Identification of *Bombyx mori* Midgut Receptor for *Bacillus thuringiensis* Insecticidal CryIA(a) Toxin

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As part of a study of the mechanism by which *Bacillus thuringiensis* insecticidal crystal protein acts, a *Bombyx mori* receptor to the CryIA(a) toxin specific for lepidopterans was examined. Histological examination showed that the toxin acted on the brush-border membrane of the midgut columnar cells and broke its infolding structure, causing cell lysis. The membrane vesicles were purified, and a 175-kDa protein binding the toxin was found that accounted for some 0.015% of membrane proteins. The protein, designated BtR175, was a glycoprotein that reacted with concanavalin A. Anti-BtR antibodies inhibited the binding of toxin to membrane vesicles in vitro and decreased the effect of the toxin to silkworms in vivo. BtR175, although found in the gut, was not found in fat bodies, integument, or silk glands. These results indicated that BtR175 was the receptor protein for the insecticidal toxin. Proteins (137 and 107 kDa) binding the CryIA(a) toxin also were found in the gut membranes of *Tenebrio morio* larvae, a coleopteran not sensitive to the toxin. The specificity of the toxin could not be explained only in term of the existence of its binding protein.

Key words: *Bacillus thuringiensis*; biopesticide; toxin; silkworm; receptor

*Bacillus thuringiensis* (Bt) toxin is the most widely used microbial pesticide, harmless to vertebrates and plants. The pest-specific toxicity arises from a crystalline inclusion comprised of one or more polypeptides (called insecticidal crystal protein, or ICP; also, δ-endotoxin) produced in the sporulating cells. When ingested by susceptible insects, the inclusions dissolve in the larval intestinal lumen, undergo limited proteolysis, and release smaller toxic polypeptide fragments with the N-terminal half as the toxin,2,3 that lyse midgut epithelial cells. The ICPs (and their toxic fragments) from different *B. thuringiensis* strains vary in their toxicity against different insects and have been classified into four classes, CryI (specific for lepidopterans), CryII (specific for lepidopterans and dipterans), CryIII (specific for coleopterans), and CryIV (specific for dipteran). The structural differences of many ICPs have been extensively investigated by cloning of their genes. Several insect-resistant transgenic plants have been generated by the introduction of truncated crystal protein genes.6 The insect factor that governs the specificity of Bt toxins seems to be specific toxin-binding sites, or receptors, in insect guts. Toxin binding to them in the brush-border membrane (BBM) vesicles of the columnar cells of the insect gut has been detected.9 However, some findings from insects resistant after the long-term use of Bt toxin cannot be explained by decreased binding of the toxin to the membranes.10 Several investigations to identify receptor molecules for Bt ICPs were done by a ligand-blotting assay that is based on toxin binding to denatured BBM vesicle proteins separated by SDS-PAGE and blotted on a membrane filter. The Bt CryIA(c) toxin binds to 120-kDa aminopeptidase N, a major BBM vesicle protein.7,8 CryIA(b) toxin binds to a 210-kDa cadherin-like protein in BBM vesicles from *Manduca sexta* midguts.9 CryIA(a) toxin binds to a 120-kDa aminopeptidase N from *Bombyx mori* midguts.10 However, it is not evident that these proteins are the main (high-affinity) toxin-binding proteins on intact microvillus membranes. Are these putative receptors actually involved in the insecticidal action of Bt toxins in vivo? Evaluation of the mechanism of specificity is important if safe toxins are to be identified and if toxin-resistant pests are to be managed.

In this study, we identified a *Bombyx mori* midgut membrane protein (designated BtR175) that binds to CryIA(a) toxin, a native BBM vesicle protein.

Materials and Methods

*Bacillus thuringiensis* and preparation of insecticidal protein. The *B. thuringiensis* used was subspp. *sotto* strain T84Al, which produces a toxin specific for lepidopterans. The nucleotide sequence of the gene encoding the ICP has been identified,11 and the deduced amino acid sequence (1180 residues) was identical with that reported for the subspp. *sotto* protein2 classified as a CryIA(a). The minimal unit with toxicity was prepared by tryptic digestion and purified as described previously.2 The toxin consisted of 590 amino acid residues (residues 29–618 of the crystal protein; molecular weight, 66,214) and had the apparent molecular weight of 58,000 by SDS-PAGE.

