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Functional characterizations of residues Arg-158 and Tyr-170 of the mosquito-larvicidal *Bacillus thuringiensis* Cry4Ba

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The insecticidal activity of *Bacillus thuringiensis* (*Bt*) Cry toxins involves toxin stabilization, oligomerization, passage across the peritrophic membrane (PM), binding to midgut receptors and pore-formation. The residues Arg-158 and Tyr-170 have been shown to be crucial for the toxicity of *Bt* Cry4Ba. We characterized the biological function of these residues. In mosquito larvae, the mutants R158A/E/Q (R158) could hardly penetrate the PM due to a significantly reduced ability to alter PM permeability; the mutant Y170A, however, could pass through the PM, but degraded in the space between the PM and the midgut epithelium. Further characterization by oligomerization demonstrated that Arg-158 mutants failed to form correctly sized high-molecular weight oligomers. This is the first report that Arg-158 plays a role in the formation of Cry4Ba oligomers, which are essential for toxin passage across the PM. Tyr-170, meanwhile, is involved in toxin stabilization in the toxic mechanism of Cry4Ba in mosquito larvae. [BMB Reports 2014; 47(10): 546-551]

INTRODUCTION

Bacillus thuringiensis (*Bt*) Cry toxins Cry4Aa, Cry4Ba, Cry11Aa, and Cry39Aa (1-3) are particularly toxic to mosquito larvae. Among these, Cry4Ba specifically targets *Aedes* and *Anopheles* mosquitoes, the major vectors for the transmission of dengue and malaria which threaten millions of people worldwide (4). Cry4Ba activation by trypsin *in vitro* results in 65 kDa comprising 47-kDa of α6-α7-linked domain II-III region and 18-20-kDa of domain I (5). Three-dimensional structure of the activated Cry4Ba shows high similarity in the overall structure of three-domain organization as the previously solved structures of Cry toxins (6). The N-terminal domain I is a helical bundle of long amphipathic and hydrophobic helices and the C-terminal domains II and III are β-sheet structures (Fig. 1A). Multiprocessing is a particular toxic mechanism of these Cry toxins. It involves toxin solubilization in the larval midgut lumen, toxin processing by gut enzymes, receptor binding, toxin oligomerization, toxin insertion and pore formation (1). The mortality of insect larvae is mainly due to osmotic lysis of midgut epithelial cells (7) by the pore-forming activity of Cry toxins *via* helices 4 and 5 in toxin domain I (8, 9). Consequently, alterations of toxin residues in helix 4,

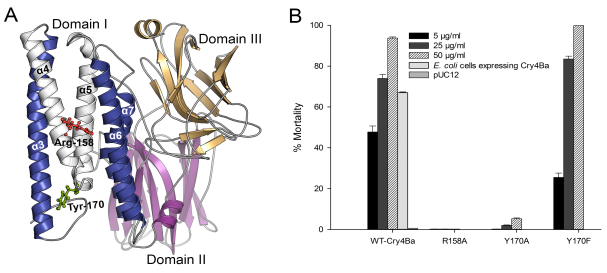

Fig. 1. Ribbon diagrams of the Cry4Ba organization and larvicidal activities of the wild type Cry4Ba and it mutants. (A) The 3D structure illustrates three-domain organization of the 65-kDa activated Cry4Ba, comprising a helical bundle of pore-forming domain I (blue) and β-sheet structures of domain II-III (purple and pale orange, respectively). Two critical residues, Arg-158 (red) in the helix 4 and Tyr-170 (green) in the surface exposure loop of helices 4-5, are represented in balls and sticks. The diagram was drawn using PyMol program (The PyMOL Molecular Graphics System, Version 1.6, Schrödinger, LLC). (B) The fourth-instar larvae were fed *E. coli* cells expressing Cry4Ba (gray) and 5 to 50 μg/ml inclusions of Cry4Ba wild type (WT-Cry4Ba) and mutants (indicated). Larvae which were fed with *E. coli* cells containing a vector backbone harboring *Cry4Ba* gene, were used as controls (pUC12). Numbers of dead larvae were recorded and represented as mean % mortality ± SEM from 3-independent experiments.

