens* was obtained from the USDA/ARS Southern Field Crop Insect Management Laboratory (Stoneville, Miss.), *M. sexta* was obtained from Carolina Biologicals (Burlington, N.C.), *S. frugiperda* was obtained from D. Isenhour (Tifton, Ga.), and *H. zea* was obtained from G. Herzog (Tifton, Ga.).

**Bacterial growth and toxin purification.** *B. thuringiensis* HD-73 was grown at 28°C in L broth until sporulation. The crystal-spore-debris mixture was centrifuged at 5,000 × *g*, and the pellet was washed in distilled $H_2O$. The crystal protein was solubilized in 0.1 M $Na_2CO_3$–20 mM dithiothreitol, pH 10.2. Trypsin was added to 0.5% and incubated overnight at 28°C. The solution was centrifuged 10,000 × *g*. The clarified supernatant was sonicated for 60 s at 250 W with a Branson sonicator and then centrifuged as described above. Solid $(NH_4)_2SO_4$ was added to 70% over a 15-min period and then mixed overnight at 5°C. The precipitate was recovered after a 20,000 × *g* centrifugation and then dissolved in 50 mM Tris-Cl–300 mM NaCl, pH 8.5. The dissolved precipitate was dialyzed overnight against 50 mM Tris-Cl–100 mM NaCl, pH 8.5, and particulates were removed by centrifugation at 20,000 × *g*. The protein sample was applied to an anion-exchange column (Sepharose-Q; Pharmacia) and eluted with a linear gradient of 100 to 600 mM NaCl in 50 mM Tris-Cl, pH 8.5. The toxin fractions were pooled and further purified over a Sephacryl S-300 (Pharmacia) column. Protein concentrations were determined by using Bio-Rad protein solutions with bovine serum albumin (BSA) as a standard (1).

**Protein iodination.** Toxin was iodinated by the chloram-

TABLE 1. Insect toxicity and membrane vesicle binding of CryIA(c)

| Insect | $LC_{50}$[a] | % Binding | $K_d$[b,c] | $B_{max}$[c,d] |
|---|---|---|---|---|
| *M. sexta* | 4.51 (3.56–5.69) | 61.1 | 1.58 (±0.06) | 20.2 (±0.04) |
| *H. virescens* | 2.26 (0.65–3.47) | 60.7 | 2.09 (±0.55) | 38.0 (±2.9) |
| *H. zea* | 464 (363–647) | 41.9 | 5.66 (±0.56) | 44.4 (±2.2) |
| *S. frugiperda* | >2,000 | 39.6 | 0.68 (±0.02) | 4.5 (±0.07) |

[a] Expressed in nanograms per square centimeter; the 95% confidence intervals are in parentheses.
[b] Expressed in nanomolar concentrations.
[c] Values are generated from the mean of duplicate samples.
[d] Expressed as picomoles per milligram of vesicle protein.

ine-T method (8). $Na^{125}I$ (0.5 mCi) and chloramine-T (0.1 mg) were added to 1 to 3 μg of toxin. After the reaction mixture was shaken gently for 60 s, the reaction was quenched with 0.125 mg of Na metabisulfite. The iodinated toxin was then separated from free iodine by using a 5-ml Sephadex G50 (Pharmacia) column. The toxin fractions were then further purified as follows: the sample was adjusted to 0.1% Triton X-100, 0.1% deoxycholate, 100 mM NaCl, and 50 mM Tris-Cl (pH 8.5). Iodinated toxin was then passed twice over a 0.5-ml Sepharose-Q (Pharmacia) column. The column was washed with 6 bed volumes of 50 mM Tris-Cl–100 mM NaCl, pH 8.5, until no radioactivity was detected in the eluate. The toxin was eluted by using 50 mM Tris-Cl–500 mM NaCl, pH 8.5, and the radioactivity was measured on a Beckman Gamma 4000 counter. Typically, the specific activities were 43.35 mCi/mg of input toxin.

**Preparation of brush border membrane vesicles.** The method of Wolfersberger et al. (22) as modified by English and Readdy (3) was used for the isolation of insect brush border membrane vesicles. Second-day fifth-instar larvae were chilled on ice for 15 min. The midguts were excised in ice-cold grinding buffer (50 mM sucrose, 2 mM Tris-Cl [pH 7.4]) and cut longitudinally, the peritrophic membrane and food contents were removed, and the midgut was frozen on dry ice. Frozen tissue was ground in a Potter-Elvenhem homogenizer in ice-cold grinding buffer containing 0.1 mM phenylmethylsulfonyl fluoride. $CaCl_2$ was added to 10 mM, and the homogenate was stirred on ice for 15 min. The mixture was centrifuged at 4,300 × *g* for 10 min at 5°C, and the pellet was discarded. The supernatant was then reclarified. The reclarified supernatant was centrifuged at 27,000 × *g* for 10 min, and the pellet was resuspended in 0.32 M sucrose. The suspension was passed through a 27-gauge needle and stored at −70°C. Rat intestinal brush border membrane vesicles were prepared by a precipitation method using Mg-EGTA (16).

