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Bacillus thuringiensis Cry1A toxin-binding glycoconjugates present on the brush border membrane and in the peritrophic membrane of the Douglas-fir tussock moth are peritrophins

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ABSTRACT

Bacillus thuringiensis (Bt) Cry1A toxin-binding sites in the Douglas fir tussock moth (DFTM) larval gut were localized using immunofluorescence microscopy. Cry1Aa, Cry1Ab and Cry1Ac all bound strongly to the DFTM peritrophic membrane (PM); weaker binding of the Cry1A toxins was observed along the apical brush border of the midgut epithelium. Comparative analysis of the Cry1A toxin-binding molecules in the PM and brush border membrane vesicles (BBMVs) showed that a similar toxin-binding complex was present in both. The Cry1A toxin-binding substance, a broad band with an apparent size of 180 kDa, consisted of a closely spaced doublet. The doublet was present in peritrophins, proteins tightly bound to the PM. Lectin binding studies of the PM and BBMV toxin-binding components revealed that they are glyconjugates with terminal α-GalNAc residues comprised exclusively of *O*-linked oligosaccharides in their glycan structures. Mild periodate oxidation, release of *O*-linked glycans by β-elimination, and enzymatic removal of terminal α-linked GalNAc residues with N-acetyl-α-D-galactosaminidase digestion abolished Cry1A toxin-binding to the PM and BBMV components. These data provide strong evidence that *O*-linked glycans are the target structures on the toxin-binding glycoconjugates for the Cry1A class of insecticidal proteins in DFTM larvae.

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1. Introduction

In North America, *Orgyia pseudotsugata*, the Douglas fir tussock moth (DFTM), feeds on needles of its host trees: Douglas fir, true fir and white fir. Outbreaks of the DFTM can cause complete defoliation and subsequent death of infested trees. A microbial biopesticide, *Bacillus thuringiensis* (Bt), has been used to manage the DFTM insect pest (Stelzer et al., 1975). However, evaluations of the effectiveness of Bt-based biopesticides against DFTM larvae have been inconsistent. The insecticidal activity of Bt is largely attributed to insecticidal proteins referred to as Cry toxins, which are produced as inclusions in the bacterial pathogen during sporulation (Schnepf et al., 1998). After the DFTM ingests Bt, the inclusion bodies release protoxins, which digestive proteases in the larval gut fluid convert to active toxins. The activated Cry toxins cross the peritrophic membrane (PM) and bind to gut epithelial cells. It is generally accepted that the pore-forming Cry toxins then insert into gut epithelial cell membranes and rupture inflicted cells (Vachon et al., 2012).

Susceptibility to purified Cry toxins is highly variable among insect species. Cry toxins rapidly kill highly susceptible type I insects (Heimpel and Angus, 1959). In contrast, type II insects, including the DFTM, are only weakly susceptible to purified Cry proteins. In the absence of spores, the purified Cry toxins induce paralysis of the gut, and the intoxicated type II insect larvae die slowly, seemingly from starvation (Dubois and Dean, 1995). However, in the presence of a small amount of spores in the Cry protein mixture the mortality of type II insects to Bt Cry proteins increases dramatically as a result of spore germination and lethal septicemia.

It is generally believed the rapid Cry toxin effect on type I insects is due to the perforation of the gut cells by the pore-forming toxins. Specific receptors on the brush border membrane of midgut cells sequester toxins on the surface of the gut cells, enabling membrane insertion, pore formation, and cytotoxic action on the gut cells (Pigott and Ellar, 2007). Considerable effort has been devoted to identifying the binding partners for various Cry toxins and to elucidating how these receptors promote the cytotoxic action of the Bt Cry toxins on the target gut cells. Although several proteins, such as cadherins, GPI-anchored aminopeptidases, and alkaline phosphatases, have been shown to interact with Cry toxins *in vitro*, a full understanding of their precise function *in vivo* is lacking. In addition, the underlying mechanism for the weak toxicity of

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the purified Cry proteins to type II insects remains elusive. It has been proposed that the absence of receptors for specific Cry toxins and also the obstruction of one or more processes, including toxin activation and entrapment, and precipitation of the insecticidal proteins in the gut environment, can contribute to weak susceptibility to Cry toxins (Oppert et al., 1994; Milne et al., 1995; Hofmann et al., 1988; Van Rie et al., 1989; Baxter et al., 2011).

The peritrophic membrane (PM) is an insoluble acelular matrix separating ingested food in the gut from the midgut epithelium. The PM consists of chitin fibrils intertwined with tightly associated proteins known as peritrophins (Tellman et al., 1999). Peritrophins are integral components of the PM. They require harsh conditions to be released from the chitinous matrix. The composition and physical properties of the PM vary significantly among different insects and different larval instars (Lehane et al., 1996; Hegedus et al., 2009; Merzendorfer and Zimoch, 2003). Chitin, a polymer of the sugar N-acetylglucosamine (GlcNAc), is an important constituent of the PM. The key enzyme in the synthesis of chitin, chitin synthase, has been localized at the tips of the microvilli (Zimoch and Merzendorfer, 2002). Chitin and peritrophins released from microvilli of gut epithelial cells rapidly coalesce along the midgut epithelium forming an insoluble lining (Martin and Kirkham, 1989; Bolognesi et al., 2001). In Lepidoptera, the PM is continuously formed by delamination from the midgut epithelium, with the microvilli acting as a mold forcing the microfibrils embedded with peritrophins into a regular pattern (Lehane, 1997). The peritrophins consist of at least two classes of glyconjugates: glycoproteins endowed with *O*-linked oligosaccharides and N-linked oligosaccharides, and proteoglycans with chains of glycosaminoglycans. The carbohydrate structures of the glycosylated peritrophins harbor multi-affinity binding capabilities, serving to entrap toxic macromolecules and microbial pathogens, protecting the vulnerable gut epithelial cells from injury and infection.