Silkworms. The silkworms used in this study were *Bombyx mori* kinshu × shouwa (Kanebo Silk Elegance Co., Ltd., Kasugai, Japan).

Electron microscopy. For investigation of the histological changes caused by the toxin, excised midgut tis-
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Proteins from silkworm larva midguts. The midgut was removed and washed with saline. The tissues were disrupted by sonication and homogenized in 9 vol. of 0.25 M sucrose and 5 mM EDTA in 5 mM Tris-HCl buffer, pH 8.1, at 25 W (25 MHz) for 15 sec. After removal of tissue pieces by passage of the mixture through four layers of wet surgical gauze, the filtrate (containing about 35% of total proteins in the tissue) was centrifuged at 1000 × g for 10 min, giving precipitate 1 (P1). The supernatant was further fractionated by successive centrifugation, at 6000 × g for 15 min, at 27,000 × g for 30 min, and at 152,000 × g for 60 min, giving precipitate fractions P2, P3, and P4, and the final supernatant, S. For further purification, P3 was resuspended in the same buffer above and centrifuged at 6000 × g for 15 min, and BBM vesicles were pelleted by centrifugation of the supernatant at 27,000 × g for 30 min.

For preparation of crude membranes from the whole midguts, minced tissues were homogenized in 9 vol. of 5 mM Tris-HCl buffer, pH 7.4, containing 5 mM EDTA (TE buffer) in a Waring blender. After passage of the mixture through four layers of wet surgical gauze, the filtrate was centrifuged at 15,000 × g for 30 min. The precipitate was washed three times with TE buffer by resuspension and centrifugation.

Protein and enzyme assays. Proteins in cell fractions were precipitated in 10% (w/v) trichloroacetic acid, dissolved in 1 N NaOH, and assayed by the method of Lowry et al. with bovine serum albumin as the standard. The concentrations of the toxin and rabbit IgG were assayed by their absorbance at 280 nm with coefficients of 1.43 and 1.35 for 1 mg/ml, respectively. Leucine aminopeptidase activity was assayed at pH 8.0 and 30°C with L-leucyl-β-naphthylamide as the substrate. Succinate dehydrogenase activity was assayed as described by Green et al. Acid phosphatase activity was assayed at pH 4.5 and 30°C with 4 mM p-nitrophenyl phosphate as the substrate in the presence of 0.15% Triton X-100 and 0.2 mg/ml bovine serum albumin. The enzyme reaction was stopped by the addition of 0.3 vol. of 1 N NaOH and the amount of p-nitrophenol released was measured at 405 nm. NADH-ferricyanide reductase activity was assayed at pH 7.5 with 0.1 mM NADH and 0.22 mM K3[Fe(CN)6] in the presence of 1 mM KCN and 0.5 mg/ml bovine serum albumin by measurement at 420 nm and reference to a solution without NADH.

I-labeling of outer surface of BBM vesicles. Proteins from the BBM vesicles were labeled with I25I. One milliliter of the reaction mixture in PBS contained vesicles (10 mg of protein), 3.6 mg of glucose, 1 μg of glucose oxidase (Sigma), 25 μg of lactoperoxidase (Sigma), and 3.7 MBq of Na25I (529 MBq/mg, Amer sham). In 30 min of incubation at 28°C, the reaction was stopped with 2-mercaptoethanol (final concentration, 0.05%), and the membrane vesicles were pelleted by centrifugation at 15,000 × g for 4°C for 30 min. The precipitate was washed five times with PBS containing 3 mM cold NaI by repeated suspension and centrifugation.

Solubilization of membrane proteins and affinity precipitation of toxin-binding proteins (BtRs) with the toxin and anti-toxin antibodies. Proteins in membrane preparations were made soluble in PBS containing 2% Triton X-100 (usually 3.0 ml per 25 mg of membrane protein) at 4°C for 1 h. Insoluble substances were removed by centrifugation at 27,000 × g for 30 min twice, and the second supernatant was incubated at 4°C with the toxin (5 μg and either crude membranes from 1 g of tissue or the subcellular fraction from 3 g of tissue) for 4 h. Then rabbit anti-toxin IgG purified by ammonium sulfate fractionation and DEAE-cellulose chromatography (2.4 mg/5 μg of toxin) was added and incubation was continued for 16 h. The solution was put on a cushion containing PBS with 1 M sucrose and 2% Triton X-100 and centrifuged at 1500 × g for 10 min, and the immunoprecipitate was washed twice with PBS containing 2% Triton X-100.

