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High Level of Soluble Expression in *Escherichia coli* and Characterisation of the Cloned *Bacillus thuringiensis* Cry4Ba Domain III Fragment

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Similar to the other known structures of *Bacillus thuringiensis* Cry δ-endotoxins, the crystal structure of the 65-kDa activated Cry4Ba toxin comprises three domains which are, from the N- to C-terminus, a bundle of α-helices, a three-β-sheet domain, and a β-sandwich. To investigate the properties of the C-terminal domain III in isolation from the rest of the toxin, the cloned Cry4Ba-domain III was over-expressed as a 21-kDa soluble protein in *Escherichia coli*, which cross-reacted with anti-Cry4Ba domain III monoclonal antibody. A highly-purified domain III was obtained in a monomeric form by ion-exchange and size-exclusion FPLC. Circular dichroism spectroscopy indicated that the isolated domain III fragment distinctly exists as a β-sheet structure, corresponding to the domain III structure embodied in the Cry4Ba crystal structure. *In vitro* binding analysis *via* immuno-histochemical assay revealed that the Cry4Ba-domain III protein was able to bind to the apical microvilli of the susceptible *Stegomyia aegypti* larval midguts, albeit at lower-binding activity when compared with the full-length active toxin. These results demonstrate for the first time that the C-terminal domain III of the Cry4Ba mosquito-larvicidal protein, which can be isolated as a native folded monomer, conceivably participates in toxin-receptor recognition.

Keywords: *Bacillus thuringiensis*, Cry4Ba δ-endotoxin, Immuno-histochemical assay, *Stegomyia aegypti,* β-sheet structure

Abbreviations: *Bt*, *Bacillus thuringiensis*; CD, circular dichroism; Cry, crystal; FPLC, fast-performance liquid chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SOE, splicing-by overlap-extension.

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Introduction

The native *Bacillus thuringiensis* Cry δ-endotoxins are synthesised during sporulation as inactive protoxins that are found within parasporal inclusion bodies. Upon ingestion by susceptible insect larvae, toxin inclusions are released from disrupted bacterial cells and then solubilised in the larval midgut lumens that are generally alkaline pH. The soluble protoxins are subsequently activated by gut proteases to yield toxic fragments that are relatively resistant to further proteolysis (Schnepf *et al.*, 1998). Binding of the active toxins to specific receptors that are present on the microvilli of larval gut epithelial cells would facilitate their insertion into the cell membrane. Finally, ion-leakage pores formed by oligomerisation of the inserted toxins would cause the target midgut cells to swell and lyse by colloid-osmotic lysis. This results in extensive damage to the midgut and insect larval death (Knowles, 1994; Whalon and Wingerd, 2003). However, the precise molecular basis of this toxicity process is still not completely described.

To date, the crystal structures of various Cry toxins including Cry1Aa (Grochulski *et al.*, 1995), Cry2Aa (Morse *et al.*, 2001), Cry3Aa (Li *et al.*, 1991), Cry3Bb (Galitsky *et al.*, 2001), Cry4Ba (Boonserm *et al.*, 2005) and more recently Cry4Aa (Boonserm *et al.*, 2006) have been resolved. Although these toxins exert their insecticidal activity against different target insect larvae, they all share a high degree of overall structural similarity and are composed of three structurally distinct domains. The N-terminal domain (I), a bundle of α-helices, has been shown to be responsible for toxin insertion into the cell membrane, leading to the formation of ion-leakage pores (Walters *et al.*, 1993; Puntheeranurak *et al.*, 2004). A number of studies have demonstrated a crucial role of α4 and α5 in pore-forming activity (Gerber and Shai, 2000; Leetachewa *et al.*, 2006). The middle domain (II) is a β-prism comprised of three anti-parallel β-sheets, each terminating in surface-exposed loops, which are the most diverse part of

the Cry toxins. This domain has been demonstrated to be involved in receptor binding, and hence determines target insect specificity (Fernandez *et al.*, 2005; Tuntitippawan *et al.*, 2005). The C-terminal domain (III) is a β-sandwich comprising two anti-parallel β-sheets arranged in a jelly-roll-like topology. The exact role of this domain is still not clearly elucidated, it however has been implicated in maintaining structural integrity of active toxin molecules (Nishimoto *et al.*, 1994), membrane permeability (Masson *et al.*, 2002), or receptor binding and specificity determination (De Maagd *et al.*, 2000).

