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Removal of Adsorbed Toxin Fragments That Modify *Bacillus thuringiensis* CryIC δ-Endotoxin Iodination and Binding by Sodium Dodecyl Sulfate Treatment and Renaturation

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We report that 10- and 25-kDa toxin fragments adhere to CryIC prepared from *Bacillus thuringiensis* insecticidal crystals, block iodination, and alter membrane binding. There is no apparent affect on CryIC toxicity against *Spodoptera exigua*. Associated peptides remained bound to CryIC in the presence of 50 mM dithiothreitol or 6 M urea. A novel detergent-renaturation procedure was developed for the purification of *B. thuringiensis* CryIC toxin. Sodium dodecyl sulfate (SDS) treatment followed by gel filtration chromatography yielded a homogeneous 62-kDa CryIC toxin. After removal of SDS and renaturation, the purified CryIC toxin was fully insecticidal to *S. exigua* larvae. ^{125}I-labeled CryIC bound with high affinity to brush border membrane vesicles from *S. exigua* larvae.

Bacillus thuringiensis produces insecticidal protein crystals made of subunits called δ-endotoxins. Of the numerous δ-endotoxins, the lepidopteran-active CryIC protein is particularly important because of its toxicity to *Spodoptera* species (16, 17, 27, 35). These insects are common agricultural pests which are not susceptible to most *B. thuringiensis* δ-endotoxins (37).

The CryI δ-endotoxins are protoxins of 130 to 140 kDa which are activated by trypsin-like enzymes in the insect midgut to 60- to 65-kDa toxins (1, 11, 14). CryI toxins bind with high affinity to proteins located in the midgut brush border membrane of susceptible insects (15, 33). For example, CryIA(c) toxin binds to a 120-kDa protein in *Manduca sexta* (13, 19). This 120-kDa protein was recently identified as aminopeptidase N (18, 28). Binding proteins enhance the ability of the toxin to form ion channels in midgut cells. A mixture of *M. sexta* aminopeptidase N and a 65-kDa toxin binding protein in phospholipid vesicles catalyzed toxin-induced pore formation 1,000-fold (28).

CryIC toxin recognizes different sites than the CryIA toxins (33). A 40-kDa protein was identified as a candidate CryIC receptor in *Spodoptera litoralis* (23, 26). The unique specificity of CryIC is apparent in insects that have acquired resistance to CryIA toxins. *Plodia interpunctella* (Indian meal moth) and *Plutella xylostella* (diamondback moth) resistant to CryIA toxins still bind and are killed by CryIC toxin (12, 34). In *P. xylostella*, resistance is reversible and correlated with the return of CryIA toxin binding sites (30).

Purified δ-endotoxin is the critical reagent in many *B. thuringiensis* experiments, and yet little is known regarding the effects of contaminating proteins. Previous reports mention peptides that copurify with toxins. Pfannenstiel et al. reported that 22- and 38-kDa peptide fragments were associated with *B. thuringiensis* subsp. *israelensis* toxin and were not removed after high-performance liquid chromatography (25). Schwartz et al. (29) found minor peptides associated with CryIC toxin prepared from *B. thuringiensis* crystals but not with CryIC produced in recombinant *Escherichia coli*. Van Rie et al. (33) extracted CryIC from recombinant *E. coli* and found that a contaminating small peptide blocked ^{125}I labeling. In this study, we found that 25- and 10-kDa peptides are tightly associated with CryIC toxin isolated from *B. thuringiensis*. These peptides were preferentially labeled with ^{125}I and caused nonspecific attachment to brush border membranes.

The primary goal of this work was to develop a method of purifying CryIC toxin that yields a homogeneous 62-kDa protein. The sodium dodecyl sulfate (SDS) treatment removed toxin-associated peptides, and the renaturation treatment restored binding and insect toxicity. The toxin-associated peptides were identified as N-terminal fragments of CryIC toxin. We also show that a ^{125}I-labeled 10-kDa peptide binds nonspecifically to *S. exigua* membrane vesicles and that adhering peptides modify the interaction of CryIC toxin with *S. exigua* brush border membrane.

