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Binding Characteristics to Mosquito-larval Midgut Proteins of the Cloned Domain II-III Fragment from the *Bacillus thuringiensis* Cry4Ba Toxin

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Receptor binding plays an important role in determining host specificity of the *Bacillus thuringiensis* Cry δ-endotoxins. Mutations in domains II and III have suggested the participation of certain residues in receptor recognition and insect specificity. In the present study, we expressed the cloned domain II-III fragment of Cry4Ba and examined its binding characteristics to mosquito-larval midgut proteins. The 43-kDa Cry4Ba-domain II-III protein over-expressed in *Escherichia coli* as inclusion bodies was only soluble when carbonate buffer, pH 10.0 was supplemented with 4 M urea. After renaturation *via* stepwise dialysis and subsequent purification, the refolded domain II-III protein, which specifically reacts with anti Cry4Ba-domain III monoclonal antibody, predominantly exists as a β-sheet structure determined by circular dichroism spectroscopy. *In vitro* binding analysis to both histological midgut tissue sections and brush border membrane proteins prepared from susceptible *Aedes aegypti* mosquito-larvae revealed that the isolated Cry4Ba-domain II-III protein showed binding functionality comparable to the 65-kDa full-length active toxin. Altogether, the data present the 43-kDa Cry4Ba fragment comprising domains II and III that was produced in isolation was able to retain its receptor-binding characteristics to the target larval midgut proteins.

Abbreviations: *Bt* (*Bacillus thuringiensis*), BBMVs (brush border membrane vesicles), CD (circular dichroism), Cry (crystal), Cyt (cytolytic), ELISA (enzyme-linked immunosorbent assay), GPI-anchored ALP (glycosylphosphatidyl inositol-anchored alkaline phosphatase, IPTG (isopropyl-β-D-thiogalactopyranoside), MAb (monoclonal antibody), PCR (polymerase chain reaction), SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis)

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Introduction

Bacillus thuringiensis (*Bt*) produces insecticidal crystal proteins (Cry and Cyt δ-endotoxins) has been wildly used as an alternative to the current chemical insecticides in the control of certain insect pests and human disease vectors (Schnepf *et al*., 1998). The Cry δ-endotoxins exhibit broad larvicidal spectra against different insect orders, Diptera, Lepidoptera, Coleoptera and Hymenoptera (Schnepf *et al*., 1998; de Maagd *et al*., 2001). These insecticidal proteins are synthesized as inactive protoxins in the form of cytoplasmic crystalline inclusions that are solubilized and subsequently processed to active toxins by gut proteases in the larval midgut lumen (Schnepf *et al*., 1998). The activated toxins bind to specific receptors on the microvilli of larval midgut epithelial cells and possibly undergo conformational changes followed by membrane insertion and pore formation, eventually resulting in the death of the larvae (Knowles, 1994; Whalon *et al*., 2003).

The X-ray crystal structures of the activated forms of several Cry δ-endotoxins, including Cry1Aa (Grochulski *et al*., 1995), Cry1Ac (Derbyshire *et al*., 2001), Cry2Aa (Morse *et al*., 2001), Cry3Aa (Li *et al*., 1991), Cry3Bb (Galitsky *et al*., 2001), Cry4Aa (Boonserm *et al*., 2006) and Cry4Ba (Boonserm *et al*., 2005), have been solved, leading to a better understanding of the molecular basis of lytic pore formation and insect specificity. These structures reveal three distinct domains with a high degree of overall structural similarity. The N-terminal domain (domain I) is an α-helical bundle that is responsible for the pore-forming activity (Masson *et al*., 1999; Puntheeranurak *et al*., 2004; Rausell *et al*., 2004; Alzate

et al., 2006). The middle and C-terminal domains (domains II and III) which comprise mostly anti-parallel β-sheets have been implicated to participate in receptor recognition and insect specificity (de Maagd *et al.*, 2000; Jurat-Fuentes and Adang, 2001; Masson *et al.*, 2002; Fernändez *et al.*, 2005; Tuntitippawan *et al.*, 2005). Although the Cry4Aa and Cry4Ba structures bear a resemblance to the other known Cry structures, the finer features are rather different. There is additional *in vitro* proteolysis by trypsin occurring in the loop connecting helices 5 and 6 of the 65-kDa activated forms for these two dipteran-active toxins, thus generating two non-covalently associated fragments of ca. 47 and 18-20 kDa (Angsuthanasombat *et al.*, 2004). For the Cry4Ba toxin, the smaller fragment of 18-20 kDa which maps to the first five helices, α1-α5, has been shown to be capable of permeabilizing liposomes and inducing ion channels in planar lipid bilayers (Puntheeranurak *et al.*, 2004).