In our earlier studies, the role in toxicity of domains I and II of the two closely-related *Bt* mosquito-larvicidal proteins, Cry4Aa and Cry4Ba, has been investigated. Of particular interest, one highly-conserved tyrosine residue located in the α4-α5 loop within domain I of both these toxins (Cry4Aa: Tyr^{202}; Cry4Ba: Tyr^{170}) was demonstrated to be an important determinant for larvicidal activity (Kanintronkul *et al.*, 2003; Pornwiroon *et al.*, 2004). We have further provided evidence for a structural requirement of both the disulphide bridge (Cys^{192}-Cys^{199}) and the proline-rich motif ($Pro^{193}Pro^{194}$-Pro^{196}) (Tapaneeyakorn *et al.*, 2005) that are exclusively found within the Cry4Aa α4-α5 loop (Angsuthanasombat *et al.*, 2004). Very recently, we have demonstrated that the highly conserved residue-Asn^{183} that is located in the middle of the transmembrane α5 of Cry4Ba plays a crucial role in toxicity and toxin oligomerisation (Likitvivatanavong *et al.*, 2006). For domain II, we have shown that Pro^{389} in the β6-β7 loop, Ser^{410} and Glu^{417} in the β8-β9 loop, and Tyr^{455} and Asn^{456} in the β10-β11 loop are involved in Cry4Ba toxicity (Tuntitippawan *et al.*, 2005). However, it remains to be tested whether these critical loop residues are involved in receptor binding. In the present study, we aimed to investigate the role in toxicity of the C-terminal domain III of Cry4Ba in isolation from the rest of the toxin. A recombinant clone that over-expresses the Cry4Ba-domain III fragment as a soluble form in *E. coli* has been successfully constructed. Subsequent purification and characterisation *via* immuno-histochemical assay demonstrated that the isolated Cry4Ba-domain III protein was able to bind along the microvilli-midgut section prepared from susceptible *S. aegypti* mosquito larvae, suggesting a possible involvement in receptor binding.

Materials and Methods

Construction of recombinant plasmid. The 552-bp DNA segment encoding the 21-kDa domain III fragment of the Cry4Ba toxin was amplified from the pMU388 recombinant plasmid (Angsuthanasombat *et al.*, 1987) by polymerase chain reaction using a high fidelity *Pfu* DNA polymerase. By PCR-based splicing-by overlap-extension (SOE) technique, the 168-bp PCR-amplified DNA fragment of the 5'-untranslated region containing the endogenous *Bt-cry4Ba* regulatory region (Boonserm *et al.*, 2004) was fused in-frame with the ATG codon of the amplified *cry4Ba-domain III* gene segment. SOE-PCR amplification was performed using the pUC universal forward primer: 5'-TTGTGAGCGGATAACAATTTC-3' and the reverse primer-DIII-R: 5'-TGTAAACAGTGCGTCGACTTATTATCGTTC TGATTCTAAATT-3', incorporating a *Sal*I site on the 3' end of the SOE product, respectively (underlined bases indicate the incorporated restriction site). The oligonucleotide primers were purchased from Sigma Proligo. The fusion DNA fragment was, respectively, treated with *Bam*HI, Klenow fragment and *Sal*I, and subsequently inserted into the *Sal*I and Klenow-blunted *Eco*RI sites of the pMEx8 expression vector (Buttcher *et al.*, 1990). The resultant plasmid, pMEx-4BD3 (Fig. 1), was transformed into *E. coli* JM109 competent cells. The correct insertion of the cloned gene was proved by *Hinc*II endonuclease digestion of the plasmid and by automated DNA sequencing, using a BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer).

