Isolated domain II and III from the *Bacillus thuringiensis* Cry1Ab δ-endotoxin binds to lepidopteran midgut membranes

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Abstract The DNA fragment encoding Cry1Ab domain II–III (45.3 kDa) was cloned and expressed. Domain II–III is expressed in low yields. In vitro binding analysis to *Manduca sexta* and *Trichoplusia ni* larval midgut tissue sections demonstrated that domain II–III fragment bound along the microvilli of the midgut epithelium, indicating that this fragment retains binding functionality in the absence of domain I. Binding of domain II–III to the midgut brush border membrane proteins from *T. ni* larvae indicated that Cry1Ab toxin and domain II–III bind to the same 150 kDa protein. In contrast, in *M. sexta* membranes, Cry1Ab toxin binds to 200 and 120 kDa proteins, and domain II–III only binds to the 200 kDa protein. Finally, binding assays with isolated brush border membrane vesicles showed that the interaction of domain II–III with the membrane vesicles is highly reversible, supporting the proposition that the integration of domain I into the membrane could participate in the irreversible binding of the toxin. These studies confirm that this part of the toxin is involved in binding interactions and could be separated as a discrete fragment that conserves at least part of its functionality.

Key words: δ-Endotoxin; Mode of action; Receptor binding; *Bacillus thuringiensis*

1. Introduction

Bacillus thuringiensis is a Gram-positive, spore-forming bacterium which produces parasporal crystals during sporulation. Crystals are predominantly comprised of one or more proteins, called δ-endotoxins or Cry proteins, known to possess insecticidal activity when ingested by certain insects. The genes for a number of Cry proteins have been cloned and sequenced. They have been classified into 19 different homology groups based on sequence similarity [1]. Each Cry protein is highly specific against its target.

A generally accepted model for the mode of action of Cry is that it is a multistage process. First, the crystals ingested by a susceptible larva dissolve in the alkaline environment of the gut, releasing soluble proteins. The inactive protoxins are cleaved by proteases yielding 60–70 kDa protease resistant, active toxin fragments [2]. The activated toxin binds to receptors located on the apical microvillus membrane of epithelial midgut cells [3–5]. After receptor binding, a change in the toxin conformation is thought to occur, allowing toxin insertion into the membrane. Oligomerization of the toxin follows, and this oligomer then forms a pore that leads to osmotic cell lysis [6–9].

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The three-dimensional structures of the Cry3A and Cry1Aa toxins (which share 33% sequence identity) have been solved [7,10]. The similarity in their structures is extensive. A toxin molecule is comprised of three domains connected by single linkers. Domain I, extending from the N-terminus, is a seven α-helix bundle, with helix α-5 in the center, encircled by the other helices; it has been considered the pore formation domain. Domain II consists of three anti-parallel β-sheets sharing similar topologies, packed around a hydrophobic core. This domain represents the most divergent part in sequence and has been described as the specificity determining domain, since reciprocal hybrid genes between closely related toxins (Cry1Aa and Cry1Ac) resulted in hybrid toxins with altered specificity [11,12]. The three protruding loops in the apex of domain II were suggested to be involved in receptor binding in Cry1 and Cry3 toxins. Mutations located in these loops demonstrated that some residues are essential for binding to membranes of different lepidopteran or coleopteran insects [13–16]. Domain III is a β-sandwich of two anti-parallel β-sheets. It has been proposed that it stabilizes the toxin by protection from proteolysis [7,17]. However, recent reports suggest that it may be involved in other processes such as receptor binding [18,19] and regulation of the pore formation activity [20].

Receptor binding is a key factor in specificity. Specific binding involves two steps, one that is reversible and the other irreversible. Recent data suggest that toxicity correlates with irreversible binding [21]. Irreversible binding might be related to the toxin insertion into the membrane but could also reflect a tighter interaction of the toxin with the receptor. Mutations in domains I and II have suggested the participation of some residues in irreversible binding [14,20,22,23].