In our earlier studies, toxicity role of each functional domain of the Cry4Ba toxin has been investigated. Several mutational studies have suggested that α4 is oriented to face the pore lumen and participates in ion permeation through the pore (Sramala *et al.*, 2001), whilst the relatively hydrophobic helix-α5 appears to be in contact with the lipid membrane and plays a crucial role in toxin-pore oligomerization (Likitvivatanavong *et al.*, 2006). We have also provided direct evidence for membrane-perturbing activity of the α4-loop-α5 hairpin isolated from the Cry4Ba toxin (Leetachewa *et al.*, 2006). More recently, we have identified functional elements by demonstrating that two highly conserved aromatic residues, Tyr^{249} and Phe^{264}, which are oriented on the same side of α7, play an important role in larvicidal activity of this mosquito-active toxin (Tiewsiri and Angsuthanasombat, 2007). For domain II, we have shown that some residues located in the β6-β7, β8-β9 and β10-β11 loops are involved in Cry4Ba larvicidal activity (Tuntitippawan *et al.*, 2005). However, it remains to be investigated whether these critical loop residues are involved in receptor binding and host specificity. We have recently demonstrated that the 21-kDa Cry4Ba-domain III, which can be isolated as a native folded monomer, conceivably participates in toxin-receptor recognition (Chayaratanasin *et al.*, 2007). In the present study, we described over-expression of the 43-kDa domain II-III fragment of Cry4Ba in *E. coli*. *In vitro* binding characteristics of the cloned domain II-III fragment to both histological midgut section and brush border membrane proteins prepared from susceptible *Ae. aegypti* mosquito-larvae were demonstrated.

Materials and Methods

Construction of the recombinant plasmid expressing the Cry4Ba-domain II-III fragment. The 1.17-kp DNA segment encoding the 43-kDa domain II-III fragment of the Cry4Ba toxin was amplified *via* polymerase chain reaction (PCR) from the pMU388 recombinant plasmid encoding the full-length *cry4Ba* gene (Angsuthanasombat *et al.*, 1987). Forward primer used (5'-CGGAATTC**GGAGG**AATAAAT**ATG**TACCCTGCGGACGACAAAATAGATAATACG-3') which contains an *Eco*RI restriction site (underlined bases), the Shine-Dalgarno (SD) sequence (double underlined bases), the start codon (bold letters) and the coding region for the loop residues ($_{272}$YPADKIDNT$_{280}$) connecting α7-domain I and β1-domain II. Reverse primer used (5'-TGTAAACAGTGCGTCGAC**TTATTA**TCGTTCTGATTCTAAATTTTG-3') contains a *Sal*I restriction site (underlined bases) and two consecutive stop codons (bold letters). PCR amplification was performed using a high fidelity *Pfu* DNA polymerase. The 1.17-kb PCR product containing *Eco*RI and *Sal*I sites on the 5' and 3' ends, respectively, was cut with corresponding restriction enzymes and subsequently inserted into the pMEx8 expression vector (Buttcher *et al.*, 1990). The resultant plasmid, pMEx-4BD23 (see Fig. 1), was transformed into $CaCl_2$-treated *E. coli* JM109 competent cells. The correct insertion of the cloned domain II-III DNA segment was proved by digestion of the plasmid with *Eco*RI and *Sal*I, and subsequently verified by nucleotide sequencing.

Expression and preparation of the Cry4Ba-domain II-III protein. *E. coli* cells harboring pMEx-4BD23 were grown at 37°C in a Luria-Bertani medium (100 μg/ml ampicillin) until OD_{600} of the culture reached 0.4-0.6. Protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM for 4 h. Cells expressing the Cry4Ba-domain II-III protein as inclusion bodies were harvested by centrifugation and subsequently disrupted by using a French Pressure Cell at 20,000 psi. Protein inclusions were extracted from crude lysate by following the method described previously (Leetachewa *et al.*, 2006), and analyzed by sodium dodecyl sulfate-(12% w/v) polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations of the partially purified inclusions were determined by using the Bradford-based protein assay (Bio-RAD), with bovine serum albumin (Sigma) as a standard. The 43-kDa domain II-III protein was verified by automated N-terminal sequencing. The protein resolved by SDS-PAGE was transferred onto a polyvinylidene difluoride membrane (Hybond P, Amersham Bioscience) and subsequently subjected to automated Edman degradation using a PPSQ-21A protein sequencer (Shimadzu, Japan).