Purification of BtRs. Complexes of BtRs with the toxin and antibodies (some 28 mg of protein) obtained from the crude membrane preparation (18.2 g of protein from 520 g of midgut tissue) were dissolved, denatured, and reduced at room temperature in 3.5 ml of 0.2 M Tris-HCl buffer, pH 8.6, containing 6 M guanidine hydrochloride, 5 mM EDTA, and 20 mM dithiothreitol for 1 h. The sample was incubated for 30 min with monooiodoacetic acid (26 mg in 236 μl of 0.5 N NaOH) for carboxymethylation and then put on a Sepharose CL-4B column (1.5 cm × 98 cm) equilibrated with 0.1 M Tris-HCl buffer, pH 8.0, containing 6 M guanidine hydrochloride and 5 mM EDTA. BtRs were eluted at near a Ve/Vo (Ve, elution volume; Vo, void volume) of 1.3, the mixture of the toxin and IgG heavy chains were eluted at a Ve/Vo of 2.15, IgG light chains were eluted...
Fig. 1. Electron Micrographs Showing Morphological Changes in Midgut Epithelial Cells of *Bombyx mori* Larvae Fed the Bt CryIA(a) Toxin. Bars, 5 μm.

(a) Section from normal midgut tissue of a fifth-instar larva. The midgut epithelium is a single layer of cells (goblet cells, GC, and columnar cells, CC, joined by the septate junctions) resting on a thin basal lamina with an underlying discontinuous muscle layer (basal parts of the epithelium are not visible). The goblet cells have a large apical cavity formed by infolding of the plasma membrane, surrounded by a thin layer of cytoplasm filled with mitochondria. The nucleus is in the basal part of the cell. The columnar cells have an apical brush border of microvilli (MV), and the apical cytoplasm contains many mitochondria. The columnar cell nucleus (N) is near the center of the cell. Some irregularly shaped protrusions (IP) from CC are seen (inset). (b and c) Sections from a midgut excised 5 min after feeding of the toxin (40 ng/g larva). Bud-like protrusions (B) have formed from the apical plasma membrane of columnar cells. The coat membranes of some protrusions are broken and the contents are leaking out. Vacuoles (V) have formed in the apical cytoplasm of columnar cells. (d) Section from a midgut excised 15 min after toxin feeding. Many bud-like protrusions (B) without anything seen inside are seen. The density of the cytoplasm in the apical and lateral parts were low. The nucleus (N), mitochondria, and cell contact regions seem intact.
at a Ve/Vo 2.34, and oxidized dithiothreitol was eluted at a Ve/Vo of 2.81. Fractions containing BtRs were pooled and dialyzed exhaustively against distilled water to yield a protein precipitate.

**Lectin-binding assay.** BtRs separated by SDS-PAGE were electroblotted on a polyvinylidene difluoride filter membrane with 25 mM Tris-glycine buffer, pH 8.3, with a semidry blotting apparatus. The filter was treated with PBS containing 10 mg/ml bovine serum albumin and then with 3 µg/ml lectins conjugated with horseradish peroxidase (Seikagaku Kogyo). Bound lectins were detected with 3,3'-diaminobenzidine and H2O2.