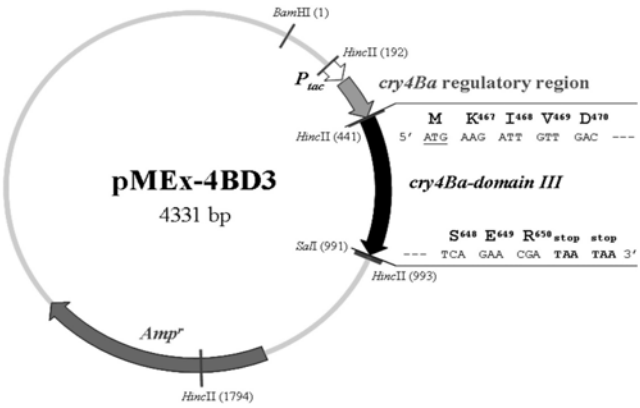

Fig. 1. Schematic map of the pMEx-4BD3 recombinant plasmid. Transcription of the *cry4Ba-domain III* gene fragment is driven by the *tac* promoter (P_{tac}) in conjunction with the *cry4Ba* regulatory region. The nucleotide and deduced amino acid sequences at the 5'- and 3'-ends of the *cry4Ba-domain III* gene are shown with the underlined and bold nucleotides representing start and stop codons, respectively. The arrows indicate the transcriptional direction of the corresponding promoters and genes. *Amp*^{r} indicates the ampicillin resistance gene. For clarity, only the restriction endonuclease sites mentioned in the text are shown.

Protein expression and purification. The *E. coli* clone harbouring the pMEx-4BD3 plasmid was grown at 37°C in Luria-Bertani medium containing 100 μg/ml ampicillin until OD_{600} of the culture reached 0.3-0.5. Protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 4 h and was subsequently analysed by sodium dodecyl sulphate-(12% w/v) polyacrylamide gel electrophoresis. *E. coli* cells, which over-expressed the Cry4Ba-domain III fragment as soluble protein, were harvested by centrifugation at 15,000 × *g*, 4°C for 10 min and resuspended in 50 mM Tris-HCl, pH 7.4. Cells were then disrupted by using French Pressure Cell at 16,000 psi. After centrifugation at 10,000 × *g*, 4°C for 60 min, the clear supernatant was collected for further purification.

Initial purification of the 21-kDa domain III protein was accomplished using an anion-exchange FPLC system (6-ml Resource Q column, Amersham Pharmacia Biotech). Chromatographic separations were achieved with a linear gradient of B in A (A = 50 mM Tris-HCl, pH 7.4; B = Buffer A containing 1 M NaCl). Proteins were eluted between 50-200 mM B gradient at a flow rate of 1 ml/min. Elution

fractions across the 100-120 mM NaCl-peak were pooled and subjected to further purification by a size-exclusion FPLC system (Superdex 200 column, Amersham Pharmacia Biotech) with buffer A at a flow rate of 0.4 ml/min. Eluted fractions containing the 21-kDa protein were pooled and the purity of the sample was analysed by SDS-PAGE.

N-terminal amino acid sequencing. Proteins separated by 15% SDS-PAGE were electroblotted onto a polyvinylidene difluoride membrane (Prosorp®, Applied Biosystems) by using Mini Trans-Blot apparatus (Bio-Rad) and subsequently visualised by Coomassie-blue staining. The 21-kDa domain III band was excised and analysed by N-terminal sequencing using an ABI 492 automated protein sequencer (Applied Biosystems).

Circular dichroism measurements. CD spectrum of the purified domain III protein prepared in 50 mM Tris-HCl, pH 7.4 was measured using a Jasco J-715 spectropolarimeter (Jasco, Inc., USA). Instrument calibration was performed with 1.0 mg/ml of (+)-10-camphorsulphonic acid (Sigma). Measurements were carried out at room temperature using a quartz cuvette with an optical path-length of 0.02 cm. Spectra in the 190-260 nm wavelength range were recorded at a scan speed of 50 nm/min using a bandwidth of 2 nm, a response time of 1 sec and a sensitivity of 50 millidegree for 3 accumulations.

Western blot analysis. Protein samples analysed by 12% SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane using the Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) at constant voltage of 50 volts, 4°C for 100 min. For blocking non-specific binding sites, the membrane was incubated with PBS containing 5% skim milk for 4 h at room temperature. The blotted proteins on the membrane were probed with the 2F-1H2 monoclonal antibody (1 : 50 dilution) which is specific to the Cry4Ba domain III fragment [for details, see (Moonsom, 2004)], and immunocomplexes were detected with alkaline phosphatase-conjugated goat anti-mouse IgG (1 : 20,000 dilution) (Pierce). The target protein band was visualised as a dark-purple color precipitate by incubating with the developing reagent [5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium] (Sigma).