Numerous individual domains from modular proteins have been isolated by either limited proteolysis or gene manipulation. Viewed in this light, the ability to create an isolated domain may facilitate its functional analysis. Domain I expressed independently [24,25] or isolated helix α-5 peptides [26,27] retain their ability to form cation channels in planar lipid bilayers when assayed at high concentrations. There have been no reports of domain II or III isolation and expression. In this work we present the isolation and characterization of a Cry1Ab isolated fragment containing domains II and III.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli strain JM101 (*sup*E *thi*-1D (*lac-pro*AB) F′ (*tra*D36 *pro*AB+ *lac*Iq *lac*ZDM15)) was used as host for electroporation [28]. *E. coli* strain BL21 (*hsd*S *gal* (lcIts857 *ind*1 *Sam*7 *nin*5 *lac*UV5-T7 gene 1)), was used for the expression of the cloned domains. Plasmid pUC18 [29] was used for DNA cloning and sequencing and for expression of the protein.

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2.2. Construction of the N-terminal deletions of Cry1Ab

The fragments for domain II or domains II–III of the *cry1Ab* gene from *B. thuringiensis* subsp. *berliner* were obtained by PCR using plasmid pTP650 [30] as a template. Primers were designed to isolate domain II or domains II–III from the *cry1Ab* gene, as a translational fusion at codon 17 of the *lacZ* coding region in pUC18. Primer A1 (5′ CGG TAC CCG GGG ATC CTC TAG AGT CGA CAA GAG AAA TTT ATA CAA AC 3′) contains a *Bam*HI restriction site at the 5′ end. Primers B1 (5′ CAG TGC CAA GCT TGC TTT ACT GCA GAC TAC GAT GTA TCC AAG AGA A 3′) and C1 (5′ AGT GCC AAG CTT AGT GAT GGT GAT GGT GAT GAA CTA AAT TGG ATA CTT GAT C 3′) contain a *Hin*dIII restriction site. Primer C1 adds six His codons at the C-terminal end to allow purification of the resultant proteins with the aid of Ni affinity columns. PCR reactions with primers A1 and B1 afforded domain II (aa 264–459). Primers A1 and C1 were used for domains II–III construction (aa 264–648). PCR reactions were performed as follows (30 cycles): 92°C, 1 min; 55°C, 1 min and 72°C, 3 min, in a RoboCycler (Stratagene). For cloning the domain II codifying region, the PCR fragment was cut with *Bam*HI and *Hin*dIII restriction enzymes and cloned into pUC18 plasmid. The resultant construction is called pDomII. For domain II–III cloning, the PCR fragment was digested with *Hin*dIII and cloned in the same vector. The correct orientation was selected by restriction analysis. The resultant construction is called pDomII–III-H6 and the corresponding toxin product domII–III-H6. Both constructions were completely sequenced by the Sequenase dideoxy DNA sequencing method (US Biochemicals).

2.3. Purification of Cry1Ab toxin and Cry1Ab protein fragments

The purification of the Cry1Ab protoxin and the generation of the toxic trypsin resistant fragment were performed as described by Höfte et al. [31]. The toxin was further purified as described by Hofmann et al. [32].

DomII–III-H6 was expressed in BL21 *E. coli* cells. Purification of the protein was carried out by Ni-NTA-agarose affinity chromatography [33]. Briefly, an overnight culture of pDomII–III-H6 transformed cells was grown at 37°C in LB medium (200 μg ml^{-1} ampicillin). This culture was used to inoculate 100 ml LB medium (1:100). The cells were grown to an OD of 0.5–0.6 (600 nm), and induced with 1 mM isopropyl thiogalactoside (IPTG). After 3 h of growth cells were centrifuged and suspended in 3 ml of buffer A (50 mM Na_2SO_4, 300 mM NaCl, pH 8). The pellet was sonicated on ice (1 min bursts, two times), centrifuged (10 min at 12000×*g*) and 0.5 ml of a 50% slurry Ni-NTA resin (Qiagen, Chatsworth, CA), equilibrated in buffer A, was added to the supernatant and stirred for 60 min (4°C). The resin containing the protein was poured into a 1 cm diameter column, and washed with buffer A, until the OD (280 nm) of the flow through was less than 0.01. Then, the column was washed with buffer B (50 mM Na_3PO_4, 300 mM NaCl, 10% glycerol, pH 6). Non-specifically bound proteins were washed with 3 ml fractions of buffer B containing 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mM imidazole. Finally, specifically bound protein was eluted with 3 ml of buffer B containing 200 mM imidazole.