**Results and Discussion**

**Morphological changes in midgut epithelial cells of Bombyx mori larvae fed B. thuringiensis toxin**

Histopathological studies21,22 of Bt toxin showed swelling of the midgut epithelium of larvae fed the toxin. These results indicated that the insect gut is the primary target of the toxin. In this study, we examined by electron microscopy the early and fine morphological changes in the midgut of Bombyx mori larvae fed a small amount of the toxin. The midgut epithelium from normal larva was shown in Fig. 1a. The first sign observed was the formation of bud-like protrusions on the apical membrane of columnar cells (Fig. 1b). The protrusions contained dense material but clear structures were not visible inside them. One such material was actin, detected by immunoelectron microscopy (data not shown). Some columnar cells (Fig. 1c) had several vacuoles in the apical cytoplasm that seemed to be oriented toward bud-like protrusions that already burst (see rents in the membrane and the low density on the inside), suggesting that vacuolization in the cytoplasm was a secondary event. These findings were seen as early as 5 min after the toxin was ingested. By 15 min, the insides of many bud-like structures looked empty and the density of the cytoplasm was low, particularly in the apical and lateral parts (Fig. 1d). Even at this stage, the nucleus, mitochondria, and cell contact regions did not show any morphological changes when compared with normal larvae (Fig. 1a). We observed some protrusions irregular in shape in the normal cells, which nevertheless looked intact (Fig. 1a, inset). While columnar cells were undergoing changes, goblet cells kept their original size and shape.

These results indicate that the toxin acted primarily on the apical membrane (the microvillus or brush-border membrane) of columnar cells of the insect gut.

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**Fig. 2.** Purification of Brush-Border Membrane (BBM) Vesicles from B. mori Midgut and Finding of Toxin-binding Proteins (BtRs) in the Vesicles.

(a) Distribution of proteins and marker enzyme activities in subcellular fractions. The apical surfaces of columnar cells were disrupted by brief sonication of small pieces of midgut tissues in a buffer. After removal of pieces of tissue by filtration through surgical gauze, membranes and organelles still in the filtrate were fractionated into precipitate fractions P1-4 and the final supernatant fraction S by centrifugations at 1000 x g for 10 min, at 6000 x g for 15 min, at 27,000 x g for 30 min, and at 152,000 x g for 60 min. Enzyme activities in the fractions assayed as markers of subcellular organelles were succinate dehydrogenase (SDH) for mitochondria, leucine aminopeptidase (LAP) for BBM, acid phosphatase (Acid-P) for lysosomes, and KCN-resistant NADH-ferricyanide reductase (NADH-Fe3+) for endoplasmic reticulum. Proportions of proteins, SDH, and LAP released into the lysate as a percentage of the amount or activity in the entire tissue were 34.9%, 40.0%, and 43.7%, respectively. (b) SDS-PAGE of BtRs in the subcellular fractions. Proteins from fractions P1-4 derived from 3 g of tissue were made soluble with Triton X-100. 70-75% of proteins in the fractions became soluble. P1, 5.88 mg of protein; P2, 8.34 mg of protein; P3, 6.48 mg of protein; P4, 1.52 mg of protein and fraction S (derived from 0.6 g of tissue; 21.94 mg of protein) were incubated with 5 µg of the toxin at 4°C for 6 h and then proteins forming complexes with the toxin were precipitated by incubation with 2.4 mg of anti-toxin IgG at 4°C for 16 h. The washed immunoprecipitates were analyzed by SDS-PAGE on a 7.5% gel. Lane C, immunoprecipitate from the reaction between 5 µg of the toxin and 2.4 mg of anti-toxin IgG as described above. IgG-H denotes heavy chains of IgG. BPB indicates the position of bromophenol blue.
Finding of BtRs on brush-border membranes of columnar cells and the mechanism of the binding of the toxin to the membrane

The Bt toxin is highly specific for certain species of insects and insect cell lines, suggesting the existence of a protein receptor on the cytoplasmic membrane of insect gut cells. We found BtRs among soluble membrane proteins by affinity precipitation with the toxin and anti-toxin antibodies (Fig. 2). Several proteins including a major 175-kDa protein (which we designated BtR175) were recovered from subcellular fractions containing leucine aminopeptidase, a microvillus membrane enzyme. The enzyme activity was in the P3 fraction, and the washed preparation contained homogeneous membranes forming vesicles with diameters of 80-400 nm (data not shown). The preparation contained ATPase (Fig. 3). The addition of 15 mM K⁺ or 0.47 mM Ca²⁺ (for the K⁺-ATPase or Ca²⁺-ATPase assay, respectively) did not change the enzyme activity. The addition of either 100 mM K⁺ or 15 mM K⁺ plus 100 mM Na⁺ (for the Na⁺, K⁺-ATPase assay) inhibited the activity somewhat. These results indicate that the preparation of BBM vesicles was virtually free from the apical membranes of the goblet cells, which contain K⁺-ATPase. The toxin did not affect the ATPase activity or morphological construction of BBM vesicles (data not shown).