The PM acts as a barrier against bacterial pathogens, but Cry toxins are able to diffuse through it (Adang and Spence, 1983). Localization studies of Cry toxin-binding sites in the gut have shown that the PM is a major site for toxin accumulation in various lepidopteran insects (Yi et al., 1996; Hayakawa et al., 2004; Chen et al., 2005; Rees et al., 2009). Both toxic and non-toxic Cry toxins are retained by the PM, suggesting that toxin binding to the PM is not relevant in determining the insecticidal effectiveness of the Bt Cry toxins (Bravo et al., 1992; Aranda et al., 1996; Denolf et al., 1993). Yet, the loss of toxin binding to the PM in Cry1Ab-resistant *Plutella xylostella* larvae suggests that PM changes may play a role in a mechanism of Bt action (Bravo et al., 1992). Studies of the histopathological effects in response to ingested Cry toxins on the insect midgut have shown alterations in the protein composition and damage to the PM, indications that the protection the PM provides to the epithelial cells has been breached (Rupp and Spence, 1985; Moonsom et al., 2008; Sousa et al., 2010). Destruction of the PM as a result of ingestion of insecticidal lectins, the insecticidal toxin from *Xenorhabdus nematophila*, plant cysteine proteases, and the baculovirus metalloprotease enhancin, has been observed (Zi-yan et al., 2006; Mohan et al., 2006; Michiels et al., 2010; Wang and Granados, 2001). The pathological signature in the midgut induced by ingestion of Cry toxins mimics the effects of several insecticidal lectins and the effects observed in the gut as result of starvation (DeLello et al., 1984). One important question that has not been resolved is whether Cry toxin binding triggers the collapse of the protective barrier, thereby facilitating bacterial invasion and fatal septicemia in type II insects.

In this study, Cry1A toxin-binding sites in the DFTM larval gut tissue were localized using fluorescence microscopy. The Cry1A toxin-binding molecules present in the PM and the BBMV were examined using toxin and lectin blotting. The effects of proteolytic digestion, and chemical and enzymatic deglycosylation on the toxin-binding molecules were investigated, providing evidence that the PM and the brush border membrane have similar toxin-binding molecules. Together, these results demonstrate that the PM and BBMV toxin-binding molecules are glyconjugates endowed with alkali-labile *O*-glycans, indispensable for their interaction with the Cry1A class of insecticidal proteins.

2. Materials and methods

2.1. Insects

Douglas-fir tussock moth (DFTM) larvae (McDunnough) (Lepidoptera: Lymantriidae), Goose Lake strain of *O. pseudotsugata*, from a colony established from egg masses collected near Goose Lake in northern California, were reared on an artificial diet using a 16:8 (light:dark) photoperiod following the methods described by Thompson and Peterson (1978). This colony was maintained at the US Forest Service facility in Hamden, Ct.

2.2. B. thuringiensis Cry1A δ-endotoxins

Cry1Aa, Cry1Ab and Cry1Ac Bt toxins were purified from clones of the *cry1Aa*, *cry1Ab* and *cry1Ac* genes expressed in *Escherichia coli*, which were obtained from the *Bacillus* Genetic Stock Center at The Ohio State University. The purified crystals were resuspended in 50 mM Na_2CO_3, 5 mM DTT and 5 mM EDTA at pH 10.4 and incubated at 37 °C for 2 h. The solubilized protoxins were digested with tosyl phenylalanyl chloromethyl ketone (TPCK)-treated bovine trypsin for 1 h at 37 °C using a trypsin to protoxin ratio of 1:20 followed by centrifugation to remove any insoluble material. The digests were treated with a protease inhibitor cocktail (Sigma, St. Louis, MO), and the activated Cry proteins were purified by gel filtration followed by ion-exchange chromatography using a Macro-Prep High Q anion-exchange support (Bio-Rad Laboratories) as previously described (Valaitis, 2008). Rabbit polyclonal antibodies specific for Cry1A toxins produced to a mixture of purified Cry1Aa, Cry1Ab and Cry1Ac toxins were used for localization in gut tissue sections and toxin overlay assays. Protein concentration was determined using the Bio-Rad protein assay dye reagent, and the purity of the Cry1A toxins was assessed by 10% SDS–PAGE. All the Cry1A toxins were filter sterilized using 0.2 μm syringe filter and stored in 50 mM Na_2CO_3, 0.5 M NaCl and 5 mM EDTA at 6 °C.

2.3. Localization of Cry1A toxin- and lectin-binding sites on DFTM tissue sections

Paraffin-embedded DFTM gut tissue sections were prepared for immunohistochemical studies according to a procedure previously described (Valaitis, 2011). Briefly, midguts from fourth instar DFTM larvae were fixed in 4% paraformaldehyde in 20 mM phosphate-buffered saline (PBS) at pH 7.4 for 24 h, dehydrated with an increasing series of ethanol, cleared in xylene, and embedded in Gem Cut Opal Paraffin. 6–8 μm thick microtome-cut transverse sections of the gut tissue were mounted on poly-L-lysine coated glass slides. Incubations with toxins and lectins were carried out using 7–10 ml of each solution per slide and a rocker platform at room temperature. The tissue sections were blocked with 2% bovine serum albumin (BSA) in 20 mM Tris-buffered saline (TBS) for 1 h. For localization of toxin-binding sites, the tissue sections were incubated with a 0.4 μg/ml Cry1A toxin solution in TBS containing 0.1% Tween-20 and 0.25 mg/ml BSA (TBST) for 2 h at pH 8.6. Unbound toxins were removed by washing the tissue sections four times with TBST, and then the slides were incubated with an anti-Cry1A antibody solution for 1 h, washed four times as described above, and incubated with Alexa Fluor 546-conjugated

goat anti-rabbit IgG secondary antibody solution for 1 h. After four additional washes with TBST, 50 μl of SlowFade Gold antifade reagent (Invitrogen) containing the blue-fluorescent nuclear counterstain DAPI was overlaid on the tissue sections and cover slips were mounted.

