A Specific Binding Protein from Manduca sexta for Bacillus thuringiensis subsp. berliner*

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Biopesticides based on the bacterium Bacillus thuringiensis have attracted wide attention as safe alternatives to chemical pesticides. In this paper, we report, for the first time, the identification and purification of a single binding protein from a lepidopteran insect, Manduca sexta, that is specific for a cryIA toxin of B. thuringiensis. The purified protein appeared as a single band of 210 kDa on a two-dimensional gel, had a pI of approximately 5.5, and stained with Schiff's reagent. The band material was sensitive to proteolytic digestion and was rich with acidic amino acids, indicating its protein nature. Radiolabeled toxin bound to the protein with a Kd value of 708 pm and could be specifically blocked by unlabeled toxin but not by toxins from other subspecies of B. thuringiensis. This study lays the groundwork to clone the toxin binding protein and to determine the molecular mechanism(s) of toxin action.

The Gram-positive spore-forming bacterium Bacillus thuringiensis synthesizes an intracellular parasporal glycoprotein crystal(s) during the sporulation cycle. The glycoprotein, which is insecticidal, is a protoxin that is activated after ingestion by an insect susceptible to the toxic product. For most glycoprotein crystals, the protoxins are processed proteolytically to yield smaller toxic components in the alkaline midgut of the insect. There is a variety of subspecies of B. thuringiensis that exhibit highly specific toxic activity against lepidopteran (moth), dipteran (mosquito), and coleopteran (beetle) larvae. Considerable nucleotide sequence information is available on what has been termed cry (crystal protein) genes of B. thuringiensis which encode a family of 13 related insecticidal proteins (cry proteins). These genes are divided further into four major classes and several subclasses depending primarily on the insecticidal spectra of the encoded proteins. Previous in vitro studies on the mechanism of toxicity have revealed a variety of symptoms including paralysis of the insect gut, disruption of midgut epithelial cell structure and function, and cessation of feeding followed by death (4, 5). Inhibition of K⁺-dependent amino acid transport has been observed when brush-border membrane vesicles (BBMV) isolated from insect midgut, were incubated with activated toxins (6, 7). Formation of pores and colloid osmotic lysis of midgut epithelial cells also has been proposed as a possible mechanism of toxin action (8). If the toxin forms pores, it is not known whether the toxin does so by interacting with a protein present in the BBMV or by insertion into the BBMV. More recent studies have shown the presence of high affinity binding sites in BBMV proteins prepared from susceptible insects (9, 10). van Rie et al. (11) using radioligand binding assays demonstrated a correlation between toxicity and toxin binding to insect BBMV. Using ligand blotting, Oddou et al. (12) demonstrated binding of different cry toxins to specific binding proteins (170, 140, and 120 kDa) in Heliothis virescens BBMV proteins. Binding of cryIA(c) toxin to a 120-kDa protein in Manduca sexta BBMV was also reported (13, 14).

Although there are several preliminary investigations on the mechanism of action of various B. thuringiensis toxins and the identification of their putative receptors, one of the biggest impediments to our understanding of both the mode of toxicity and the specificity of these toxins is the almost complete lack of information about the nature of the insect target receptor(s). In the present study, we have identified, purified, and partially characterized a protein of 210 kDa to which the cryIA(b) toxin of B. thuringiensis subsp. berliner binds in BBMV of M. sexta. Previously, we first reported the sequence (15) of the cryIA(b) gene of B. thuringiensis subsp. berliner, which is considered the holotype sequence for the gene (3). The 210-kDa protein, present in BBMV of M. sexta (tobacco hornworm), was absent in BBMV of Leptinotarsa decemlineata (Colorado potato beetle, a coleopteran) and Aedes aegypti (mosquito, a dipteran) BBMV. Significantly, the latter two insects lacking the 210-kDa protein in BBMV were not susceptible to the cryIA(b) toxin.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions—B. thuringiensis subsp. berliner was the gift of M.-M. Lecadet, Institut Pasteur, Paris. Growth, sporulation, and parasporal crystal purification were as described by Tyrell et al. (16). B. thuringiensis subsp. tenebrionis and israelensis were obtained from R. M. Faust, Beltsville Agricultural Research Center, Beltsville, MD. Subspecies tenebrionis was grown in liquid broth medium, and crystals were purified using buoyant density centrifugation in 68% Renografin as described previously (17). Crystal protein dissolved in 68% Renografin, whereas spores and debris sedimented at the bottom of the gradient. The dissolved crystal protein was recrystallized by dialyzing against water containing 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF).