For unfolding and refolding steps, the domain II-III protein inclusions (2 mg/ml) were initially washed by incubating in 50 mM Na_2CO_3 buffer (pH 10.0) containing 3 M urea at 37°C for 1 h. The pre-washed inclusions were centrifuged at 10,000 × *g*, 4°C for 15 min and subsequently solubilized in the carbonate buffer supplemented with 4 M urea at 37°C for 1 h. The solubilized domain II-III protein was refolded by stepwise dialysis against 300 volumes of the carbonate buffer with decreasing urea concentrations of 3, 2, 1, 0.5, and 0.25 M at 4°C for 1 h at each step, and was finally dialyzed twice against the carbonate buffer for 2 h each. After centrifugation at 10,000 × *g*, 4°C for 15 min, the clear supernatant containing the refolded domain II-III protein was analyzed by SDS-PAGE (12% gel).

The refolded domain II-III fragment was purified by size-exclusion liquid chromatography (Superpose 12 HR 10/30 column, Amersham Pharmacia Biotech) with the carbonate buffer (pH 10.0) at a flow rate of 0.4 ml/min. Eluted fractions containing the 43-kDa domain II-III protein were concentrated by dialysis absorption with polyethyleneglycol (M.W. 15,000-20,000).

Structural characterization of the purified domain II-III protein. Circular dichroism (CD) spectrum of the purified domain II-III protein (0.3-0.4 mg/ml) prepared in the carbonate buffer, pH 10.0 was measured using a Jasco J-715 CD spectropolarimeter (Jasco Inc., USA) as described previously (Leetachewa *et al*., 2006). Secondary structure contents were estimated from the CD spectra using the CONTINLL program on the Dichroweb site (www.cryst.bbk.ac.uk/cdweb/html/ home.html) (Lobley *et al*, 2002; Whitemore and Wallace, 2004).

Monoclonal antibody (MAb) production. An 8-week-old BALB/c mouse was immunized with the 65-kDa FPLC-purified Cry4Ba protein as follows: 25 mg immunogen emulsified in a complete Freunds adjuvant (Gibco BRL) was first injected into the peritoneal cavity. For second and third injections with 2 week-intervals, the immunogen was prepared in an incomplete Freunds adjuvant. Production of anti-Cry4Ba polyclonal antibodies in mouse sera was determined by an indirect Enzyme-Linked Immunosorbent Assay (ELISA) using the purified 65-kDa Cry4Ba protein as an antigen (100 ng/well). Spleen cells collected from the immunized mouse were fused with non-secreting mouse myeloma cells (X63AG8.653). Culture supernatant of hybrid cells was screened for antibody reactivity against the 65-kDa Cry4Ba protein by using indirect ELISA. Antibody-secreting hybridomas were subsequently sub-cloned by limiting dilution for 1-2 rounds. Specificity of the resultant MAbs was tested by Western blot analysis probed with the full-length activated Cry4Ba protein, the cloned Cry4Ba-domain II-III fragment and the cloned domain III fragment. The culture supernatant containing MAb (2F-1H2) that is specific to the Cry4Ba-domain III epitope was obtained and used for *in vitro* binding characterization of the Cry4Ba toxin and its derivatives.

Western blot analysis. Protein samples resolved by SDS-PAGE in Tris-glycine buffer (192 mM glycine, 25 mM Tris-HCl, pH 8.3) were electrophoretically transferred onto a nitrocellulose membrane using the Mini Trans-Blot electrophoretic transfer cell (Bio-RAD) in transferring buffer (190 mM glycine, 0.04% SDS, 20% methanol and 25 mM Tris-HCl, pH 8.3) at constant voltage of 30 volts, 4°C for 2 h and subsequently at 70 volts for additional 4 h. For blocking non-specific binding sites, the membrane was incubated with PBS (phosphate buffered saline), pH 7.4 containing 5% bovine serum (Research Organics). The proteins immobilized on the membrane were probed with 1 : 50 dilution of the 2F-1H2 hybrid culture supernatant containing anti-Cry4Ba domain III MAb. The immuno-complexes were incubated with biotin-conjugated rabbit anti-mouse IgG (1 : 200,000 dilution) (Pierce) and subsequently detected with horseradish peroxidase-conjugated streptavidin (1 : 50,000 dilution) (Pierce). The target protein band was visualized as a dark-blue color precipitate by incubating with the developing reagent [0.6 mg/ml 3,3'-diaminobenzoidine (DAB, Sigma-Aldrich), 0.4 mg/ml $NiCl_2$, 0.03% H_2O_2].