**Insect bioassays.** Toxicity assays were performed on neonatal larvae as follows. CryIA(c) toxin was diluted in phosphate-buffered saline (58 mM $Na_2HPO_4$, 17 mM $NaH_2PO_4$, 6.8 mM NaCl [pH 7.4]) with 0.1% BSA. Samples (10 μl containing 0 to 2,000 ng of toxin) were applied to the diet surface and allowed to dry. First-instar larvae were placed onto diet and reared at 26°C. Mortality and insect weight were scored after 4 (*M. sexta*) or 7 (*H. virescens*, *H. zea*, and *S. frugiperda*) days. The results were analyzed by probit analysis (2, 4).

**CryIA(c) toxin binding to membrane vesicles.** Brush border membrane vesicles (14 μg of protein) were incubated at room temperature for 30 min with 0.14 nM $^{125}$I-toxin, either alone or in combination with various amounts of unlabeled toxin, in 0.1 ml of phosphate-buffered saline containing 0.1% BSA. The samples were then centrifuged for 5 min at 13,000 × *g*, and the unbound toxin was removed by aspiration. The pellet containing brush border membrane vesicle and the bound toxin was washed twice with 0.5 ml of phosphate-buffered saline containing 0.1% BSA. Bound radioactivity was then counted in a Beckman Gamma 4000 detector. Homologous competition experiments were performed as described above with various amounts of unlabeled toxin (0.01 to 1,000 nM) added. Scatchard and Hill analyses were performed using the EBDA/LIGAND program obtained from Biosoft/Elsevier (Milltown, N.J.).

**Protein blot analysis.** Brush border membrane vesicles (20 μg of protein) were heated in cracking buffer (50 mM Tris-Cl, 1% sodium dodecyl sulfate [SDS], 40 mM dithiothreitol, 0.02% bromophenol blue, 10% glycerol) for 3 min at 95°C and analyzed by SDS–10%-polyacrylamide gel electrophoresis (11). The proteins were transferred to nitrocellulose membrane (17) by using a Hoeffer TE 50 electroblot apparatus. The membrane was subsequently rinsed twice for 5 min each time in Tris-buffered saline (50 mM Tris-Cl, 150 mM NaCl [pH 7.5]) containing 0.2% Tween 20 and then incubated for 1 h in Tris-buffered saline with 3% BSA. Approximately $10^8$ cpm of $^{125}$I-toxin (0.14 nM) was added to 50 ml of Tris-buffered saline with 0.1% BSA and incubated with the filter for 3 h at room temperature with constant shaking. Nonbound radioactivity was aspirated, and the filter was washed four times (20 min for each wash) in Tris-buffered saline with 0.1% Tween 20. The filter was blotted dry and exposed for 1 h to Kodak X-Omat AR X-ray film with a DuPont Cronex Lightning Plus intensifying screen at −70°C.

Competition assays were performed as described above, except that either unlabeled toxin (100 nM), *N*-acetylgalactosamine (50 mM), or *N*-acetylglucosamine (50 mM) was added to the toxin-binding mixture. Films were scanned with a laser densitometer (Molecular Dynamics).

## RESULTS

**Insect bioassays with CryIA(c) toxin.** The insects in this study were chosen because they represent species which differ in their susceptibilities to *B. thuringiensis* HD-73 crystals (7, 13). The active ingredient of HD-73 crystals, CryIA(c) toxin, was bioassayed against four lepidopteran larvae (Table 1). *H. virescens* and *M. sexta* were highly susceptible to CryIA(c) toxin, and *S. frugiperda* was resistant. For *S. frugiperda*, the actual 50% lethal dose was greater than the maximum dose (2,000 ng/cm$^2$) tested, indicating a susceptibility to CryIA(c) toxin over 1,000-fold less than that of *H. virescens*. It was also observed, by using $^{125}$I-CryIA(c) toxin, that iodination or detergent treatment (0.1% Triton X-100, 0.1% deoxycholate) had little effect on toxicity against *M. sexta* larvae (data not shown). These assays established the relative toxicities of the CryIA(c) toxin preparation against the insects described above.

