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Isolation and Partial Characterization of Gypsy Moth BTR-270, an Anionic Brush Border Membrane Glycoconjugate That Binds *Bacillus thuringiensis* Cry1A Toxins With High Affinity

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BTR-270, a gypsy moth (*Lymantria dispar*) brush border membrane molecule that binds *Bacillus thuringiensis* (Bt) Cry1A toxins with high affinity, was purified by preparative gel electrophoresis. Rabbit antibodies specific for the Bt toxin-binding molecule were raised. Attempts to label BTR-270 by protein-directed techniques were futile, but it was degraded by proteases with broad specificity indicating the presence of a peptide. Carbohydrate was detected by labeling with digoxigenin hydrazide following periodate oxidation. Mild alkaline hydrolysis destroyed toxin and antibody binding, suggesting *O*-linked glycans are involved in the activity. GC/MS composition analysis showed that the predominant sugars were galactose, glucose, and N-acetyl galactosamine with lesser amounts of N-acetyl glucosamine, glucuronic acid, xylose, and fucose. The carbohydrate moiety accounted for 73% of its total mass. Amino acid analysis showed a high content of aspartic/asparagine, threonine, and serine residues in the protein moiety. The purified glycoconjugate was not visualized using Coomassie or silver staining procedures, but stained "blue" using the cationic dye Stains-all. BTR-270 was labeled with biotin and used as a diagnostic probe for screening and identifying toxins that bind to the receptor. Toxin-binding kinetics obtained using a biosensor demonstrated that the receptor binds Cry1Aa and Cry1Ab toxins with high affinity, and displays a weaker affinity for Cry1Ac, in correlation with the toxicity of these toxins towards gypsy moth. Arch. Insect Biochem. Physiol. 46:186–200, 2001. **© 2001 Wiley-Liss, Inc.**

Key words: Gypsy moth; BTR-270; *Bacillus thuringiensis* Cry1A toxin receptor

INTRODUCTION

Many strains of *Bacillus thuringiensis* (Bt) produce crystalline insecticidal (Cry) proteins that exhibit selective insecticidal activity for certain insect larvae but are harmless to people, pets, and most beneficial insects (Höfte and Whiteley, 1989).

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Bt insecticidal proteins can be grouped into different classes based upon their insecticidal spectrum, and these toxins have been separated into families based on protein sequence relationships (Crickmore et al., 1998). The lepidopteran-specific Cry1A toxins are produced as protoxins that are activated in the gut of insect larvae by proteolytic processing. Bt toxins bind to specific receptors present on the apical brush border membrane of midgut epithelial cells in susceptible insects (Hofmann et al., 1988; Van Rie et al., 1990). After initial binding, the toxin inserts irreversibly into midgut cell brush border membranes (Ihara et al., 1993, Liang et al, 1995), forming ion channels presumably through oligomerization in the membrane, resulting in disruption of midgut function and integrity.

The Cry1A insecticidal proteins are composed of three distinct domains (Grochulski et al., 1995; Schnepf et al., 1998). Domain I consists of an α-helical structure characteristic of membrane-spanning regions in other protein toxins and is believed to have the role of partitioning the toxin into the membrane and channel formation (Li et al., 1991). Domains II and III (consisting mostly of B-sheets) are involved in receptor binding. Some regions that are important in receptor interaction and toxin specificity have been identified (Schnepf et al., 1990; Ge et al., 1991; Wu and Aronson, 1992; Dean et al., 1996; de Maagd et al., 1996, 1999; Burton et al., 1999). Results of all studies to date have shown that a high-affinity interaction of toxin with membrane receptors is requisite for insecticidal activity, and a correlation between toxicity and toxin-binding to insect brush border membrane vesicles (BBMV) has been observed in insects (Hofmann et al., 1988; Van Rie et al., 1989; Lee et al., 1992).

Several Cry1A toxin-binding proteins have been identified as putative Bt receptors. Midgut aminopeptidase N (APN) isozymes interacting with one or more Bt toxins in *Manduca sexta* (Knight et al., 1994; Sangadala et al., 1994; Luo et al., 1996; Denolf et al., 1997), *Heliothis virescens* (Gill et al., 1995; Luo et al., 1997), *Plutella xylostella* (Denolf et al., 1997), *Bombyx mori* (Yaoi et al., 1997) and the gypsy moth, *Lymantria dispar* (Valaitis et al., 1995; Lee et al., 1996; Garner et al., 1999) have been isolated and characterized. Kinetic studies of two distinct APNs purified from gypsy moth revealed that only one isozyme (APN-1) possessed a Cry1Ac specific toxin-binding activity (Valaitis et al., 1997). Cry1Ac binding to APNs is inhibited by N-acetyl galactosamine (GalNAc) (Knowles et al., 1991; Knight et al., 1994; Masson et al., 1995; Valaitis, et al., 1997) indicating a lectin-like recognition of APN by a domain III region present on Cry1Ac toxin (Burton et al., 1999). The precise role of toxin-binding APNs in the insecticidal activity of Bt remains unclear. Studies have shown that APNs from different lepidopteron species expressed in insect cells have either a low affinity or do not bind Bt toxin (Garner et al., 1999; Denolf et al., 1997; Luo et al., 1999). A recent study of the binding and toxicity of Cry1Ac domain III mutant toxins with *M. sexta* APN revealed that binding to APN is not directly related to toxicity (Jenkins et al., 1999). In addition, rapid shedding of the gypsy moth APN in the larval midgut occurs in response to larval intoxication with Cry1A toxins, suggesting that release of the toxin-binding membrane-anchored APN may serve as a defense mechanism in the gypsy moth (Valaitis, 1995).

A different Cry1Ab toxin-binding molecule unaffected by GalNAc has been identified in the gypsy moth brush border membrane (Valaitis et al., 1995, Lee and Dean, 1996; Lee et al., 1996). The presence of similar large molecular weight Cry1Ab toxin-binding molecules has also been detected in other insects (Oddou et al., 1991, 1993; Lee et al., 1996). In *M. sexta* and *B. mori* cadherin-like Cry1A toxin-binding proteins have been characterized (Vadlamudi et al., 1995; Nagamatsu et al., 1998).

We report here isolation and partial analysis of BTR-270, previously referred to as the 210-kDa toxin-binding peptide in gypsy moth, which binds Cry1A toxins with high affinity. Our findings show that BTR-270, which fails to stain with Coomassie, is an anionic glycoconjugate that stains blue with cationic dye Stains-all.

MATERIALS AND METHODS

Insects

Gypsy moth, *Lymantria dispar* (New Jersey strain) larvae, were obtained from the Otis Methods Development Center (USDA, Otis AGB, MA) and reared on artificial wheat germ diet (ICN Biomedicals, Irvine, CA). Larvae were decapitated,

and the guts were pulled out of the larvae onto absorbent paper. The foregut was excised and the luminal content enveloped in the peritrophic membrane was removed. Guts were rinsed with 20 mM Tris-Cl (pH 6.8), blotted dry, and frozen. Brush border membrane vesicles (BBMV) were prepared from day 4–5 5th instar gypsy moth larval guts utilizing two cycles of the $CaCl_2$ precipitation procedure of English and Readdy (1989). Protein concentrations were determined by the Coomassie-dye binding assay (Bio-Rad), and carbohydrate concentrations were measured by the phenol-sulfuric acid assay (Chaplin and Kennedy, 1986).