***In vitro* binding assays *via* immunohistochemical staining**. Paraffin-embedded histological sections of 5-day-old *Ae. aegypti* larval gut tissue were prepared as previously described (Chayaratanasin *et al*., 2007). Immunohistochemical staining was performed following the method described previously (Chayaratanasin *et al*., 2007) using the 65-kDa activated Cry4Ba toxin (12.5 mg/ml) or the purified domain II-III fragment (12.5 and 37.5 mg/ml), and sequentially probed with the 2F-1H2 anti-Cry4Ba domain III MAb (1 : 20 dilution), biotin-conjugated rabbit anti-mouse IgG (1 : 8,000 dilution) and peroxidase-conjugated streptavidin (1 : 50,000 dilution).

Preparation of larval gut brush border membrane vesicles (BBMVs). The guts containing food boluses of 5-day-old *Ae. aegypti* larvae (about 10,000 guts) were pulled out of the dissected bodies, and homogenized in ice-cold gut extracting (GET) buffer (10 mM Tris-HCl, pH 8.0, 1 mM EGTA, 300 mM mannitol, 1 mM PMSF). BBMVs were prepared according to the method of Wolfersberger *et al*. (1987) with some modifications. An equal volume of ice-cold 24 mM $MgCl_2$ was added to the gut homogenate and the mixture was kept on ice for 15 min. After centrifugation at 2,500 × *g*, 4°C for 5 min, the supernatant was transferred to another centrifuge tube. The pellet was suspended in ice-cold GET buffer with 0.5 volume of the gut homogenate. The suspension was added with 24 mM $MgCl_2$ and the above protocol was repeated. All the supernatant fractions from $MgCl_2$ treatment were pooled and centrifuged at 16,000 × *g* for 1 h. The final BBMV pellet was suspended in GET buffer and kept at −70°C.

Toxin-overlaying assay. Protein samples resolved by SDS-PAGE (12% gel) were transferred onto a nitrocellulose membrane as described above. The membrane was incubated with blocking buffer (5% bovine serum in PBS, pH 7.4) containing either 3 μg/ml (~45 nM) of the full-length activated Cry4Ba toxin, 2 μg/ml (~45 nM) of the purified domain II-III protein or 6 μg/ml (~290 nM) of the isolated domain III protein at room temperature for 1 h. After 3 time-washing with PBS (pH 7.4) containing 0.1% tween-20, BBMV protein-Cry4Ba complexes were incubated with the 2F-1H2 MAb (1 : 200 dilution), and subsequently detected with biotin-conjugated rabbit anti-mouse IgG (1 : 200,000 dilution) and peroxidase-conjugated streptavidin (1 : 50,000 dilution). A positive signal was developed by chemiluminescent based-Pico Supersignal kit (Pierce) and imaged on Medical X-ray film blue (AGFA).

Mosquito-larvicidal activity assays. Larvicidal activity assays were performed following the method described previously (Chayaratanasin *et al*., 2007). 2-day-old *Ae. aegypti* mosquito-larvae were fasted for 4 h prior to the assays. The assays were performed at room temperature (25°C) in 48-well titration plate (11.3 mm well diameter, Costar) containing 1 ml of *E. coli* suspension (10^8 cells) or partially purified domain II-III inclusions (5 μg), with 10 larvae per well and a total of 100 larvae for each tested sample. Percent mortality was recorded after 24-h incubation period. Bacterial cells harboring the pMEX8 vector was used as a negative control.

Results and Discussion

Expression in *E. coli*, refolding and purification of the cloned Cry4Ba-domain II-III fragment. As can be inferred from the Cry4Ba crystal structure, there are several hydrogen bonds formed between the α7-β1 loop in domain I and β-strands of the inner sheet or α9 in domain III (Boonserm *et al*., 2005). This configuration suggests a need for the α7-β1 loop to retain structural integrity of domain III in addition to