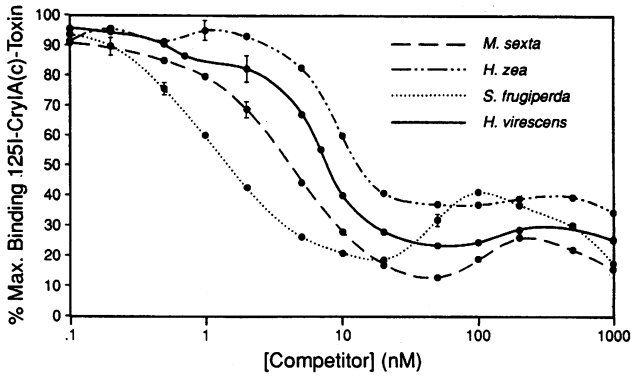


FIG. 1. Composite competitive binding assay using CryIA(c) toxin and insect brush border membrane vesicles. Vesicles from each insect were incubated with 0.14 nM $^{125}$I-CryIA(c) toxin and with increasing concentrations of unlabeled toxin. Binding is expressed as a percentage of the maximum amount of toxin bound by using labeled toxin alone, without subtracting nonspecific binding. The curves were generated by the LIGAND computer program. Each point represents the mean of duplicate samples. Standard error between samples is shown by brackets.

**Binding of CryIA(c) toxin to brush border membranes.** After determining the potencies of CryIA(c) toxin, we determined its binding characteristics in relation to brush border membrane vesicles. Homologous competition experiments (unlabeled toxin competing against $^{125}$I-labeled toxin for binding sites) were used to quantify CryIA(c) toxin binding. The reaction conditions employed were those developed by Hofmann et al. (6). The brush border membrane vesicles of each species specifically bound 40 to 60% of the total $^{125}$I-labeled CryIA(c) toxin (0.14 nM). Rat brush border membrane vesicles, used as a control, exhibited only low, nonspecific binding (1.8%). Figure 1 shows the toxin competition curves for each species. When subjected to competition with unlabeled toxin, binding decreased at concentrations of the competitor in the ranges of 0.2 to 10 nM for *S. frugiperda*, 1 to 50 nM for *M. sexta*, 2 to 50 nM for *H. virescens*, and 2 to 50 nM for *H. zea*. Each binding curve shows an increase in $^{125}$I-CryIA(c) toxin bound between 100 and 1,000 nM of total toxin. The increase in toxin bound at high concentrations is most pronounced with *S. frugiperda* vesicles. We used the computer program LIGAND to determine the dissociation constants ($K_d$) and the binding-site concentrations ($B_{max}$) for the insect brush border membrane vesicles (listed in Table 1). LIGAND analysis showed that each Hill coefficient was approximately 1, indicating compatibility of a single-site model with the binding data. The values of dissociation constants ranged from 0.68 nM (for *S. frugiperda*) to 5.66 nM (for *H. zea*). The dissociation constants of *M. sexta* (1.58 nM) and *H. virescens* (2.09 nM) were similar to those previously reported (18, 19). The binding-site concentrations varied from 4.5 to 44.4 pmol/mg of membrane vesicle protein (Table 1). *S. frugiperda*, the most resistant insect tested, had the lowest concentration of binding sites (4.5 pmol/mg). These findings support a model which requires *B. thuringiensis* toxin-binding sites for toxicity (6). However, as suggested by *S. frugiperda* and, to some extent, *H. zea* vesicle-binding data, toxin binding alone is not sufficient for toxicity.

**Protein blots.** To study the toxin-binding-site complexes further, we employed a protein blot technique. After SDS-gel electrophoresis, membrane vesicle proteins (20 μg) were transferred to nitrocellulose membranes and incubated with $^{125}$I-CryIA(c) toxin. To compare blots of vesicle proteins with membrane vesicle assays, we determined $^{125}$I-CryIA(c) toxin binding in the presence or absence of potential competitors. The intensity of the major band was estimated with a scanning laser densitometer. Figure 2 shows that unlabeled toxin reduces $^{125}$I-CryIA(c) toxin binding to *M. sexta* membranes on protein blots (to 18% of $^{125}$I-CryIA(c) toxin binding without competition). *N*-Acetylgalactosamine also reduces binding, but to a lesser degree (50% of labeled toxin alone in vesicle assays and 40% on blots). *N*-Acetylglucosamine has no measurable effect. These results indicate that transferred proteins maintain, to some degree, specificity for toxin binding. Labeled CryIA(c) toxin binds to vesicle proteins in each insect (Fig. 3). *M. sexta* displayed a major band at 120 kDa (Fig. 3, lane 5), and *S. frugiperda* had only one band at 148 kDa (Fig. 3, lane 2). *H. virescens* displayed a major band at 155 kDa and minor bands of 120, 105, 90, 81, and 64 kDa. *H. zea* (Fig. 3, lane 3) showed multiple bands of relatively equal intensity. *M. sexta*, *H. virescens*, and *H. zea*

