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Comparison of Toxin Overlay and Solid-Phase Binding Assays To Identify Diverse CryIA(c) Toxin-Binding Proteins in *Heliothis virescens* Midgut

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The binding proteins, or receptors, for insecticidal *Bacillus thuringiensis* subsp. *kurstaki* δ-endotoxins are located in the brush border membranes of susceptible insect midguts. The interaction of one of these toxins, CryIA(c), with proteins isolated from *Heliothis virescens* larval midguts was investigated. To facilitate the identification of solubilized putative toxin-binding proteins, a solid-phase binding assay was developed and compared with toxin overlay assays. The overlay assays demonstrated that a number of proteins of 170, 140, 120, 90, 75, 60, and 50 kDa bound the radiolabeled CryIA(c) toxin. Anion-exchange fractionation allowed the separation of these proteins into three toxin binding fractions, or pools. Toxin overlay assays demonstrated that although the three pools had distinct protein profiles, similar-size proteins could be detected in these three pools. However, determination of toxin affinity by using the solid-phase binding assay showed that only one of the three pools contained high-affinity binding proteins. The K_d obtained, 0.65 nM, is similar to that of the unsolubilized brush border membrane vesicles. Thus, the solid-phase binding assay in combination with the toxin overlay assay facilitates the identification and purification of high-affinity *B. thuringiensis* toxin-binding proteins from the insect midgut.

The gram-positive, spore-forming bacterium *Bacillus thuringiensis* produces insecticidal inclusion proteins, or δ-endotoxins, which accumulate during sporulation (2, 8). Upon ingestion by susceptible insect larvae, the parasporal inclusion dissolves in the alkaline midgut environment; the proteins are released and activated by proteolytic cleavage. The toxins then interact with the midgut apical membranes, disrupting membrane integrity and eventually leading to insect death.

The precise mechanism of toxic action is not known. The current model of *B. thuringiensis* toxin action involves ion pore formation that leads to osmotic balance disruption in the midgut epithelial layer (5, 10). This selective action on midgut epithelium is facilitated by the presence of high-affinity toxin binding sites in the brush border membranes of midgut columnar cells. The insecticidal activity of *B. thuringiensis* toxins is generally correlated with toxin binding to the brush border membrane vesicles (BBMV) isolated from midguts of susceptible insects, while nonsusceptible insects usually lack specific binding sites (7, 24). In the lepidopteran *Heliothis virescens*, differences in the toxicity of the CryIA(a), CryIA(b), and CryIA(c) toxins correspond to variances in the binding site concentrations (R_t), not the binding affinities (K_d), of these toxins (23).

The importance of high-affinity toxin binding sites in insects is further illustrated by the low insecticidal activity in insects resistant to *B. thuringiensis* toxins. For example, CryIA(b)-resistant *Plutella xylostella* BBMV had no detectable binding to this particular toxin, while the binding parameters for a related CryIC toxin remained unchanged (3). In CryIA(b)-resistant *Plodia interpunctella* BBMV, there was decreased toxin affinity but no change in binding sites (25). However, in *H. virescens*, resistance to the CryIA(b) and CryIA(c) toxins is not correlated with significant changes in either the R_t or the K_d (6, 13).

Although a number of high-affinity binding sites have been identified for *B. thuringiensis* toxins, the proteins that bind these toxins have not been characterized. In attempts to identify the actual binding proteins involved, BBMV proteins have been electrophoretically separated and probed with appropriate toxins. Using this toxin overlay method, Knowles et al. (11) reported that CryIA(c) binds to several *H. virescens* BBMV proteins, the most prominent of which were 68 and 50 kDa. *N*-Acetylgalactosamine partially blocked toxin binding and the lectin soybean agglutinin bound to proteins of the same mass, hinting that the binding proteins might be glycosylated. Oddou et al. (15, 16) detected two CryIA(c)-binding proteins of 140 and 120 kDa, while Garczynski et al. (4) detected several ^{125}I-CryIA(c)-binding polypeptides of 140, 105, 90, 81, and 64 kDa. In contrast, CryIA(c) binding studies using *H. virescens* BBMV (23) had predicted the existence of three distinct high-affinity binding sites. In another insect, *Manduca sexta*, only one CryIA(c)-binding protein, a 120-kDa glycoprotein, has been reported (4, 9, 11, 19), while a 210-kDa protein is involved in CryIA(b) toxin binding (22). For CryIC toxin binding, a 65-kDa protein is reportedly involved in *Spodoptera littoralis* (18).

Recently, two of the *B. thuringiensis* toxin-binding proteins have been partially purified. The *M. sexta* high-affinity CryIA(b)-binding protein is a glycosylated 210-kDa protein with an acidic pI (22). Sangadala et al. (19) and Knight et al. (9) identified the *M. sexta* CryIA(c)-binding protein as a 120-kDa glycosylated aminopeptidase N-like protein. Phospholipid vesicles reconstituted with this protein demonstrate enhanced Rb^{2+} permeability when challenged with the toxin (19).