Localization of lectin binding sites in the DFTM whole gut tissue section was performed using a 0.2 μg/ml solution of biotinylated-lectins in TBS containing 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 0.05% Triton-X 100 at pH 7.6. After 1 h incubation with the lectins, the slides were washed four times with TBS containing 0.1% Triton X-100 and incubated with the orange-fluorescent Alexa Fluor 546 streptavidin (Invitrogen) diluted 1:30,000 with TBS for 1 h. Then, coverslips were mounted using the SlowFade reagent with DAPI as described above. All slide preparations were examined using a Zeiss Axiophot microscope equipped with a ProgRes Capture Pro digital camera.

2.4. BBMV and PM preparations

PM and BBMV were isolated from 4th instar larvae reared on an artificial diet. The PM was removed from the guts used for the BBMV preparation. 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 PM was removed. The guts were washed in 20 mM Bis–Tris, 0.15 M mannitol, 5 mM EGTA at pH 7.0 treated with an EDTA-free protease inhibitor cocktail (Sigma). BBMV were prepared using two cycles of the Mg^{2+} precipitation and differential procedure, as described by Wolfersberger et al. (1987), with the exception that the solutions were supplemented with 1 mM PMSF. A portion of the BBMV preparation was resuspended in 20 mM Bis–Tris, 0.15 M mannitol and 5 mM EGTA at pH 6.8 and then solubilized by addition of 10 mM EDTA and 2% CHAPS at room temperature. The detergent solubilized fraction was separated from the CHAPS-insoluble material by centrifugation at 14,000*g* for 20 min at 20 °C.

The PM dissected from the DFTM larvae were washed thoroughly with 20 mM Bis–Tris, 50 mM EDTA and 1 mM PMSF at pH 6.8 and blotted dry on absorbent paper. The integral PM proteins, peritrophins, were extracted from the chitinous matrix by transferring the PM into 20 mM Bis–Tris containing 2% SDS and 5% 2-mercapthoethanol at pH 6.8 and immediately heating at 100 °C for 10 min. After cooling to room temperature, the solution was clarified by centrifugation at 10,000*g* for 10 min at 20 °C. Four volumes of methanol were added to the supernatant, and the precipitated peritrophins were collected by centrifugation. The peritrophin pellet was dissolved in deionized water containing 1 mM DTT.

2.5. Cry1A toxin- and lectin blots

After SDS–PAGE of 5 μg of the BBMV and PM samples, the proteins were electrophoretically transferred onto PVDF membranes. The membranes were blocked by incubation with 2% BSA in TBS at pH 7.8 for 1 h. For Cry1A toxin-binding studies the membranes were incubated with 30 ml solution containing 10 nM of Cry1A toxin in TBS containing 0.25 mg/ml BSA for 1 h at pH 8.6. Unbound toxin was removed by washing with TBS containing 0.25 mg/ml BSA and 0.1% Triton X-100 (TBST) four times for 5 min each. The washed membranes were then incubated with anti-Cry1A rabbit polyclonal antibodies diluted 1:3000 for 1 h, followed with alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibodies (Sigma) diluted 1:30,000. Detection was performed by staining with BCIP and NBT. Lectin binding studies were carried out using the same protocol except all the Tris-buffered solutions at pH 7.6 contained 0.1% Triton X-100 and were supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$.

2.6. Chemical and enzymatic treatments

Peritrophins extracted from the DFTM PM were subjected to SDS–PAGE and transferred to PVDF membrane. To examine the effect of mild periodate oxidation, a PVDF blot was incubated with 10 mM sodium periodate in 50 mM sodium acetate containing 0.05% Triton-X100 at pH 4.5 for 1 h in the dark. The untreated and the periodate-treated PVDF blots were washed thoroughly with TBS and then blocked with a 2% solution of BSA. The control and periodate-treated blots were equilibrated in TBS with 0.25% BSA at pH 8.6 and then subjected to Cry1Ab toxin binding analysis using the toxin overlay assay described above. A PVDF replicate of the BBMV and PM samples was stained using Coomassie blue or a combined Alcian blue and Coomassie staining protocol.

An equal aliquot (2 μg) of the peritrophins extracted from the DFTM PM was subjected to various enzymatic and chemical treatments. Digestion with 50 ng of TPCK-treated bovine trypsin, proteinase K and chitinase (Sigma) was carried out for 16 h at 30 °C in TBS at pH 7.6, β-elimination of *O*-linked glycans was performed by incubation of the sample in 20 mM NaOH at 30 °C for 16 h, digestion with N-acetyl-α-D-galactosaminidase (GalNAcase) from chicken liver (Sigma) was performed using 10 mU of GalNAcase in 20 mM sodium acetate at pH 4.0 at 30 °C for 4 h, and chemical degradation of the carbohydrate moiety was carried out by incubation of the peritrophins with 10 mM sodium periodate in 50 mM sodium acetate at pH 4.5 for 1 h in the dark at room temperature. Unreacted periodate was destroyed by the addition of ethylene glycol, and dilute NaOH was added to the samples in sodium acetate buffer to bring up the pH to approximately 6.8 prior to SDS-gel electrophoresis. The effect of these treatments was assessed by 7% SDS–PAGE, transfer to PVDF membrane followed by Cry1A toxin and lectin blotting.