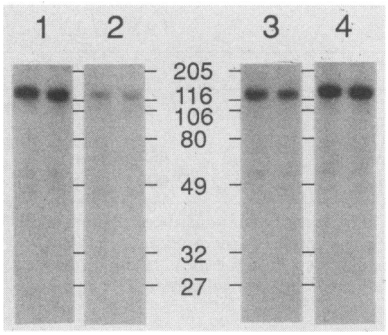


FIG. 2. Competitive binding of CryIA(c) toxin to *M. sexta* brush border membrane proteins on blots (SDS–10% polyacrylamide gels of *M. sexta* brush border membrane proteins). Duplicate lanes containing 10 μg of protein were treated with 0.14 nM $^{125}$I-CryIA(c) toxin (lanes 1) alone or toxin containing 100 nM unlabeled toxin competitor (lanes 2), 50 mM *N*-acetylgalactosamine (lanes 3), or 50 mM *N*-acetylgalactosamine (lanes 4). For details of the procedure used, see Materials and Methods. Lines indicate the positions of prestained $M_r$ standards (molecular weights are in thousands) (Bio-Rad).

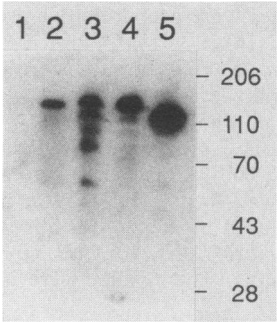


FIG. 3. Protein blot identification of brush border membrane vesicle-binding proteins specific to CryIA(c) toxin [SDS–10% polyacrylamide gel of membrane vesicles (20 μg), incubated with trypsin-activated $^{125}$I-CryIA(c) toxin]. Lane 1, rat; lane 2, *S. frugiperda*; lane 3, *H. zea*; lane 4, *H. virescens*; lane 5, *M. sexta*. Lines indicate the positions of prestained $M_r$ standards (molecular weights are in thousands) (Bethesda Research Laboratories, Inc.).

all had the 120-kDa band present, but at differing intensities, while *S. frugiperda* did not exhibit a 120-kDa polypeptide. Rat brush border membrane proteins, run as a control, exhibited no detectable binding (Fig. 3, lane 1).

## DISCUSSION

We have determined the binding characteristics and molecular sizes of putative high-affinity binding sites for $^{125}$I-CryIA(c) toxin in the midgut brush border membrane vesicles of selected sensitive and naturally resistant insects. Complementary approaches were taken to characterize target sites in the insect midgut: homologous competition binding assays and protein blot binding.

As an initial step, we established insecticidal activity of CryIA(c) toxin. Results for the highly susceptible insects *M. sexta* and *H. virescens* were similar to those of previous reports (18). *H. zea* was 100-fold less sensitive to CryIA(c) toxin than *H. virescens*. MacIntosh et al. (13), using *B. thuringiensis* HD-73 crystals, reported a 10-fold difference between those insects. *S. frugiperda* was not killed at the highest dose tested (2,000 ng/cm$^2$), which is consistent with the tolerance of other *Spodoptera* species for CryIA(a) and CryIA(c) proteins (19).

Competition binding assays indicated high-affinity binding sites for each species tested. *M. sexta* and *H. virescens*, both highly susceptible species, have binding sites for CryIA(c) toxin (18; this report). *H. zea* is 100-fold less sensitive than *M. sexta* and *H. virescens* but has a similar concentration and affinity of binding sites. Even brush border membrane vesicles from naturally resistant *S. frugiperda* have a relatively small number (4.5 pmol/mg) of high-affinity ($K_d$ value of 0.68 nM) binding sites for CryIA(c) toxin. Computer analysis of equilibrium binding data for each insect fits a single-site model. However, this analysis may underestimate the number of binding sites since it does not resolve multiple binding sites when they have similar $K_d$ values or when there is a large difference in the binding-site concentrations of high- and low-affinity sites (12). Membrane assays using related CryIA toxins as competing ligands overcome these limitations. By this approach, three to four binding sites were revealed in *H. virescens* vesicles (18, 19).