Although several proteins bind the CryIA(c) toxin (4, 11, 15, 16), the *H. virescens* proteins that bind the CryIA(c) toxin with

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high affinity have not been positively identified. In this study, we demonstrated that toxin overlay analysis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-separated BBMV proteins does not necessarily identify proteins with the highest toxin affinity. To aid in the characterization and purification of CryIA(c) toxin-binding proteins from *H. virescens* BBMV, we have developed a simple, reproducible solid-phase binding assay. In contrast to the toxin overlay assay, the solid-phase assay facilitates determination of binding affinities of detergent-solubilized BBMV proteins.

MATERIALS AND METHODS

Isolation of the CryIA(c) crystal. *Bacillus thuringiensis* subsp. *kurstaki* HD-73, which expresses only the CryIA(c) toxin in the parasporal inclusion (1), was cultured in GYS medium as previously described (14). After complete cell lysis was achieved, usually in 2 to 3 days, the cultures were harvested by centrifugation at 8,000 × *g* for 30 min. The pellets were washed with 1 M NaCl–10 mM EDTA, suspended in the same solution, and sonicated for 4 min. The suspension was placed onto continuous 40 to 70% (vol/vol) Renografin gradients (Squibb Diagnostics) and centrifuged in an SW 28 rotor at 15,000 rpm for 30 min. The isolated crystals were washed several times with 10 mM EDTA and stored in phosphate-buffered saline (PBS)–10 mM EDTA at −70°C until needed.

The purified crystals were solubilized in a solution containing 0.1 M Na_2CO_3 (pH 10.5) and 10 mM dithiothreitol for 1 h, and the mixture was centrifuged for 20 min at 16,000 × *g* to remove any unsolubilized material. Sequencing-grade porcine pancreatic trypsin (Calbiochem) was then added at a toxin-to-trypsin ratio of 5:1 (wt/wt), and the mixture was incubated for 6 h at 18°C. The 60-kDa protein was separated from the trypsin and proteolytic fragments on an FPLC Superose 12 column (Pharmacia) equilibrated with 0.1 M Na_2CO_3 buffer, pH 10. One-milliliter fractions were collected, and the contents were analyzed by SDS-PAGE (12). Fractions containing the 60-kDa toxin were pooled, and the purified toxin was stored at −70°C until needed.

Radioiodination of the CryIA(c) toxin. The CryIA(c) toxin was iodinated according to the method of MacIntosh et al. (13). Briefly, 100 μg of the purified 60-kDa toxin was incubated with 1 mCi of $Na^{125}I$ (Amersham) and two Iodobeads (Pierce) for 15 min at room temperature. Unincorporated iodine was removed by chromatography with PD10 columns (Pharmacia) or by dialysis with 50-kDa-cutoff tubing. Densitometric analyses of SDS-PAGE gel autoradiograms indicated that at least 90% of the label was present in the toxin. The specific activity was calculated by using enzyme-linked immunosorbent assay (23). The CryIA(c) toxin used in this study had specific activities within the range of 170 to 200 Ci/mmol.

Bioassays. The toxicities of the CryIA(c)-activated toxin and iodinated toxin were determined by using neonate *H. virescens* larvae. Serial dilutions of the purified and the iodinated toxin were prepared on the basis of the protein concentration or the specific activity of the iodinated toxin. Mortality of the larvae was recorded after 7 days. The 50% lethal concentration for the purified toxin was 0.09 ± 0.02 μg/ml of diet, which is similar to the values obtained by MacIntosh et al. (13) and Van Rie et al. (23). The 50% lethal concentration for the iodinated toxin was 0.08 ± 0.02 μg/ml, indicating that the ^{125}I-labeled toxin was as active as the unlabeled toxin.

Preparation of BBMV. BBMV from *H. virescens* and *M. sexta* were prepared from fifth-instar larval midguts according to the $MgCl_2$ precipitation method of Wolfersberger et al. (27), except that 1 mM phenylmethylsulfonyl fluoride was included in the solutions. Midguts and BBMV aliquots were stored in liquid N_2 until use. Protein concentrations were determined by using the detergent-compatible protein assay kit (BioRad) with bovine serum albumin (BSA) as a standard. BBMV leucine aminopeptidase and alkaline phosphatase activities were typically 5 to 8 times that of the midgut homogenate.

