The *Bacillus thuringiensis* Cry4Ba toxin weakens the protective function of the midgut peritrophic membrane in *Aedes aegypti* larvae

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Abstract

Background

*Bacillus thuringiensis (Bt)* toxin Cry4Ba specifically targets the mosquito larval midgut epithelial cells for its insecticidal toxicity. Prior to reaching the midgut cells, the toxin must penetrate the midgut peritrophic membrane (PM), an acellular gut protective structure lining the midgut epithelium. However, the mechanism of toxin passage through the PM is currently unknown. To examine the correlation between the permeability of *Ae. aegypti* larval PM and larval toxicity of the Cry4Ba toxin, larvae were fed with Cry4Ba and an inactive mutant, respectively. The toxin was subsequently localized in the larvae to examine the passage of Cry4Ba through the PM.

Results

In Cry4Ba fed larvae, Cry4Ba was localized in the PM, within the gastric caeca, and in the midgut epithelial cell surface at 10 min after ingestion of the toxin. Furthermore, in vivo PM permeability assays using FITC-dextran (2000 KDa) demonstrated that the permeability of the PM in Cry4Ba fed larvae was significantly increased. However, in larvae fed with the inactive Cry4Ba mutant, the toxin was restricted within the endoperitrophic space of the larval gut, indicating that alanine substitution at arginine 158 (Arg\textsuperscript{158}) in domain I resulted in failure of the toxin to pass through the PM.

Conclusion

We therefore conclude that the PM became more permeable upon larval exposure to Cry4Ba, thus weakening its protective function. The amino acid residue Arg\textsuperscript{158} in Cry4Ba may play an important role in its ability to penetrate the mosquito larval PM.

Background

Mosquito-borne diseases such as dengue, malaria and viral encephalitis have emerged as global health problems since the 1950s, affecting millions of people, particularly in
developing countries in Africa and Asia [1]. Due to its environmental compatibility and host specificity compared to chemical pesticides, the Gram-positive soil bacterium Bt has become an important biological control agent against the mosquitoes. Bt produces insecticidal parasporal crystalline inclusions during sporulation phase. These crystalline inclusions are composed of one or more insecticidal proteins which are classified as Cry (crystal) and Cyt (cytolytic) toxins based on their protein sequences [2-3]. The 130-kDa Cry4Ba toxin produced from Bt subsp. israelensis exhibits specific toxicity towards Ae. aegypti and Anopheles gambiae larvae [4-5]. To date, solved structures of seven Cry toxins show high similarity in the overall structure of their three-domain organization [2, 6-7]. The N-terminal domain (I) is a helical bundle where the central α-helix 5 is surrounded with six amphipatic helices. This domain has been shown to play a role in membrane insertion and pore formation [8-10]. Moreover, domains II and III comprising β-sheet structures have been reported to be involved in receptor recognition and binding [11-12], stabilization of structural integrity [13] and membrane permeabilization [14].

Cry protoxins are produced in Bt as insoluble inclusion bodies. These inclusion bodies are solubilized under alkaline conditions in the midgut of insect larvae and are activated by digestive enzymes. The activated toxin then binds to a specific receptor residing at brush border microvilli of the midgut epithelium after passing through the midgut lining, which is a non-cellular, chitin and protein containing structure known as the peritrophic membrane (PM) [2]. The toxin-receptor binding leads to 1) toxin structural rearrangement, followed by insertion of the toxin into the midgut epithelial cells to form ion-leakage pores, which eventually results in larval mortality due to lysis of midgut epithelial cells [15-16], or 2) triggering intracellular signaling cascades leading to cell death [17]. Cry toxins exhibit high host specificity. The host specificity has been attributed to interaction of the toxin with specific receptors located on the larval midgut epithelium [12, 18]. Accordingly, most
research has been focused on identification of these midgut binding proteins. In the digestive tract of insects, the PM serves as a barrier for protection of gut cells from physical, chemical and biological damage. Cry toxins must traverse the PM barrier in order to react with the targeted midgut epithelial cells. Improving the insecticidal activity of Cry toxins by Cry toxin feeding along with PM targeting agents [19] or trapping of Cry1A toxin in the PM contributes to Bt resistance in insects [20-21], suggesting that the Cry toxin’s passage through the PM is an important factor in Bt pathogenesis in insects. However, the mechanism for the passage of Cry toxins across the PM is largely unknown. In this study, we examined the interaction of a Bt Cry4Ba with the PM in Ae. aegypti larvae. We found that the permeability of the PM was significantly increased in Ae. aegypti larvae upon exposure to Cry4Ba. In addition, we also observed that the amino acid residue Arg158 in the Cry4Ba plays an important role in the passage of the toxin through the larval PM.