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Glu-129, Arg-131 and Asp-136 caused complete loss of Cry1Aa toxicity due to a defective ability to create ion channels (10, 11). This Arg-131 of Cry1Aa has been found to be perfectly matched with Arg-158 in the same helix in Cry4Ba (12). Replacement of residue Arg-158 with alanine (R158A), glutamine (R158Q) and glutamic acid (R158E) resulted in loss of Cry4Ba toxicity towards *A. aegypti* larvae (12, 13) indicating the crucial role of Arg-158 in Cry4Ba-larvicidal activity. Other mutations were made at conserved Tyr-170 in the loop connecting helix 4 and helix 5, revealing a role of aromaticity in Cry4Ba toxicity at this position (14). By structural analysis and comparison with other pore-forming Cry toxins, Arg-158 and Tyr-170 may be involved in ion-channel regulation and toxin insertion into the larval midgut membrane. However, the role of these residues in the pathogenic pathways of the toxins is still unclear. In this report, the functions of residues Arg-158 and Tyr-170 were determined by toxin localization and stabilization in mosquito larvae, toxin binding to the midgut epithelial membrane, toxin oligomerization, and PM permeability alteration using Cry4Ba wild type and mutants.

RESULTS AND DISCUSSION

Larvicidal activities of mutants R158A and Y170A compared with Cry4Ba wild type

It has been shown previously that mutations at residues Arg-158 in the middle of helix 4 and Tyr-170 in the loop between helices 4-5, affect Cry4Ba toxicity (12, 14). Here, the fourth-instar larvae of *A. aegypti* were fed with protein inclusions of mutants R158A and Y170A, from 5 to 50 μg/ml, yet they survived (Fig. 1B). In contrast, larvae which were treated with *E. coli* cells expressing Cry4Ba wild type and its toxin inclusions showed a moderate to high mortality rate of approximately 67% and 45-90% (5-50 μg/ml toxin inclusions), respectively. It should be noted that protein-expression levels of mutants in the bacteria cells varied, and they also differed from the Cry4Ba wild type. Thus, larvae were fed with the same amount of protein inclusions to compare toxicity of the Cry4Ba wild type and the mutants. Larval treatment with 50 μg/ml inclusions of mutant Y170F showed mortality close to the Cry4Ba wild type. Altogether, the results confirm that Arg-158 and Tyr-170 are crucial for Cry4Ba toxicity against *A. aegypti* larvae.

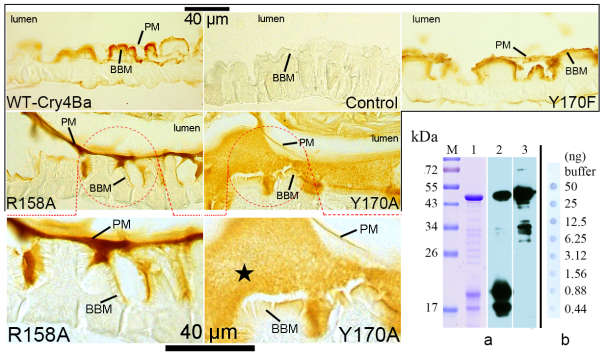

Fig. 2. Cry4Ba localization in toxin treated larvae. The tissue sections prepared from the toxin-treated (indicated) and non-treated larvae (control) were immunostained with Cry4Ba-specific antibodies; C4bPAbs followed by secondary antibodies and color developing (see Methods). The positive binding at BBM of midgut epithelium and at the PM was represented by a dark-brown color. The control tissues show no reactivity with the antibodies. The last two panels at the bottom are enlarged images (areas in the red circles) demonstrating toxin binding signal at the BBM and the lumen-facing PM of mutants Arg-158 and Y170A. Star indicates the toxin accumulated in the ectoperitrophic space between the PM and the BBM. Results are a representation of 3-independent experiments. The inset (a) shows SDS-PAGE separation of 47 kDa and 18-20 kDa of Cry4Ba (lane 1) and their specific reactions with newly-generated antibodies C4bPAbs (lane 2), and anti- Cry4Ba domain III mAb 2F1H2 (lane 3). (b) Blue dots represent reactivities of antibodies C4bPAbs with various amounts of Cry4Ba protein.

Toxin localization in the mosquito larval gut

Larvae were fed with Cry4Ba wild type and mutants. Protein localization was followed by polyclonal antibodies C4bPAbs specific to 47-kDa and 18-20-kDa fragments of the 65-kDa activated Cry4Ba (Fig. 2-inset a). These antibodies were capable of detecting 0.44 ng or less of the native Cry4Ba protein (Fig. 2-inset b). Thus, they are useful for monitoring toxins bound to the brush border membrane (BBM), where the amounts are assumed to be very low. In the larval gut, Cry4Ba needs to transverse across the PM which lies between the lumen and the BBM (15, 16). Upon larval feeding with the toxin, immunohistochemistry demonstrated localization of Cry4Ba wild type and the active mutant Y170F at both the PM and midgut epithelium. In contrast, the inactive mutant R158A was obviously detected on the PM yet was barely detected on the BBM (Fig. 2). The results suggest, therefore, that the mutant R158A may carry a defect in terms of the toxin's passage across the membrane. However, we could not omit the possibility that the mutant itself may not be able to bind to the midgut epithelium, resulting in a low signal. For the mutant Y170A, it was found to accumulate heavily in the space between the PM and the epithelium, suggesting that the mutant passes the PM but may not be able to bind to the BBM (Fig. 2).