Detergent solubilization and anion-exchange chromatography of the CryIA(c)-binding proteins. Freshly prepared *H. virescens* BBMV were suspended in a solubilization buffer (20 mM Tris [pH 7.5], 1 mM EDTA, 1 mM Mg_2SO_4, 0.01% NaN_3, and 10% glycerol) with 2% CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate [wt/vol]) (Boehringer Mannheim Biochemicals [BMB]) and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μM protinin, 0.7 μM leupeptin, and 1 μM pepstatin). Various other detergents were tested in this solubilization buffer. These included 2% sodium deoxycholate (wt/vol) (BMB), 2% *n*-octylglucoside (wt/vol) (BMB), 2% Triton X-100 (vol/vol) (Sigma), and 2% digitonin (wt/vol) (BMB). In all cases, 60 to 75% of the total BBMV protein was solubilized. After mixing for 1 h at 4°C, the preparations were centrifuged for 1 h at 100,000 × *g*. The supernatants were applied to a 1-ml FPLC MonoQ column (Pharmacia) with a 10-ml Superloop and subjected to a 0 to 500 mM KCl linear gradient in solubilization buffer with 1% CHAPS. One-milliliter fractions were collected, and 5-μl aliquots were subjected to a solid-phase assay.

Solid-phase assay. In order to localize CryIA(c) binding activity in the various fractions, 5-μl samples were spotted onto nitrocellulose (BA85; Schleicher & Schuell), allowed to dry for 1 to 2 h at room temperature, and blocked with 3% BSA in PBS (20 mM sodium phosphate, 150 mM NaCl [pH 7.5]). The blots were incubated with 1 nM ^{125}I-CryIA(c) toxin in binding buffer (20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) [pH 7.5], 150 mM NaCl, 0.1% BSA, 0.1% Tween 20, and 0.01% NaN_3) for 1 h, rinsed three times for 10 min each time with the binding buffer, dried, and analyzed by autoradiography on Kodak X-Omat film.

A modified solid-phase binding assay (21) was used to determine toxin affinity. Five-microliter aliquots of sample were spotted onto nitrocellulose squares (1 by 1 cm), dried, placed into 24-well tissue culture dishes, and then blocked as described above. The squares were washed twice with 1 ml of PBS and then once with binding buffer. The squares were then incubated with 200 μl of binding buffer containing 1 nM ^{125}I-CryIA(c) or 1 nM ^{125}I-CryIA(c) plus various amounts of unlabeled competitor. The dishes were incubated on a rocker for 16 h at 4°C, after which the medium either was removed and counted or was aspirated. The nitrocellulose squares were washed once with 1 ml of binding buffer and three times with 1 ml of PBS, with 30 min for each wash. The squares were dried on filter paper and counted in an LKB gamma detector. Scatchard analyses were performed with the LIGAND program (17). All experimental data obtained are the averages for three replicates. Similar results were obtained from two other identical experiments.

Specific toxin binding was measured in a similar manner. Various amounts of *H. virescens* BBMV were spotted onto the squares, blocked, and treated as outlined above. The squares were incubated with 1 nM ^{125}I-CryIA(c) to determine total binding; in order to assess the nonspecific binding, the squares were incubated with the labeled toxin and excess (1,000 nM) unlabeled toxin. Specific binding was calculated by subtracting the nonspecific binding from the total binding.

Gel electrophoresis, electroblotting, and toxin overlay assay. SDS-PAGE was performed with a discontinuous buffer system (12) and 10% polyacrylamide gels. Samples were boiled in sample buffer for 5 min. Sample buffer containing an eightfold excess of SDS was used with samples containing detergents. Separated proteins were electrotransferred for 1 h at 50 V to Immobilon membranes (Millipore) by using a Tris-glycine buffer as suggested by the manufacturer. After the transfer, membranes were blocked in PBS containing 3% BSA. The membranes were then incubated in binding buffer containing ^{125}I-CryIA(c) toxin with or without unlabeled competitor (500-fold) for 1 h at room temperature. The blots were washed four times with binding buffer, dried, and analyzed by autoradiography.

RESULTS

Identification of *H. virescens* CryIA(c)-binding proteins by toxin overlay analysis. *B. thuringiensis* subsp. *kurstaki* HD-73 produces parasporal inclusions that contain a major 130-kDa polypeptide (Fig. 1A, lane 2), which upon trypsinization and purification yielded a 60-kDa protein (lanes 3 and 4). This protein, radiolabeled with high specific activity (Fig. 1A, lane 5) while retaining full toxicity, was then used for identification of midgut toxin-binding proteins.

Proteins isolated from *H. virescens* midgut, BBMV, and CHAPS-solubilized BBMV were separated by SDS-PAGE and electroblotted onto nitrocellulose. Toxin overlay analyses showed that all three preparations contained CryIA(c)-binding proteins. The clearest binding was observed with CHAPS-solubilized BBMV, in which proteins of 170, 140, 120, 90, 75, 60, and 50 kDa bound radiolabeled toxin (Fig. 1B, lane 9). The 170-kDa protein bound toxin with the greatest intensity. Labeled toxin was removed from most of the proteins by competition with excess unlabeled toxin, with the possible exception of the 170-kDa protein, suggesting that cold toxin can compete with the radiolabeled toxin for binding (Fig. 1B, lane 10).