3. Results and discussion

3.1. Localization of Cry1A toxin- and lectin-binding sites in DFTM larval gut

Fluorescent micrographs of the Cry1Aa, Cry1Ab, and Cry1Ac toxin-binding sites in the 4th instar DFTM gut tissue sections are shown in Fig 1. The Cry1A toxin-binding sites were localized with orange-fluorescent secondary antibodies, and nuclei present in the epithelial cells were stained with the blue-fluorescent nucleic acid stain DAPI. All three of the Cry1A toxins showed very strong binding to the PM in the gut tissue sections, and a weaker interaction with the apical surface of the midgut epithelial cells. Delamination of the newly formed PM from the apical surface of the midgut epithelium is evident in several regions along the brush border of the gut cells. Toxin binding along the apical surface of the midgut epithelium surface where the PM is formed is strong. In contrast, toxin binding to the anterior surface of the epithelium where the PM has recently detached is significantly weaker; suggesting that newly synthesized components of the peritrophic membrane along the microvillar surface of the epithelial cells may be the toxin binding molecules observed with the brush border membrane. Probing the gut tissue with soybean agglutinin (SBA) lectin resulted in an intense fluorescence of the PM, indicating an abundance of glycans containing terminal GalNAc residues. The intense fluorescence of the PM was very similar to the image observed with the localization of the Cry1A toxin binding sites. SBA exhibits high selectivity for terminal α-GalNAc residues in oligosaccharides, which can be found attached to glycoproteins and glycolipids. Wheat germ agglutinin (WGA) lectin stained the same matrix, confirming that the SBA- and toxin-binding structure in the gut is the PM (micrograph not shown). WGA lectin has been widely used to localize

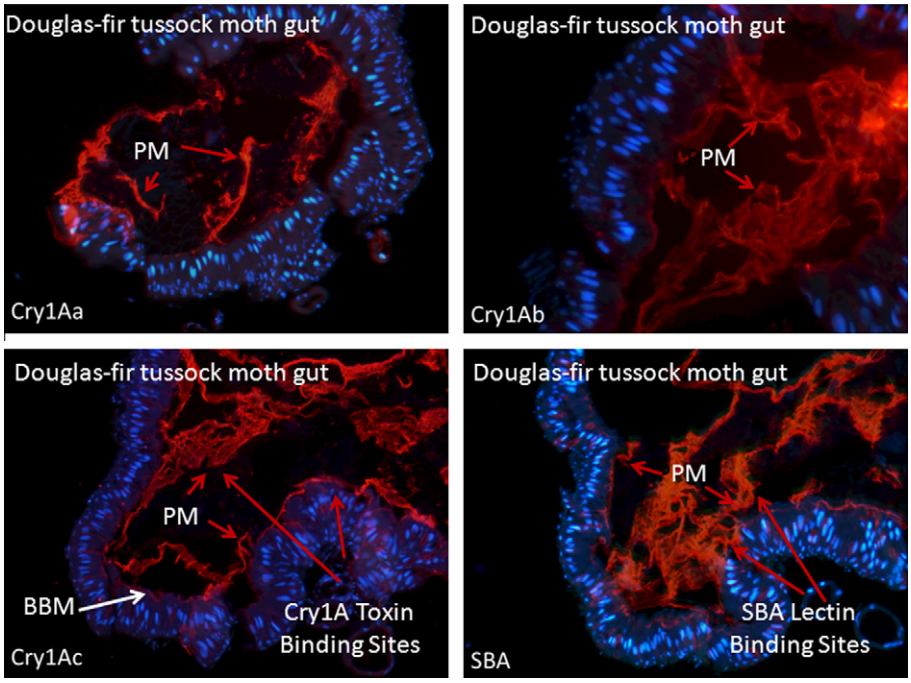

Fig. 1. Localization of Cry1A toxin and soybean lectin (SBA) binding sites in cross sections of the midgut of Douglas-fir tussock moth (DFTM) using fluorescence microscopy. The Cry1A toxin and SBA binding sites were labeled with orange fluorescent secondary antibodies and orange fluorescent streptavidin, respectively. Nuclei in the epithelial cells were stained blue. Note: Cry1A toxin binding sites on the peritrophic membrane (PM) and the brush border membrane (BBM) are also labeled with the GalNAc-specific SBA lectin.

the PM in insects due to its high affinity for chitin, an integral component of the PM. Some lectins that display insecticidal activity bind and alter the physical properties of the PM, resulting in the disruption of feeding and digestion, and kill the midgut epithelial cells. All the insecticidal lectins examined to date exert their biological activity by binding to specific carbohydrate moieties (Murdock and Shade, 2002).

The 3D structural analyses of Bt Cry toxins have shown that the toxins have three domains. Two of structural regions of Cry toxins, domain II and III, may have built-in carbohydrate-recognition sites (Knight et al., 2004). The function of domain II as the major determinant for specific receptor binding, essential for toxicity, has been well-established. Analysis of the tertiary structure of domain II revealed a β-prism structural fold, which is typically associated with carbohydrate-binding proteins. However, a carbohydrate-binding function has yet to be confirmed. Domain III, located in the C-terminus of the Cry toxins, has also been implicated in receptor binding, and also contains a structural motif that is found in carbohydrate-binding proteins. Biochemical studies have clearly established that domain III of the Cry1Ac toxin interacts with GalNAc residues, and toxin binding can be inhibited with competing GalNAc. To date, the carbohydrate ligands recognized by domain III of the closely related Cry1Aa and Cry1Ab toxins have not been characterized. Hypothetically, the two carbohydrate-recognition domains in the Cry toxins can provide for multivalent interactions with remarkably high affinity for specific carbohydrate receptors (Kiessling and Pohl, 1996). Carbohydrates act as receptors for many pathogenic bacterial toxins that drive toxins into the target cells (Chen et al., 2009).