The presence of CryIA(c) toxin binding sites in susceptible and resistant insects supports a hypothesis in which toxin receptors are necessary, but insufficient, for insecticidal activity. This report is not the first to question the correlation between toxin potency and binding sites. Recently, Wolfersberger found that the dissociation constants for CryIA(b) and CryIA(c) toxin binding were inversely correlated with their potencies against *Lymantria dispar* (gypsy moth) larvae (21). However, CryIA(c) toxin binding to *S. frugiperda* vesicles is not completely analogous to that for *L. dispar* vesicles because of the greater tolerance of *S. frugiperda* for high CryIA(c) toxin doses. This paper and Wolfersberger's work (21) report high-affinity binding and low toxicity with CryIA(c) toxin. In contrast, Van Rie et al. (19) reported no binding of CryIA(a) toxin to brush border membrane vesicles from *Spodoptera litoralis*. Also, we cannot dismiss the possibility that a critical concentration of binding sites is needed for toxicity, but the 5- to 10-fold-lower concentration of binding sites in *S. frugiperda* vesicles appears insufficient to account for the higher level of resistance.

As others have proposed (6), recognition of a binding site in the insect midgut is an important part of *B. thuringiensis* toxemia, but there are other determinants of potency. This proposal fits the current models for CryI toxin action via pore formation, which leads to colloid osmotic lysis. Pore formation could be direct, through toxin integration into the cell membrane (10), or indirect, by disrupting receptor function (14). It is possible that the CryIA(c) toxin-binding sites in *S. frugiperda* are structurally different and that although binding does occur, the toxin does not integrate into the membrane and cause cell lysis. Alternatively, CryIA(c) toxin may have a binding domain that recognizes *S. frugiperda* membrane proteins but a cytolytic domain that does not function in this species. In either case, CryIA(c) toxin binding to *S. frugiperda* midgut brush border membrane sites is not sufficient to cause insect mortality.

A protein blot technique, recently reviewed by Soutar and Wade (15), was used by Knowles and Ellar (9) to identify specific proteins from cultured lepidopteran and dipteran cells that bound $^{125}$I-labeled CryIA toxin. The toxin bound to proteins in each of the cell lines tested. The CryIA toxin however killed only the CF1 cells, suggesting that binding alone is not sufficient for toxicity. Their study also implies that the protein bands observed are glycosylated, containing *N*-acetylgalactosamine residues. This is consistent with our findings that *N*-acetylgalactosamine is an active competitor for toxin binding (Fig. 2; also unpublished data).

Haider and Ellar (5) used $^{125}$I-labeled CryIA(b) toxin to probe protein blots of cultured lepidopteran (CF1 and *H. zea*) and dipteran (*Aedes albopictus* and *Drosophila melanogaster*) cell lines. The toxin bound specifically to blots from the lepidopteran cell lines and revealed 120- and 68-kDa bands. These results agree in part with the 120- and 64-kDa bands we observed in *M. sexta*, *H. virescens*, and *H. zea*. However, we also detected other bands from the brush border membrane that were not detected in the lepidopteran cell lines studied by Haider and Ellar. Their data also demonstrated a correlation between binding of toxin to proteins on blots and insecticidal activity toward the cell lines.

From the vesicle binding experiments reported here and the heterologous competition experiments of Van Rie et al. (18), *M. sexta* was expected to have one CryIA(c) toxin-binding site. This lends support to the hypothesis of the 120-kDa protein as a binding site (Fig. 3). We observed two additional proteins that bind less CryIA(c) toxin on blots (Fig. 2 and 3). Minor *M. sexta* bands vary in intensity between experiments and vesicle preparations. *H. virescens* and *H. zea* display binding proteins of similar sizes on blots. Both species have a major 155-kDa band and four to five minor bands. In *H. zea*, all bands are relatively equal in intensity, while in *H. virescens*, the 155-kDa band is predominant. This diversity of CryIA(c) toxin-binding proteins in *Heliothis* species was expected. Van Rie et al. (18) found that CryIA(c) toxin recognizes more binding sites than CryIA(a) and CryIA(b) do. They interpret this to mean that CryIA(c) toxin recognizes at least three classes of binding sites. Multiple proteins shown on blots of *H. virescens* brush border membrane vesicles support their interpretation. *S. frugiperda* is the only species that has a 148-kDa toxin-binding protein, but faint bands corresponding to binding molecules seen in other species are detected.

We have shown that CryIA(c) toxin binds with high affinity to sites in the midgut of susceptible and resistant insects. Protein blot experiments suggest specific candidates for toxin binding molecules. The information presented in this study now can be used to facilitate the cloning of the candidate toxin-binding proteins, a step which may allow

elucidation of specific modes of action of *B. thuringiensis* toxin.

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