Toxin affinity of *H. virescens* BBMV and solubilized preparations. Previous data on *B. thuringiensis* toxin affinity have relied on the physical separation of toxin-bound BBMV from unbound toxin either by retention of BBMV through glass fiber filters or by centrifugation of BBMV (7, 13, 23). Since the *H. virescens* BBMV proteins are detergent solubilized during receptor purification procedures, it was imperative that a binding assay that could accommodate these solubilized proteins be developed. Attempts to utilize filtration through glass fiber filter assays (23) by using solubilized BBMV preparations were unsuccessful. Hence, the solid-phase binding assay described here was utilized. To ensure that results comparable to those

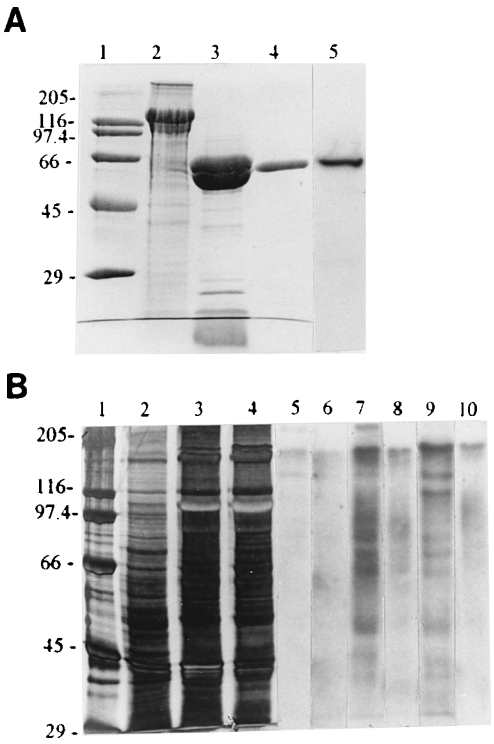

FIG. 1. (A) Purification and radiolabeling of CryIA(c) toxin. Lanes 1 through 4 were visualized with Coomassie blue staining. Lane 1, molecular mass standards (numbers on the left are kilodaltons); lane 2, *B. thuringiensis* subsp. *kurstaki* HD-73 crystal isolate; lane 3, trypsinized crystal; lane 4, FPLC-purified CryIA(c) toxin. Lane 5 contained 2×10^6 cpm of ^{125}I-CryIA(c) toxin and was visualized by autoradiography (30-min exposure). (B) SDS-PAGE and electroblot analysis of *H. virescens* protein midgut isolates. Lanes 1 through 4 were silver stained, and lanes 5 through 10 were blotted onto Immobilon and probed with 1 nM ^{125}I-CryIA(c) (lanes 5, 7, and 9) or with 1 nM ^{125}I-CryIA(c) and excess (500 nM) unlabeled toxin (lanes 6, 8, and 10). Lane 1 contained molecular mass standards (numbers on the left are kilodaltons); lanes 2, 5, and 6 contained 20 μg of midgut proteins; lanes 3, 7, and 8 contained 20 μg of BBMV; and lanes 4, 9, and 10 contained 20 μg of CHAPS-solubilized BBMV.

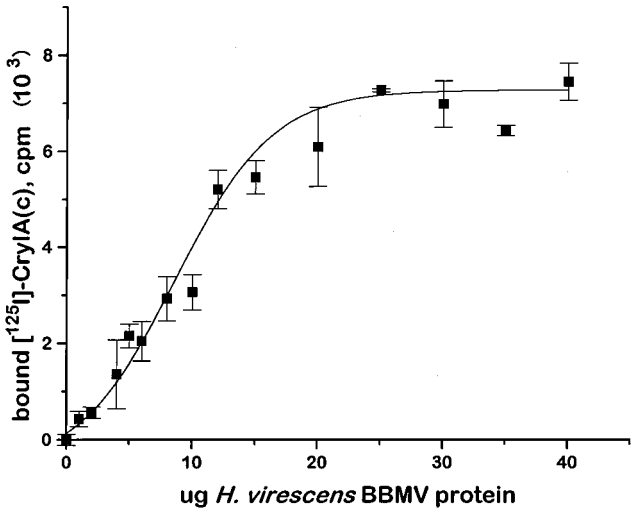

FIG. 2. Specific binding of ^{125}I-CryIA(c) toxin as a function of *H. virescens* BBMV concentration. Specific binding was calculated by subtracting binding in the presence of excess unlabeled toxin from total binding at each point. Labeled CryIA(c) (7,000 cpm), representing 15% of the total available counts, saturated 20 μg of BBMV proteins. In other experiments, a maximum of 25 to 30% of the available counts were bound to *H. virescens* BBMV. Each point represents the mean of triplicate samples ± standard deviation (see Materials and Methods for details).