3.2. Specific binding of Cry1Ab and Cry1Ac to DFTM BBMV and PM

The toxin-binding molecules present in the DFTM BBMV and PM were identified by Cry1A toxin blot studies performed using 10 nM Cry1A toxins in TBS at PH 8.6. An Alcian blue-Coomassie blue stained profile of the BBMV and PM samples blotted onto PVDF membrane is shown in the left panel of Fig 2. The double staining protocol revealed two closely-spaced bands in the 180 kDa PM toxin-binding complex, which were not detected by Coomassie blue staining alone. The reaction of the cationic dye with acidic groups enables staining of highly glycosylated molecules such as proteoglycans, which stain poorly with conventional protein staining techniques. A broad Cry1Ab toxin-binding band with a similar size of approximately 180 kDa was detected in the BBMV and in the PM samples by the toxin overlay assay is shown in the center panel in Fig 2. Results obtained with a Cry1Aa toxin blot (not shown) were similar to the Cry1Ab toxin blot. After treatment of BBMV with 2% CHAPS in the presence 50 M EDTA at pH 6.8, the broad toxin-binding band was partitioned into the detergent insoluble BBMV fraction. In contrast, most of the BBMV proteins, including two of the major constituents of the brush border microvillar membranes, the 110 kDa and 100 kDa APNs were solubilized by the CHAPS detergent. The 110 and 100 kDa protein bands in the BBMV were identified as APNs respectively, by mass spectrometry (data not shown).

A strong interaction of Cry1Ac toxin was also observed with the 180 kDa Cry1Ab toxin-binding complex in the BBMV and PM, shown in the right panel in Fig 2. Cry1Ac toxin also bound to a series of smaller 90–150 kDa molecules in the PM, which were not detected in the Cry1Ab toxin blot and the Alcian blue-Coomassie stained blot. The CHAPS-soluble 110 kDa APN in the BBMV was also recognized with the Cry1Ac toxin. Cry1Ac toxin binding to the 110 kDa APN was partially inhibited by competing GalNAc. Cry1Ac toxin has a unique ability to form reversible complexes mediated by a monovalent recognition of a GalNAc residue, a common recognition epitope found in GPI-anchored aminopeptidases and alkaline phosphatases, identified as Cry1Ac toxin-binding proteins on the brush border membrane of midgut epithelial cells in various insect species. The recognition of the monosaccharide by a GalNAc-binding pocket in domain III in Cry1Ac toxin may be

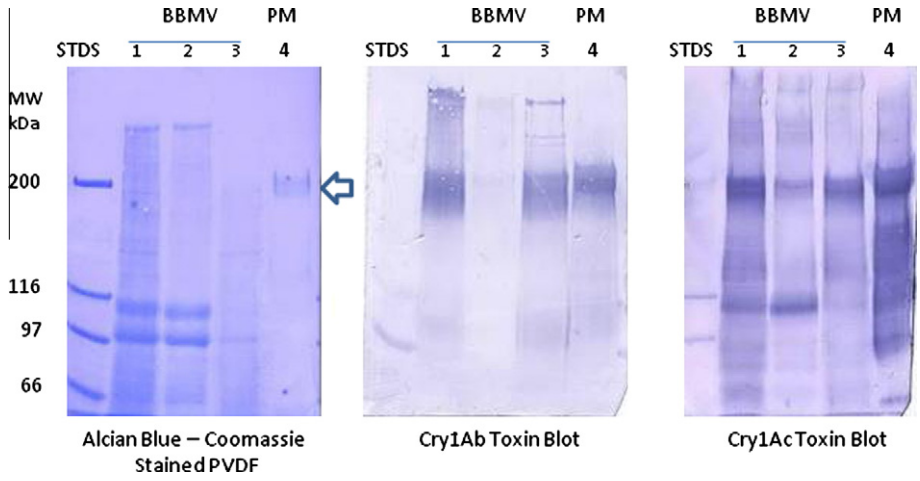

Fig. 2. Cry1Ab and Cry1Ac toxin-binding molecules in the BBMV and PM from DFTM. Left panel. Successive Alcian blue-Coomassie stained profile of molecular weight markers (STDS), DFTM BBMV (1), CHAPS-soluble BBMV fraction (2), CHAPS-insoluble BBMV fraction (3), and peritrophins (PM) extracted from DFTM PM. Center panel. Cry1Ab toxin blot of DFTM BBMV and PM samples. Right panel. Cry1Ac toxin blot of DFTM BBMV and PM samples. The broad 180 kDa toxin-binding complex PVDF stained panel is marked with an arrow.

responsible for its interaction with various proteins that are not involved in the mechanism of Bt action (Jenkins et al., 1999; Kitami et al., 2011). Neither Cry1Ac nor Cry1Ab toxin binding to the 180 kDa complex in the BBMV and the PM were inhibited by GalNAc, suggesting that the Cry toxins recognize a different structure in the 180 kDa complex. The strong interaction of the Cry1Ab and Cry1Ac toxins with the common 180 kDa complex in the BBMV and PM, suggest that it may be the most relevant target of the Cry1A class of toxins governing their activity in DFTM larvae.