The BtRs detected were a mixture of polypeptides of 86 to 197 kDa, and the relative weights, with 100 for BtR175, were 6.5, 12.4, 13.1, 1.7, 13.8, and 6.1 for 197-, 170-, 159-, 150-, 105-, and 82-kDa polypeptides, respectively. BtR175 accounted for 0.1-0.2 µg/mg BBM vesicle protein.

Partial disruption of the apical surfaces of columnar cells by sonication was a good method when very pure BBM vesicles were being prepared, but recovery was low. We used also a crude membrane preparation that was about 48% proteins, with 97% of the leucine aminopeptidase activity and 92% of the succinate dehydrogenase activity (a marker enzyme for mitochondria) of whole midgut tissue including goblet and muscle cells. Only these proteins were detected as BtRs in proteins obtained from the crude membranes prepared and made soluble. BtR175 amounted to 1.5 µg/78 mg of protein per gram of wet midgut tissue. BtRs were separated from the toxin and antitoxin antibodies, and were used for preparation of anti-BtR antibodies (Fig. 4). Compared with the immunoprecipitate, the proportions of proteins smaller than the 175-kDa protein increased during isolation of BtR by gel-filtration chromatography in the presence of 6 M guanidine hydrochloride (lane 2 in Fig. 4a). When the immunoprecipitate was being made soluble in a solution of 4 M denaturing agent, the 197-kDa and 175-kDa proteins almost disappeared (data not shown). These results suggested that the washed immunoprecipitate still contained a minute amount of some endogenous proteases, and that the smaller polypeptides were derived from the major protein BtR175. The polypeptides in the BtR preparation reacted with concanavalin A (which has affinity with α-mannoside), but not with peanut agglutinin (has affinity with Gal-β-1,3-GalNAC), and reacted very weakly with wheat germ agglutinin (has affinity with GlcNAC) or ricin agglutinin (has affinity with terminal Gal or GlcNAC). Thus, BtR175 was suggested to be N-glycosylated at several sites. Rabbit anti-serum raised against the BtR preparation specifically immunoprecipitated a few micrograms of BtR175 and BtR197 among 40 mg of proteins made soluble from the crude midgut membrane preparation (lane 2 in Fig. 4b).

BBM vesicles were incubated with various concentrations of the toxin before the membrane was treated with Triton X-100. Then, membrane-bound toxin was made soluble, recovered by immunoprecipitation, and analyzed by SDS-PAGE and densitometry (Fig. 5a). The amount of bound toxins increased in a dose-dependent way and reached a maximum at about 2 µg/ml toxin. BtR175 also was recovered with the toxin, and the amount increased as the amount of bound toxins increased. When BtRs were immunoprecipitated with anti-BtR antibodies, about 70% of the membrane-bound toxin was recovered with BtRs. The addition of anti-BtR antibodies in the reaction mixture of BBM vesicles and toxin inhibited much the binding of the toxin to the vesicles (Fig. 5b). These results indicated that toxin-binding to microvillus membrane was mediated for the most part by BtR175 protein.

Participation of BtR175 in the action of the Bt toxin

The toxin bound specifically to BtR175 on intact microvillus membranes of columnar cells. Does the bound toxin cause cell to lysis? Fifth-instar larvae of silkworms were reared on an artificial diet containing normal serum or anti-BtR serum and were then force-fed with the toxin (Table I). Treatment of B. mori larvae
with anti-BtR serum before feeding of the toxin decreased the action of the toxin, but treatment with normal serum did not affect the action of the toxin.

**Distribution of BtR175 in the B. mori tissues**

Insects have open blood-vascular systems, and all organs are floating in the hemolymph. Bt ICP is an oral toxin that probably dissolves within the lumen of the gut to act the midgut cell. A large dose of the minimal toxin unit (the soluble toxic fragment used in this study), however, is not effective when injected into the hemocoe.[21] So, we searched for BtRs in membrane fractions of fat bodies, integument, and silk glands (Fig. 6). No BtR was detectable in those tissues, suggesting that the gut is the target organ and that BtR175 is the Bt toxin-receptor.