MATERIALS AND METHODS

Biological materials. A *B. thuringiensis* strain harboring a single δ-endotoxin gene (*cryIC*) was provided by Ecogen, Inc. (Langhorne, Pa.). This strain was constructed by transferring a *cryIC* gene (16) on a self-replicating plasmid (2) into a crystal-negative *B. thuringiensis* host. *S. exigua* was obtained from the USDA/ARS Southern Field Crop Insect Management Laboratory (Stoneville, Miss.).

Bacterial growth and toxin isolation. *B. thuringiensis* was grown at 30°C in 1 liter of L broth until sporulation and cell lysis. The crystal-spore-debris mixture was centrifuged at 7,500 × *g* for 30 min, and the pellet was washed two times with distilled H_2O. The pellet was treated with 30 ml of 50 mM KOH–1% 2-mercaptoethanol for 30 min to dissolve crystals and then centrifuged at 27,000 × *g* to remove insoluble debris. Protoxin was precipitated by lowering the pH to 5.0 with HCl. The precipitate was recovered after centrifugation at 27,000 × *g* and then dissolved in 5 ml of 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS; pH 10.0). L-1-Tosylamide-2-phenylethylchloromethylketone (TPCK)-treated trypsin (5 mg) was added, and the mixture was incubated at room temperature for 15 min. The resulting toxin was precipitated at pH 5.0 and recovered by centrifugation at 27,000 × *g* for 30 min. This toxin is called $CryIC_{crude}$ ($CryIC_c$). The $CryIC_c$ precipitate was stored at −20°C in distilled H_2O.

Toxin was also prepared from crystals purified by buoyant

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TABLE 1. Insect toxicity of CryIC preparations

Toxin	Description	LC_{50}[a]
$CryIC_c$	Trypsin-activated toxin	186 (127–247)
$CryIC_p$	CryIC with 10- and 25-kDa peptides	192 (136–254)
$CryIC_s$	CryIC in 0.1% SDS	599 (426–1187)
$CryIC_r$	CryIC purified in SDS and then renatured	188 (109–261)
^{125}I-$CryIC_r$	Iodinated $CryIC_r$	200 (136–287)

[a] Expressed in nanograms per square centimeter; the 95% confidence intervals are in parentheses.

density gradient centrifugation using Renografin-76 (Squibb) (22). The spore-crystal pellet was sonicated in distilled H_2O and then washed two times in 1 M NaCl containing 0.1% Triton X-100. This suspension was layered on 64% Renografin and centrifuged at 43,000 × *g* for 1 h in a swinging-bucket rotor. The central region containing crystals was collected, washed, and further purified by using 60, 67% Renografin step gradients and the same centrifugation conditions. Crystals were washed two times in distilled H_2O, lyophilized, and stored dry at −20°C. Toxin was prepared from crystals by the procedure described above. Activation of toxin was done by using 1 mg of trypsin per 2 mg of protoxin in 1 ml of CAPS (pH 10.0).

Toxin purification methods. Toxin descriptions and designations are listed in Table 1. (i) $CryIC_{peptides}$ ($CryIC_p$) was prepared by dissolving $CryIC_c$ toxin in 50 mM CAPS (pH 10.0) and then separating it over a Sephacryl S-300 (Pharmacia) column (1.5 by 40 cm) equilibrated with the same buffer.

(ii) For anion-exchange chromatography, the $CryIC_c$ toxin was dissolved in 20 mM piperazine (pH 9.5) and applied to a Q-Sephadex (Pharmacia) column, and proteins were eluted with a 0 to 1 M NaCl gradient in 20 mM piperazine (pH 9.5).

The following treatments and purification steps were taken to remove adhering peptides from $CryIC_p$. $CryIC_p$ was precipitated at pH 5.0 and washed as described above for $CryIC_c$. (i) $CryIC_p$ was dissolved in 50 mM CAPS (pH 10.0) containing 50 mM dithiothreitol, incubated for 30 min, and then fractionated in the same buffer on a Superose 12 fast-performance liquid chromatography (FPLC; Pharmacia) column. (ii) $CryIC_p$ was dissolved in 6 M urea (pH 7.0), incubated at room temperature for 30 min, and then passed through the Superose column in the same buffer. Urea and dithiothreitol in the samples were removed by dialysis overnight against 50 mM CAPS (pH 10.0) at 4°C. (iii) $CryIC_p$ was dissolved in 50 mM CAPS (pH 10.0) containing 1% SDS, incubated at room temperature for 10 min, and then applied to the column equilibrated in 50 mM CAPS (pH 10.0) containing 0.1% SDS. Toxin eluted in 0.1% SDS is called $CryIC_{SDS}$ (abbreviated $CryIC_s$).