reported in the literature were obtained, binding assays were initially performed with unsolubilized midgut and BBMV proteins. ^{125}I-CryIA(c) was observed to bind in a saturable manner (Fig. 2). When the assay was used in homologous competition experiments, decreasing amounts of the labeled CryIA(c) bound with increasing concentrations of the unlabeled toxin (Fig. 3). With crude midgut proteins, analyses using the LIGAND program (17) revealed a high-affinity binding site with a K_d of 5.0 nM and an R_t of 12.3 pmol/mg of midgut protein (Table 1). For BBMV, these values were 0.97 nM and 16.3 pmol/mg of BBMV protein, respectively. CHAPS-solubilized BBMV also gave very similar K_ds and R_ts and a good displacement curve (Table 1; Fig. 4). The analysis was also performed with *M. sexta* BBMV, and K_ds and R_ts of 0.78 nM and 2.85 pmol/mg of BBMV protein, respectively, were obtained (Table 1). The values for non-detergent-solubilized BBMV are very similar to those reported by other investigators (4, 6, 7, 13, 23).

Partial purification of CryIA(c)-binding protein by ion-exchange chromatography. In order to purify the CryIA(c)-binding proteins, various detergents were tested for their ability to solubilize BBMV. All the detergents solubilized 60 to 75% of the total protein. In order to distinguish between the utilities of the five detergents, toxin binding assays were performed. Samples solubilized with either Triton X-100 or *n*-octylglucoside gave anomalous results in that the labeled toxin could not be displaced by competition (Fig. 4A and B), even with 1,500-fold excess unlabeled CryIA(c). Only two steroid-type detergents, CHAPS and deoxycholate, provided meaningful toxin binding data, with K_ds of 0.74 and 0.68, respectively (Table 1). On the basis of the R_ts, a measure of the number of binding sites present, CHAPS was selected as the solubilization detergent of choice.

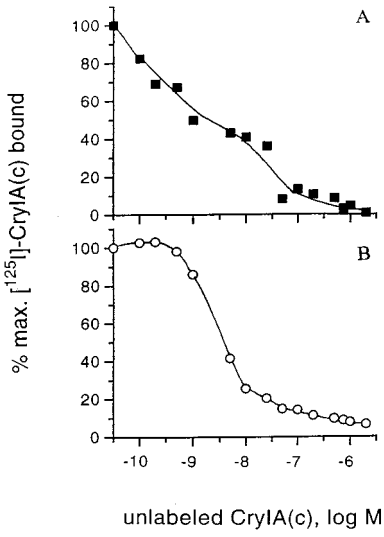

FIG. 3. Competitive binding of ^{125}I-CryIA(c) to *H. virescens* midgut protein (A) and BBMV proteins (B). *H. virescens* midgut and BBMV proteins (20 μg) were incubated with 1 nM ^{125}I-CryIA(c) toxin in the presence of unlabeled competitor at the indicated concentrations. The binding levels were calculated as percentages of the amount bound after incubation with the labeled toxin alone. Each point represents the mean of triplicate determinations. The counts per minute minus the background values for 100% binding for midgut and BBMV proteins were 7,380 and 11,480, respectively.

TABLE 1. Competitive binding data for *H. virescens* and *M. sexta* protein preparations

Protein source	K_d (nM)[a]	R_t (pmol/mg)
H. virescens		
Midgut homogenate	5.0 ± 4.0	12.3 ± 5.5
BBMV	0.97 ± 0.16	16.3 ± 9.1
Solubilized BBMV		
CHAPS	0.74 ± 0.33	19.2 ± 2.8
Deoxycholate	0.68 ± 0.50	6.5 ± 1.4
Triton X-100	ID	ID
Digitonin	ID	ID
n-Octylglucoside	ID	ID
MonoQ isolates		
Flowthrough	NB	NB
Pool I	ID	ID
Pool II	ID	ID
Pool III	0.65 ± 0.2	56.5 ± 1.4
M. sexta BBMV	0.78 ± 0.23	2.85 ± 0.21

[a] NB, no binding above background levels detected; ID, value could not be determined.

Following CHAPS solubilization, BBMV proteins were subjected to anion-exchange chromatography. Approximately 40% of the total protein did not bind the MonoQ matrix. The flowthrough fractions had little toxin binding during solid-phase analysis (Fig. 5). The bound proteins were eluted by using a linear 0 to 500 mM KCl gradient, and aliquots from each fraction were tested for their ability to bind labeled CryIA(c) (Fig. 5). On the basis of these solid-phase binding assay results, three groups, designated I (fractions 13 to 14), II (fractions 19 to 21), and III (fractions 26 to 29), were pooled and analyzed by SDS-PAGE and CryIA(c) binding.