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1The abbreviations used are: BBMV, brush-border membrane vesicles; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride (Immobilion-P membrane).
at 4 °C. The recrystallized protein was washed and stored in water at 4 °C. Growth of subspecies israelensis and purification of its parasporal crystals were as described by Hurley et al. (18).

**Toxin Purification**—Purified crystals from *B. thuringiensis* subsp. *berliner* were solubilized at 37 °C for 2 h in 50 mM Na2CO3 buffer (pH 10) containing 5 mM dithiothreitol. Solubilized crystals were dialyzed against 100 mM NH4HCO3 (12). The solubilized crystals were centrifuged at 80,000 g for 1 h at 4 °C. The resulting 60-kDa polypeptide was further purified on fast protein liquid chromatography using an anion exchange column (9). Activated and purified toxins from *B. thuringiensis* subsp. *berliner*, *tenebrionis*, and *israelensis* are referred to hereafter as cryIAb, cryIIA, and cryVd toxins (3), respectively. The amount of toxic protein was determined using Bradford Reagent method.

**Radioiodination**—The cryIAb toxin was radioiodinated using the chloramine-T method (23). Ten μg of cryIAb was iodinated with 100 μCi of chloramine T and 5 μl of Na125I (0.5 mCi) in 100 μl of phosphate-buffered saline (PBS). The reaction mixture was shaken gently at room temperature for 30 s, and the reaction was stopped by the addition of 50 μl of 4% sulfosalicylic acid. The iodinated toxin was removed by gel filtration on a Sephadex G-50 column equilibrated with PBS. Specific activities of the labeled toxin varied from 10 to 15 mCi/μg.

**Preparation of BBMV**—*M. sexta* eggs were purchased from Carolina Biological Supply Co., and the larvae were reared on an artificial diet also obtained from Carolina Biological Supply Co. *L. decemlineata* eggs were kindly provided by J. Kemp, New Mexico State University, and the larvae were reared on potato plants. *Aedes aegypti* larvae were provided by G. Hunt, Arthropod-Borne Disease Research Laboratory, Agricultural Research Service, United States Department of Agriculture, Laramie, WY. BBMV from third- and fourth-instar larvae of *M. sexta*, *L. decemlineata*, and *A. aegypti* were prepared according to the method described by Wolfsberger et al. (7) except that 1 mm PMSF was added to the buffers. The final pellet was resuspended in 150 mM NaCl, 2.7 mM KCl, and 9.5 mM NaH2PO4 buffer containing 0.5 mM Na2EDTA and 0.5 mM cysteine, 0.5 mM Na2EDTA, and 0.5 mM sodium azide) and mixed for 1 h at 4 °C. Sepharose beads were pelleted and washed four times with dilution buffer and finally rinsed with TBS. The binding complex was dissociated from the beads by heating in SDS-solubilization buffer, and the binding protein was visualized by ligand blotting with cryIAb toxin as described in the legend to Fig. 6.

**Ligand Binding Assays**—Binding assays were performed as described by Hofmann (9). For competition binding assays, duplicate samples of BBMV (10 μg) were incubated with 0.31 nM 125I-cryIAb toxin or CrylAb binds to the BBMV containing 1 μM PMSF and 1 μM Nonidet P-40. Solubilized proteins were diluted with 0.5× binding buffer containing 0.5 mM EDTA, and the sample was mixed for 2 h at 4 °C. Sepharose beads were pelleted and washed four times with dilution buffer and finally rinsed with TBS. The binding complex was dissociated from the beads by heating in SDS-solubilization buffer, and the binding protein was visualized by ligand blotting with cryIAb toxin as described in the legend to Fig. 6.