2.4. SDS-PAGE and Western blotting

Proteins were separated by SDS-10% (w/v) PAGE as described by Laemmli [34]. Western blot analysis was performed as described [35], using a poly-Cry1Ab antiserum raised against Cry1Ab toxin (1:50000) [30], followed by incubation with a secondary goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (1:2500).

2.5. In vitro binding of Cry1Ab toxin and domain II–III fragment on tissue sections

Histological sections of *Manduca sexta* or *T. ni* last instar midgut tissue were prepared as previously described [36]. The in vitro binding assays were performed using 20 μg ml^{-1} of Cry1Ab toxin or domII–III-H6 protein fragment, detected with the polyclonal anti-Cry1Ab solution, 1 mg ml^{-1} and peroxidase-conjugated goat anti-rabbit (Sigma). Sections were stained with the peroxidase substrate as described [36]. Cry1Ab toxin or domII–III-H6, primary antibodies, enzyme conjugated and secondary antibodies were sequentially omitted to exclude false positive results from tissue binding analysis.

2.6. Protein ligand blot analysis

Brush border membrane vesicles (BBMV) from *T. ni* or *M. sexta* fourth instar larvae were prepared as described by Wolfersberger et al. [37]. Protein blot analysis of BBMV preparations was done as described [38]. 20 μg of BBMV protein was separated by 9% SDS-PAGE and electrotransferred to nitrocellulose membranes. After renaturation and blocking, blots were incubated for 3 h with 0.5 μg ml^{-1} of biotinylated Cry1Ab toxin or biotinylated domII–III-H6 in washing buffer (0.5% Tween 20 in TBS) at room temperature. Unbound toxin was removed by washing three times for 10 min in washing buffer and bound toxin was identified by streptavidin-peroxidase conjugate (1:1000 dilution) for 1 h and visualized using luminol (ECL, Amersham) as described by the manufacturers.

2.7. Binding assays on isolated brush border membrane vesicles

Binding of biotinylated toxins to BBMV was performed as previously described [38]. 20 μg of BBMV protein was incubated with 10 nM biotinylated Cry1Ab toxin or biotinylated domII–III-H6 for 1 h in the presence or absence of 100-fold unlabeled Cry1Ab toxin. Unbound toxin was then washed and proteins were electrophoresed on PAGE, and transferred onto nitrocellulose membranes. The biotinylated proteins which were isolated with the vesicles were visualized by incubating with streptavidin-peroxidase conjugate (1:4000 dilution) and luminol as above.

3. Results and discussion

3.1. Expression of domain II–III-H6 in E. coli

The three domains of Cry3A and Cry1Aa toxins are rather closely packed together and the interfaces between domains are highly conserved [7,10]. The largest number of interdomain contacts are between domains I and II. Based on the sequence alignment of Cry proteins in combination with inspection of the structure of Cry1Aa toxin (Fig. 1), we selected zones of the *cry1Ab* gene to place the boundaries for domain II and domain II–III fragment expression.