Toxin and Antibody Preparation

Recombinant *B. thuringiensis* Cry1Aa, Cry1Ab, and Cry1Ac toxins expressed in *E. coli* were prepared from purified crystals (Lee et al., 1992). Crystals were solubilized in 50 mM carbonate (pH 10.5) and 10 mM DTT by incubation for 2 h at 37°C. The protoxins were activated by brief digestion with trypsin, and the toxins were purified by Superdex-200 gel filtration in 50 mM bicarbonate buffer (pH 8.4) using FPLC (Pharmacia). The purity of the toxins was assessed by SDS-PAGE. A mixture containing 2 mg of each of the three purified Cry1A toxins was used to produce a rabbit polyclonal antibody specific for Cry1A toxins used in immunodetection of toxin-binding molecules in Western blots of gypsy moth samples. Cry1C crystals were obtained from Dr. Luke Masson (Biotechnology Research Institute, NRC, Montreal, Canada). Cry1B, Cry2A, Cry2B, and Cry3A were obtained from the *Bacillus* Genetic Stock Culture Collection at The Ohio State University, Columbus, Ohio.

Purification of BTR-270 by Preparative Gel Electrophoresis and Immunization of Rabbits

BBMV prepared from gypsy moth larvae were solubilized in 50 mM Tris-Cl buffer (pH 6.8) containing 1% SDS, 5% glycerol, and 1% 2-mercaptoethanol (pH 7.4) in a boiling water bath for 5 min. Samples containing 3.5 to 5 mg of BBMV protein were layered onto 13.5 × 14.5 cm slab gels 0.5 cm thick containing 90 ml of a 5% polyacrylamide support (2.7% bis-acrylamide) with a 2 ml 5% polyacrylamide (2.7% bis-acrylamide) stacking gel. Electrophoresis was carried out at 20 mA overnight for 16–18 h. After electrophoresis, gels were rinsed in de-ionized water for 3 min twice, and immersed in a solution of 0.2 M zinc chloride for approximately 30 s until the background became deep white with transparent protein bands. The modified reverse-staining procedure of Castellanos-Serra et al. (1997) produced reproducible staining patterns of gypsy moth BBMV in analytical and preparatory gels. Preliminary studies were carried out targeting analysis of the high molecular weight proteins by excision and assessment of their ability to bind Cry1A toxins. The Cry1Aa and Cry1Ab toxin-binding molecule in 5% SDS-PAGE gels under reducing conditions was located slightly above the uppermost protein band, and was coined "BTR-270" due to estimated size based on extrapolation of molecular weight data obtained from SDS-PAGE. Reducing agents had no affect on the electrophoretic mobility of BTR-270. The excised gel pieces from the prep gels of BTR-270 were homogenized with 3 volumes of 0.2 M EDTA, pH 7.0. After centrifugation at 10,000*g* for 10 min, the pellet was re-extracted twice with additional EDTA solution. The supernatants were combined, and BTR-270 in this fraction was concentrated and equilibrated with 10 mM EDTA (pH 7.0) by Amicon PM-30 ultrafiltration. The extraction of BTR-270 from the gel matrix was monitored by SDS-PAGE of gel extracts and Western blot analysis using Bt toxins and an antibody specific for Cry1A toxins. BTR-270 isolated from approximately 34 prep gels was used for immunization of two rabbits to raise a specific antibody for the Cry1Ab Bt toxin-binding molecule.

Western Blot Ligand Blotting and Immunoanalysis

Five percent SDS-PAGE gels were routinely used for analysis of gypsy moth BTR-270 and BBMV by Western blots. Samples were electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane after SDS-PAGE using a Bio-Rad mini trans-blot cell. Excess sites on the membrane were blocked by incubation with 5% milk powder in 20 mM Tris-buffered saline, pH 8.6 (TBS). Anti-270 rabbit serum (1:30,000 dilution) was used to probe PVDF blots for immunodetection of the Cry1Ab toxin-binding molecule. Visualization of the bound antibodies was achieved by incubation with alkaline phosphatase-conju-

gated goat anti-rabbit IgG followed by staining with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT).

To identify Cry1A toxin-binding molecules, PVDF membrane blots were incubated with 0.2 μg/ml of toxin in TBS containing 0.2 mg/ml of BSA for 1 h at room temperature. After removing unbound toxin by washing with TBS containing 0.1% Tween-20 (TTBS), bound toxin was detected by sequential incubation with rabbit anti-Cry1A antiserum (1:3,000), and alkaline phosphatase-conjugated goat anti-rabbit IgG. Including 0.2 mg/ml BSA in the incubation and wash solutions minimized nonspecific interaction. Color was developed using BCIP and NBT.

Chemical Labeling of Purified BTR-270

A DIG glyan detection kit obtained from Boehringer Mannheim (Indianapolis, IN) was used to detect BTR-270 bound to PVDF. Briefly, BTR-270 that bound to PVDF after SDS-PAGE and Western blotting was oxidized with 10 mM sodium metaperiodate in sodium acetate buffer (pH 5.5) for 20 min in the dark at room temperature. After washing with 50 mM phosphate, 150 mM NaCl buffer (pH 6.5), the membrane was incubated with digoxigenin-3-O-succinyl-ε-aminocaproic acid hydrazide (DIG-hydrazide). Digoxigenin that covalently bound to the aldehyde groups generated in periodate-treated sample was detected using anti-digoxigenin-alkaline phosphatase conjugate. BTR-270 was also labeled prior to electrophoresis and Western blotting. To detect proteins, reaction of the activated ester, digoxigenin-3-O-succinyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (Boehringer Mannheim), with amino groups was used with subsequent labeling with the anti-digoxigenin conjugate.

Development of an Immunoaffinity Procedure for Purification of BTR-270

IgG from anti-270 rabbit serum was purified by affinity chromatography using Protein G Sepharose Fast Flow (Pharmacia). The IgG fraction was coupled to CNBr-activated Sepharose 4B, and used for purification of BTR-270 from gypsy moth BBMV. To inactivate the proteolytic activity and inhibit degradation of the IgG fraction bound to the Sepharose matrix, the pH of BBMV solubilized in 0.1% Nonidet P-40 was reduced to pH 3.0 with dilute acetic acid, neutralized, and then heated for 3 min at 65°C. After cooling to room temperature, the sample was centrifuged in a microfuge for 3 min, and the insoluble fraction was removed. The soluble BBMV fraction was applied to the immunoaffinity matrix equilibrated with 20 mM Tris-Cl, 0.15 M NaCl (pH 8.0) buffer, and allowed to react for 1 h at room temperature on a rotary shaker. The unbound fraction was collected, and the column was washed with TBS buffers containing low salt (0.15 M NaCl) and high salt (0.5 M NaCl). BTR-270 was eluted from the affinity matrix with 50 mM carbonate buffer.