**Search for toxin-binding proteins in the guts of insects and mice not susceptible to Bt Cry1A(a) toxin**

The LD90 of the Cry1A(a) toxin against *Bombix mori* larvae is 12.9 ng/g larva.[20] This toxin is not harmful to mice at 18 μg/g body weight.[22] *Tenebrio moritor*, a coleopteran, was also insusceptible to the toxin (LD90 > 15 μg/g body weight). *Mamestera brassicae*, a lepidopteran, was not killed by the Cry1A(a) toxin at 600 ng/g larva (LD90 from the literature is 46 μg/g body weight). The BBM vesicles from guts of those animals were treated with 125I, and then the proteins were made soluble. Proteins binding to the Cry1A(a) toxin were analyzed (Fig. 7). To our surprise, 137-kDa and 107-kDa proteins were found in the gut membrane of the toxin-insusceptible *T. moritor*. Does the binding of the toxin to these proteins somehow not allow the toxin to act? Was the Cry1A(a) toxin processed to nontoxic fragments before coming into contact with the proteins in guts? The results showed that the specificity of Bt toxin could not be explained only in term of the existence of toxin-binding proteins in the gut membrane.

In this study, we identified a 175-kDa glycoprotein, BtR175, as the receptor of *B. thuringiensis* Cry1A(a) toxin on the microvillus (brush-border) membrane of *B. mori* midgut columnar cells. BtR175 was a minor membrane protein (0.01-0.02% of BBM vesicle proteins) but more than 70% of the binding of Cry1A(a) toxin to the intact BBM vesicles was mediated by BtR175. Inhibition of the binding in vivo to the midgut with anti-BtR antibodies completely blocked the insecticidal action of the Bt toxin. Recently, *B. mori* aminopeptidase N (M., 120,000) was suggested to be a possible candidate as the Bt Cry1A(a) toxin-receptor on the basis of ligand-blot-
Fig. 5. Relation between Binding of Toxin to BBM Vesicles with Formation of BrR175-Toxin Complexes (a) and Inhibition of Binding of Toxin to BBM Vesicles by Anti-BtR Antibodies (b).

(a) BBM vesicles (6 mg of protein) were incubated at 22°C with various concentrations of the toxin in 12 ml of PBS containing 0.1% bovine serum albumin for 30 min. The treated vesicles were pelleted by centrifugation at 27,000 × g and 4°C for 30 min and then washed once with the same buffer above by resuspension and centrifugation. Proteins in the BBM vesicles were made soluble in 2.6 ml of PBS containing 2% Triton X-100 (insoluble substances were removed by centrifugation at 27,000 × g for 30 min) and incubated at 4°C with an excess of antitoxin IgG for 16 h. The resultant immunoprecipitate was collected by centrifugation, washed, and analyzed by SDS-PAGE on a 7.5% gel. Bands of the toxin and BrR175 in the gel stained with Coomassie Brilliant Blue were measured by densitometry in comparison with bands of known amounts of the toxin, and the values were used for estimation of the amount of toxin bound to BBM vesicles and BrR175 bound with toxin, respectively. The values measured from analysis of the immunoprecipitate obtained when an excess of anti-BtR antibodies was used instead of antitoxin IgG in the procedure described above were used for estimation of the amount of toxin bound to BrRs and BrR175 in BBM vesicles. (b) BBM vesicles (3 mg of protein) in 4.3 ml of PBS containing 2.0 mg/ml bovine serum albumin with no additions, with normal serum (1.6 ml), or with anti-BtR serum (0.4 ml) was brought to 5.9 ml with PBS containing 2 mg/ml bovine serum albumin. After incubation of the mixture at 22°C for 30 min, toxin (60 μg/0.1 ml) in PBS containing 2.0 mg/ml bovine serum albumin was added and the mixture was incubated at 22°C for 30 min. The mixture was centrifuged at 27,000 × g for 30 min and the pellet of BBM vesicles was washed once with 5 ml of PBS by resuspension and centrifugation. The proteins in the vesicles were made soluble in PBS containing 2% Triton X-100 and incubated at 4°C with 7.5 mg of antitoxin IgG for 16 h. The resultant immunoprecipitate was washed and analyzed by SDS-PAGE on a 7.5% gel. Lanes contain samples from the reaction with no serum (1), normal serum (2), and anti-BtR serum (3). IgG-H denotes heavy chains of IgG. BPB indicates the position of bromophenol blue.