The corresponding Cry1Ab domain II (24.3 kDa) and domain II–III (45.3 kDa) coding fragments were cloned by PCR amplification. These constructions were expressed in *E. coli* BL21. The product of pDomII–III-H6 construction (domII–III-H6) is expressed in very low yields (Fig. 2). In contrast, the isolated domain II was not detected. These data may indicate that interactions between domains are necessary to stabilize the structure of domain II. Also, it has been suggested that some regions within domain III are important determinants for proteolysis protection. Wabico and Yasuda [17] have reported the introduction of termination codons in the *cry1Ab* gene. Mutations encoding 606 aa or more were toxic, whereas those encoding 605 aa or less were unstable, suggesting that some residues of domain III are important for the structural integrity of the molecule.

3.2. Binding of dom-II–III-H6 analysis to midgut tissue sections

Cry1Ab toxin and domII–III-H6 were used for in vitro binding analysis to midgut tissue sections of *M. sexta* and *T. ni* larvae. The tissue sections were incubated with the native toxin or with domII–III-H6, and bound proteins were detected by antibody incubation as described in Section 2. We found that both proteins bound evenly along the top of the microvilli of the midgut epithelium of *M. sexta* larvae (Fig. 3A,B) and also to the complete microvilli membrane of *T. ni* midgut cells (Fig. 3D,E). These data indicate that domains II–III expressed independently retain binding functionality in the absence of domain I, since domII–III-H6 is able to interact with the tissue section microvilli similar to the complete toxin. No binding to other structures such as Malpighian tubules, basement membrane or muscle cells was observed.

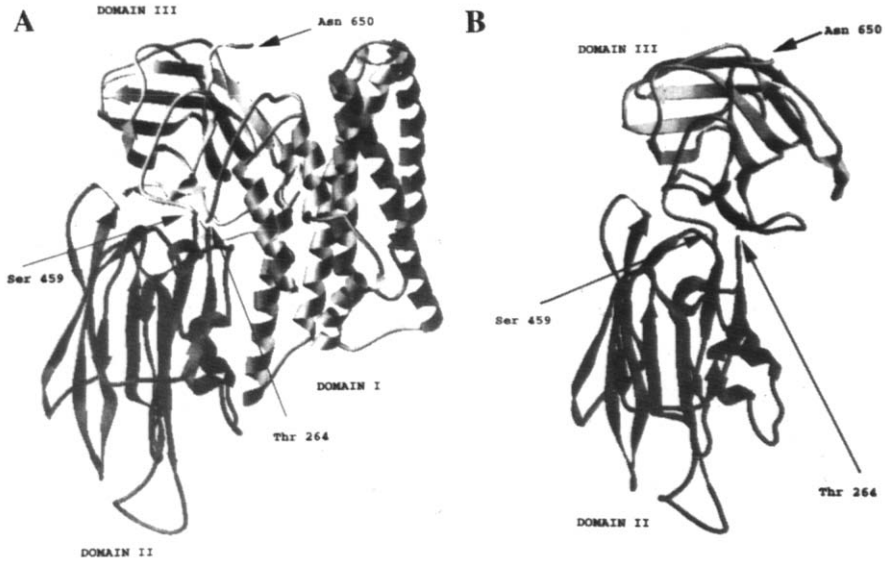

Fig. 1. Crystal structure of Cry1Aa toxin (modified from Grochulski et al. [10]). A: Schematic ribbon representation of the three domain (domain I–III) organization of the toxin. The positions of the boundaries for domain II (Thr^{264}-Ser^{459}) and domain II–III (Thr^{264}-Asn^{650}) are indicated. B: Representation of isolated domain II–III.

3.3. Protein blots

The 210 kDa cadherin-like glycoprotein from *M. sexta* has been identified as Cry1Ab toxin binding protein [39,40] and an aminopeptidase N from *M. sexta*, *Heliothis virescens*, *Plutella xylostella* and *Lymantria dispar* as the Cry1Ac toxin binding protein [41–45]. However, it has been demonstrated that Cry1Ab also binds with high affinity to the purified 120 kDa aminopeptidase N Cry1Ac toxin binding protein purified from *M. sexta* [46]. Some authors have shown that also in ligand blot experiments performed with BBMV from *M. sexta* the Cry1Ab toxin binds to both 120 and 210 kDa proteins [14,23].