**Toxin Binding Protein**—A combination of immunoprecipitation and two-dimensional gel electrophoresis, as described by Wada et al. (28), was used to further purify the cryIAb toxin binding protein. Brush-border membrane vesicles (5 mg/ml) in buffer A containing 1 μM PMSF were incubated with 20 μg of unlabeled cryIAb toxin for 20 min at 37 °C to saturate the toxin binding sites. After incubation, the suspension was chilled on ice, washed with a buffer containing 50 mM Tris-HCl, 250 mM NaCl, 1 mM PMSF, 6 mM EDTA, 10 μM aprotinin, 2 μM leupeptin, 2 μM pepstatin A, and 2 μM antipain (buffer B). Washed crylAb saturated BBMV then were solubilized by stirring in buffer B containing 5% Nonidet P-40 at 4 °C for 1 h, and the insoluble material was removed by centrifugation at 13,000 rpm for 30 min at 4 °C. The supernatant was precleared with cryIAb antiserum and protein A-Sepharose beads. Precleared supernatant was incubated with 2 μl of cryIAb antiserum to the crylAb antigen and 5 μl of protein A-Sepharose beads and the sample was mixed for 1 h at 4 °C. The protein A beads were pelleted and washed four times with buffer B containing 0.5% Nonidet P-40 and 0.02% SDS. The binding protein-crylAb complex was eluted from the beads by heating the sample in 2% sample buffer for 2 min at 100 °C. The extract was mixed with 5 volumes of 9 M urea, electrophoresed in 10-cm SDS polyacrylamide gels and then analyzed by two-dimensional gel electrophoresis according to O'Farrell et al. (29). Isoelectric focusing was done in the first dimension in a 4% acrylamide gel containing 8 M urea, 2% Nonidet P-40, and 2% 3.5-10 ampholytes. Electrophoresis was carried out in the second dimension in an SDS-polyacrylamide (7%) slab gel. Binding protein present in the two-dimensional gel was visualized by ligand blotting with 125I-cryIAb toxin after transferring the protein in the gel to PVDF.

**Amino Acid Analysis**—Multiple two-dimensional gels were run and the 210-kDa protein was transferred to PVDF membranes (30), stained with Coomassie Blue, and the protein spots were excised from the membranes. The 210 kDa binding protein spots (0.3 μg) were hydrolyzed with 6 N HCl for 35 min at 155 °C under vacuum. Amino acid composition was determined after high performance liquid chromatography by the O-phthalaldehyde method (31) using a C18 reverse-phase column. Blank spots from the same membranes were excised and hydrolyzed under similar conditions, and the amino acid values from the blank spots were used to correct the amino acid composition of the 210-kDa binding protein.

**Protease Digestion**—60 μg of BBMV protein was incubated with four different proteolytic enzymes (trypsin, proteinase K, endoproteinase Glu-C, and papain) from Sigma. The digestion was carried out at 0.5 mg/ml at room temperature for 4 h. Buffers used for trypsin and proteinase K digestion were prepared according to Hofmann et al. (27). Papain digestion was carried out in 2.5 mM sodium phosphate buffer containing 0.5 mM cysteine, 0.5 mM Na2EDTA, and 0.5 mM

**Radioligand Blotting**—Gel electrophoresis was carried out according to Laemmli (25). Fifty μg of BBMV proteins were solubilized in sodium dodecyl sulfate (SDS)-solubilization buffer and electrophoresis was carried out at 4 °C. BBMV proteins then were transferred to Immobilon-P membranes (PVDF) in 25 mM Tris-HCl, 192 mM glycine, and 20% methanol buffer at 100 mA for 16–18 h at 4 °C in a Bio-Rad Trans-Blot apparatus. Lanes containing molecular weight markers were stained with Amido Black, and lanes containing BBMV proteins were incubated at room temperature for 4 h in TBS (10 mM Tris, 0.9% NaCl) containing 5% non-fat dry milk powder, 0.5% Tween 20, and 0.02% sodium azide (pH 8.0) (blocking buffer). The blots were washed with 1× TBS for 90 min at room temperature (5 min of blocking buffer containing 125I-cryIAb toxin (1× 106 cpm). The strips then were washed four times with blocking buffer (20 min/wash) and finally rinsed with TBS, dried, and exposed to Kodak X-OMAT AR x-ray film at −70 °C for 1–2 days. For competition experiments, strips were incubated in 0.5 ml of the blocking buffer containing 1× 106 cpm of 125I-cryIAb toxin along with an appropriate concentration of unlabeled toxin as stated in the appropriate figure legends. The position of radioactive bands was determined from the autoradiogram and the amount of bound 125I-cryIAb toxin was determined by excising the radioactive bands and measuring the radioactivity in a Beckman γ counter.