Probing Immobilized *Bacillus thuringiensis* Toxins With Biotin-Labeled BTR-270

Biotin-hydrazide from Sigma (St. Louis, MO) was used to chemically label BTR-270 for development of a diagnostic probe for rapid *in vitro* identification of Bt toxins that bind the Cry1Ab Bt toxin-binding receptor with high affinity. After mild oxidation of BTR-270 with 10 mM periodate in 100 mM sodium acetate buffer for 20 min at room temperature at pH 5.5, excess sodium metaperiodate was destroyed by the addition of sodium bisulfite. The oxidized molecule was allowed to react with 0.5 μg/ml of biotin hydrazide in 100 mM sodium acetate buffer at pH 5.5 for 1 h at room temperature. The reaction was quenched with 1 M Tris, pH 7.8, and unreacted reagents were removed from the biotin-labeled BTR-270 by ultrafiltration followed by precipitation of the biotin-labeled probe with 3 volumes of ethanol at –20°C overnight. A 1:5 serial dilution of Cry1A, Cry2A, Cry1B, Cry1C, and Cry3A Bt toxins ranging from 1 μg to 1.28 pg were immobilized on PVDF membrane. The membrane was washed, blocked with 5% milk powder in TBS, and then probed with 0.5 μg /ml of biotin-labeled BTR-270 measured by the phenol-sulfuric acid assay in TBS for 2 h at room temperature. Bound BTR-270 was detected using an alkaline-phosphatase-conjugated monoclonal antibody specific for the biotin moiety obtained from Sigma.

Chemical and Enzymatic Treatments of BTR-270

To examine the nature of BTR-270, the purified Cry1Ab-binding molecule was subjected to various enzymatic and chemical treatments. The

conditions employed for the treatments are described in Figure 4. The effects of enzymatic digestions, mild alkaline and acid hydrolysis, hydrazinolysis, chemical deglycosylation using trifluoramethanesulfonic acid (Oxford glycosystems), oxidation with sodium meta-periodate, and reduction of BTR-270 were assessed by probing PVDF blots with Cry1Ab toxin followed by detection of bound toxin using the immunochemical procedure described above.

Extraction of BTR-270 by Phenol-Carbonate Partitioning of BBMV and Staining Using Stains-All

Purified BBMV were solubilized in 50 mM carbonate containing 1% CHAPS. An equal volume of phenol was added to the solution with stirring for 15 min at room temperature. The aqueous and phenol layers were separated by centrifugation at 1,800*g* for 15 min. Three volumes of 5% potassium acetate in ethanol were added to the aqueous phase, and to the phenol layer. After mixing, the suspensions were stored overnight at –20°C. The precipitate obtained from the aqueous phase was collected by centrifugation for 20 min at 15,000*g* and dissolved in water. The pellet obtained from the phenol layer was solubilized in SDS-PAGE sample buffer by heating for 5 min at 100°C. After SDS-PAGE, ligand blots were used to assess the partitioning of BTR-270 and BBMV proteins.

Staining with the carbocyanine dye Stains-all (1-ethyl-2- [3-ethylnaphtho [1,2-*d*] thiaxolin-2-ylidene)-2-methylpropenyl] naphtha- [1,2-*d*] thiazolium bromide) was as follows. After SDS-PAGE, the gel was fixed, stained with Coomassie blue, destained completely with 30% ethanol containing 5% acetic acid, and then equilibrated in 25% methanol. Stains-all was prepared as a 0.1% stock solution in formamide. The gel was stained with 0.0025% Stains-all in 30 mM Tris, pH 9.0, containing 25% methanol and 7.5% formamide overnight in the dark.

Amino Acid and Carbohydrate Analyses

Acid-stable amino acid analysis of purified BTR-270 was performed at Scientific Research Consortium, Inc. (St. Paul, MN). The amino acid analysis was obtained using a Beckman Model 7300 amino acid analyzer with a cation-exchange column and detection with post column color development with ninhydrin reagent. The carbohydrate composition of purified BTR-270 was analyzed at the Complex Carbohydrate Research Center in Athens, Georgia. The sample was hydrolyzed using 1M methanolic-HCl for 16 h at 80°C. The released sugars were derivatized with Tri-Sil, and the sample was analyzed by GC/MS using a Supelco column.

Analysis of Toxin Binding to BTR-270 Using Surface Plasmon Resonance

Binding kinetics and affinities were determined by surface plasmon resonance, using a BIAcore™ 2000 biosensor system (Biacore AB, Uppsala, Sweden) (Jönsson et al., 1991). BTR-270 was oxidized 20 min on ice with 1 mM sodium metaperiodate, and dialyzed overnight in 10 mM sodium acetate, pH 4.0. The receptor was immobilized on a CM5 chip by an aldehyde coupling method using a standard BIAcore protocol. All toxins were size-purified using a Superdex 200 column (Pharmacia). Various randomized concentrations of toxin (100–1500 nM) were injected at a flow rate of 50 µl/min over 90 RUs of immobilized BTR-270. For dissociation, toxin was replaced with buffer at the end of injection. Regeneration was accomplished with a 6-µl pulse of 10 mM NaOH, pH 11.5 at 100 µl/min. A flow cell containing only immobilized ethanolamine served as a control. BIAevaluation 3.0 was used for kinetic analysis, which allowed for global fittings of overlaid toxin response curves from all concentrations after subtraction of the control flow cell curve.

RESULTS

Purification of BTR-270 From Gypsy Moth Midgut Brush Border Membrane Vesicles

Bt toxin blots have shown a high molecular weight Cry1Aa and Cry1Ab toxin-binding molecule in gypsy moth larval midgut brush border membrane vesicles (BBMV) previously referred to as the 210-kDa peptide (Lee et al., 1996). In this study, separation of the low-mobility BBMV bands was optimized for purification of the toxin-binding molecule using a low polyacrylamide concentration in preparatory gel electrophoresis. A reversible zinc chloride protein staining technique using mini gels facilitated locating the toxin-binding component in relation to the BBMV protein profile in the gel, and to optimize sample load. The

toxin-binding band was located slightly above the uppermost protein band of BBMV fractionated by SDS PAGE using a 5% polyacrylamide gel. An apparent size of 270 kDa was derived from SDS-PAGE analysis, and it was coined BTR-270. The toxin-binding band was refractive to Coomassie blue, zinc chloride, and silver nitrate staining. Reducing agents had no affect on the electrophoretic mobility of BTR-270. Attempts to detect purified BTR-270 bound to PVDF membrane by probing blots with amine-directed (digoxigenin- and biotinamidocaproate N-hydroxysuccinimide esters) and sulfhydryl-directed (biotin-maleimide) reagents were unsuccessful (results not shown).