All three pools contained a wide range of proteins (Fig. 6, lanes 2 through 4). When the pools were probed with ^{125}I-CryIA(c), proteins of 170 and 120 kDa (pool I, lane 5) and 140, 120, 90, 60, and 50 kDa (pool II, lane 6) were revealed. In pool III, proteins of 170, 130, 125, and 90 kDa bound toxin (Fig. 6, lane 7).

To determine the affinity of the various proteins observed to bind ^{125}I-CryIA(c) in toxin overlay assays (Fig. 6), the three MonoQ pools (I, II, and III) were further characterized in homologous competition binding assays using the solid-phase binding assay described above. Pool I, instead of displaying competition, showed apparent cooperativity; at very high cold-toxin concentrations (>100 molar excess), more radiolabeled CryIA(c) toxin was bound (Fig. 7A). Likewise, pool II displayed little competition, except at the highest unlabeled-toxin concentration tested (>100 molar excess) (Fig. 7B). Pool III, when analyzed in this manner, displayed high-affinity, saturable binding sites (Fig. 7C). The K_d of 0.65 nM for pool III is similar to that obtained for *H. virescens* BBMV, and this pool has an R_t of 56.5 pmol/mg of protein, a 3.5-fold purification from BBMV (Table 1).

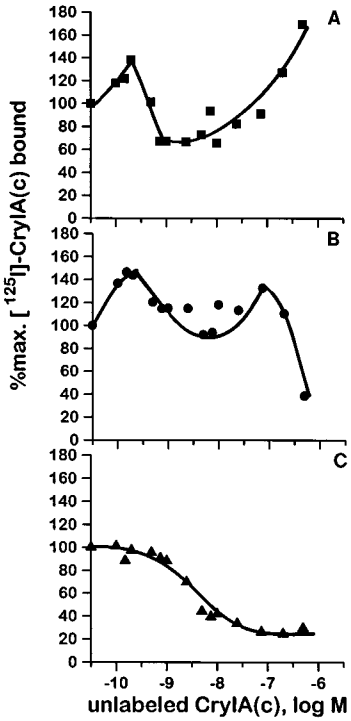

FIG. 4. Competitive binding of ^{125}I-CryIA(c) to detergent-solubilized BBMV. Solubilized BBMV (20 μg) were incubated with 1 nM ^{125}I-CryIA(c) in the presence of unlabeled competitor at the indicated concentrations. *n*-Octylglucoside-solubilized (A), Triton X-100-solubilized (B), and CHAPS-solubilized (C) BBMV were compared for toxin binding. Deoxycholate gave a displacement curve similar to that obtained with CHAPS. Each point represents the mean of triplicate determinations. The counts per minute minus the background values for 100% binding in panels A, B, and C were 1,390, 1,520, and 4,150, respectively. The background binding levels with all three detergents, about 3,000 cpm, were much higher than those with unsolubilized BBMV, about 1,000 cpm.

DISCUSSION

The initial step for the insecticidal activity of *B. thuringiensis* is believed to be the recognition of the toxin by highly specific binding proteins, or receptors, in the larval midgut. In previous studies (4, 11, 15, 16) and in this study, several proteins from *H. virescens* larval midguts were shown to bind CryIA(c) toxin by using a toxin overlay on proteins separated by SDS-PAGE and electroblotted onto nitrocellulose or Immobilon.

These toxin overlay assays, while ideal for identifying the number and molecular sizes of proteins that bind the toxin, do not readily distinguish between the relative toxin affinities of the various proteins that bind the CryIA(c) toxin. Moreover, it is difficult to assess the significance of toxin binding in these toxin overlay experiments because often the CryIA(c) toxin binds proteins that are abundant. For example, as shown in Fig. 1B and 6, the 170-kDa protein, which is an abundant protein, binds significant amounts of CryIA(c) toxin. Even when the BBMV samples are diluted, this protein consistently binds the CryIA(c) (data not shown). Hence, the significance of this 170-kDa protein in CryIA(c) toxicity is unclear. Indeed, the homologous competition experiments performed in this study (Fig. 7) suggest that this protein, which is enriched during anion-exchange chromatography, does not have any high-affinity binding sites. In contrast, the 140- and 120-kDa proteins, less abundant in BBMV preparations, could potentially be identified as having some specificity for the CryIA(c) toxin. These proteins have also been demonstrated by others (15) to bind the CryIA(c) toxin in *H. virescens*. High concentrations of these proteins, as observed in pool II, bind significant levels of radiolabeled CryIA(c), which are not readily displaced by cold toxin in homologous binding assays unless very high toxin concentrations are used. These data suggest that the 120- and 140-kDa proteins have some affinity for the toxin.