Midgut-membrane binding ability and stability of Cry4Ba

Immunohistochemistry staining was applied to examine whether mutants R158A and Y170A lost their binding to BBM. Larval-tissue sections were covered with Cry4Ba, and bound toxins were followed by polyclonal antibodies C4bPAbs. It was shown that both mutants R158A and Y170A bound to the BBM in a similar way to the Cry4Ba wild type and the active mutant Y170F (Fig. 3A). Consequently, it can be concluded that residues Arg-158 and Tyr-170 in domain I are not involved in *in vivo* binding of Cry4Ba to the BBM. This conclusion is aligned with previous reports, that residues located in domain II-surface exposure loops contribute mostly to bind-

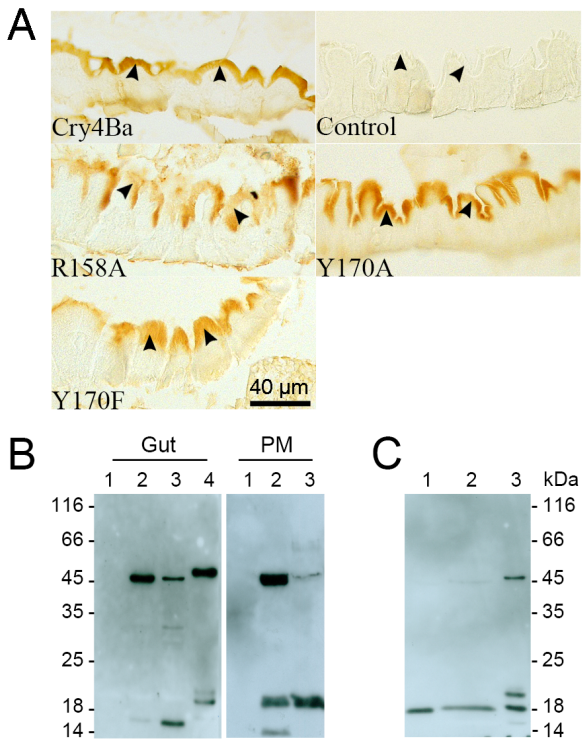

Fig. 3. Cry4Ba binding to the larval midgut epithelium and toxin proteolytic processing in the larvae. (A) Larval tissue sections were covered by Cry4Ba wild type (Cry4Ba) and mutants (indicated). A bound toxin was detected with antibodies C4bPAbs, this was followed by secondary antibodies; biotin conjugated anti-mouse IgG /streptavidin-HRP (see Methods). The dark-brown color represents toxin binding at BBM (arrow heads) from 3-independent experiments. A control is a larval section which was reacted with antibodies, but without toxin reaction (control). (B) Western blot analysis of a toxin associated with PM or gut in the larvae treated with Cry4Ba wild type (lane 2) and R158A (lane 3) with Cry4Ba specific antibodies C4bPAbs. Antibodies show negative reactivity with proteins which were isolated from the PMs and guts of normal larvae (lane 1). (C) Reactivity of the antibodies C4bPAbs with Y170A protein associated with gut (lane 1) and PM (lane 2). The 47 kDa and 18-20 kDa of the trypsin activated Cry4Ba *in vitro* (B-lane 4, C-lane 3) were used as positive controls.

ing of Cry1 (17, 18), Cry4Ba (19, 20) and Cry11 toxins (21) to the BBM in the larval hosts.