Results and discussion

Production of specific antibodies to Cry4Ba

Proteolytic processing of the 130-kDa Cry4Ba protoxin by trypsin yields two fragments of 18 kDa and 47 kDa, which are mapped to domain I and domain II linked domain III in the Cry4Ba crystal structure, respectively [22]. In the current study, the soluble Cry4Ba protoxin was digested with non-L-1-tosylamido-2-phenylethyl chloromethyl ketone (TCPK) treated trypsin. SDS-PAGE analysis of the 65-kDa activated toxin showed the 47-kDa and 20 kDa fragments, and that the 18 kDa fragment resulted from further cleavage of the 20-kDa fragment by chymotrypsin contamination (Fig. 1A). Mice were challenged with Cry4Ba fragments (see methods) and specific antibodies were determined by Western blot analysis with the activated 65-kDa Cry4Ba. Polyclonal antibodies (PAbs) specific to the 65-kDa Cry4Ba were obtained (Fig. 1A). Antibodies specific to an individual domain of Cry4Ba were subsequently purified by Cry4Ba domain II-III linked resins (Fig. 1B). As expected,
Western blot analysis demonstrated antibodies specific to domain II and domain III (Fig. 1B) and specific antibodies to the 18 kDa and 20 kDa fragments of Cry4Ba domain I (Fig. 1C) in the resin-bound and unbound fractions, respectively.

**Localization of Cry4Ba in *Ae. aegypti* larvae**

To examine the passage of Cry toxin from the midgut lumen to the target sites, *Ae. aegypti* mosquito larvae were fed with Cry4Ba inclusion bodies for 10 min, 30 min and 1 h. Pathological observation by haematoxylin and eosin (H&E) staining of toxin-fed larval sections showed drastic morphological changes in epithelial cells, such as vacuolization of the cytoplasm, microvilli impairment and the presence of proteinaceous material in the ectoperitrophic space of the larvae after 1 h of toxin feeding (Fig. 2A). These histological changes were not observed in epithelial cells of normal larvae, (Fig. 2B), therefore suggesting the pathological effect of Cry4Ba in the toxin fed larvae. Moreover, PAbs specific to Cry4Ba were employed for tracking proteolytic processing and *in vivo* binding of the Cry4Ba toxin.

Immunostaining of Cry4Ba treated larval sections with antibodies specific to Cry4Ba domain II and domain III (Fig. 2F, G), domain I specific antibodies (Fig. 2E) readily showed intensive toxin binding along the path of the food bolus, as well as toxin binding at apical microvilli of the midgut and gastric caeca after toxin feeding for 10 min. These positive signals were not detectable in the toxin fed larval sections reacted with biotin-secondary antibodies/streptavidin-HRP (Fig. 2D) or normal larval sections probed with pooled M1-M2 antisera followed by biotin conjugated secondary antibodies/streptavidin-HRP (Fig. 2I), suggesting binding specificity of the Cry4Ba. Several *in vitro* binding analyses have shown that a primary site of the Cry4Aa-, Cry4Ba-, Cry11Aa- and Cry11Ba-mosquito larvicidal toxins is located at apical microvilli of the larval midgut epithelium [23-26]. However our current results showed that the 65-kDa Cry4Ba binds to both the brush border of midgut epithelium and the PM. Our previous studies of the structural and functional relationships of
charged residues in α4 of the Cry4Ba domain I have shown that the alanine substitution at arginine-158 (R158A) completely abolishes its toxicity to Ae. aegypti larvae [27]. Further studies suggested that this residue may participate in ion permeation through the pore at the epithelial membrane of the midgut [28-29]. In order to investigate in vivo action of the Cry4Ba toxin in the midgut of Ae. aegypti, in this study, mosquito larvae were fed inclusion bodies of the wild type and the inactive Cry4Ba mutant. The toxin in the midgut was subsequently localized with antibodies specific to the Cry4Ba. Immunohistochemical staining of R158A fed larval sections clearly demonstrated that R158A was restricted within the endoperitrophic space and in gastric caeca (Fig. 2H). Moreover, no R158A binding was observed on apical microvilli of the gastric caeca or the epithelial cell membrane, indicating that this mutant was unable to cross the PM. As gastric caeca produce digestive enzymes, the presence of antibody positive signal in the gastric caeca of the R158A mutant-treated larvae (Fig. 2H) may suggest inactivation of the Cry4Ba toxin by enzymatic digestion. Since the mechanism of solute passage across the PM barrier is as yet not understood, we herein demonstrate that toxin alters PM permeability, allowing passage of the toxin through the PM and thus enabling the toxin to reach its receptor at the midgut epithelial membrane. Furthermore the Arg158 in the α4 of domain I accounts for PM alteration of the Cry4Ba, thus mutation/change at this residue leads to impermeability of the PM and insect insusceptibility to the Cry4Ba toxin. Alternatively, the increase in permeability of the PM could be due to abnormal synthesis of the PM resulting from midgut-cell damage upon Cry4Ba feeding. Since the increase in PM permeability was immediately detected after 10 min of larval exposure with the Cry4Ba, such processes must be quite rapid.