Thus, although toxin overlay analyses are easy to perform, they may not necessarily give a correct profile of the proteins involved in mediating *B. thuringiensis* toxicity. This may be

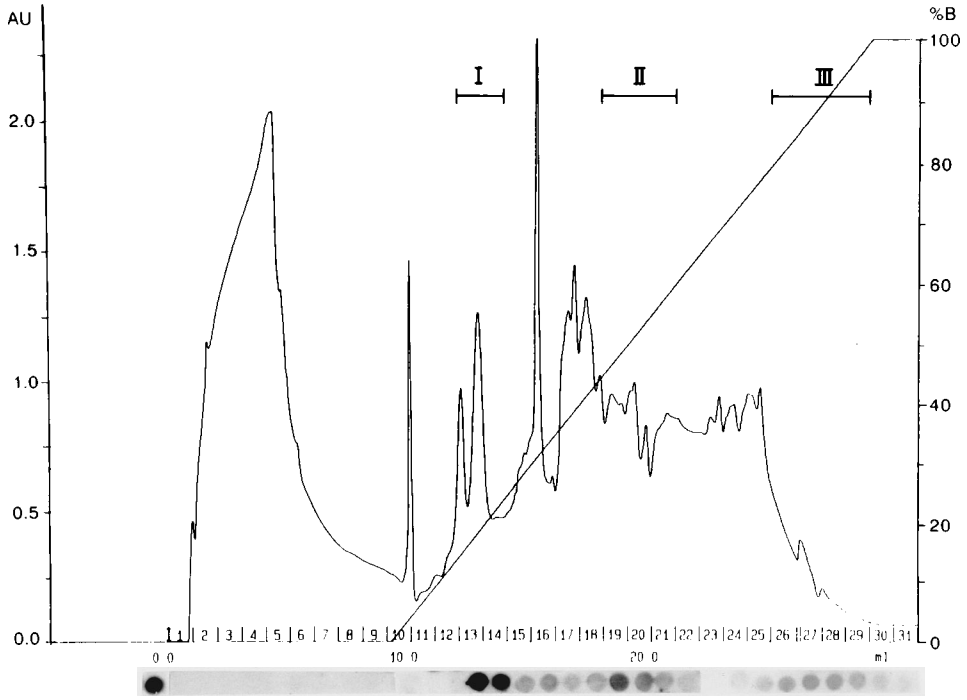

FIG. 5. SMART System MonoQ elution profile of CHAPS-solubilized BBMV. Twenty-five milligrams of solubilized BBMV was loaded onto a MonoQ 5/5 column and eluted with a linear KCl gradient from 0 to 500 mM. Five microliters of each 1-ml sample was spotted onto nitrocellulose and probed with 1 nM ^{125}I-CryIA(c). A sample of the column load was spotted at 0 ml. Bars indicate the pool assignments for the column isolates. AU, absorbancy units; %B, percent 500 mM KCl.

particularly important in insects, such as *H. virescens*, in which a number of proteins can potentially bind a single toxin. Hence, care has to be taken to identify the proteins that are likely to play an important role in toxicity rather than just displaying a toxin binding profile.

By using the solid-phase assay developed here, it was possible to obtain affinity parameters for CryIA(c) binding to midgut homogenates, BBMV, and detergent-treated BBMV, demonstrating the utility of the assay for BBMV and solubilized membranes. The K_ds and R_ts for non-detergent-solubilized BBMV are very similar to those reported by other investigators using either centrifugation or filtration assay methods (4, 13, 23, 24). However, unlike in earlier reported data (23, 24), a second lower-affinity binding site ($K_d \sim 100$ to 700 nM) is observed with some *H. virescens* preparations.

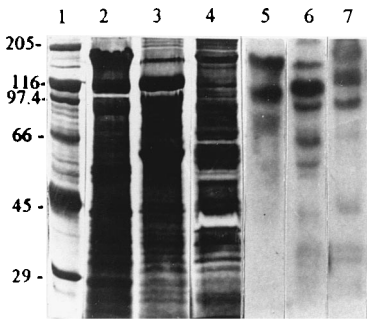

FIG. 6. SDS-PAGE and electroblot analysis of MonoQ column isolates. Lanes 1 through 4 were silver stained, and lanes 5 through 10 were blotted onto Immobilon and probed with 1 nM ^{125}I-CryIA(c) (lanes 5 through 7). Lane 1, molecular mass standards (numbers on the left are kilodaltons); lane 2, 20 μg of pool I; lane 3, 20 μg of pool II; lane 4, 20 μg of pool III; lanes 5, 6, and 7, 15 μg each of pools I, II, and III, respectively.