Purification and sequence analysis of 10- and 25-kDa peptides. During the purification of $CryIC_s$ on the Superose 12 column, 10- and 25-kDa peptides coeluted in a broad peak. The 10- or 25-kDa protein peak was pooled and then concentrated by lyophilization. The dried proteins were dissolved in distilled H_2O and rechromatographed on a Sephacryl S300 (Pharmacia) gel filtration column (1.5 by 40 cm) in 50 mM CAPS (pH 10.0) containing 0.1% SDS. The 10- and 25-kDa peptides which eluted in separate fractions were collected and then stored at −20°C for later analysis. For N-terminal amino acid sequence analysis, proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (21) using 10 to 20% polyacrylamide gels and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad) in 0.5× Towbin buffer (12.5 mM Tris, 96 mM glycine [pH 8.3], 10% methanol) (31). Blotted membranes were rinsed with distilled H_2O, stained with Ponceau S (Sigma) as described in the manufacturer's (Bio-Rad) instructions, and sequenced on an Applied Biosystems model 477A protein sequenator at Tufts University (Boston, Mass.).

Removal of SDS and renaturation of toxin. Toxin eluted in 0.1% SDS was treated as described by Zahler et al. (38). Four volumes of cold acetone (−20°C) were added to the $CryIC_s$ toxin and then placed at −20°C for 20 min. The resulting precipitate was collected by centrifugation at 27,000 × *g* for 20 min at 4°C. The pellet was dissolved in 6 M guanidine hydrochloride and dialyzed against 50 mM CAPS (pH 10.0) containing 5% (vol/vol) glycerol, 0.1 M NaCl, and 1 mM EDTA at 4°C overnight to renature the toxin. SDS-treated and renatured toxin is called $CryIC_{renatured}$ ($CryIC_r$). The 10-kDa peptide was treated by the same renaturation method used in the binding studies.

Iodination. To ^{125}I-labeled toxin, two Iodobeads (Pierce) were added to 100 μl of phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 2.7 mM KCl, 136.9 mM NaCl [pH 7.4]) containing 0.5 mCi of $Na^{125}I$ and allowed to react for 5 min at room temperature. Ten micrograms of toxin (in 50 mM CAPS [pH 10.0]) was added to the reaction vial. The mixture was incubated for 15 min at room temperature. The reaction solution was applied to a Sephadex G-50 (Pharmacia) column to remove free iodine. The radioactivity was measured on a Beckman Gamma 4000 counter. Specific activities were 8.8 μCi/μg of input toxin for SDS-purified toxin and 6.6 μCi/μg for the dithiothreitol- and urea-treated toxins.

Preparation of BBMVs. Brush border membrane vesicles (BBMVs) from *S. exigua* were prepared by the method of Wolfersberger et al. (36) as modified by Ferre et al. (12). Fifth-instar larvae were chilled on ice for 15 min. The midguts were excised in ice-cold MET buffer {0.3 M mannitol, 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], 17 mM Tris-Cl [pH 7.5]} and then frozen on dry ice. Frozen tissue was ground in a Potter-Elvenhem homogenizer in ice-cold MET buffer containing 0.1 mM phenylmethylsulfonyl fluoride. An equal volume of cold 24 mM $MgCl_2$ was added, and the mixture was incubated on ice for 15 min. The mixture was centrifuged at 2,000 × *g* for 10 min at 4°C, and the pellet was discarded. The supernatant was clarified by centrifugation at 2,000 × *g* for 10 min and then centrifuged at 27,000 × *g* for 30 min. The pellet was resuspended in MET buffer and stored at −80°C until use.

Membrane vesicle binding assays. Immediately before use in binding assays, BBMVs were thawed on ice and then centrifuged at 15,000 × *g* for 5 min. The storage buffer was replaced with PBS containing 0.1% bovine serum albumin (BSA). Binding assays were performed as described by Garczynski et al. (13).