**Alteration of PM permeability in Ae. aegypti larvae upon ingestion of Cry4Ba**

The PM separates the Ae. aegypti larval gut into endoperitrophic (lumen side) and ectoperitrophic (epithelial side) spaces. A PM-like caecal membrane is located at the entrance
to gastric caeca [30]. Previous studies have shown that the PM of *Ae. aegypti* larvae are impermeable for passage of the FITC labeled 2000-kDa dextran, thus presence of fluorescence from FITC in the gastric caeca in FITC-dextran (2000 kDa)-fed larvae is indicative of PM disruption in larvae [31]. In the present study, *Ae. aegypti* larvae were fed with Cry4Ba along with 2000-kDa FITC-dextran to examine the PM permeability changes upon exposure to the toxin. In toxin-fed larvae fluorescence from FITC-dextran became apparent in the ectoperitrophic space and inside gastric caeca at 1 h after feeding with Cry4Ba, whereas fluorescence was restricted to the midgut lumen only in larvae fed with FITC-labeled dextrans alone (Fig. 3). These observations confirmed that the PM became more permeable in *Ae. aegypti* larvae once exposed to Cry4Ba.

**Conclusions**

We produced antibodies specific to Cry4Ba individual domains. Specific antibodies were applied for monitoring binding and processing of Cry4Ba wild type and inactive R158A Cry4Ba mutant *in vivo* to achieve a better understanding of the lethal mechanism of the Cry4Ba toxin. Our current findings suggest that the PM becomes more permeable and consequently its protective function weakens upon Cry4Ba feeding allowing the toxin passage to the midgut epithelium. The amino acid residue arginine-158 in α4 of domain I may play an important role in toxin passage through the PM as well as the toxicity in mosquito larvae.

**Methods**

**Expression and purification of Cry4Ba toxin**

Cry4Ba toxin was expressed and purified as previously described [29]. Briefly, *Escherichia coli* cells strain JM109 harboring the pMU388 plasmid encoding for Cry4Ba or a Cry4Ba mutant, R158A, were grown at 37°C in Luria–Bertani medium containing 100 µg/ml ampicillin until they reached the log phase (OD_{600} = 0.5). Protein expression was induced
with 0.1 mM isopropanyl-β-D-thiogalactopyranoside (USB, Cleveland, OH, USA) for 10 h at 30°C. Cells were disrupted by a French Press Cell at 20000 psi. The crude lysate was centrifuged at 6000g, 4°C for 10 min. After pellet washing, inclusion bodies were solubilized in 50 mM sodium carbonate, pH 10.0 at 37°C for 1 h followed by digestion with non-TCPK treated trypsin at enzyme to toxin ratio of 1:20 at 37°C for 16 h. The enzymatic activity was stopped by addition of 1 mM phenylmethane sulfonyl fluoride (Sigma-Aldrich, Steinheim, Germany). The activated Cry4Ba was subjected to gel filtration chromatography equipped with a Sephadex G200 column (GE Healthcare, Uppsala, Sweden). Protein fractions were eluted with carbonate buffer (pH 10.0) at a flow rate of 0.4 ml/min and monitored by spectrophotometry at 280 nm. Eluted fractions containing the 65 kDa Cry4Ba were pooled and concentrated to a final concentration of 5 mg/ml. The purified protein was analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The 47-kDa or 20-kDa protein band was recovered by electroelution.