In order to investigate the specific proteins, responsible for the interaction between domII–III-H6 and the BBMV of both insects, we analyzed the toxin binding protein complexes employing the ligand blot technique. Membrane vesicle proteins from *T. ni* and *M. sexta* larvae were resolved by SDS-PAGE, transferred to nitrocellulose membranes and incubated with biotinylated Cry1Ab toxin or domII–III-H6. After washing the unbound toxin, the biotinylated proteins were visualized by incubation with streptavidin-peroxidase conjugate. The results indicate that both proteins bound to a 150 kDa protein in *T. ni* membranes (Fig. 4A, lanes 1 and 3) and that this interaction is specific since binding of biotinylated domain II–III-H6 can be eliminated by incubation of the biotinylated protein fragment with the BBMV in the presence of a 100-fold excess of unlabeled Cry1Ab toxin (Fig. 4A, lane 2). In *M. sexta* membranes the Cry1Ab toxin binds to 120 kDa and 210 kDa proteins (Fig. 4B, lane 1) while domII–III-H6 only binds to a 210 kDa protein (Fig. 4B, lane 3). We do not have a definitive explanation for the lack of binding to the 120 kDa protein by domII–III-H6, a possibility is that domain I may be involved in the interaction with the 120 kDa protein or that the structure of domII–III-H6 could be altered affecting this interaction.

3.4. Homologous competition binding analysis

To study the binding characteristics of domII–III-H6 with native membrane preparations, binding experiments with biotinylated domII–III-H6 to freshly isolated *T. ni* BBMV were performed. BBMV were incubated with biotinylated Cry1Ab toxin or domII–III-H6 in the presence or absence of a 100-fold excess of Cry1Ab native toxin. Subsequently, the unbound toxin was washed off and the BBMV containing the bound toxin were analyzed by SDS-PAGE. After electrophoresis and blotting on nitrocellulose membranes, the biotinylated crystal proteins were visualized by incubation with the streptavidin-peroxidase conjugate. Fig. 5 shows the binding of biotinylated Cry1Ab toxin (Fig. 5, lane 1) and no binding of biotinylated domII–III-H6 to the BBMV (Fig. 5, lane 4). It

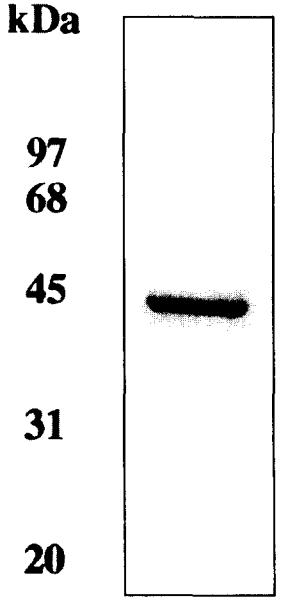

Fig. 2. Western blot analysis of domII–III-H6 expression in *E. coli* strain BL21 using a poly-Cry1Ab antiserum raised against Cry1Ab toxin (1:50000).