***In vitro* binding analysis *via* immuno-histochemical assay.** To prepare sections of larval tissues, the head of 5-day-old *S. aegypti* mosquito larvae was removed, and the dissected larvae were incubated with fixative reagent [sodium-phosphate-buffered saline, pH 7.4 (PBS) containing 4% paraformaldehyde and 10% sucrose] prior to partial dehydration in a series of ethanol baths (80, 95 and 100%) for 1 h each. The larval tissues were subsequently infiltrated, embedded at 38°C in polyester wax and stored at 4°C. Slices of 10-μm were made using a microtome and attached on mounting glasses coated with 2% 3-aminopropyltrithoxysilane (Sigma).

Immunohistochemical detection was performed following the method described previously (Moonsom, 2004). The larval sections were de-paraffinated in 100% xylene solution for 2 min and subsequently rehydrated in decreasing concentrations of ethanol, and finally rinsed with water. Endogenous peroxidase activity was eliminated by incubating the sections in PBS containing 0.1% Triton X-100 and 5% H_2O_2 for 30 min. Non-specific binding was blocked by incubating with normal rabbit serum (1 : 200 dilution) for 45 min. The solution was removed and the slides were covered for 45 min with either 0.6 μM protein, 50 mM Na_2CO_3 (pH 9.0), or 50 mM Tris-HCl (pH 7.4). After washing 3 times with 0.1% Triton X-100 in PBS (T-PBS), the bound toxin was probed with the 2F-1H2 monoclonal antibody (1 : 20 dilution) for 45 min. After washing 3 times in T-PBS, biotin-conjugated rabbit anti-mouse IgG (1 : 200 dilution) (Pierce) was added and incubated for 45 min. The slides were then washed 3 times in T-PBS, and incubated with peroxidase-conjugated streptavidin (5 μg/ml) for 45 min. After removal of unbound streptavidin, colour development was initiated by incubating with 3,3'-diaminobenzidine (DAB, SK-4100, Vector) for 2 min at room temperature, and the reaction was stopped by rinsing with distilled water. The sections were then dehydrated with a series of ethanol baths, clarified in xylene solution, mounted with mounting medium (Fisher Scientific Company), covered with cover glasses and analysed by light microscopy.

Mosquito-larvicidal activity assays. Bioassays were performed as previously described (Sramala *et al.*, 2000), using 2-day-old *S. aegypti* larvae (hatched from eggs supplied by the mosquito-rearing facility of the Institute of Molecular Biology and Genetics, Mahidol University, Thailand). Both rearing and bioassays were carried out at room temperature (25°C). The assays were done in 1 ml *E. coli* suspension (10^8 cells suspended in distilled water) in a 48-well plate (11.3-mm well diameter, Costar), with 10 larvae per well and a total of 100 larvae for each test sample. *E. coli* cells containing the recombinant plasmid (pMU388) and the vector (pMEx8) were used as the positive and negative controls, respectively. Mortality was recorded after 24-h incubation period.

Results and Discussion

Expression and purification of the cloned Cry4Ba-domain III fragment. In the present study, we have constructed the recombinant pMEx-4BD3 plasmid (Fig. 1) in which the *cry4Ba-domain III* gene segment was fused in-frame with the *Bti-cry4Ba* regulatory element and cloned into the pMEx8 expression vector. The *Bti-cry4Ba* regulatory region, possessing the endogenous *Bti-cry4Ba* promoter and the Shine-Dalgarno sequence (Boonserm *et al.*, 2004), was previously demonstrated to confer the high-level expression in *E. coli* cells of both Cry4Aa and Cry4Ba toxins, leading to the formation of a toxin inclusion (Angsuthanasombat *et al.*, 1987; Boonserm *et al.*, 2004). Following the ATG codon, the deduced amino acid sequence of the cloned *cry4Ba-domain III* gene begins from Lys^{467} to Arg^{650} (see Fig. 1), with Lys^{467} corresponding to the N-terminal margin of domain III in the crystal structure (Boonserm *et al.*, 2005). N-terminal sequencing of the expressed gene product (see below) confirmed that its N-terminus (Met-Lys-Ile-Val-Asp---) was identical to the expected terminus of the cloned Cry4Ba-domain III fragment.