Gel electrophoresis. CryIC toxin preparations (either 20 μg for gels to be stained with Coomassie blue or 100,000 cpm for ^{125}I-labeled proteins) were analyzed by SDS-PAGE (20). Gels were either stained with Coomassie brilliant blue R-250 or exposed for autoradiography. Coomassie blued-stained gels were scanned with a laser densitometer (Molecular Dynamics). Autoradiography was carried out at −80°C with Kodak XAR-5 film and an intensifying screen.

Insect bioassays. Toxin preparations were diluted with PBS containing 0.1% BSA. Eight toxin concentrations were tested with 24 neonate *S. exigua* larvae per concentration. Samples (50 μl) were applied uniformly to the diet surface and then allowed to dry. For toxin in 0.1% SDS, an equivalent amount of SDS was added to buffer controls. One larva was placed onto the diet surface and reared at 26°C. Insect mortality was

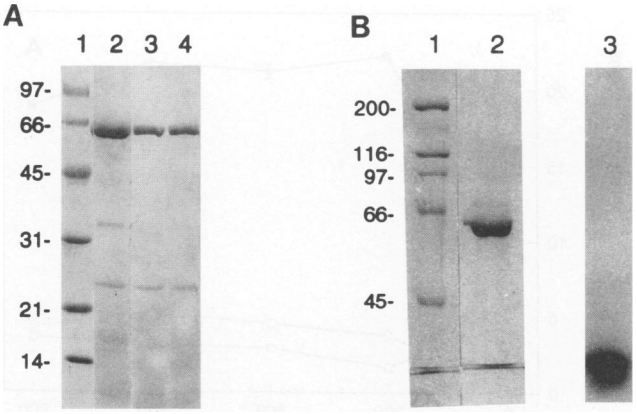

FIG. 1. SDS-PAGE of crude and partially purified CryIC. (A) CryIC prepared from a spore-crystal mixture. Lanes: 2, $CryIC_c$; 3, $CryIC_c$ passed through a Sephacryl S-300 gel filtration column in 50 mM CAPS (pH 10); 4, $CryIC_c$ applied to a Q-Sephadex ion-exchange resin and eluted with 0 to 1 M NaCl in 20 mM piperazine (pH 9.5). The 5 to 20% polyacrylamide gel was stained with Coomassie blue. (B) CryIC prepared from purified crystals. Lanes: 2, $CryIC_c$ after treatment in 50 mM dithiothreitol–50 mM CAPS (pH 10) and FPLC chromatography on Superose 12 gel filtration resin; 3, autoradiogram of the ^{125}I-labeled CryIC preparation shown in lane 2. The gel was 10% polyacrylamide. Molecular mass markers (in kilodaltons) are shown in lane 1 of both panels.

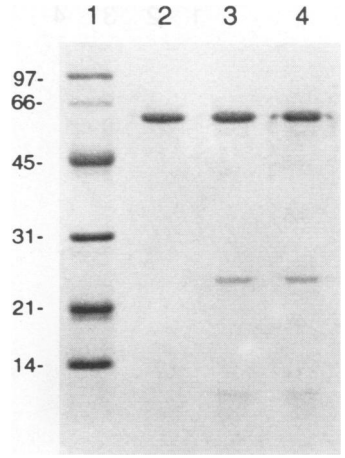

FIG. 2. Effect of various chemical treatments on the CryIC-peptide complex. Lanes: 1, molecular mass markers (in kilodaltons); 2, $CryIC_r$ (1% SDS treated and renatured); 3, $CryIC_p$ (50 mM dithiothreitol); 4, $CryIC_p$ (6 M urea). After the indicated treatment, proteins were fractionated by using FPLC and Superose 12 gel filtration. The 5 to 20% gel was stained with Coomassie blue.

scored after 7 days, and the results were analyzed by probit analysis (9).

Determination of protein concentration. The protein contents of BBMVs and CryIC toxin were measured as described by Bradford (4) with BSA as a standard. The concentration of CryIC in the presence of SDS or urea was determined with the bicinchoninic acid protein assay reagent (Pierce) with BSA as a standard. Toxin concentrations were identical when determined by both methods.