There are high numbers of proteases in gut juice, while trypsin and chymotrypsin are the main enzymes in the midgut during the development of *A. aegypti* larvae (22). In this circumstance, there is a high chance that a Cry4Ba mutant may be degraded by gut proteinases, resulting in low toxins binding to the BBM. For verification of this, Western blot analysis was applied to determine those toxin fragments that associate with PMs and guts in toxin fed larvae. Polyclonal antibodies C4bPAbs revealed approximate 45-kDa and 18-kDa, non-covalently associated fragments, of the 65-kDa, Cry4Ba wild type associated with PM and gut components in toxin treated larvae (Fig. 3B-lanes 2). These sizes of proteolytically-processed fragments larvae were slightly different from 47 kDa and 18-20 kDa (Fig. 3B-lane 4, 3C-lane 3); which were previously obtained by toxin digestion with trypsin *in vitro* (5), suggesting Cry4Ba toxin processing *in vivo* that differs from *in vitro*. Compared to the Cry4Ba wild type, the amount of fragments of 45 kDa and 18 kDa of R158A were greatly reduced in both the PM and midgut (Fig. 3B-lanes 3). In addition, the 65-kDa protein which is equivalent to the size of Cry4Ba monomer was found to be associated with the PM (Fig. 3B-lane 3 PM). This size perhaps resulted from inter-domain interaction or aggregation of the toxin on the PM. Furthermore, Western blot analysis demonstrates rigorous degradation of Y170A, possessing proteolytic fragments which are smaller than 18-kDa (Fig. 3C-lanes 1, 2). Although the mutants R158A and Y170A are well characterized by previous reports as having equal protein folding, and as having *in vitro* proteolytic stability just like the wild type (12, 14), the current results clearly demonstrate these mutants are unstable, and drastically degraded in the fed larvae. It is possible that replacement of Tyr-170 with non-aromatic residues may result in toxin instability (23), that consequently enhances toxin susceptibility to gut proteases and loss in toxicity of the mutant Y170A towards mosquito larvae.

Correlation between Cry4Ba oligomerization and PM-permeability alteration activity

The PM has a permeability function for trafficking solutes (16, 24); the passage of Cy4Ba across the PM may result from PM-permeability alteration or from physical damage by the toxin itself. We investigated further for a role of Arg-158 and Tyr-170 in PM-permeability alteration, by feeding larvae with the Cry4Ba toxin along with 2000-kDa of dextran-FITC; a tracker. It should be noted that dextran-FITCs at this size are unable to pass across the PM (25). Therefore, presence of such fluorescent particles in the ectoperitrophic space or in gastric ceca is an indicator of PM-permeability alteration or PM-structure disruption. In the larvae which were fed with the 2000-kDa dextran-FITC alone, most show the fluorescent dyes inside the midgut lumen (Fig. 4Aa-4Ac). In contrast, the larvae fed with Cry4Ba show the fluorescent signal in the ectoperitrophic space or at the gastric ceca (Fig. 4Ad-4Af), suggesting PM permeability was altered by the toxin. The larvae fed with mutant Y170A (47-51%) showed comparable permeability alteration activity to those fed with the Cry4Ba wild type (48%-56%) (Fig. 4B). This suggests a negligible effect of alanine substitution at Tyr-170 of Cry4Ba on PM-permeability alteration. Remarkably, only 9-22% of the larvae that were exposed to Arg-158 mutants (R158A, R158E and R158Q), were positive for fluorescence in such compartments (Fig. 4B), demonstrating a significant reduction in PM-permeability alteration ability of these mutants. Hence, the results reveal a novel function of Arg-158 in the PM-permeability alteration in the patho-