3.3. Lectin blot analysis

Lectin binding studies indicated the presence of carbohydrate residues on the toxin-binding components in the 180 kDa complex in the BBMV and PM (Fig 3). Glycans are present on glyconjugates as *O*-glycans and N-glycans. Lectins, such as snowdrop (GNA) and Concanavalin A (Con A), are good indicators for the presence of N-linked carbohydrate structures. GNA and ConA binding to the 180-kDa toxin-binding components showed negative results, indicating that these toxin-binding components were not N-glycosylated. However, GNA and Con A reacted strongly with the 100 kDa APN and proteins with a size of 230 kDa and 70 kDa in the BBMV, evidence for N-glycans on these proteins. SBA reacted strongly with the 180 kDa toxin-binding components in the BBMV and PM, indicating the presence of carbohydrate structures with terminal α-GalNAc residues. A strong interaction was also obtained with other GalNAc-specific lectins, *Griffonia simplicifolia* (GS-1) and *Wisteria floribunda* (WFA) (data not shown). A group of smaller molecules (130–150 kDa) in the PM were also recognized with the SBA lectin, and SBA binding to the 110 kDa APN in the BBMV was detected. The interaction of the toxin-binding components in the 180 kDa complex with the peanut (PNA) lectin, which preferentially binds to terminal galactose molecules, was significantly weaker. The development time with PNA lectin blot had to be extended to obtain the staining seen in Fig 3. The lectin binding profiles of the 110 kDa and the 100 kDa APNs were clearly distinct. The lectin binding data indicate that the Cry1Ac toxin-binding 110 kDa APN is not N-glycosylated, but is modified by *O*-linked car-

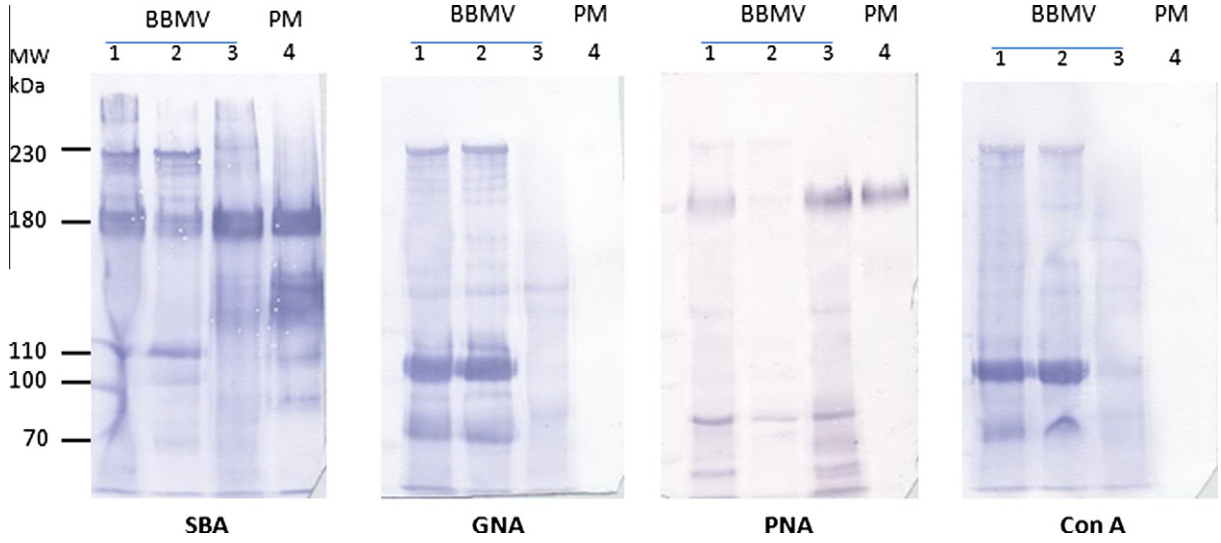

Fig. 3. Soybean (SBA), snowdrop (GNA), peanut (PNA) and Concanavalin A (Con A) lectin binding to DFTM BBMV and PM samples. Samples are the same as annotated in Fig 3. SBA reacted strongly with the 180 kDa toxin-binding complex in the CHAPS-insoluble BBMV fraction (3) and the DFTM peritrophins (PM). The interaction with PNA was weaker .Probing with GNA and Con A showed negative results, indicating that the toxin-binding components 180 kDa complex were not N-glycosylated.

bohydrate structures with terminal α-GalNAc residues. In contrast, the 100 kDa APN is not *O*-glycosylated, but has N-linked oligosaccharides. The strong reaction with SBA, and the absence of N-linked glycans in the 180 kDa components, provides evidence that carbohydrate moiety present on the toxin-binding molecules consist exclusively of *O*-linked glycans. Although protein staining failed to detect the 180 kDa doublet in the BBMV, the presence of the toxin-binding molecules was readily detected by the toxin overlay assay. Consequently, these results suggest that the toxin-binding molecules are present at a relatively low level in the BBMV. The similarity in size, toxin- and lectin-binding properties of the toxin-binding molecules in the BBMV with those in the PM suggest that they are the same.

Although the PM has been identified as a major site of toxin binding in various lepidopteran insects, investigation of the toxin-binding molecules in the PM has been the subject of only few studies (Hayakawa et al., 2004; Rees et al., 2009). Toxin-binding studies of Western blots of the PM in five insect species were shown to bind to a diverse group of proteins regardless of the presence of GalNAc residues by Rees et al. (2009). PM proteins with an apparent size of 250 kDa, 190 kDa, and 150 kDa were identified as Cry1A toxin-binding molecules in *Bombyx mori* (Hayakawa et al., 2004). Cry1Ac binding to some of the PM proteins was partially inhibited by GalNAc, but Cry1Aa toxin binding to the same molecules was not. Whether a carbohydrate-mediated mechanism was involved in the Cry1Aa toxin binding to the PM components in *B. mori* was not determined.