RESULTS

Partial purification of CryIC toxin. Protoxin was extracted from a spore-crystal mixture and then cleaved by trypsin to the expected 62-kDa protein and small peptides (Fig. 1A, lane 2). Crude $CryIC_c$ toxin was passed through a gel filtration or anion-exchange column. Instead of pure toxin, the 62-kDa toxin fractions had contaminating proteins of 25 and 10 kDa plus several other peptides (Fig. 1A, lanes 3 and 4). Because we could not iodinate the CryIC preparations shown in Fig. 1 (data presented below), we investigated methods to eliminate copurifying peptides. We were initially concerned that the adhering peptides were caused by our use of a spore-crystal mixture instead of purified crystals. Crystals were purified on Renografin density gradients, and CryIC toxin was prepared. Figure 1B (lane 2) shows the toxin derived from crystals after separation on a Superose 12 gel filtration column in 50 mM dithiothreitol. This column procedure was recommended previously for *E. coli*-derived CryIC as a means to eliminate peptides that block iodination (33). The small peptides migrated near the dye front on the SDS–10% polyacrylamide gel (Fig. 1B, lane 2), but their effects on iodination are clear (Fig. 1B, lane 3). When toxin was iodinated, the label was not attached to 62-kDa toxin but to the small peptide near the dye front. Use of a spore-crystal mixture was not a factor in toxin purity. These results showed that peptides adhered to CryIC toxin and blocked toxin iodination.

SDS treatment, identification of peptides, and CryIC renaturation. To identify a treatment that dissociated toxin from contaminating peptides, $CryIC_c$ was treated with 50 mM dithiothreitol, 6 M urea, or 1% SDS and then fractionated by gel filtration chromatography. Figure 2 shows a stained SDS-polyacrylamide gel of the toxin fractions. Neither dithiothreitol nor urea eliminated the 25- and 10-kDa peptides (Fig. 2, lanes 3 and 4). SDS treatment followed by gel filtration in the presence of 0.1% SDS resulted in a single protein of 62 kDa (Fig. 2, lane 2).

After separation from CryIC toxin in 0.1% SDS, the 25- and 10-kDa peptides were further purified and used to obtain a partial amino acid sequence. Both peptides were found to be N-terminal fragments of CryIC toxin. The first nine amino acid residues of the 25-kDa peptide matched residues 28 to 36 of the CryIC protoxin (10), while the 10-kDa N terminus corresponded to residues 50 to 58 of CryIC protoxin. We did not determine the first residue of trypsin-activated CryIC toxin, but the N terminus predicted by comparison with other CryI toxins is residue 29.

A drawback of SDS is its strong denaturant effect that destroys the function of many proteins. $CryIC_s$ had only 31% of the normal $CryIC_c$ toxicity to *S. exigua* larvae in bioassays (Table 1). Pfannenstiel et al. (24) reported a similar reduction in toxicity for SDS-treated *B. thuringiensis* var. *israelensis* δ-endotoxins. SDS can be removed from protein by a number of procedures. We circumvented this problem by a method in which SDS is stripped from the protein with guanidine and then the guanidine is removed by dialysis (38). This toxin preparation is designated $CryIC_{renatured}$ ($CryIC_r$).

When $CryIC_r$ was labeled with ^{125}I, a single 62-kDa peptide was visualized by autoradiography (Fig. 3). In contrast, preparations of CryIC with adhering peptides (both $CryIC_c$ and $CryIC_p$) had the ^{125}I residue attached to the 10-kDa peptide. On the basis of the relative intensities of the stained 10-, 25-, and 62-kDa bands seen in Fig. 2, a small amount of adhering peptide was sufficient to completely block iodination of CryIC toxin.