**Immunoprecipitation of Ligand Binding**—BBMV proteins transferred and blocked as described above were incubated overnight in 5 ml of blocking buffer containing unlabeled cryIAb toxin (1 μg/ml). Unbound toxin was removed by washing with PBS. Bound toxin was immunchemically visualized as described previously (26). The primary antibody was prepared in rabbits by injecting the purified active 60-kDa toxin in a Freund's Complete adjuvant (FCA) and Freund's Incomplete adjuvant (FIA). The antibody specificity of anti-cryIAb antiserum was confirmed by Western blotting of cryIAb toxin.

**Immunoprecipitation of cryIAb Binding Protein**—Immunoprecipitation was carried out according to Oddou et al. (12). Three μl of cryIAb antiserum were added to 100 μl of protein A-Sepharose CL-4B equilibrated in dilution buffer (TBS containing 0.1% Nonidet P-40, 5 mM EDTA, and 0.02% sodium azide) and mixed for 1 h at 4 °C. After washing three times with dilution buffer, cryIAb toxin (10 μg in 100 μl) was added, and the mixture was incubated for an additional hour at 4 °C and then washed again three times with dilution buffer. BBMV proteins (100 μg) were solubilized in 100 μl of TBS containing 1 mM PMSF and 1% Nonidet P-40. Solubilized proteins were diluted with dilution buffer containing 100 μl of protein A-Sepharose beads linked to cryIAb toxin, and the sample was mixed for 2 h at 4 °C. Sepharose beads were pelleted and washed four times with dilution buffer and finally rinsed with TBS. The binding complex was dissociated from the beads by heating in SDS-solubilization buffer, and the binding protein was visualized by ligand blotting with cryIAb toxin as described in the legend to Fig. 6.
RESULTS

Specific Toxicity of *B. thuringiensis* Toxins—To demonstrate the specific toxicity of the three different toxins of *B. thuringiensis*, we bioassayed *M. sexta*, *L. decemlineata*, and *A. aegypti*. As can be seen in Table 1, each toxin was specific for only one insect. No toxicity was exhibited by cryIA(b) toxin against *L. decemlineata* and *A. aegypti* at the highest concentration used in the bioassay (2.5 × 10^4 ng/ml) for both insects. Thus, the toxin was not toxic to *M. sexta* at 100 ng/ml, *aegypti* at the highest concentration tested (500 ng/cm^2 and 2.5 × 10^4 ng/ml, respectively). The cryVD toxin was not toxic to *M. sexta* and *L. decemlineata* at the highest concentrations used (500 ng/cm^2 and 2.5 × 10^4 ng/ml, respectively). Because of these extreme specificities, we used the cryIIIa and cryVD toxins as controls in our ligand binding and blotting experiments as well as BBMV proteins from *L. decemlineata* and *A. aegypti* in the ligand blotting experiments.