3.4. The effect of periodate oxidation and deglycosylation

The effects of mild periodate ($NaIO_4$) oxidation of the DFTM BBMV and PM samples on a Western blot was examined to determine if the carbohydrate moiety on the toxin-binding molecules was important for their ability to bind Cry1A toxin. The conditions employed (10 mM $NaIO_4$ at pH 4.5 for 1 h) have been widely used to selectively degrade the carbohydrate moiety of glycoproteins without perturbing the protein structure in studies of carbohydrate-binding proteins, including the Cry1Ac toxin-binding *Manduca sexta* aminopeptidase N (Knight et al., 2004). The Coomassie-stained profile of the BBMV samples and the peritrophin extract from the PM is shown in the left panel in Fig 4. The control, Cry1Ab toxin blot is shown in the middle panel, and the PVDF blot subjected to the periodate treatment is shown in the right panel. When a large amount of the BBMV and PM samples were subjected to SDS-gel electrophoresis followed by Cry1Ab toxin blotting, smearing in the PM sample and a higher molecular weight Cry1Ab toxin-binding band (>250 kDa) were observed. The smearing of the toxin-binding substance in PM suggests that the components may be highly glycosylated. Smearing is a common feature observed with mucins, proteins that have a high content of carbohydrate residues and large clusters of *O*-linked glycans. The higher molecular weight toxin-binding band detected in the BBMV samples may be due to oligomerization or aggregation of the toxin-binding molecules with other BBMV components. The Cry1Ab toxin binding components in the BBMV and PM were not detected by Coomassie blue staining indicating that they may be acidic glyconjugates, which stain weakly with traditional protein stains. Treatment of the PVDF blot with periodate abolished Cry1Ab toxin binding to the BBMV and PM molecules, suggesting that the carbohydrates associated with the toxin-binding molecules provide the binding sites for the Cry1A toxins. These results are consistent with several studies demonstrating the important role of specific carbohydrates for the recognition of Bt toxin receptors by Cry toxins in various insects and the nematode, *Caenorhabditis elegans* (Valaitis et al., 2001; Griffitts et al., 2001; Jurat-Fuentes et al., 2002; Knight et al., 2004; Sarkar et al., 2008; Ning et al., 2010).

The 180 kDa Cry1A toxin-binding molecules in the PM, which were extracted with harsh denaturing conditions, place them in a class of integral PM components referred to as peritrophins. The toxin-binding peritrophins were resistant to digestion with trypsin, but were degraded with the more robust proteinase K (Fig 5). Their resistance to trypsin digestion was not surprising since PM is naturally exposed to trypsins present in the insect larval gut. Degradation with proteinase K suggests that these glycosylated molecules are either glycoproteins or proteoglycans. Digestion with chitinase resulted in slight loss of the toxin-binding molecules, which may be due to the presence of some chitin in the peritrophin sample providing protection to degradation or the presence of some contaminating protease(s) in the chitinase. Periodate oxidation of the peritrophin sample prior to electrophoresis

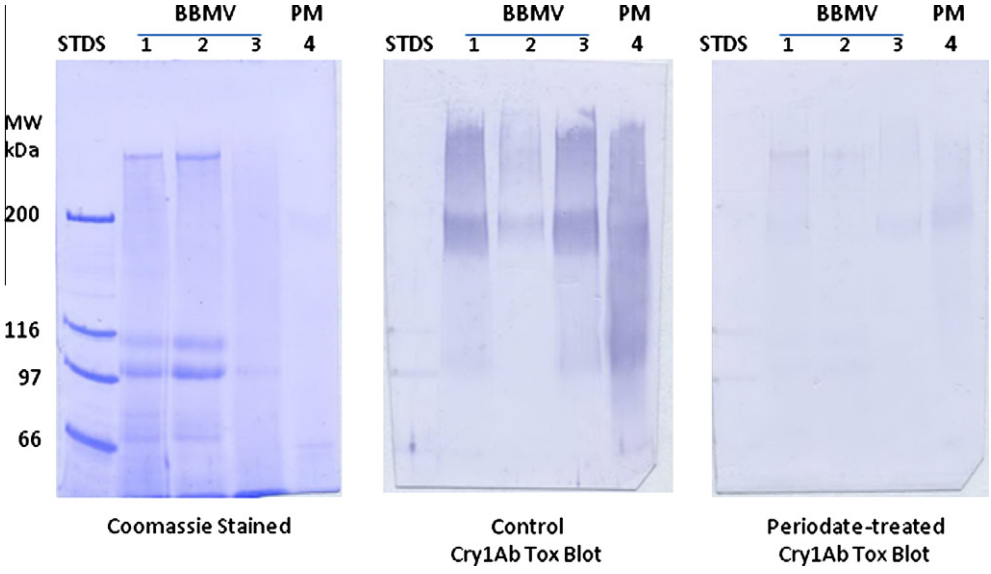

Fig. 4. Effect of mild periodate oxidation on the toxin-binding properties of DFTM BBMV and PM. Samples are the same as annotated in Fig 3. A Coomassie-stained profile of the samples is shown in the left panel, the control Cry1Ab toxin blot of the samples is shown in the center panel and the effect of mild periodate oxidation, which abolished toxin-binding to the BBMV and PM molecules, is shown in the right panel.