Upon IPTG induction, the 21-kDa Cry4Ba-domain III fragment (calculated molecular mass = 20,504.32 Da) was highly produced almost exclusively as a soluble protein (Fig. 2A, lane 4), indicating that it could exist in the native

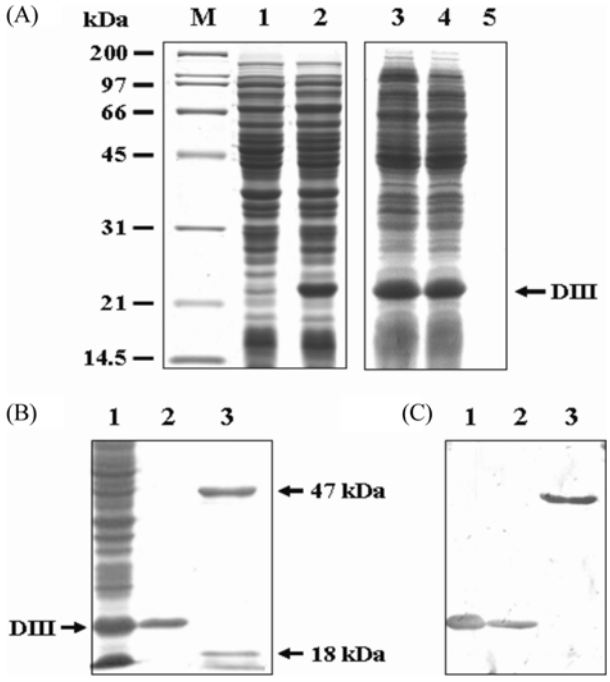

Fig. 2. (A) SDS-PAGE analysis (Coomassie brilliant blue-stained 12% gel) of protein expression of the cloned Cry4Ba-domain III fragment. Crude extracts of *E. coli* cells containing the pMEx8 vector (lane 1) or pMEx-4BD3 plasmid (lanes 2 and 3). Lanes 4 and 5 represent the supernatant and pellet fractions, respectively, after centrifugation of the crude lysate from lane 3. Lane M represents the molecular mass standards. Arrow indicates the band corresponding to the Cry4Ba-domain III protein. (B) Crude lysate of the recombinant cells containing pMEx-4BD3 (lane 1), the purified domain III protein (lane 2) and the purified trypsin-activated Cry4Ba toxin composed of 47- and 18-kDa fragments (lane 3) as analysed by SDS-PAGE. (C) Western blot analysis of (B) probed with the 2F-1H2 monoclonal antibody specific to the Cry4Ba-domain III.

conformation, albeit detaching from the N-terminal domain I-II fragment (see Fig. 3). This is contrary to the previous report that the cloned Cry1Ab- and Cry1Ac-domain III proteins fused with a hexa-histidine tag were shown to be over-produced in the form of insoluble aggregates in *E. coli* (Vazquez-Padron *et al.*, 1998). Differences in the cloning region for the domain III termini or the additional fusion tag used in their expression system may contribute to the discrepancies. As can be inferred from the Cry1Aa crystal structure, domain III, which comprises of β12-β23 (residues 463 to 609), directly contacts an additional β-strand (β1a; residues 254 to 264) which is conserved among the Cry1A toxins (Grochulski *et al.*, 1995). This configuration suggests a structural role for β1a to maintain the integrity of domain III in Cry1Aa, but not in Cry4Ba (see Fig. 3B). Thus the lack of this β-strand could possibly result in improper folding of the cloned Cry1Ab- and Cry1Ac-domain III proteins, leading to the formation of an insoluble aggregate *in vivo*.

For purification, the soluble Cry4Ba-domain III protein was initially subjected to anion-exchange FPLC and the peak fractions containing a major protein band of 21 kDa were eluted at a concentration of ca. 106 mM NaCl (see Fig. 4A). The highly-pure domain III protein was obtained by further purification on size-exclusion chromatography. The elution profile from the FPLC analysis revealed that the 21-kDa Cry4Ba-domain III protein was eluted as a single peak corresponding to the elution volume of the 23-kDa lysozyme protein marker (see Fig. 4B), suggesting that it exists as a monomer. These optimised purifying conditions allowed us to obtain sufficient amounts of the purified domain III protein for subsequent investigations of its properties.