Binding of cryIA(b) Toxin to BBMV Proteins—Competitive binding of 125I-cryIA(b) toxin to BBMV proteins of *M. sexta* was performed in the presence of increasing concentrations of unlabeled cryIA(b), cryIIIA, and cryIVD toxins. Forty percent of 125I-cryIA(b) toxin bound to BBMV proteins in the absence of competitor. Unlabeled cryIA(b) toxin was capable of blocking the radioactively labeled toxin, reaching a plateau at 40 nM (Fig. 1A). This result indicates that the binding was specific and saturable. Scatchard plot of the binding data revealed a single high affinity binding site with a Kd value of 1.075 pm and a binding site concentration of 3.162 pmol/mg of BBMV protein. The cryIIIA and cryIVD toxins did not compete for the binding site of the cryIA(b) toxin. Consistent with these data, binding of the 125I-cryIA(b) toxin increased linearly with increasing concentrations of BBMV proteins from 100 to 1000 μg/ml (Fig. 1B). The Kd value for 125I-cryIA(b) was similar to that of the natural toxin (Table 1), demonstrating that the radioiodinated toxin is fully active.

Identification of 125I-cryIA(b) Binding Protein by Radioligand Blotting—BBMV proteins of *M. sexta* were blotted onto PVDF, and a binding protein was identified by incubating the membrane with 125I-cryIA(b). Under both reducing (Fig. 2, lane 1) and nonreducing (Fig. 2, lane 2) conditions, cryIA(b) toxin exclusively bound to a protein band of 210 kDa. The profile of stained BBMV proteins of *M. sexta*, *L. decemlineata*, and *A. aegypti* was quite broad (Fig. 3A, lanes 2-4). Molecular sizes ranged from greater than 200 kDa for *M. sexta* and *L. decemlineata* to much less than 30 kDa for all three insects. Binding of 125I-cryIA(b) toxin was observed only in *M. sexta* BBMV protein preparations (Fig. 3B, lane 2) but not in BBMV proteins prepared from *L. decemlineata* and *A. aegypti* (Fig. 3B, lanes 3 and 4). The major protein that recognized the cryIA(b) toxin is approximately 210 kDa (see arrow in Fig. 3B, lane 2). The smaller bands that were faintly labeled with the toxin probably are degradation products resulting from endogenous proteases in the BBMV. This particular preparation was several days old, whereas the preparation used in Fig. 2 was only a few minutes old.

**Table 1**

| Toxin   | *M. sexta* | *L. decemlineata* | *A. aegypti* |
|---------|------------|-------------------|--------------|
| cryIA(b) | 7.5 ng/cm^2 | –                 | –            |
| 125I-cryIA(b) | 10 ng/cm^2 | 10 μg/ml          | –            |
| cryIIIA | –          | 10 μg/ml          | –            |
| cryIVD  | –          | –                 | 180 ng/ml    |

LIC0 (LC50) = lethal concentration that kills 50% of the insect population; negative values represent no toxicity at the concentrations indicated under "Results." Values of *B. thuringiensis* cry toxins
FIG. 1. Binding of $^{125}$I-cryIA(b) toxin to BBMV proteins of M. sexta larvae. A, BBMV of M. sexta (100 µg/ml) were incubated with 0.31 nM $^{125}$I-cryIA(b) toxin in the presence of unlabeled cryIA(b) (open squares), cryIIIA (closed squares), and cryIVD (open circles) toxins. A Scatchard plot of the data is shown in the inset. B, $^{125}$I-cryIA(b) toxin (3.1 nM) was incubated with increasing concentrations of BBMV proteins in the presence of 100 nM unlabeled cryIA(b) toxin. Each point represents the mean of duplicate samples with a standard error of mean less than 2.25.

FIG. 2. Binding of $^{125}$I-cryA(b) toxin to protein blots of M. sexta BBMV proteins. Solubilized BBMV proteins were separated by SDS-polyacrylamide gel electrophoresis (10%), transferred to an Immobilon-P membrane, and the membrane was incubated with $^{125}$I-cryA(b) toxin. Dried membrane strips were subjected to autoradiography. Lane 1, sample prepared under reducing conditions; lane 2, sample prepared under nonreducing conditions. The position of reduced standards are indicated on the left: myosin (205 kDa), β-galactosidase (116 kDa), rabbit muscle phosphorylase b (97 kDa), bovine plasma albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa).

pH gradient and has an estimated pl of 5.5 ± 0.1 and an apparent molecular mass of 210 kDa. The purified 210-kDa binding protein stained with Schiff's reagent (data not shown), suggesting glycosylation of the binding protein.