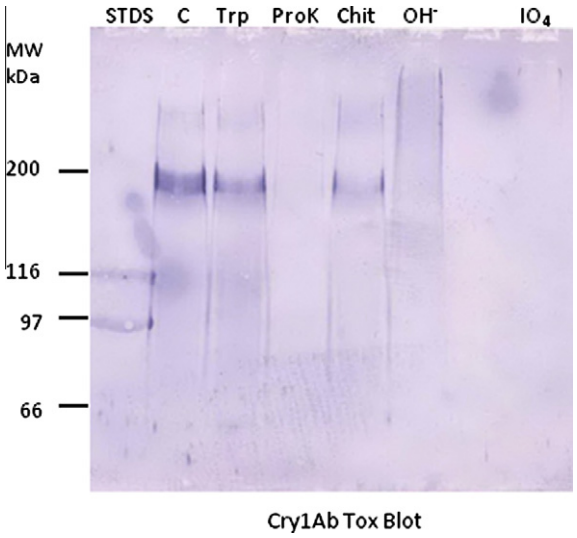

Fig. 5. Enzymatic digestions and chemical treatments of DFTM toxin-binding peritrophins. Digestion with trypsin (Trp) and chitinase (Chit) had little effect on the toxin-binding doublet in the 180 kDa complex. Toxin-binding was abolished by proteinase K digestion (Prok), β-elimination of O-glycans (OH^-) and periodate oxidation (IO_4). Control sample is indicated by (C).

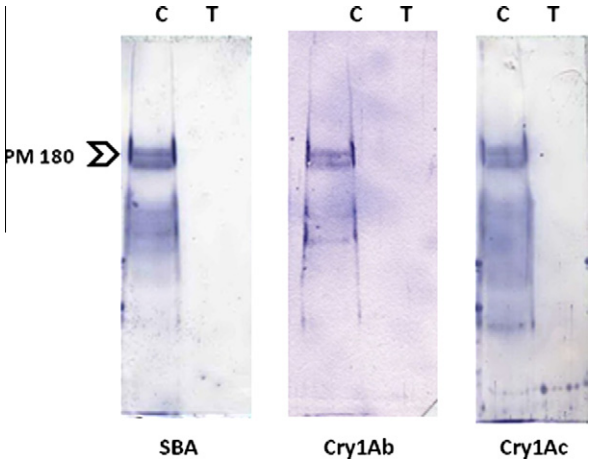

Fig. 6. Effect of N-acetyl-α-D-galactosaminidase (GalNAcase) treatment on the SBA lectin- and Cry1A toxin-binding properties of the 180 kDa (PM 180) DFTM peritrophins. Control (C) and GalNAcase-treated (T) PM 180 samples.

resulted in loss of toxin binding, which is consistent with the inhibitory effect of mild periodate oxidation on the toxin-binding properties of the peritrophins transferred to PVDF membrane shown in Fig 4. Further evidence that carbohydrates are important for toxin binding to the peritrophins was obtained by the selective removal of *O*-glycans from the peritrophins by β-elimination. The mild alkaline treatment of the peritrophins resulted in the loss of toxin binding to the peritrophins in the 180 kDa complex, leaving only a light smear at the top of the gel. The residual toxin-binding substance remaining may be due incomplete deglycosylation. The β-elimination procedure is the most popular method employed to specifically release *O*-linked oligosaccharides from glycoproteins, and has been adapted for analysis of glycoproteins transferred to PVDF (Duk et al., 1997). Under mild alkaline conditions the *O*-linkage between GalNAc and serine or threonine residues in the protein is cleaved by the β-elimination reaction without affecting the polypeptide or the release of N-glycans, which requires more drastic conditions. These results clearly demonstrate that alkali-labile *O*-glycans on the peritrophins provide essential binding sites for their interaction with the Cry1A toxins. Whether these toxin-binding peritrophins are mucins or proteoglycans remains unclear, since glycosaminoglycan chains in proteoglycans will also be released from their core proteins by the same type of β-elimination reaction.

To further explore the carbohydrate binding specificity for the Cry1A toxins, the peritrophins were digested with N-acetyl-α-D-galactosaminidase (α-GalNAcase), and the results were examined by SBA lectin and Cry1A toxin blotting shown in Fig 6. Treatment of the DFTM peritrophins with α-GalNAcase, which specifically cleaves terminal α1-3 linked GalNAc residues from glycoproteins, abolished Cry1Ab and Cry1Ac toxin, and SBA lectin binding to the 180 kDa complex. Toxin and lectin binding to the smaller components present in the peritrophin sample was also eliminated. Loss of SBA binding as a result of α-GalNAcase digestion of the peritrophins was expected due to the removal of terminal α-GalNAc residues recognized by the lectin. The loss of Cry1Ac toxin binding was also not surprising since GalNAc has been shown to mediate the binding promiscuity of Cry1Ac toxin with a variety of GalNAc-containing glycoproteins. However, the loss of Cry1Ab toxin binding as a result of α-GalNAcase digestion was not expected, since Cry1Aa and Cry1Ab toxin binding to the 180 kDa peritrophins were not inhibited by competing GalNAc. These results suggest that a more complex carbohydrate structure is recognized by the Cry1Aa and Cry1Ab toxins, which was compromised by the removal of the terminal GalNac residues. Taken together, the inhibitory effects of mild periodate oxidation, β-elimination of *O*-glycans and α-GalNAcase digestion; demonstrate that terminal α-GalNAc-containing *O*-glycans on the peritrophins play an essential role for their interaction with the Cry1A insecticidal proteins. Further studies are needed to elucidate the specific sugar sequences in the carbohydrate moiety of the Cry1A toxin-binding peritrophins that serve as the binding sites for the Cry1A toxins, and to identify the domains in the Cry1A toxins that are responsible for the carbohydrate binding activity.

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