Development of an Immunoaffinity Column for Purification of Bt Receptor

Western blots of BBMV, peritrophic membrane, luminal digestive fluid, fat body, hemocytes, and hemolymph tissue samples from gypsy moth probed with antibodies raised to BTR-270 revealed a direct correlation of BTR-270 with Cry1A toxin-binding, and it was localized to the midgut brush border membrane fraction (data not shown). The specificity of the antibody for BTR-270 was exploited for development of an immunoaffinity procedure for purification of the molecule from detergent-solubilized BBMV. Due to intrinsic proteolytic activities present in BBMV, which can destroy the immobilized antigen-binding IgG, inactivation of these enzymes was necessary. This was accomplished by a combination of brief acid (pH 3.0) and heat (65°C) treatment of detergent-solubilized BBMV. After incubation of BBMV samples with the affinity matrix, there was little toxin-binding material left in the unbound fraction of the BBMV sample. However, no change in the Coomassie-stained SDS-PAGE profile of the BBMV proteins was observed (data not shown). Elution of BTR-270 bound to the immunoaffinity matrix was achieved using 50 mM sodium carbonate (pH 11.5). The eluate from the affinity column was contaminated with a trace amount of rabbit IgG, which was removed by passing it through an anti-rabbit IgG agarose immunoaffinity column. BTR-270 in the preparation was confirmed by assay for antigen activity probing Western blots with anti-270 antibody (Fig. 1, left panel), by probing blots with Bt toxin, and by labeling with biotin hydrazide following periodate oxidation, and detection using an anti-biotin antibody (Fig. 1, right panel).

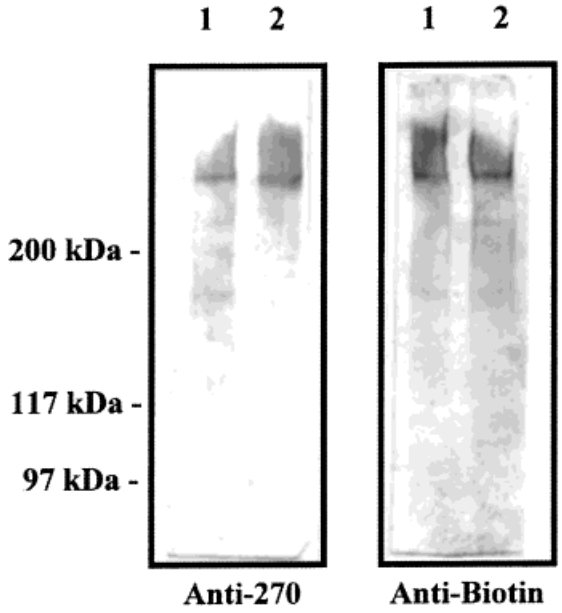

Fig. 1. Western blot analysis of immunoaffinity-purified BTR-270. Two samples of purified BTR-270 were transferred to PVDF after SDS-PAGE using a 5% gel. **Left:** BTR-270 was detected by labeling with anti-270 antibody. **Right:** BTR-270 was labeled with biotin hydrazide after mild oxidation with periodate followed detection using an anti-biotin monoclonal antibody.

Chemical Labeling of BTR-270

A GC/MS analysis of purified BTR-270 revealed that the Bt receptor is glycosylated. This finding resulted in developing a new means for detecting the receptor using an established method for detection of glycoconjugates. Aldehyde groups generated by mild periodate oxidation in the glycosidic moiety of BTR-270 were reacted with hydrazide derivatives generating a labeled conjugate that could be detected by sensitive immunochemical means. Digoxigenin was incorporated into periodate-oxidized BTR-270 by condensation with digoxigenin-hydrazide followed by immunodetection of the labeled molecule (Fig. 2, anti-digoxigenin panel). No incorporation of digoxigenin occurred without a prior oxidation step. Results in Figure 2 show that purified BTR-270 (270) binds Cry1Aa and Cry1Ab toxins, is refractive to Coomassie blue staining, and shows no binding to Concanavalin A. To exploit the selective toxin-binding properties of BTR-270 for development of a probe for screening and analysis of Bt toxins, it was important to ascertain that oxi-

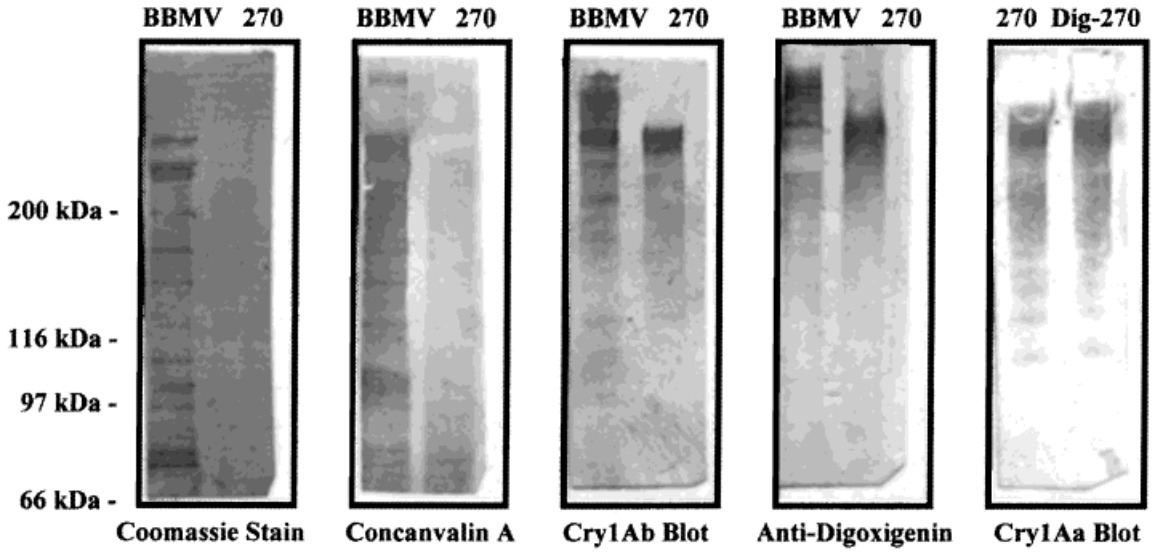

Fig. 2. Incorporation of digoxigenin into BTR-270 following periodate oxidation. Purified BTR-270 could not be detected using Coomassie or by probing blots with Concanavalin A. However, BTR-270 was labeled with digoxigenin after mild periodate oxidation (anti-digoxigenin panel) indicating the presence of a glycoconjugate. Probing a Western blot of native (270) and digoxigenin-labeled BTR (Dig-270) using Cry1Aa toxin revealed that toxin binding was not impaired by incorporation of digoxigenin.

dation and incorporation of label into BTR-270 did not alter the activity of the receptor. No impairment of toxin binding was observed in either the oxidized (data not shown), and subsequently, the digoxigenin labeled receptor (Dig-270) (Fig. 2, Cry1Aa Blot, right panel). The ability of digoxigenin labeled BTR-270 to react with the anti-270 antibody was also not compromised.