Amino Acid Analysis—Table II shows the amino acid composition of 210-kDa spots blotted from two-dimensional gels to PVDF membranes. Because of the possibility of contamination during the purification steps, three blank spots of identical size to the 210-kDa spots on the PVDF membranes were excised, hydrolyzed, and analyzed. The resulting amino acid compositions were approximately 10% of the amino acid compositions of the 210-kDa spots.
FIG. 4. Ligand blotting of M. sexta BBMV proteins with 125I-cryIA(b) in the absence and presence of unlabeled cryIA(b), cryIVD, and cryIIIA toxins. Lanes containing 50 μg of BBMV proteins were blotted and probed with 0.25 nM of 125I-cryIA(b) toxin alone (lane 1) or 125I-cryIA(b) toxin containing 2, 8, 16, 33, and 83 nM of unlabeled cryIA(b) toxin (lanes 2-6, respectively) and 83 nM of cryIVD and cryIIIA toxins (lanes 7 and 8). A Scatchard plot of the binding observed in lanes 1-5 is shown in the inset. Molecular size markers are the same as those in Fig. 2.

FIG. 5. Ligand blot of M. sexta BBMV proteins with unlabeled cryIA(b) toxin. Fifty μg of BBMV proteins were blotted and incubated as described under "Materials and Methods." Detection of cryIA(b) binding protein was carried out using polyclonal antisera to the 60-kDa activated cryIA(b) toxin of B. thuringiensis subsp. berliner. Protein markers on the left are the same as in Fig. 2.

For the composition of the sample, and therefore, the blank spot values were used to correct the amino acid values of the 210-kDa protein spots. The binding protein had a high content of acidic amino acids (Asp, Glu) and hydrophobic amino acids (Ala, Val, Leu, Ile). No attempts were made to estimate the amounts of cysteine and tryptophan.

Protease Treatment of M. sexta BBMV Proteins—BBMV proteins from M. sexta were treated with trypsin, proteinase K, papain, and endoproteinase Glu-C, subjected to SDS-polyacrylamide gel electrophoresis, and then ligand-blotted with 125I-cryIA(b) (Fig. 9). Lane 1 shows the control band pattern when BBMV proteins were kept at room temperature for 1 h without any enzyme treatment. Lane 1 contained several protein bands, including the 210-kDa band. As was indicated above in the BBMV competition experiments (Fig. 3), we believe that the lower molecular mass components probably are proteolytic products of the 210-kDa protein resulting from the action of endogenous proteases present in the BBMV. All four enzymes destroyed the ability of the 210-kDa protein to bind the 125I-cryIA(b) toxin. Trypsin and papain digestion (lanes 2 and 4) resulted in binding the cryIA(b) toxin to three lower molecular mass polypeptides (50-65 kDa). Upon digestion with endoproteinase Glu-C, three polypeptides of mass ranging from 60 to 120 kDa bound to the 125I-cryIA(b) toxin (lane 5). Proteinase K completely destroyed the binding of 125I-cryIA(b) toxin to M. sexta BBMV proteins (lane 3).

FIG. 6. Immunoprecipitation of the cryIA(b) toxin binding protein. The cryIA(b) binding protein was precipitated by adding protein A-Sepharose-anti-cryIA(b) antisera toxin complex to solubilized M. sexta BBMV proteins. The precipitate was dissolved in SDS-solubilization buffer, and the dissociated proteins were analyzed by SDS-polyacrylamide gel electrophoresis. Detection of the binding protein was carried out by ligand blotting with both anti-cryIA(b) toxin antiserum (lane 1) and 125I-cryIA(b) toxin (lane 2).
The gel was stained with Coomassie Brilliant Blue R-250. Protein solubilized proteins were analyzed on a 10% SDS-polyacrylamide gel. The munoprecipitated cryIA(b) binding protein complexes. The markers on the left are the same as in Fig. 2.