Functional assay of renatured CryIC. Renatured toxin, i.e., $CryIC_r$, was compared with native CryIC ($CryIC_c$ and $CryIC_p$)

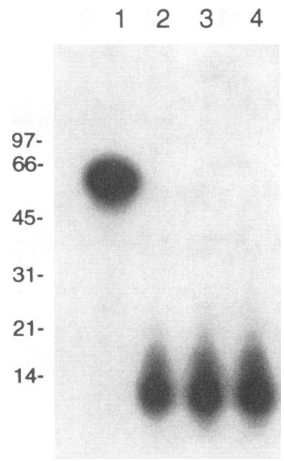

FIG. 3. Autoradiography of ^{125}I-labeled CryIC preparations. Lanes: 1, CryIC$_r$; 2, CryIC$_p$; 3; CryIC treated and purified in 6 M urea; 4, ^{125}I-labeled CryIC$_c$. Approximately 10^5 cpm was loaded per lane on an SDS–5 to 20% polyacrylamide gel. The autoradiogram was obtained after a 2-h exposure to film at −80°C. The positions of molecular mass markers (kilodaltons) are indicated on the left.

by the following tests: (i) bioassay of CryIC toxins against *S. exigua* larvae and (ii) ^{125}I-CryIC binding to *S. exigua* BBMV. The results presented below show that CryIC$_r$ was biologically active.

The results are presented in Table 1. If SDS was removed and toxin was successfully renatured, then the 50% lethal concentrations (LC_{50}) of CryIC$_c$, CryIC$_r$, and CryIC$_p$ should be equivalent. The data show that while the CryIC$_s$ LC_{50} value was threefold higher, renatured CryIC$_r$, CryIC$_c$, and CryIC$_p$ had nearly identical LC_{50} values. Also, iodination of CryIC$_r$ did not change its toxicity to *S. exigua*. These data demonstrate the biological activity of CryIC$_r$ and show that iodination did not decrease CryIC$_r$ toxicity significantly.

The next step in evaluating CryIC$_r$ was in binding assays with *S. exigua* BBMV. ^{125}I-CryIC$_r$ was added to increasing concentrations of *S. exigua* vesicles. Figure 4A shows maximal binding (23% of input ^{125}I-CryIC$_r$) at a vesicle concentration of 200 μg of protein per ml. The curve shape and extent of binding are characteristic of CryI toxins that bind saturably and with high affinity to receptor sites. Figure 4A also shows the results of a binding experiment using CryIC$_p$ (labeled at the 10-kDa peptide) as the ligand. The rationale was that if the 10-kDa peptide remained associated with toxin, CryIC$_p$ binding would be similar to CryIC$_r$ binding. Figure 4A shows that only 5% of the input label bound to BBMV. We purified and ^{125}I labeled the 10-kDa peptide and showed that it bound to the same extent as CryIC$_p$ (Fig. 4A). This interaction between CryIC, small peptides, and membranes was interesting and explored further.

Figure 4B shows the results of an experiment in which ^{125}I-CryIC$_r$ and the amount of vesicles were kept constant and increasing amounts of unlabeled CryIC$_r$ were added. CryIC$_r$ bound with high affinity, and binding was reduced by increasing amounts of unlabeled CryIC$_r$. Data were analyzed by the computer program LIGAND. The dissociation constant (K_d) for CryIC$_r$ was 4.8 nM, and the binding site concentration (B_{max}) was 2.1 pmol/mg of vesicle protein.

We next performed a heterologous binding experiment between ^{125}I-CryIC$_r$ and unlabeled CryIC$_p$. CryIC$_p$ reduced ^{125}I-CryIC$_r$ binding, but the calculated affinity was significantly