A broad band was observed with an increasing sample load of BTR-270 in Western blots probed with Cry1A toxins or antibody that suggested the receptor is highly glycosylated. In general, the purified BTR-270 is stable stored at neutral pH at 4°C. However, degradation of the purified receptor was observed in mildly alkaline solutions. In Figure 3, A Cry1Aa blot of three preparations (Fig. 3, lanes 1–3) of purified BTR-270, and two different loads (1–7 μl) of each preparation (Fig. 3, lanes a, b) is shown. A typical profile of a fresh preparation of BTR-270 (Fig. 3, lanes 3a,b) shows a single toxin-binding band at a position centering between the top of the separating gel and the 200-kDa myosin protein standard in 5% gels. However, with increasing sample load a broad band with some ladder-type banding was observed in some preparations characteristic of some polysaccharide (Plötz et al., 2000) and glycosaminoglycan structures (Kim et al., 1996) that have repetitive oligosaccharide units (Fig. 3, lanes 1a through 2b). A broad toxin-binding smear with some banding can also be observed in BBMV samples probed with Cry1Aa toxin.

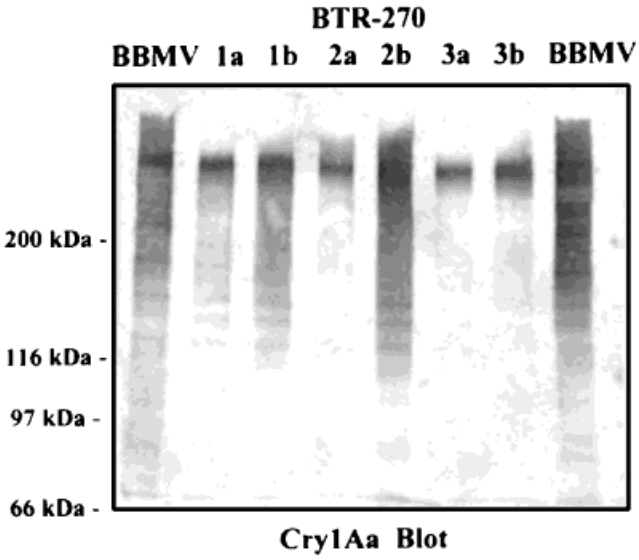

Fig. 3. Observation of a broad toxin-binding band and a ladder-like banding pattern in BTR-270 preparations. Western blot of BBMV and three preparations of BTR-270 (**lanes 1–3**) with two different loads (**lanes a**, 1 μl; **lanes b**, 7 μl) probed with Cry1Aa toxin showed that with high sample load, a broad smear and a banding pattern characteristic of a highly glycosylated molecule with a repetitive structure can be observed in BTR-270 and BBMV samples.

Effect of Proteases, Mild Hydrolysis, and Chemical Treatments on BTR-270

Results of enzymatic and chemical treatments of BTR-270 in Figure 4 show that BTR-270 is resistant to bovine trypsin (Fig. 4, lane 2) and chymotrypsin (Fig. 4, lane 3), *S. aureus* V8 protease (Fig. 4, lane 4), gypsy moth trypsin (Fig. 4, lane 5), submaxillaris protease (Fig. 4, lane 6), papain (Fig. 4, lane 7), endoporteinase Lys-C (Fig. 4, lane 8), and elastase (not shown). However, subtilisin (Fig. 4, lane 9), pronase E (Fig. 4, lane 10), and proteinase K (not shown) degraded BTR-270 judged by the Western blot results.

Treatment with O-glycosidase (Fig. 4, lane 12) and N-glycosidase F (Fig. 4, lane 13) had no apparent effect on the electrophoretic mobility, antigenicity, or toxin binding. However, extensive oxidation with sodium periodate destroys the ability of BTR-270 to bind toxin (Fig. 4, lane 14), and antibody (data not shown). The antigenic and toxin-binding properties are also destroyed by oxidation of PVDF-bound BTR-270 (data not shown). BTR-270 was insensitive to cleavage with 2% cyanogen bromide in formic acid for 24 h (Fig. 4, lane 15), and was not affected by treatment with hydrofluoric acid at 4°C for 48 h (Fig. 4, lane 18). The receptor was destroyed by hydrazinolysis (Fig. 4, lane 16), methanolysis (Fig. 4, lane 17), and mild alkaline or acid hydrolysis (Fig. 4, lanes 19, 20). Furthermore, the antigenic and toxin binding properties of BTR-270 were destroyed by very mild alkaline hydrolysis (50 mM NaOH at 37°C for 3 h) suggesting that an alkali-labile O-linked glycan or a hydroxyester-linked fatty acid released by β-elimination is important for the activity of the intact molecule.

Isolation of BTR-270 by Phenol Extraction of BBMV

In order to develop a more rapid and efficient protocol for purification BTR-270, extraction of BBMV using various solvent systems was evaluated. A phenol extraction of CHAPS-solubilized BBMV was found to be effective for isolation of BTR-270 from the proteins and lipids of BBMV that are partitioned into the phenol phase. The Coomassie-stained gel and Cry1Ab toxin blot in Figure 5 show the proteins partitioned the phenol layer and the isolation of toxin-binding BTR-270 in the aqueous phase. Extraction of BBMV from *Manduca sexta* yields a similar, but antigenically distinct Cry1Ab toxin-binding molecule in the aqueous phase (data not shown).