Sepharose beads by heating in 2% SDS sample buffer, and the solubilized proteins were analyzed on a 10% SDS-polyacrylamide gel. The CryIAW binding protein complexes were dissociated from protein A- beads with 2% SDS sample buffer, heated for 2 min at 100 °C, and noprecipitated cryIA(b)-binding protein complexes. The protein-cryIA@) toxin complex was eluted from protein A-Sepharose mixed with urea solution as described under "Materials and Methods." The first dimension was isoelectric focusing in a 3.5-10 pH gradient 4% acrylamide gel containing 2% ampholytes, and the corresponding 210-kDa band was cut from the gel, eluted, and subjected to amino acid analysis. Values shown are the mean of two analyses corrected relative to the blank values.

Table II

| Amino acid | Mol % |
|------------|-------|
| Asp        | 14.6  |
| Glu        | 13.9  |
| Ser        | 2.3   |
| His        | 1.3   |
| Gly        | 7.1   |
| Thr        | 8.0   |
| Arg        | 6.9   |
| Ala        | 8.2   |
| Tyr        | 3.7   |
| Met        | 1.9   |
| Val        | 9.1   |
| Phe        | 5.2   |
| Ile        | 7.6   |
| Leu        | 10.3  |

Fig. 7. SDS-polyacrylamide gel electrophoresis of the immunoprecipitated cryIA(b) binding protein complexes. CryIA(b)-binding protein complexes were dissociated from protein A-Sepharose beads by heating in 2% SDS sample buffer, and the solubilized proteins were analyzed on a 10% SDS-polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R-250. Protein markers on the left are the same as in Fig. 2.

Fig. 8. Two-dimensional gel electrophoresis of the immunoprecipitated cryIA(b)-binding protein complexes. Binding protein-cryIA(b) toxin complex was eluted from protein A-Sepharose beads with 2% SDS sample buffer, heated for 2 min at 100 °C, and mixed with urea solution as described under "Materials and Methods." The first dimension was isoelectric focusing in a 3.5-10 pH gradient 4% acrylamide gel containing 8 M urea, 2% ampholytes, and 2% Nonidet P-40. The second dimension was polyacrylamide gel electrophoresis in a 7% slab gel. A, the gel was stained with Coomassie Blue, and the corresponding 210-kDa band was cut from the gel, eluted, and subjected to amino acid analysis. B, a duplicate gel was blotted to PVDF and probed with 125I-cryIA(b) toxin. The membrane was washed, dried, and autoradiographed for 24 h. Protein markers are the same as in Fig. 2.

DISCUSSION

A first step in insecticidal action of B. thuringiensis toxins is thought to be their recognition of target molecules. Existing evidence indicates that the target molecules are within or on the surface of BBMV of epithelial cells which line the midgut of a susceptible insect. We have identified and purified a single protein of 210 kDa in BBMV of M. sexta that specifically recognizes and binds the cryIA(b) toxin of B. thuringiensis subsp. berliner. Trypsin-activated cryIA(b) toxin bound to M. sexta BBMV saturaably, and the binding was displaceable only with unlabeled cryIA(b) toxin and not with cryIIIA or cryIVD toxins. The Aequorea 97-(lane 1) contained untreated BBMV proteins that were maintained at room temperature for 1 h. Protein markers are the same as in Fig. 2.

Fig. 9. Digestion of M. sexta BBMV proteins with various proteases. BBMV proteins (50 μg) were digested with trypsin (lane 2), protease K (lane 3), papain (lane 4), and endoproteinase Glu-C (lane 5). Samples were blotted and probed with 125I-cryIA(b). Lane 1 contained untreated BBMV proteins that were maintained at room temperature for several days (results not shown). Because the 210-kDa protein recognized and bound the cryIA(b) toxin under both reducing and nonreducing conditions, it is not a disulfide-linked oligomer. The 210-kDa toxin binding molecule was susceptible to proteolysis and, therefore, is likely to be a protein. Degradation occurred in preparations that were allowed to stand for only 1 h at room temperature. Similar degradation occurred in membrane vesicles that were stored at −70 °C for several days (results not shown).