Structural and functional characterisation of the purified domain III protein. The 21-kDa purified domain III fragment was assessed for secondary structural elements by far-UV CD spectroscopy. CD spectrum of the domain III protein from repeated scans within 190-260 nm showed evidence of excitation coupling in the π-π* transition with a positive peak at 197 nm and a negative peak at 218 nm [molar ellipticity [θ] = 13.49 and −19.87 ($\times 10^7$) deg cm^2/dmole, respectively]. These spectral characteristics are typical for a protein having a predominant anti-parallel β-sheet structure, indicating that the 21-kDa isolated domain III fragment distinctly adopted a β-structure.

In the Cry4Ba crystal structure, the C-terminal domain III is made of two anti-parallel β-sheets forming a face-to-face sandwich ending with a short α-helix (see Fig. 3A) (Boonserm *et al.*, 2005). It is noteworthy that this domain contains three of the highly conserved regions (Fig. 3B) and shows the highest structural similarity among the known Cry toxin structures when compared to the two other domains (Boonserm *et al.*, 2005). It has been also noted that domain III of the Cry1A toxins and the carbohydrate-binding domain of several proteins, such as 1,4-β-glucanase CenC, galactose oxidase and sialidase, share high overall structural similarity (for reviews, see De Maagd *et al.*, 2003). Moreover, domain III of the Cry1A toxins was demonstrated to bind aminopeptidase N (APN) receptors of the target insect larvae through a *N*-acetylgalactosamine (GalNAc) moiety (Jenkins *et al.*, 1999; Jenkins *et al.*, 2000). A putative GalNAc-binding pocket on Cry1Ac-domain III is conceivably located on the outer sheet, comprising Gln^{509}, Arg^{511}, Gly^{546} and Trp^{545} (Jenkins *et al.*, 1999). Despite the fact that a receptor specific for the Cry4Ba toxin, possibly APN, has not yet been identified, a similar binding pocket was apparently observed in Cry4Ba-domain III in which Asn^{552}, Arg^{520}, Gly^{563} and Phe^{490} were lined the pocket (see Fig. 3C). This could imply an important, but as yet unrevealed, role of domain III in the Cry4Ba toxin activity.

Many reports demonstrated that domain III plays a significant role in receptor binding, particularly in specificity determination of different insect species (De Maagd *et al.*, 1996; De Maagd *et al.*, 2000). For example, this has been shown by replacement with Cry1Ca-domain III to generate hybrid Cry1Ab-Cry1Ca or Cry1Ea-Cry1Ca toxins with altered specificity characteristics (De Maagd *et al.*, 2000). For the