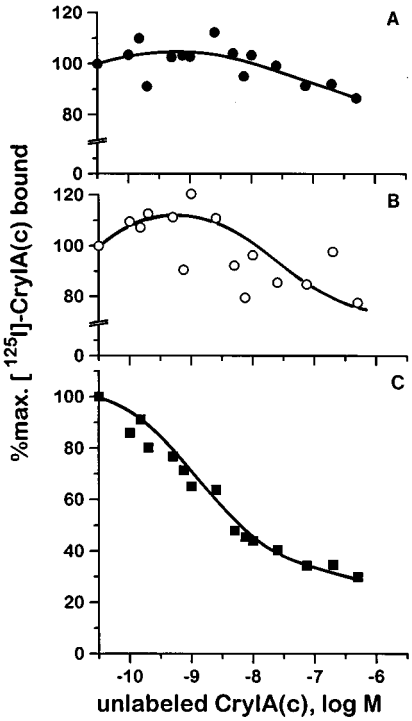

FIG. 7. Competitive binding of ^{125}I-CryIA(c) to the MonoQ isolates. Twenty micrograms of the MonoQ isolates was incubated with 1 nM labeled CryIA(c) in the presence of unlabeled competitor at the indicated concentrations. Pool I (A), pool II (B), and pool III (C) were compared. Each point represents the mean of triplicate determinations. The counts per minute minus the background value for 100% binding for panels A and B is about 1,000, while for panel C, the 100% binding value is 2,210 cpm.

The solid-phase assay was then used to partially purify protein fractions that have differing toxin affinity. Only CHAPS solubilization and, to a lesser extent, deoxycholate solubilization yielded functional binding proteins in adequate quantities. Other detergents, such as digitonin, Triton X-100, and *n*-octylglucoside, solubilized BBMV proteins but interfered with the solid-phase binding assay. The basis for this disruption is not evident. It is possible that membrane lipids or proteins modulate toxin-binding protein interactions. Hence, BBMV solubilization by these detergents denatures the protein-lipid complexes. Moreover, *n*-octylglucoside could interfere with toxin binding since the CryIA(c) toxin apparently binds carbohydrate moieties on membrane proteins (11).

The CryIA(c) binding activity of CHAPS-solubilized *H. virescens* BBMV separated into three distinct pools on MonoQ anion-exchange chromatography. Two of these pools did not display homologous competition. In pool I, increasing cold-toxin concentration resulted in increased toxin binding. Usually binding of this nature implies either the absence of binding-site saturation and/or cooperative binding, in which two or more membrane components could interact, allowing increased toxin binding. The specificity of the proteins could not be discerned, since unlabeled toxin could not displace the labeled toxin by competition, suggesting strongly that proteins in this pool bind the CryIA(c) toxin nonspecifically. In pool II, only very high toxin concentrations, greater than 10^{-7} M, caused some displacement in toxin binding, suggesting the presence of a relatively low-affinity binding protein. Pools I and II together contain the 170-, 140-, 120-, and 90-kDa proteins that are identified in toxin overlay assays as the main toxin-binding proteins.

In contrast to pool II, which binds the CryIA(c) toxin with low affinity, pool III has higher-affinity toxin-binding proteins with receptor-like characteristics similar to those observed with whole BBMV. A number of proteins were observed in this fraction, including proteins of 170, 120, and 90 kDa. Although proteins of similar sizes were identified in pools I and II, these proteins may not necessarily be the same as those isolated in pool III. Furthermore, it is also possible that proteins that show displaceable binding are not readily detected in toxin overlay experiments. We currently are using pool III to further purify CryIA(c) toxin-specific protein(s) in order to further elucidate the mode of action of *B. thuringiensis* CryIA(c) in *H. virescens* midguts.

The differences observed between the toxin overlay and the solid-phase assays using these three pools could in part be explained by the separation of protein complexes during anion exchange. These complexes could be composed of a number of proteins and/or protein-lipid complexes. Potentially high toxin affinity is observed only with protein complexes such as that observed in pool III. When these complexes are separated into individual components, as in pools I and II, lower-affinity binding is observed. Consequently, the toxin overlay assay is useful for identifying individual components within these complexes, whereas the solid-phase assay can be used to determine the affinity of individual proteins or protein complexes.

Although the solubilized *H. virescens* midgut BBMV proteins can be separated into distinct protein profiles, it is not clear whether similar separation will be observed with proteins from other insects. With *M. sexta*, in which only a single major protein is observed to bind the CryIA(b) and CryIA(c) toxins, it is possible that toxin overlay experiments and toxin affinity determinations could identify the same protein. Indeed, with the CryIA(b) toxin, a 210-kDa high-affinity binding protein is identified by toxin overlay assays (22). Recent results show that the CryIA(c) similarly binds a 120-kDa aminopeptidase N-like protein in *M. sexta* (9, 19).

Finally, although high-affinity toxin-binding proteins appear to be involved in *B. thuringiensis* action and in resistance development (3, 20, 25), a significant role for large numbers of low-affinity binding sites cannot be excluded. In at least one insect (26), the *B. thuringiensis* toxicity is not correlated with high-affinity binding sites. Moreover, CryIA toxin resistance development in *H. virescens* is not correlated with a change in the K_ds or R_ts (6, 13).

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