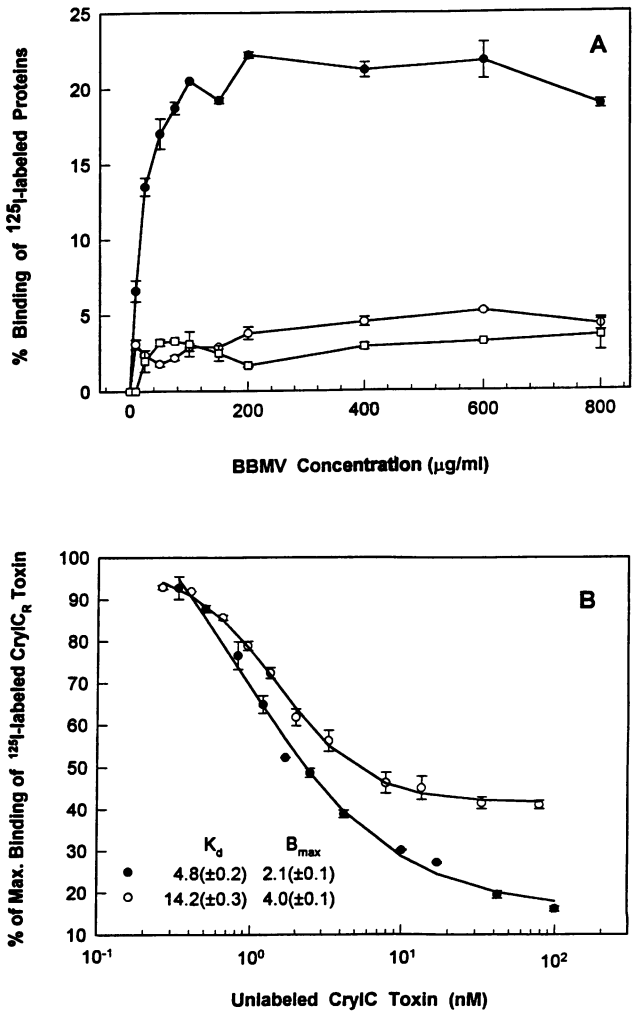

FIG. 4. CryIC binding to *S. exigua* vesicles. (A) Binding of ^{125}I-CryIC$_r$, and ^{125}I-labeled 10-kDa protein as a function of vesicle concentration. The indicated concentrations of vesicles were incubated with 0.1 nM ^{125}I-CryIC$_r$ (●), 0.1 nM CryIC$_p$ with attached ^{125}I-labeled 10-kDa peptide (○), or purified ^{125}I-labeled 10-kDa peptide (□). (B) Inhibition of ^{125}I-CryIC$_r$ binding by unlabeled CryIC preparations. Vesicles were incubated with 0.1 nM ^{125}I-CryIC$_r$ plus the indicated concentrations of unlabeled CryIC$_r$ (●) and CryIC$_p$ (○). The CryIC$_p$ preparation was 79% toxin as determined by scanning densitometry. Binding is expressed as a percentage of the maximum amount of toxin bound upon incubation with labeled toxin alone. The binding parameters (mean ± standard error) estimated by LIGAND analysis are presented in the inset. K_d is expressed in nanomolar concentrations, and B_{max} is expressed in picomoles per milligram of vesicle protein. For both panels, each point is the mean of duplicate samples. Standard error between samples is shown by bars.

lower than that with CryIC$_r$ (14.2 versus 4.8 nM). In contrast to the homologous preparation CryIC$_r$, CryIC$_p$ was not able to displace 40% of the bound toxin. The amount of CryIC$_r$ bound remained at 40% in the presence of 500 nM unlabeled CryIC$_p$. These data showed that adhering toxin fragments modified CryIC toxin interaction with vesicles. We also incubated CryIC$_p$ with associated ^{125}I-labeled 10-kDa peptide with vesicles and used unlabeled CryIC$_p$ as the competitor. As expected from the results in Fig. 4A, this complex showed a low level of

nonspecific binding, but the binding was not displaced by a 1,000-fold excess of unlabeled $CryIC_p$ toxin (data not shown).

In summary, $CryIC_r$ bound saturably and with high affinity to *S. exigua* BBMV. The K_d and B_{max} were close to those reported for high-affinity CryIC in *S. litoralis* (33). These binding data supported the bioassay results which showed $CryIC_r$ to be successfully renatured. A surprising observation was that the 10- and 25-kDa toxin peptides modified CryIC binding. Furthermore, labeled 10-kDa peptide bound nonspecifically to *S. exigua* vesicles.

DISCUSSION

This study describes a method to purify CryIC in SDS that removes adhering 10- and 25-kDa toxin fragments. These toxin fragments blocked iodination at tyrosine residues. How a 10-kDa toxin fragment with a single tyrosine residue (predicted from the *cryIC* DNA sequence) blocks labeling at all of the 12 to 13 tyrosine residues in the CryIC toxin is not clear. We found that increasing the $Na^{125}I$ concentration in the labeling reaction or using the chloramine-T method as described in reference 13 did not yield ^{125}I-CryIC (data not shown).