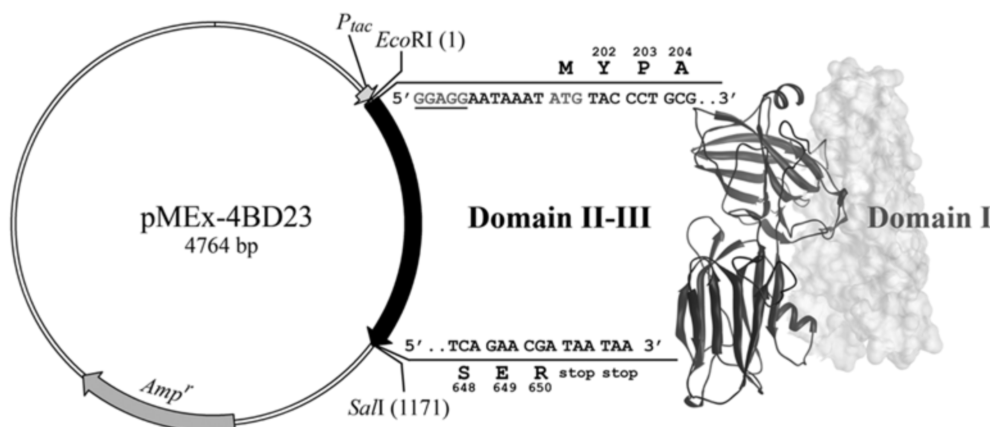

Fig. 1. Schematic construct of the pMEx-4BD23 recombinant plasmid for expressing the corresponding Cry4Ba-domain II-III coding fragment under control of the *tac* promoter (P_{tac}). The nucleotide and deduced amino acid sequences at the 5'- and 3'-ends of the *cry4Ba-domain II-III* gene are shown with the SD sequence (underlined nucleotides). The arrows indicate the transcriptional direction of the corresponding promoter and genes. Amp^r indicates the ampicillin resistance gene. For clarity, only the restriction endonuclease sites mentioned in the text are shown. The right-hand figure represents the crystal structure of the 65-kDa activated Cry4Ba toxin, possessing three domains. Domain I is shown in a space-filling model, while domains II and III including the α7-β1 loop are illustrated in a schematic ribbon.

the large number of interdomain contacts occurring between domains II and III. In the present study, we therefore decided to express separately the Cry4Ba-domain II-III fragment including the loop residues (Tyr^{272}-Pro-Ala-Asp-Lys-Ile-Asp-Asn-Thr^{280}) connecting α7 and β1 (see Fig. 1). Purification of the cloned domain II-III fragment has made it possible to attempt structure-function studies of this protein.

When the plasmid clone (pMEx-4BD23, Fig. 1) was expressed in *E. coli* under regulation of the *tac* promoter together with the SD sequence (GGAGG) derived from the *Bti*-regulatory element (Boonserm *et al.*, 2004), the corresponding Cry4Ba-domain II-III fragment was produced as sedimentable inclusion bodies. Analysis of the inclusion preparation by SDS-PAGE revealed a major band at 43-kDa (see Fig. 2, lane 1) which specifically reacted in Western blots probed with the 2F-1H2 anti-Cry4Ba domain III MAb (Fig. 2, lane 2). The N-terminal sequence of the 43-kDa protein obtained by automated Edman degradation was found to be identical to the expected N-terminus (Met-Tyr-Pro-Ala-Asp-Lys-Ile-Asp-Asn-Thr---) of the cloned Cry4Ba-domain II-III fragment. Since the calculated molecular mass of the polypeptide encoded in the cloned domain II-III fragment is 42,498.60 Da, these results provide evidence that the 43-kDa product we have obtained from over-expressing *E. coli* corresponds to the domain II-III fragment of the Cry4Ba toxin.

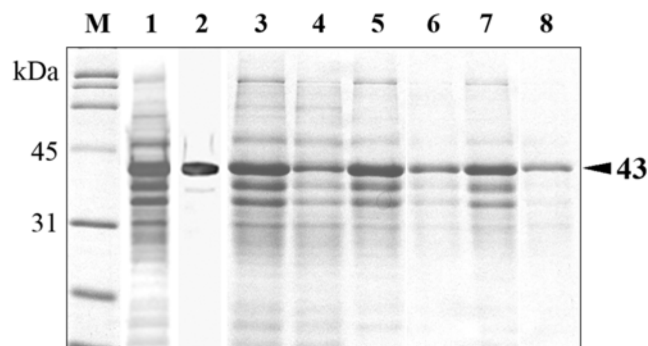

Fig. 2. SDS-PAGE analysis (Coomassie brilliant blue-stained 12% gel) of unfolding and refolding of the Cry4Ba-domain II-III fragment. Lane 1 represents total fraction of the partially purified Cry4Ba-domain II-III inclusions incubated in 50 mM Na_2CO_3 buffer (pH 10.0). Lane 2 represents Western blot analysis of lane 1 probed with the 2F-1H2 MAb specific to the Cry4Ba domain III fragment. Lane 3 is total fraction of the domain II-III inclusion in carbonate buffer supplemented with 3 M urea. Lanes 4 and 5 are soluble and insoluble protein fractions, respectively, after centrifugation of total fraction from lane 3. Lanes 6 and 7 are soluble and insoluble protein fractions, respectively, after incubating the insoluble materials of lane 5 in carbonate buffer containing 4 M urea. Lane 8 represents the refolded domain II-III protein after stepwise dialysis of the soluble proteins from lane 4. M is standard molecular mass markers.