Binding of the cryIA(b) toxin to the 210-kDa protein was observed (Fig. 5) when BBMV proteins were ligand blotted with unlabeled cryIA(b) toxin, and the binding complex was visualized using anti-cryIA(b) antiserum. Immunoprecipitation with the same antiserum and subsequent ligand blotting with cryIA(b) toxin also showed binding of the toxin to the
210-kDa protein (Fig. 6). The radioligand binding and blotting experiments, taken together with immunochemical detection, unequivocally demonstrate that the 210-kDa protein is an exclusive binding protein for the cryIA(b) toxin. This binding is toxin-specific because it could be blocked by unlabelled cryIA(b) toxin but not by other cry toxins. Furthermore, the binding is species-specific for M. sexta but not for A. aegypti and L. decemlineata.

Protease digestion of the cryIA(b) binding protein produced interesting results (Fig. 9). Trypsin and papain treatment yielded polypeptides of 50–65 kDa in parallel to disappearance of the 210-kDa protein, suggesting a precursor-product relationship. Interestingly, the putative 50–65-kDa fragment was capable of recognizing the toxin. Therefore, the toxin binding site is likely to be located in this fragment. It has been reported that treatment of Pieris brassicae (cabbage white butterfly) and H. virescens (tobacco budworm) BBMV proteins with trypsin did not affect the ability of cryIA(b) toxin to bind to BBMV or to a protein in protein blots (12, 28). Endoprotease Glu-C treatment produced binding fragments of 120, 100, and 60 kDa with concurrent disappearance of the 210-kDa protein, indicating the presence of susceptible aspartic acid or glutamic acid residues. The amino acid composition of the protein purified by two-dimensional gel electrophoresis revealed an abundance of these two amino acids (Table II). Protease K treatment resulted in the total loss of toxin binding, probably due to the exhaustive digestion of the cryIA(b) binding protein.

We have demonstrated that M. sexta contains within its midgut a 210-kDa protein which is capable of binding the cryIA(b) toxin of B. thuringiensis subsp. berliner but not the cryIIIa and cryIVD toxins of B. thuringiensis subsp. tenebrionis and B. thuringiensis subsp. israelensis, respectively. This result signifies that the insect host range specificity of B. thuringiensis toxins is predicated on receptor-mediated differences in the midgut of the insect as well as on properties of the toxins themselves. Whether the 210-kDa protein of M. sexta binds other cryI toxins is not known. Other investigators (13, 14) have reported that the cryIA(c) toxin of B. thuringiensis subsp. kurstaki binds to a 120-kDa polypeptide of M. sexta BBMV and to polypeptides of various sizes in other lepidopteran insects. It has been reported (12) that cryIA(a) and cryIA(b) toxins bind to a protein of the same size (170 kDa) and that cryIA(c) toxin binds to two proteins of smaller size in H. virescens BBMV. Also, it has been reported (10) that the cryIA(b) toxin can compete with the cryIA(c) toxin for the same binding site in H. virescens BBMV. Apparently, there are different binding proteins, depending on the insect species, for cryI toxins of B. thuringiensis. In some instances, cryI toxins can compete for the same binding site. Such molecules appear to have binding sites for both toxins. Also, it is possible that distinct binding proteins form noncovalent complexes in BBMV and become capable of recognizing various toxins competitively. Consequently, any lack of competition in blotting experiments (12) could be due to separation of these proteins into individual subunits.

To our knowledge, we are the first to report the purification of a single insect BBMV protein that specifically binds the cryIA toxin of B. thuringiensis. Whether the 210-kDa protein reported herein exclusively binds the cryIA(b) toxin is not known. Oddou et al. (12) observed that a 170-kDa protein in H. virescens BBMV bound a cryIA(b) toxin from B. thuringiensis subsp. kurstaki. Possibly, the cryIA(b) binding proteins of M. sexta and H. virescens are related but not identical. Obviously, the cryIIIa and the cryIVD toxins do not bind the 210-kDa protein of M. sexta, and therefore, we are investigating further the biochemical properties of this protein to gain a better understanding of its binding specificity and, eventually, to determine the molecular basis of insecticidal action of the cryIA(b) toxin of B. thuringiensis subsp. berliner.

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