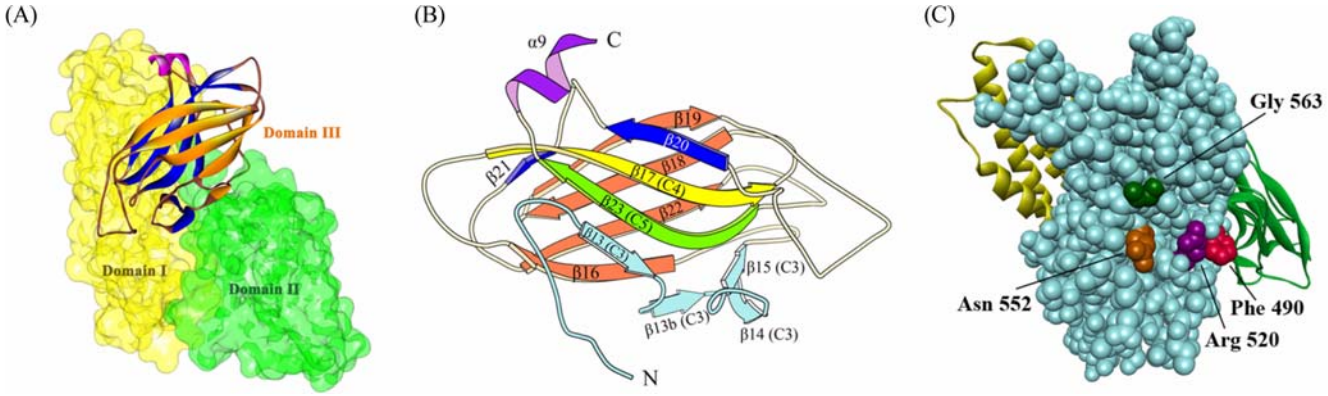

Fig. 3. (A) Crystal structure of the 65-kDa activated Cry4Ba toxin (Boonserm *et al.*, 2005), possessing three domains. Domains I and II are drawn in a space-filling model. Domain III is illustrated in a schematic ribbon displaying the inner and outer sheets with different shades. (B) Ribbon representation of a different view of the Cry4Ba-domain III by facing the inner sheet upwards, showing three of the highly conserved blocks (C3, C4 and C5). (C) The Cry4Ba crystal structure with domains I-II (schematic ribbon) and domain III (space-filling model). The outer sheet of domain III facing upwards shows a putative Cry1Ac-like pocket. The residues lining the lip of the pocket are labeled. The 3D structures were generated by WebLab Viewer (http://www.msi.com) for (A) and (C), and Molscript (http://www.avatar.se/molscript/) for (B).

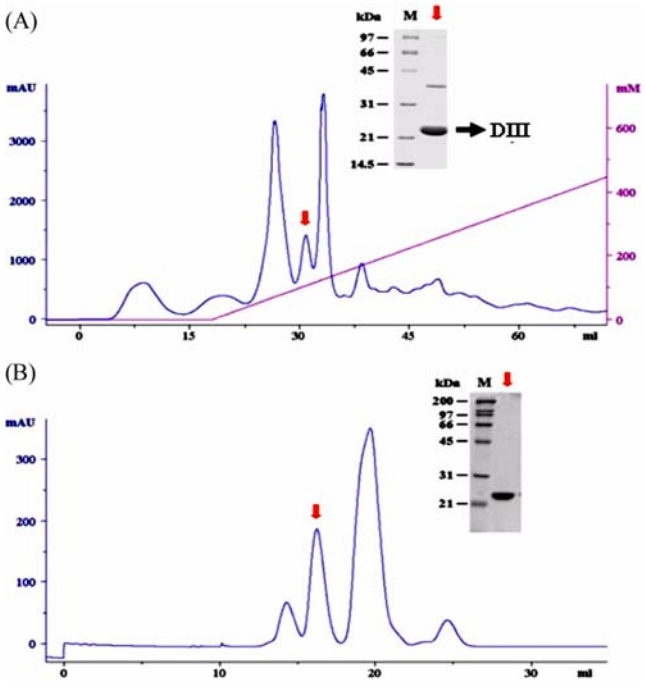

Fig. 4. Protein purification chromatograms and SDS-PAGE analysis of the cloned Cry4Ba-domain III protein. (A) Chromatographic elution profile from the 6-ml Resource Q ion-exchange column, showing absorbance at 280 nm (mAU), a continuous gradient of NaCl concentrations (mM) and elution volume (ml). (B) Elution profiles from the Superdex 200 size-exclusion column. Inset, SDS-PAGE analysis (Coomassie brilliant blue-stained 12% gel) of a selected peak fraction (arrow) containing the purified Cry4Ba-domain III fragment. M represents molecular mass standards.

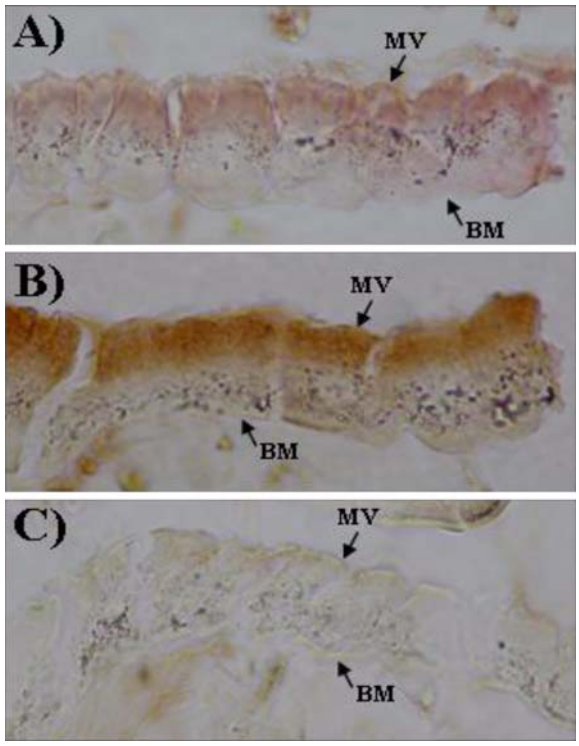

Fig. 5. Immuno-histochemical staining of 5-day-old *S. aegypti* gut tissue sections. Slides were incubated with either (A) the purified Cry4Ba-domain III protein or (B) the purified activated full-length Cry4Ba toxin. (C) Control slide omitting the tested proteins. BM, basement membrane; MV, microvilli.