Dissimilar to the protein inclusion of the 130-kDa Cry4Ba protoxin which is soluble (up to 85% solubility) in carbonate buffer, pH 10.0, the Cry4Ba-domain II-III inclusion was readily soluble only in the presence of 4 M urea, giving at least 50% solubility (see Fig. 2, lanes 6 and 7). This indicates that the absence of the N-terminal domain I, which might be needed for correct folding of the rest of the toxin, could disturb the structural characteristics that consequently result in improper folding of the isolated Cry4Ba-domain II-III fragment. Protein misfolding can be attributed to the intracellular concentration of aggregation-prone intermediates that would lead to the formation of an insoluble aggregate *in vivo*. It should be noted that the domain II-III inclusion was pre-washed with 3 M urea-carbonate buffer prior to solubilizing in

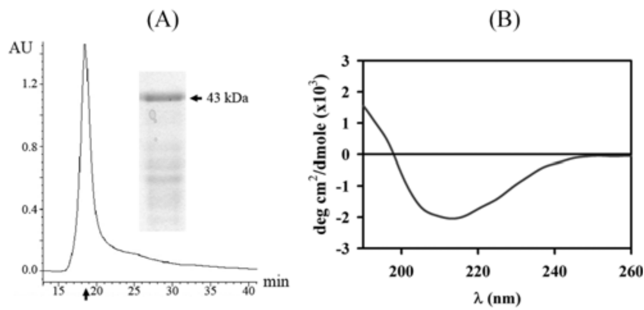

Fig. 3. (A) Protein purification chromatogram of the refolded Cry4Ba-domain II-III protein. FPLC-elution profile from the Superose 12 size-exclusion column, showing absorbance at 280 nm (AU) and elution volume (ml). Inset, SDS-PAGE analysis (Coomassie brilliant blue-stained 12% gel) of the peak fraction (arrowed) containing the 43-kDa purified Cry4Ba-domain II-III fragment. M represents molecular mass standards. (B) CD Spectrum of the FPLC-purified domain II-III protein in carbonate buffer, pH 10.0.

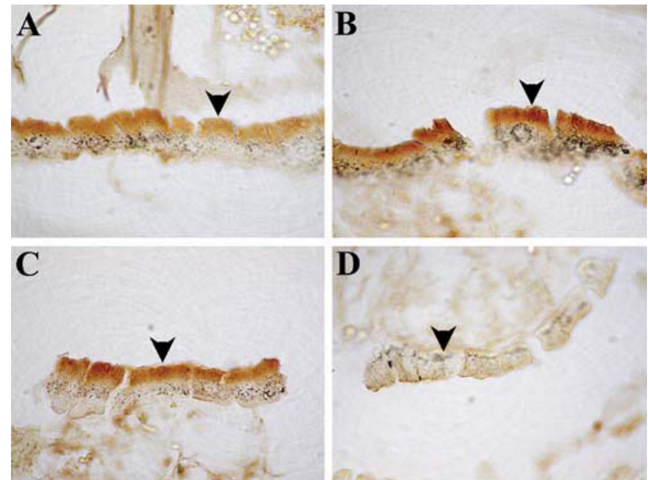

Fig. 4. Immunolocalization of proteins bound to histological sections of 5-day-old *Ae. aegypti* larval midgut tissue. Slide sections were incubated with the purified Cry4Ba-domain II-III protein at concentrations of 12.5 μg/ml (A) and 37 μg/ml (B), or with the 65-kDa full-length activated Cry4Ba toxin at 12.5 μg/ml (C). Negative control slide omitting the tested proteins (D) shows no immunohistochemical staining. Arrows indicate apical microvilli.

the 4 M urea buffer, giving a cleaner soluble faction (see Fig. 2, lanes 4 and 6). The solubilized domain II-III protein was refolded by stepwise dialysis against buffers with decreasing urea concentrations and finally the refolded protein was obtained in urea-free carbonate buffer, pH 10.0 (Fig. 2, lane 8).