**Mouse immunization**

Animal handling and immunization protocols were approved by the Animal Care and Use Committee, Faculty of Tropical Medicine, Mahidol University (FTM-ACUC 006/2009). Pre-immune serum was collected after centrifugation of whole blood at 5000g for 15 min and stored at -20°C. To prepare the antigen, 25 µg of protein were mixed with aluminum potassium sulphate (alum) (Pierce, Rockford, IL, USA) at alum/protein ratio of 1:4 and the protein mixture was injected into an animal every 2 weeks. Two mice were immunized with the purified 65-kDa Cry4Ba in the intraperitoneal route (IP) 2 times followed by 3 times with an individual fragment of 20 kDa (M1) or of 47 kDa (M2). Mice (2 mice/group) were IP injected three times with an individual fragment of the 20 kDa (M3) and the 47 kDa (M4) followed by a single dose of IP injected with a single dose of the 65-kDa Cry4Ba protein followed by an individual fragment corresponding to the previous immunization. Blood was
drawn every 2 weeks after each immunization to monitor the presence of Cry4Ba polyclonal antibodies using Western blot analysis.

**Affinity purification of Cry4Ba specific antibodies**

The recombinant Cry4Ba domain II (Moonsom S, unpublished data), domain III [32] and domain II-III fragments [23] were expressed and purified following the procedures as described previously. The purified 47 kDa of Cry4Ba domain II-III fragments were coupled onto activated cyanogen bromide resins following the manufacturer’s instructions. An equal volume of antiserum was incubated with domain II-III linked resins at room temperature for 1 h. The unbound serum was collected by spinning resins at 3000g for 20 sec. After washing, resin bound antibodies were eluted with 0.1 M glycine-HCl (pH 2.7) and neutralized with 1M Tris-HCl (pH 8.0). Finally, eluted antibodies (B fraction) were dialyzed (M.W. cut-off 15 kDa, Millipore, Bedford, MA, USA) against PBS.

**Western blot analysis**

Protein samples were separated by SDS-PAGE (8-16% gradient gel) and transferred to a nitrocellulose membrane using Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA) via constant voltage of 30 volts for 1 h followed by 80 volts for 2 h at 4°C. After 1-h blocking with 5% BSA-PBS at room temperature, the membrane was probed with the following antibodies: 1:1000 dilution of pre-immune serum, and 1:5000 dilution of immunized serum, affinity-unbound and affinity-bound antisera. The membrane was washed 3 times with PBS containing 0.1% Tween-20 (TPBS) for 5 min each time.

Immunocomplexes were detected with 1:6000 dilution of horseradish peroxidase (HRP) labeled anti-mouse immunoglobulin (Igs) antibodies (DAKO, Glostrup, Sweden) at room temperature for 1 h. After washing, protein bands were visualized by enhanced chemiluminescence (Pierce).

**Binding assays via immunohistochemical staining**
Ae. aegypti larvae (5 days old) were fed with 50 μg/ml of Cry4Ba inclusions for 10, 30 and 60 min (25 larvae/time). The mouth and siphon regions were removed and larvae were fixed at 4°C for 2 h in PBS-4% paraformaldehyde (fixative solution) containing 5% sucrose followed by 4-h incubation in the fixative solution containing 10% sucrose. Paraffin larval section preparation and immunostaining were performed as described by Chayaratanasin et al. [32]. Longitudinal-section slices (10 μm) were made using a microtome (American Optical Corporation, Buffalo, NY, USA) and attached onto positively charged glass slides (Yan Cheng Huida Medical Instrument, Jainesu, China). The sections were subsequently deparaffinized, rehydrated in an ethanol series and rinsed with water. After elimination of endogenous peroxidase activity with 3% H2O2, non-specific binding on the section was blocked with 1:200 diluted normal rabbit serum. Tissue slides were then probed with 1:5000 dilution of antiserum, or affinity purified antibodies followed by 1:8000 dilution of biotin conjugated rabbit anti-mouse IgG (Pierce) and HRP conjugated streptavidin (1:50000 dilution, Pierce). A dark brown color indicating a positive signal was developed with 3, 3′-diaminobenzidine; DAB substrate kit (Vector Laboratories, Burlingame, CA, USA). Morphological changes of toxin treated larvae were observed by staining larval sections with H&E dyes.