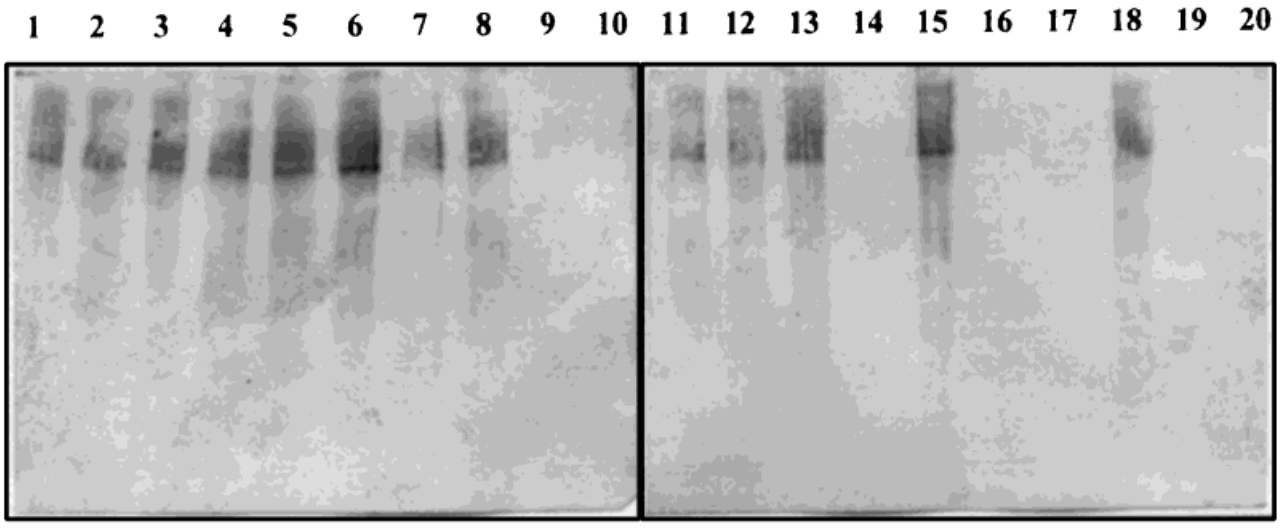

Fig. 4. Effect of proteases and chemical treatments on BTR-270. Cry1Ab toxin blots were used to assess the effect of enzymatic and chemical treatments of BTR-270. The samples in the lanes correspond to (**lane 1**) BTR-270 control, (lanes 2–13) BTR-270 after digestion for 24 h at 37°C with (**lane 2**) bovine trypsin, (**lane 3**) chymotrypsin, (**lane 4**) *S. aureus* V8 protease, (**lane 5**) gypsy moth trypsin, (**lane 6**) submaxillaris protease, (**lane 7**) papain, (**lane 8**) endoproteinase Lys-C, (**lane 9**) subtilisin, (**lane 10**) pronase E, (**lane 11**) BTR-270 control, (**lane 12**) O-glycosidase, (**lane 13**) N-glycosidase F, and (**lane 14**) 50 mM periodate, (**lane 15**) 2% cyanogen bromide in 70% formic acid, (**lane 16**) anhydrous hydrazine (60°C, 5 h), (**lane 17**) 1 N methanolic-HCl (60°C, 17 h), (**lane 18**) 48% hydrofluoric acid (4°C, 24 h), (**lane 19**) 10 mM NaOH (100°C, 30 min), and (**lane 20**) 10 mM HCl (100°C, 30 min).

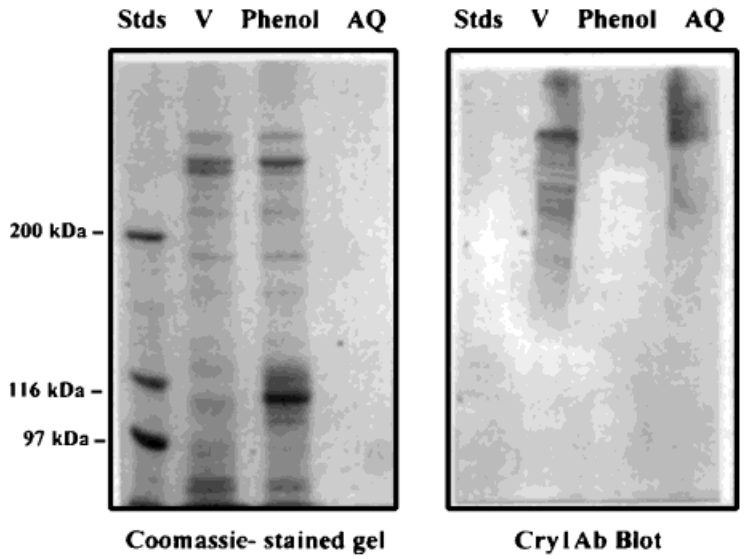

Fig. 5. SDS-PAGE and Western blot analysis of phenol extraction of gypsy moth BBMV. Phenol extraction of CHAPS-solubilized BBMV in 50 mM carbonate results in isolation of BTR-270 in the aqueous phase (AQ) while most of BBMV proteins and lipids are extracted into the phenol layer.

Visualization of BTR-270 With Stains-All

The most common methods for detection of proteins on polyacrylamide gels are the use of Coomassie blue, and the more sensitive silver nitrate stains. However, purified BTR-270 did not stain using these procedures. Due to their high negative charge, proteoglycans, mucins, and phosphoproteins have been found that interact poorly or not at all with Coomassie blue and silver stains. Cationic dyes such as Stains-all have been used to detect these proteins (Goldberg and Warner, 1997). The highly acidic proteins stain blue, while other proteins stain red. After gel electrophoresis of a sample aliquot of BBMV and purified BTR-270, the gel was stained with Coomassie blue. The gel was destained completely, and then stained using Stains-all. The results in Figure 6 show that purified BTR-270, which appears as a diffuse high molecular band in the gel, stains intensely blue while the proteins in the standards (Stds) and BBMV lane stain pink or red, indicating that BTR-270 is a highly acidic molecule.

Amino Acid and Carbohydrate Composition of BTR-270

Amino acid analysis revealed that BTR-270 has a very high content (20.4%) of aspartic/asparagine (Asx) residues, and a high content of serine (11.2%) and threonine (14.6%). A comparison to the amino acid composition of *Drosophila* syndecan (SDC), *Trichplusia ni* intestinal invertebrate mucin (IIM), and an *L. dispar* cadherin-like protein (LdCAD) is shown in Table 1. An amino acid composition similarity search of the protein databases could not identify BTR-270. There was some similarity of the amino acid composition to *Drosophila* syndecan. Comparison of BTR-270 to IIM and LdCAD shows substantial differences in their amino acid compositions.

The results of the carbohydrate analysis of BTR-270 are given in Table 2. Glucose, galactose,

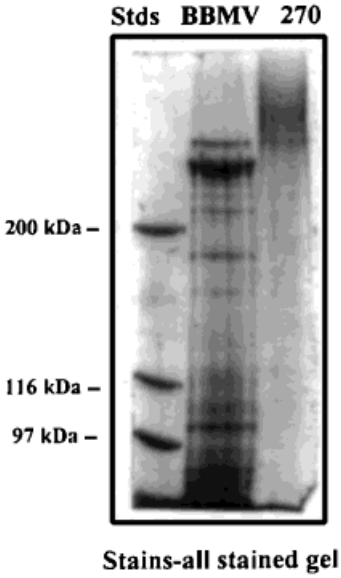

Fig. 6. Visualization of purified BTR-270 using Stains-all. BTR-270 is not detected by Coomassie blue staining after gel electrophoresis. However, using Stains-all BTR-270 stains intensely blue while the standards (Stds) and the BBMV proteins are visible in the gel stain red, indicating that the Bt receptor is a highly anionic glycoconjugate.

TABLE 1. Amino Acid Composition of BTR-270, Compared to *Drosophila* Syndecan (SDC), *Trichplusia ni* Intestinal Mucin (IMM), and Gypsy Moth Cadherin (LdCAD)*

Residue (Mol. %)	BTR-270	SDC	IMM	LdCAD
Ala	5.2	7.59	5.9	5.9
Arg	1.8	3.04	1.5	4.8
Asx	20.4	16.96	8.1	14.2
Gly	8.7	8.61	4.5	5.4
Glx	6.1	8.86	9.3	12.1
Cys	—	0.25	2.5	0.8
His	2.0	3.54	2.5	2.3
Ile	4.7	6.58	2.4	7.8
Leu	4.3	4.30	3.8	7.4
Lys	3.0	3.80	0.8	1.0
Met	0.5	1.27	0.2	2.0
Phe	2.4	2.03	2.1	4.8
Pro	6.0	8.10	16.9	5.2
Ser	11.2	9.62	3.4	5.1
Thr	14.6	8.61	18.7	8.6
Trp	—	0	1.4	0.8
Tyr	2.3	2.03	1.4	3.8
Val	6.6	4.81	4.7	7.9

*Data for *Drosophila* Syndecan from Spring et al. (1994); data for *Trichplusia ni* intestinal mucin from Wang and Grandados (1997); data for gypsy moth cadherin from unpublished sequence submitted to GenBank (accession number AF317621); –, not determined.

and N-acetyl galactosamine were the major components of the glycan moieties found in BTR-270. A lesser amount of glucuronic acid, N-acetyl glucosamine, mannose, fucose, and xylose was also detected. The relatively high level of N-acetyl galactosamine, which is mainly found in *O*-linked glycans, suggests that BTR-270 is covered with mucin-type oligosaccharides. The relatively small amount of mannose found in *N*-linked carbohydrate structures suggests that *O*-linked structures predominate. The presence of xylose and glucuronic acid residues could indicate the presence of proteoglycan-type acidic mucopolysaccharides.