Upon purification using size-exclusion FPLC with carbonate buffer, pH 10.0 as eluent, the 43-kDa refolded domain II-III protein was eluted in the void volume of the column (Fig. 3A). This suggested that a high molecular mass (>300 kDa) oligomer or aggregate was present rather than the monomeric form of the refolded protein. Notwithstanding this observation, this aggregate could represent a stable and functional oligomerization state of the domain II-III fragment with the ability to bind to its receptors. The 43-kDa purified domain II-III protein was also assessed for secondary structural elements by far-UV CD spectroscopy. CD spectrum of the domain II-III protein from repeated scans within 190-260 nm showed characteristics conceivably for a protein having a predominant anti-parallel β-sheet structure (46% β-sheet, 5% α-helix, 22% turns and 26% random coil). This indicates that the 43-kDa isolated domain II-III fragment distinctly adopted a β-structure, corresponding to the domain II-III structure embodied in the Cry4Ba crystal structure (Boonserm *et al.*, 2005). We conclude that the refolded 43-kDa domain II-III protein likely exists, albeit in an oligomeric form, in its native folded conformation.

Functional characterization of the isolated Cry4Ba-domain II-III protein. No significant activity was observed in bioassays against *Ae. aegypti* mosquito-larvae using either *E. coli* cells (10^8 cells/ml) expressing the cloned domain II-III fragment or its inclusion bodies (5 μg/ml). Lack of toxicity is most likely due to the absence of the N-terminal α-helical bundle (Boonserm *et al.*, 2005) which is required for exerting larvicidal activity *via* membrane insertion and pore formation. Recently, we have demonstrated that the 47-kDa purified Cry4Ba toxin fragment, which corresponds to α6-loop-α7 linked with domains II and III, was incapable to induce calcein permeability of lipid vesicles when compared to the 65-kDa full-length activated toxin (Leetachewa, *et al*., 2006). In other studies with the Cry4Aa cleavage products genetically fused with glutathione S-transferase (GST), significant larvicidal activity was observed only when both of the fusion proteins, GST-20-kDa (α1-α5) and GST-45-kDa containing domains II and III, were co-ingested by the larvae (Yamagiwa *et al.*, 1999).

It has been shown by *in vitro* binding analysis *via* immuno-histochemical assays that the primary site of action of several mosquito-specific Cry toxins (*i.e.* Cry4Aa, Cry4Ba, Cry11Aa and Cry11Bb) is located in the apical microvilli of the mosquito-larval midguts (Ravoahangimalala and Charles, 1995; Ruiz *et al.*, 2004). Here, we have employed a similar approach *via* immuno-histochemical staining to analyze the binding capability of the isolated Cry4Ba-domain II-III protein towards the mosquito larval midgut. In the assay, histological sections of *Ae. aegypti* larval midgut tissue incubated with the purified domain II-III protein showed an intense brown staining along the apical microvilli of the midgut epithelium (Fig. 4A,B), and the staining signal was comparable to that observed upon incubation with the 65-kDa full-length activated toxin (Fig. 4C). Control larval midgut section in which the domain II-III protein or the activated toxin was omitted showed a very weak staining of non-specific signals on the apical microvilli membrane (see Fig. 4D). These data directly demonstrate that the 43-kDa purified Cry4Ba-domain II-III

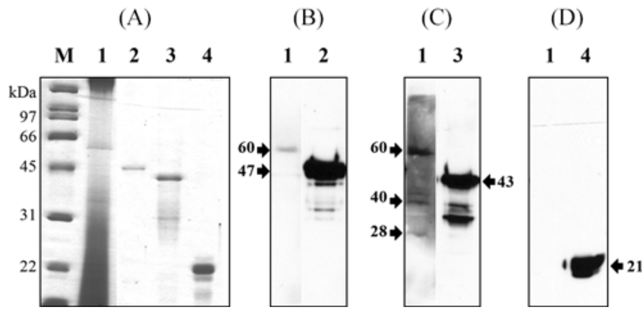

Fig. 5. Toxin-overlaying assay on *Ae. aegypti* BBMV proteins. (A) SDS-PAGE analysis (Coomassie brilliant blue-stained 12% gel) of 50 μg BBMV proteins (lane 1), 1 μg the full-length activated Cry4Ba toxin comprising two non-covalently associated fragments of ca. 47 and 18-20 kDa (lane 2), 2 μg the purified Cry4Ba-domain II-III fragment (lane 3) and 3 μg the purified Cry4Ba-domain III fragment (lane 4). The protein samples were blotted to a nitrocellulose membrane and subsequently incubated with the full-length activated Cry4Ba toxin (B), the domain II-III fragment (C) or the domain III fragment (D). Bound toxins were detected by means of streptavidin-peroxidase conjugate after probing with the 2F-1H2 anti-Cry4Ba domain III MAb. A positive signal was developed by a chemiluminescent reaction and imaged on an X-ray film. M is protein molecular mass standards.