**PM permeability assay**

Five-day old Ae. aegypti larvae were fed with 0.5 mg/ml of fluorescein isothiocyanate (FITC, Sigma-Aldrich) labeled 2000-kDa dextrans alone or with Cry4Ba-protoxin inclusions (50 μg/ml) in 24-well tissue culture plates at room temperature (5 larvae/group). Larvae were collected every hour, rinsed with water and fixed in 4% paraformaldehyde-PBS. Localization of fluorescent tracer was monitored under a fluorescence microscope and imaged using an Olympus inverted fluorescence microscope equipped with a digital imaging system. Images were analyzed via MagnaFire software (Optronics, Goletta, CA, USA).
Authors’ contributions

SL carried out protein preparations, antibody characterization, purification, immunohistochemical analysis and drafted the manuscript. SM conceived of the study, participated in its design and coordination, helped to draft the manuscript and carried out the permeability assay. UC carried out immunohistochemical analysis and participated in mouse immunization. NY carried out mouse immunization and participated in antibody characterization. PW conceived and participated in the study of the peritrophic membrane, and performed critical reading and discussion of the manuscript. CA helped in critical reading and discussion of the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1 - Specificity of antibodies produced to Cry4Ba

In the leftmost lane, the purified activated Cry4Ba protein was separated by SDS-PAGE (8-16% gradient gel) and Western blotted onto a membrane. (A) The Cry4Ba protein was probed with 1:1000 dilution of antisera collected after dose-4 immunization (Im) of M1, M2
M3 and M4 mice, respectively. The protein in the Pi lane of each panel was reacted with pre-immunized serum of each mouse. Bound antibodies in all reactions were detected with 1:6000 diluted anti-mouse Igs-HRP followed by chemiluminescent developing. (B) The leftmost panel shows SDS-PAGE of a prestained protein marker; M, a total protein of the cloned domain II, domain III in bacterial crude extract and the domain II-III fragments of the Cry4Ba protein. Proteins were blotted onto the membrane and reacted with a resin-bound (B) fraction of antibodies affinity purified from pooled M1-M2, M3 and M4 antisera, respectively. (C) In the second from left lane, the activated Cry4Ba protein was separated by 8-16% gradient SDS-PAGE and electrotransferred onto a membrane. The blotted Cry4Ba was probed with a resin-unbound fraction of antibodies affinity purified from M3 serum (lane 2), total M3 serum (lane 3) and pooled M1-M2 sera (lane 4). All antisera were used at 1:5000 dilution. A bound antibody was tracked by anti-mouse Igs-HRP (1:6000 dilution) and developed by chemiluminescence.

**Figure 2 - H&E staining and immunolocalization of Cry4Ba protein on Ae. aegypti larval sections**

(A) Tissue sections of normal and (B) 1-h Cry4Ba fed larvae were stained with H&E, demonstrating morphological changes and vacuolization (indicated by white arrows) of epithelial cells. (C) The paraffinized section of 10-min Cry4Ba fed larvae was reacted with pooled M1-M2 antisera, (D) antibody diluting buffer, (E) antibodies specific to 20-kDa domain I, (F) antibodies specific to domain II-III, (G) M4 antiserum, respectively. (H) A tissue section of R158A fed larvae and (I) normal larvae reacted with M4 antiserum. Toxin-antibody complexes were visualized with biotin conjugated anti-mouse IgG/streptavidin-HRP followed by color developing with DAB substrate. A positive antibody reaction is represented by a dark brown color. Degenerated brush border microvilli and secreted material are indicated by an arrow head and star, respectively. ec, epithelial cells; mv, microvilli; lu,
lumen; fb, food bolus; gc, gastric caeca; pm, peritrophic membrane. Scale bar is in millimetre. Results were represented from 3 independent experiments.

**Figure 3 - Cry4Ba toxin alters PM permeability of mosquito larvae**

Larvae were fed with 2000-kDa dextran-FITC alone (lower panels) or with Cry4Ba-protoxin inclusions (upper panels) for 1 h and 6 h, and fixed with paraformaldehyde. Fluorescence localization at anterior midgut and caeca of larva is shown. Fluorescent dye localization at the caeca is indicated by asterisks.
Figure 1