TABLE 2. Carbohydrate Composition of *Lymantria dispar* BTR-270

Carbohydrate residue	Mol %
Fucose (Fuc)	1.5
Xylose (Xyl)	0.8
Glucuronic acid (GlcA)	8.7
Mamnose (Man)	3.2
Galactose (Gal)	15.5
Glucose (Glc)	46.5
N-acetyl galactosamine (GalNAc)	16.4
N-acetyl glucosamine (GlcNAc)	7.4

The glucose may be derived from a glycogen-like contaminant.

Development of a Rapid In Vitro Assay for Screening Toxins Using BTR-270 as a Diagnostic Probe

A key step in the mechanism of Bt action is the binding of toxin to its receptor. Exploiting the selective toxin-binding properties of the receptor can facilitate the development of new Bt-based insecticidal proteins that are more potent or specific for target insect pests. A simple assay for rapid screening of Bt toxins was developed utilizing biotin-labeled BTR-270 as a diagnostic probe. Figure 7 shows the results of probing a 1:5 serial dilutions of eight different Bt toxins immobilized on PVDF with biotin-labeled probe followed by immunodetection of BTR-270 bound to toxins with alkaline-phosphatase conjugated anti-biotin antibody. This assay demonstrates that BTR-270 binds Cry1Aa, Cry1Ab, and Cry1B toxins, and Cry1Ac with less affinity. No binding of BTR-270 to Cry2B, Cry1C, and Cry3A was observed as expected with these toxins that are non-toxic to *L. dispar*. No binding to the toxic Cry2A was observed by this technique. These results demonstrate that the biotin-labeled receptor re-

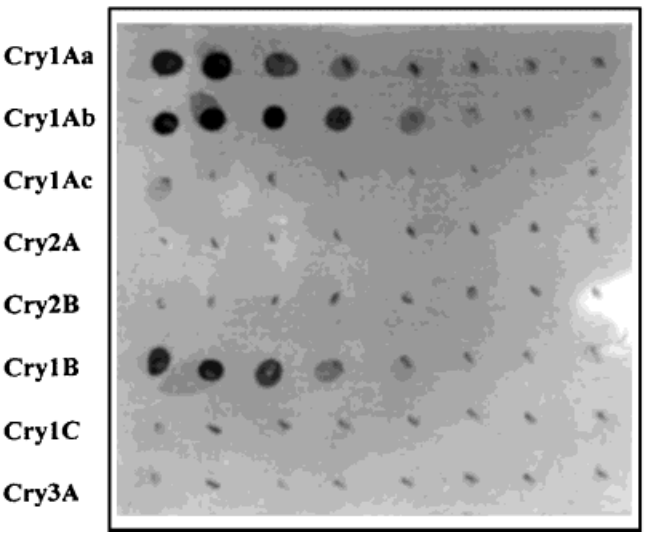

Fig. 7. Biotin-labeled BTR-270 as a diagnostic probe for screening Bt toxins. Serial dilutions (1:5) of eight Bt toxins ranging from 1 μg to 1.2 pg were spotted on a PVDF membrane from left to right. After incubation with biotin-labeled BTR-270 in TBS and washing, toxins that bind the labeled receptor were detected using an anti-biotin antibody.

tained its selective and tight interaction with toxin, and may be exploited as a convenient probe to identify toxins that bind to the receptor.

Kinetic Analysis of Bt Toxin-Binding to Purified BTR-270 From Gypsy Moth

To further evaluate the specific interaction of Bt toxins with BTR-270, real-time binding was examined using surface plasmon resonance spectroscopy on a BIAcore biosensor. BTR-270 was directly immobilized to the sensor surface by aldehyde coupling, and toxins were injected at multiple concentrations (Fig. 8). All response curves fit statistically well to a one-site binding model (A + B $\Leftrightarrow$ AB) based on χ^2 analysis, and visual inspection of the simulated fitted curves. Cry1Aa toxin bound with high affinity, yielding a $k_a = 1.5 * 10^4\ M^{-1}\ s^{-1}$, $k_d = 2.6 * 10^4\ s^{-1}$, and $K_D = 1.7 * 10^{-8}$ M ($\chi^2 = 0.05$). The total binding response with Cry1Ab toxin was similar, although it displayed a slightly lower affinity of $k_a = 1.4 * 10^4\ M^{-1}\ s^{-1}$, $k_d = 6.6 * 10^{-4}\ s^{-1}$, and $K_D = 4.9 * 10^{-8}$ M ($\chi^2 = 0.23$). Consistent with our ligand blot results, Cry1Ac bound with much lower affinity to BTR-270, despite its similar off-rate to other toxins, producing apparent rate constants of $k_a = 1.5 * 10^3\ M^{-1}\ s^{-1}$, $k_d = 6.0 * 10^{-4}\ s^{-1}$, and $K_D = 3.9 * 10^{-7}$ M ($\chi^2 = 0.04$). The standard error for all rate constants was less than 1%.

To analyze the stoichiometric binding ratio of Cry1A toxins to BTR-270, a saturating concentration of toxin was injected (3,000 nM). All three toxins obtained an Rmax of approximately 32 RUs when binding to 90 RUs of the receptor (270 kDa) and Cry1A toxins (65 kDa); the stoichiometry of binding can be calculated by dividing the interactants' weight ratio by the relative response at saturation, yielding a binding stoichiometry of 1 receptor: 1.46 toxins. However, the exact weight of BTR-270 is not known, and there is evidence that it is highly glycosylated. Thus, it is reasonable to assume its actual molecular weight is likely to be smaller than its size estimated by SDS-PAGE, and the resulting binding stoichiometry is closer to 1:1. This is further supported by the fittings of toxin response curves to a one-site binding model.