fragment is able to bind independently to the apical microvilli of the susceptible larval midgut epithelium, conceivably similar to the full-length activated toxin. This is in agreement with similar studies that the cloned Cry1Ab-domain II-III fragment expressed separately, albeit in very low yields, was found to interact with susceptible lepidopteran larval microvilli similar to the full-length activated toxin (Flores *et al.*, 1997). More recently, we have shown that the 21-kDa Cry4Ba-domain III protein was able to bind to the apical microvilli of *Ae. aegypti* larval midgut, but with lower-binding activity when compared with the full-length activated toxin (Chayaratanasin *et al.*, 2007). These findings together with the data here have strengthened the notion that the receptor-binding capability of the Cry toxins is contributed mostly by domain II rather than domain III, although domain III has been demonstrated to play a significant role in receptor binding, particularly in specificity determination (de Maagd *et al*., 1996; de Maagd *et al*., 2000).

Experiments were further carried out to explore the larval midgut specific proteins, which are responsible for the interaction between the Cry4Ba-domain II-III fragment and the BBMVs of *Ae. aegypti* larvae. Toxin-overlaying assay together with an enhanced chemiluminescence system was employed to analyze the toxin-binding BBMV protein complexes on nitrocellulose membranes. The results revealed that the domain II-III fragment reproducibly reacted to *Ae. aegypti* larval BBMV proteins of ca. 60-kDa, and apparently also, with less intensity, to membrane vesicle proteins of ca. 40- and 28-kDa (Fig. 5C, lane 1). Under the conditions used, the full-length activated toxin at molar concentration equivalent to the domain II-III fragment (~45 mM) was also found to bind merely to a 60-kDa BBMV protein with less intensity when compared to the domain II-III fragment (Fig. 5B, lane 1). However, no detectable binding of the isolated domain III fragment to the BBMV proteins was observed (Fig. 5D, lane 1), albeit its weak interaction with the apical microvilli of *Aedes* larval midgut (Chayaratanasin *et al.*, 2007). At this stage, there is no definitive reason for the difference in binding characteristics to the BBMV proteins between the full-length activated toxin and its isolated domain II-III fragment, but obviously domain I could contribute to this discrepancy.

Currently, four different toxin-receptors for the lepidopteran-active Cry1A toxins have been identified from various susceptible insect larvae: a cadherin-like protein, a glycosylphosphatidyl inositol (GPI)-anchored aminopeptidase-N, a GPI-anchored alkaline phosphatase (ALP) and a 270-kDa glycoconjugate (for reviews, see Gómez *et al*., 2007). For the mosquito-larvicidal Cry proteins, BBMV proteins of ca. 62-65-kDa from *Ae. aegypti* larvae were identified to be receptors of both Cry4Ba and Cry11Aa (Buzdin *et al*., 2002). Other studies suggested that the Cry4Aa, Cry4Ba and Cry11Aa toxins all share a common class of binding sites on *Ae. aegypti* BBMV proteins, although they exhibit an overall low affinity (de Barros Moreira Beltrão and Silva-Filha, 2007). As was also reported for the Cry11Aa toxin, the 65-kDa binding-protein from *Ae. aegypti* larvae was characterized as a GPI-anchored ALP protein (Fernändez *et al*., 2006). Notwithstanding the lack of characterization of the 60-kDa Cry4Ba-binding protein as demonstrated here (see Fig. 5B,C), this BBMV protein may indeed serve as a receptor for Cry4Ba. However, it remains to be characterized whether this 60-kDa Cry4Ba-binding protein is a GPI-anchored ALP protein as mentioned earlier for the Cry11Aa toxin.

Overall, this study demonstrated the achievement of over-expression in *E. coli*, unfolding-refolding and purification of the 43-kDa Cry4Ba-domain II-III protein. We have provided binding characteristics of this purified domain II-III fragment to both histological midgut tissue sections and BBMV proteins prepared from susceptible *Ae. aegypti* mosquito-larvae. Moreover, by using toxin-overlaying assay, a 60-kDa toxin-binding protein was identified as a putative receptor for the Cry4Ba toxin. However, a detailed understanding of the interaction between the toxin *via* the isolated domain II-III fragment and its characterized receptors on the susceptible larval gut epithelium, employing other experimental techniques *e.g.* BIAcore biosensor, needs further investigation.

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