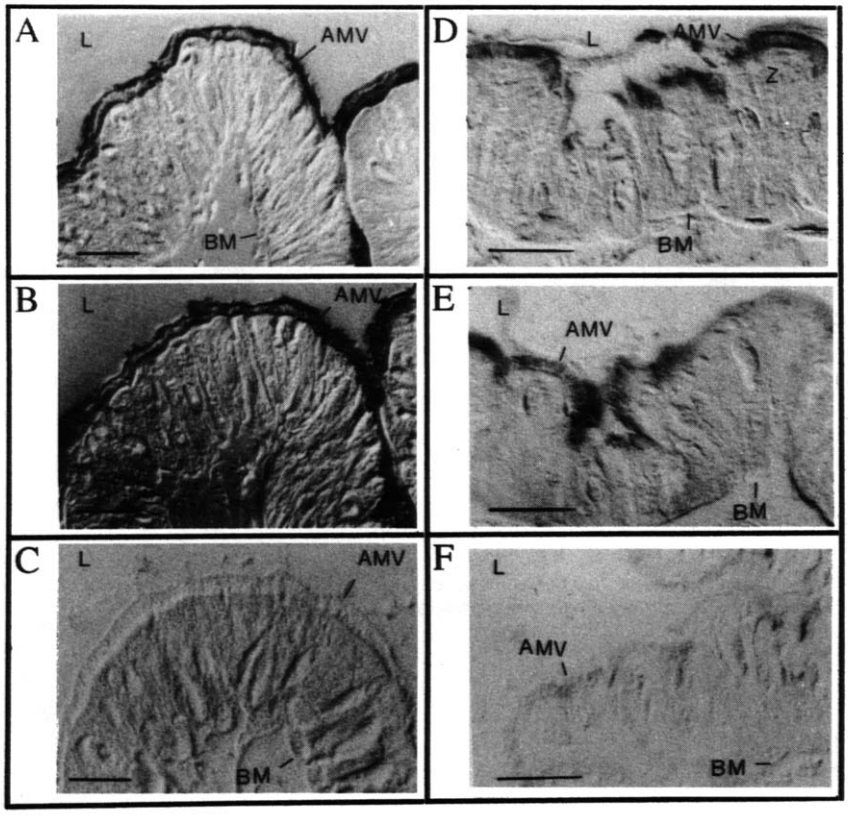

Fig. 3. Immunolocalization of proteins bound to midgut tissue sections of *M. sexta* (A–C) and *T. ni* (D–F) larvae. Midgut sections were incubated with Cry1Ab toxin (A, D) or domII–III-H6 (B, E) solutions. DomII–III-H6 and Cry1Ab toxin bound to the top of the *M. sexta* apical microvilli (A, B). In contrast, they bound to the whole microvillus membrane of *T. ni* tissue sections (D, E). None of them bound to other organs such as trachea or Malpighian tubules. Negative control with no ICP incubation (C, F) showed no staining. BM, basement membrane and connective tissue; L, lumen; AMV, apical microvilli. Bar = 20 μm.

has been proposed that residues F371 and G374 from domain II of Cry1Ab play an important role in the irreversible interaction of the toxin with its receptor, since mutants affected in these residues lost toxicity and showed reduced irreversible binding without affecting initial binding [23]. However, mutants in these residues have not lost total irreversible binding, suggesting the existence of other important contacts with the receptor or the membrane in other parts of the toxin molecule that could also participate in the irreversible binding. Our data indicate that the interaction of domII–III-H6 with the BBMV is quite reversible; this could be due to the absence of domain I which might be necessary to obtain irreversible binding to the membrane due to membrane insertion. However, we cannot exclude the possibility that domII–III-H6 could be altered in some way that disrupts its irreversible binding. The determination of the three-dimensional structure of domII–III-H6 could be a way to analyze if it has a different structure. Also, a future determination of on- and off-rate binding constants to native purified receptor would determine if the binding of domII–III-H6 is the same as the whole toxin.

Several proteins are organized as discrete modules which may have different functions. It has been proposed that the different domains of modular proteins could have evolved independently and that domain swapping may contribute to the versatility of protein function [47,48]. The Cry proteins are a family that have biocidal activities against very different targets. These proteins are modular in structure. It has been reported that the vitelline membrane outer layer protein I