Contaminating peptides in CryIC preparations have been reported previously (29, 33). Van Rie et al. (33) concluded that peptides associated with CryIC (isolated from *E. coli*) impeded ^{125}I labeling. Those researchers (33) found that exposure to 50 mM dithiothreitol followed by gel filtration eliminated this difficulty. Neither dithiothreitol nor 6 M urea had any effect on our *B. thuringiensis*-derived toxin. The discrepancy between our results and those of Van Rie et al. (33) may be caused by several factors. One factor may be the different host organisms. For example, to isolate CryIC and investigate toxin-induced ion channels, Schwartz et al. (29) prepared CryIC from both *B. thuringiensis* crystals and from *E. coli* inclusions. Only CryIC prepared from *B. thuringiensis* crystals had contaminating peptides. Fermentation conditions may also be a factor in δ-endotoxin production. Culture media do not only have the obvious impact on yield but also seem to cause subtle changes in toxin chemistry. Recently, Bhattacharya et al. (3) showed that sugars are nonenzymatically attached to CryI proteins released into culture media. Those researchers (3) convincingly presented arguments as to how glycosylation can account for toxin aggregation and altered toxicity to insects.

SDS-treated and renatured $CryIC_r$ was active in insect and binding assays. The capacity of CryIC to refold to a biologically active state was remarkable but predictable. Choma and Kaplan (6) denatured CryIA(c) with guanidine hydrochloride and then restored toxicity to cultured cells by removal of guanidine hydrochloride. In our experiments, $CryIC_r$ bound with high affinity (K_d = 4.8 nM) to *S. exigua* vesicles. Van Rie et al. (33) measured ^{125}I-CryIC binding to *S. litoralis* BBMV. Analysis by the LIGAND program revealed high-affinity (K_d = 0.18 nM) and low-affinity (K_d = 13.9 nM) binding sites (33). Our data do not eliminate the possibility of multiple CryIC sites in *S. exigua*. The slight upward deflection of the $CryIC_r$ binding curve (Fig. 4B) at 10 nM could be interpreted as being due to a population of low-affinity binding sites. Assays using different CryI toxins are necessary to aid in choosing between one- and two-site models of CryIC binding to *S. exigua*.

The distinct binding characteristics obtained with unlabeled $CryIC_r$ and $CryIC_p$ were dramatic. Substituting $CryIC_p$ with adhering peptides for $CryIC_r$ decreased the affinity (K_d) from 4.8 to 14.2 nM. Also, a 1,000-fold excess of $CryIC_p$ did not displace 43% of the ^{125}I-$CryIC_r$. We obtained identical results with a second independent set of vesicles and toxin preparations (data not shown). Three plausible explanations for these results follow. (i) $CryIC_r$ binding fits a two-site model in which $CryIC_p$ competes for the low- but not the high-affinity site. This pattern is observed with CryI toxins that have some, but not all, midgut sites in common (32, 33). (ii) Adhering 10- and 25-kDa toxin fragments change the affinity of CryIC for the receptor. (iii) The nonspecific interaction of 10- and 25-kDa sub-toxin peptides with the membrane alters $CryIC_r$ binding.

Amino acid sequencing revealed that the 10- and 25-kDa peptides originated from the CryIC molecule. The peptides correspond in molecular size to known proteolytic fragments of CryIA toxins. CryIA toxins are cleaved under conditions of excess trypsin, or while partially denatured, to peptides of 30 to 34 kDa (5, 7, 8). Intensive proteolysis of *B. thuringiensis* subsp. *alesti* CryIA δ-endotoxin leads to the release of 10-kDa peptides from the toxin molecule (5). These peptides were hydrophobic and formed stable aggregates (5). We observed CryIC toxin to be particularly susceptible to overdigestion by trypsin (unpublished data). Our observation that the 10-kDa toxin fragment binds nonspecifically to BBMV is not surprising since it originates from the hydrophobic domain predicted to enter the membrane upon insertion of toxin (21).

The purification-renaturation method presented here will be useful in the preparation of CryIC toxin and may have further applications for other classes of δ-endotoxins. The method further demonstrates the structural stability of CryI toxins. While this study showed that adhering sub-toxin peptides modify CryIC labeling and membrane binding, adhering peptides probably affect other in vitro and possibly in vivo toxicity of CryIC δ-endotoxin.

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