mosquito-specific Cry toxins, *in vitro* binding studies *via* immuno-histochemical assays demonstrated that the primary site of action of Cry4Aa, Cry4Ba, Cry11Aa or Cry11Bb toxins 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 analysis to investigate further into the binding ability of the isolated Cry4Ba-domain III protein towards the mosquito larval midgut. As initially analysed by Western blotting (Fig. 2C), the cloned domain III fragment could specifically cross-react with the 2F-1H2 monoclonal antibody (Moonsom, 2004), giving a strong signal comparable to that of the 47-kDa fragment which corresponds to α6-loop-α7 linked with domains II and III of the 65-kDa activated Cry4Ba toxin (see Fig. 2C).

In immuno-histochemical assay, the *S. aegypti* larval midgut section incubated with the isolated Cry4Ba-domain III protein exhibited a light-brown staining along the apical microvilli of epithelial cells (Fig. 5A), whilst an intense brown signal was observed upon incubation with the 65-kDa activated Cry4Ba toxin (Fig. 5B). Control larval gut section in which the domain III protein or the activated toxin was omitted showed a very weak staining of non-specific signals on the microvilli (see Fig. 5C). These data provide direct evidence that the 21-kDa purified Cry4Ba-domain III fragment, which could exist as a native monomer by itself, was able to bind to the apical microvilli of the susceptible larval midgut, conceivably participates in receptor recognition. For toxicity-competition assays, the mortality data recorded after 24-h incubation, however, revealed that no significant reduced effect was observed on larvicidal activity of *E. coli* cells (10^8 cells/ml) expressing the full-length Cry4Ba toxin when 2-day old *S. aegypti* larvae were pre-fed with the cells (10^8 cells/ml) expressing the cloned domain III fragment at either 2-h or 4-h pre-incubation. This indicated that the cloned domain III could not inhibit the binding and toxicity of the full-length Cry4Ba toxin, albeit its conceivable binding activity detected via immuno-histochemical assay. This result could suggest that the isolated domain III binds to the sites different from the ones for the full-length toxin utilising mainly domain II for the binding. Another possibility is that multiple receptor molecules are involved in the interaction of the Cry4Ba toxin with the *S. aegypti* larval midgut epithelia as also suggested for the Cry11Aa toxin (Fernandez *et al*., 2005). Previously, the cloned domain II-III fragment was shown to bind to midgut membranes of susceptible lepidopteran larvae in a rather similar fashion to the full-length activated Cry1Ab toxin (Flores *et al*., 1997). These results together with the observation here suggest that the receptor-binding ability of the Cry toxins is contributed mostly by domain II rather than domain III, although the nature of the binding determinant on mosquito specific toxin-receptors might be very different from those found in other insect larvae. Other studies *via* toxin mutagenesis supported this notion by showing that mutating receptor-specifying residues in domain II was more detrimental to the binding affinity of Cry1Ac to APN, a major receptor for this toxin in gypsy moth, than mutating receptor-binding residues in domain III (Jenkins *et al*., 2000).

In conclusion, this study demonstrated the novel achievement of the high-level soluble expression and purification of the 21-kDa native monomeric Cry4Ba-domain III protein without additional requirements of either a fusion tag system or unfolding-refolding of proteins *in vitro*. Moreover, we have provided functional evidence that this purified domain III fragment is conceivably involved in receptor recognition. However, a detailed understanding of the interaction between the Cry toxin *via* the isolated domain III fragment and its receptor on the cell membrane target needs further investigation. This cloned fragment would prove very useful for better defining the function of domain III. For these specific assays, it would be preferable to use an isolated specific receptor or the brush-border membrane vesicles from the susceptible larval gut epithelium.

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