Table I. Inhibition of the Insecticidal Action of Bt CrylA(a) Toxin by Anti-BtR Serum

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<th>Treatment with serum</th>
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a Five Bombyx mori larvae (0.9-1.0 g) were reared for 4 h on artificial feed (0.85 g) treated with nothing (−), normal serum, or anti-BtR serum.
b Two microliters of a toxin solution (15 μg/ml) in 10 mM Tris-glycine buffer, pH 8.3, containing 1 mg/ml bovine serum albumin was force-fed to each larva. As a control, the same amount of the buffer solution (−) was given instead of the toxin (+).
c After administration of the toxin, the larvae were reared on artificial feed. At the indicated times, the number of larvae lying down were counted.

In our binding assay, native proteins made soluble from BBM vesicles were used and even proteins that bound with high-affinity and that were present at low concentrations could be directly analyzed. Minor 105-kDa and 118-kDa proteins were found in the BrR preparation. However, they were partially degraded products from the major binding protein, BtR175, and were not aminopeptidases. BtR175 denatured by SDS-PAGE blotted onto a membrane filter as in a ligand-blotting assay lost most of its ability to bind to the toxin, and so the native polypeptide conformation of the protein may be responsible for the activity. Aminopeptidase seems to have low affinity for binding to the toxin. Thus, binding of CrylA(a) toxin to the B. mori aminopeptidase N probably does not reflect of the action of the Bt toxin in vivo, although we cannot be sure of the physiological reason for interaction between Bt CrylA(c) toxin and aminopeptidases from several lepidopteran insects.1,8,36-39 In Lymantria dispar BBM vesicles, CrylA(c) toxin binds to a 120-kDa protein, aminopeptidase, and CrylA(a) and CrylA(b) toxins bind to a 210-kDa protein.20 Cells transfected by the cDNA of Manduca sexta CrylA(b) toxin-binding protein (210 kDa) recognizes all three toxins, CrylA(a), CrylA(b), and CrylA(c).30 Bt HD-73 toxin binds to a 120-kDa protein in Manduca sexta BBM vesicles, and is a protein containing biotin that is not aminopeptidase.31 Receptors should be identified by binding assay to the intact BBM vesicles together with some in vivo experiments using insect bodies.
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Stained with CBB

Fig. 6. Distribution of BtR175 in Tissues of Bombyx mori Larvae.

Membrane fractions (corresponding to P2 plus P3 in Fig. 1) were prepared from midgut, integument, silk glands, and fat bodies. The membrane proteins (6.0 mg) were made soluble with Triton X-100, treated with the toxin as described in Fig. 2, and then with antitoxin IgG in the same way. The immunoprecipitates were analyzed by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R-250 (CBB). For immunoblot analysis, proteins transferred to a polyvinylidene difluoride filter membrane were treated with rabbit anti-BtR antibodies and then with anti-rabbit IgG antibodies conjugated with horseradish peroxidase. Detection was with 3,3'-diaminobenzidine as the substrate. IgG-H denotes heavy chains of IgG. BPB indicates the position of bromophenol blue.

Immunoblot with anti-BtR antibodies

Fig. 7. Search for CrylA(a) Toxin-binding Proteins in Insusceptible Insects and Mouse.

BBM vesicles prepared from the gut of Bombyx mori (1), Mamestera brassicae (2), and Tenebrio morio (3), and from the small intestine of mice (4) were labeled with ^125I as described in Materials and Methods. Membrane proteins made soluble with Triton X-100 were analyzed for binding to the Bt CrylA(a) toxin as described in Fig. 2. The gel was stained with Coomassie Brilliant Blue R-250 (CBB). Lane 5 contains complexes of toxin-antitoxin antibodies. IgG-H denotes heavy chains of IgG. BPB indicates the position of bromophenol blue.
References