The coleopteran-specific CryIIIA toxin was also injected, but did not bind to the receptor (data not shown), indicating that the Cry1A bind-

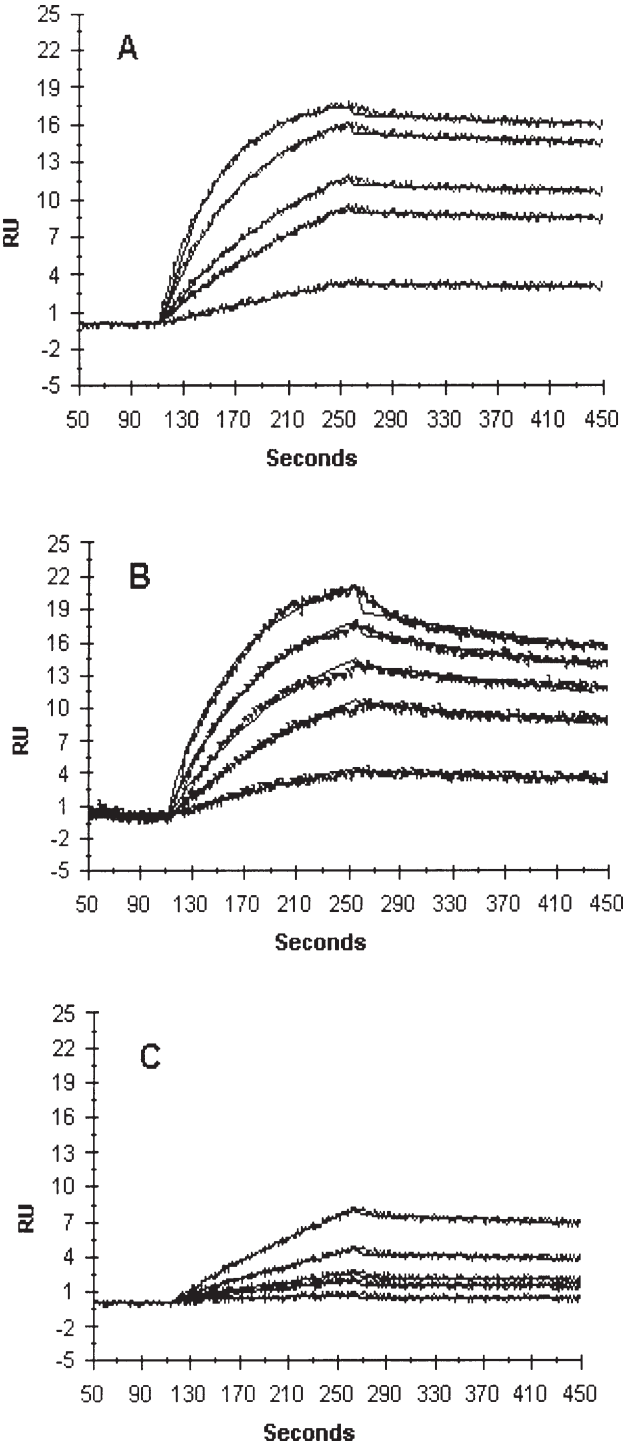

Fig. 8. Real-time binding of Cry1A toxins to BTR-270. Toxins were injected over 90 RUs of immobilized BTR-270 at concentrations of 1,500 (highest response curve), 1,000, 500, 350, and 100 nM (lowest response curve). All experimental curves are overlaid with simulated fittings (smooth line) obtained from the Langmuir one-site binding model. **A:** Cry1Aa. **B:** Cry1Ab. **C:** Cry1Ac.

ing to BTR-270 is selective. In addition, the toxicity of Cry1Aa, Cry1Ab, and Cry1Ac toxins to *L. dispar* correlates with their respective affinities for BTR-270.

DISCUSSION

In this study, a high molecular weight toxin-binding molecule (BTR-270) previously referred to as the 210-kDa peptide (Lee et al., 1996) was purified by preparatory gel electrophoresis, and an immunoaffinity procedure. The evidence presented in this study indicates that BTR-270 is a highly glycosylated protein. BTR-270 does not stain with Coomassie but stains blue with Stains-all, which is characteristic of acidic proteins that include mucins and proteoglycans.

Amino acid analysis of BTR-270 revealed a high content of aspartic-asparagine residues, which may account for the highly anionic nature of BTR-270. A high content of serine and threonine residues that could serve as sites for *O*-glycosylation was also found. An amino acid composition similarity search of databases to identify the protein was unsuccessful. However, there was some similarity found to syndecan, a cell-surface heparan sulfate proteoglycan in *Drosophila*. *Drosophila* syndecan displays a broad smear with an apparent size of 120–160 kDa due to its heparan sulfate chains (Spring et al., 1994). Syndecans bind a variety of extracellular ligands mediated by their glycans composed of glucuronic acid and N-substituted glucosamine residues linked to protein core by an *O*-glycosidic bond between serine and xylose (Höök et al., 1984). The glucuronic acid and xylose detected in BTR-270 was suggestive for the presence of proteoglycan-like oligosaccharides.

However, several features suggest that BTR-270 probably is an anionic mucoprotein and not a proteoglycan, and the toxin-binding molecule may be a component of a brush border membrane glycocalyx-like material. Extraction of gypsy moth BBMV with phenol yields a completely water-soluble molecule that retains its toxin-binding activity. Phenol-water extraction, originally used to extract lipopolysaccharides, has been applied for the preparation of mucins (Gold et al., 1981), and glycocalyces (Caulfield et al., 1987). The glycocalyx of intestinal brush cells is a carbohydrate-rich material with a polyanionic nature that is composed of glycoconjugates that differ in glycosylation patterns in all species examined (Gebet et al., 2000). At present there is no comprehensive analysis of the protein components of the intestinal epithelial glycocalyx. The major constituent of the brush border glycocalyx of the rabbit intestinal epithelium was shown to be a large transmembrane mucin with abundant heterogeneous oligosaccharide chains (Maury et al., 1995). The rabbit mucoprotein is similar to BTR-270 in several respects including its high carbohydrate content, sensitivity to mild alkaline hydrolysis, and the inability to detect the glycoconjugate by Coomassie blue staining. In invertebrates, a large intestinal mucin-type glycoprotein (IIM) has been isolated and characterized from the cabbage looper (Wang and Granados, 1997). IIM was primarily localized in the peritrophic membrane of the cabbage looper and was detected in the area surrounding the brush border membrane of midgut epithelial cells. In contrast, BTR-270 is associated with the brush border membrane, and does not appear to be an intrinsic component of the peritrophic membrane. In general, mucins contain a high degree of *O*-linked oligosaccharide chains that are susceptible to cleavage by mild alkaline treatment. The observation that periodate oxidation, deglycosylation, and mild alkaline treatments destroy the activity of BTR-270 suggests that an alkali-labile carbohydrate moiety is important for receptor function. Carbohydrates display enormous structural and functional diversity and are important as elements for the recognition and function of proteins. Characterization of the alkali-labile oligosaccharide motifs found in BTR-270 will be valuable to unravel the function of the carbohydrate portion of the receptor in toxin adhesion and the pathological processes.

The SPR binding studies presented here show that several Bt toxins highly active against gypsy moth can specifically bind BTR-270 with high affinity. These affinities represent the highest measured for a Bt toxin receptor by SPR analysis yet reported. The affinities of Cry1Aa, Cry1Ab, Cry2Aa, Cry1Ac, and Cry3Aa toxins to BTR-270 correlate with their respective toxicity to *L. dispar*. With further characterization of BTR-270, it will be possible to determine the structural features that are critical in toxin interaction and in the pathological process of Bt.

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