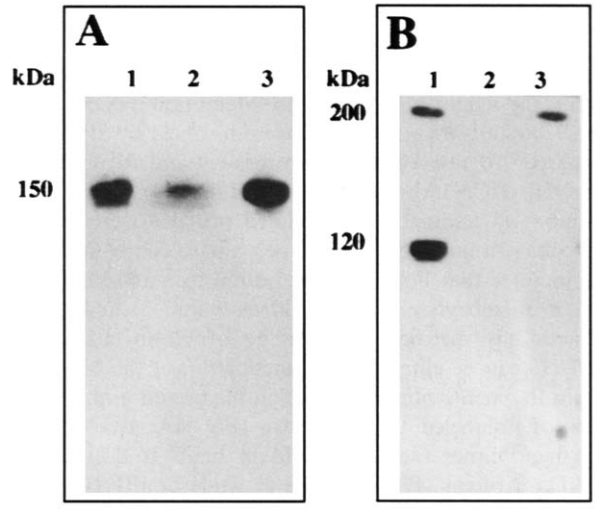

Fig. 4. Ligand blotting assay on *T. ni* and *M. sexta* BBMV. BBMV proteins were loaded onto a SDS-PAGE and blotted to a nitrocellulose membrane. *T. ni* (A) or *M. sexta* (B) BBMV were incubated with biotinylated domII–III-H6 (lanes A1, B3) and biotinylated Cry1Ab toxin (lanes A3, B1) and bound toxins were detected by means of streptavidin-peroxidase conjugate as described in Section 2. Lane A2 showed the competition of the biotinylated domII–III-H6 fragment with a 100-fold excess of unlabeled Cry1Ab toxin. Lane B2 showed the competition of the biotinylated Cry1Ab toxin with a 100-fold excess of unlabeled Cry1Ab toxin.

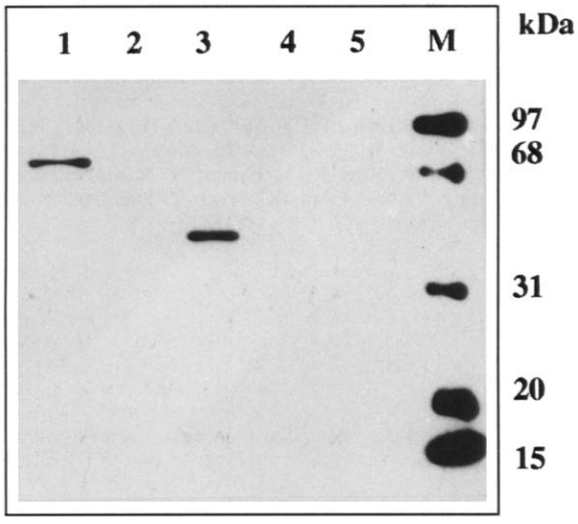

Fig. 5. Homologous competition binding assays on *T. ni* BBMV. Biotinylated Cry1Ab toxin (lanes 1 and 2) and biotinylated domII–III-H6 (lanes 4 and 5) were incubated with BBMV in the absence (lanes 1 and 4) or the presence of a 100-fold excess of Cry1Ab unlabeled toxin (lanes 2 and 5). After 1 h incubation, unbound toxins were removed and vesicles containing bound toxins were loaded onto a SDS-PAGE and blotted to a nitrocellulose membrane. Labeled proteins were visualized by means of streptavidin-peroxidase conjugate. Lane 3, biotinylated domII–III-H6 directly loaded on the SDS-PAGE.

(VMO-I) and domain II from Cry have similar three-dimensional structures and it has been proposed that the β-prism fold may be a structural domain associated with carbohydrate binding functionality [49]. Both proteins may have a carbohydrate binding site, since binding of Cry1Ac toxin to its receptor is inhibited by *N*-acetylgalactosamine [50] and the VMO-I binds hexasaccharides of *N*-acetylglucosamine [51]. Unfortunately, the inability to express domain II in isolation did not allow us to test if this domain is the major contributor to specific binding.

However, in this work, we present evidence that domains II–III expressed independently are able to interact with the membranes of lepidopteran insects in a rather similar way to the complete Cry1Ab toxin, confirming that this part of the toxin is involved in binding interactions and that they could be separated as a discrete fragment that conserves at least part of its functionality.

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