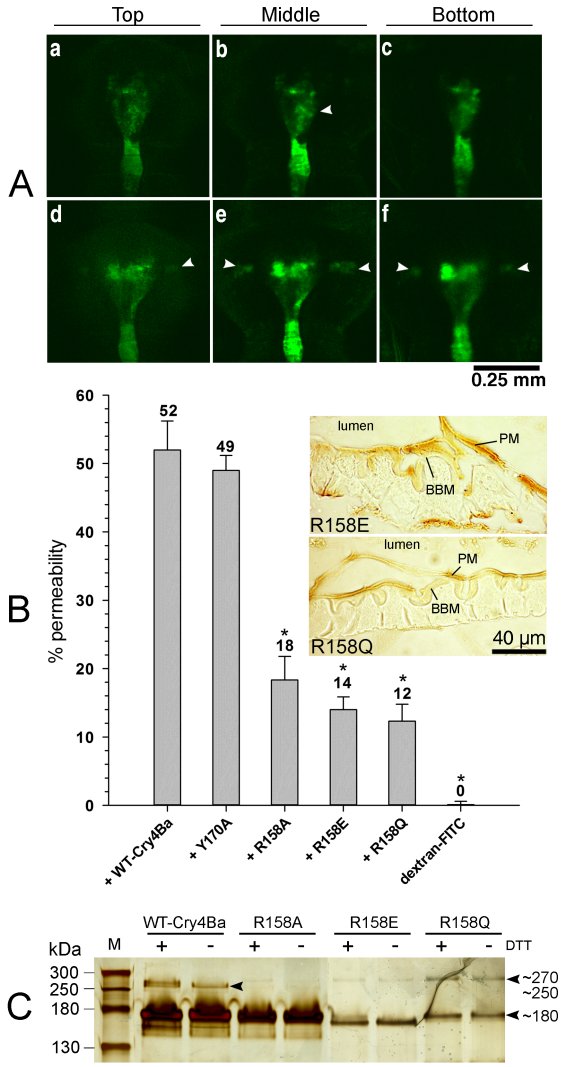

Fig. 4. Analysis of Cry4Ba PM-permeability alteration and oligomers. (A) Larvae were fed with 2000 kDa of the dextran-FITC alone (dextran-FITC), or plus the Cry4Ba wild type (WT-Cry4Ba) and mutants (+R158A/E/Q and +Y170A). Fixed larvae were analyzed by fluorescent and confocal microscopies. Confocal images of 30-μm continuous sections demonstrate fluorescence tracker accumulating inside the lumen of the larvae treated with dextran-FITC alone (Aa-Ac) in the ectoperitrophic space or in the gastric ceca of larvae fed with the dextran-FITC plus Cry4Ba toxin (Ad-Af). Confocal images which were captured at the top, middle and the bottom layer of larval body were labeled as (a/d), (b/e) and (c/f), respectively. (B) Larvae with positive fluorescence at the gastric ceca were recorded and represented by mean (numbers) % permeability alteration ± SD. The inset shows localization of R158Q and R158E at the PM of the midgut in toxin treated larvae. (C) shows high molecular weight oligomers (indicated) of Cry4Ba wild type and mutants (R158A, R158Q and R158E) formed in carbonate buffer pH 10.0, and analyzed by SDS-PAGE in the presence (+) and absence (-) of DTT; a reducing agent.

genic pathway of the Cry4Ba toxin. Defects in function of the mutants such as this result in abnormal traffic across the PM and subsequent binding of mutants to midgut epithelium (Fig. 4, inset). It has been shown recently that Arg-158 plays a role in the formation of non-sulfide mediated high molecular weight oligomers which are correlated to Cry4Ba toxicity, while the mutant R158A failed to form the correct size of these oligomers (submitted for publication). Here we found that substitution of Arg-158, either with a positive (R158Q) or negative charge (R158E) residue, significantly affected Cry4Ba oligomerization (Fig. 4C), thus indicating the crucial role of this residue on high molecular weight oligomer formation.

In summary, Arg-158 plays a central role in formation of the oligomers which function in PM-permeability alteration, and allow Cry4Ba to pass to midgut epithelium where the toxin can cause cell death. In addition, aromatic residue Tyr-170 prevents the toxin from degradation in the proteinase rich condition of the larval gut.

MATERIALS AND METHODS

Expression, activation and purification of the Cry4Ba toxin

Escherichia coli cells JM109 harboring the pMU388 plasmid encoding for Cry4Ba and its mutants R158A (12), R158E, R158Q (13), Y170A and Y170F (14) were grown at 37°C in Luria-Bertani medium containing 100 μg/ml ampicillin. Protein expression and production of toxin inclusions were performed as previously described (9). Protoxin inclusions (130 kDa) were solubilized in 50 mM sodium carbonate (pH 10.0) and digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma-Aldrich, St. Louis, MO), resulting in a 65 kDa of the activated toxin, which was subjected to protein purification by gel filtration chromatography equipped with a Superose 12 column (GE Healthcare, Uppsala, Sweden). The purified 65-kDa protein was resolved by (12% gel) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands of the 47 kDa and the 18-20 kDa were then recovered by electroelution.

Larvicidal assay

A. aegypti eggs were supplied by the mosquito-rearing facility of the Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, Thailand. Biological activity of Cry4Ba was performed in a 24-well culture plate (Costar, Corning, NY). Twenty five of the fourth-instar larvae (a total of 100/each concentration) were fed with 5, 25 and 50 μg/ml inclusions, and compared to *E. coli* expressing protein inclusions of Cry4Ba wild type and mutants. After 24 h, numbers of dead larvae were recorded and presented as mean percent mortality of at least 3-independent experiments ± standard error of mean (SEM).

Production of specific antibodies to Cry4Ba

Polyclonal antibodies specific to the whole molecule of

Cry4Ba were selectively produced in order to increase sensitivity of toxin detection. A protein (25 μg) was mixed with Alum (Thermo Scientific, Rockford, IL) at Alum/protein ratio of 1 : 4 and injected into an animal every 2 weeks. Animal handling and immunization protocols were approved by the Animal Care and Use Committee, Faculty of Tropical Medicine, Mahidol University (FTM-ACUC 006/2009). Immunized blood was drawn every 2 weeks after each immunization to monitor the presence of Cry4Ba-specific antibodies by Western blot analysis. Antisera with high titers from 2 mice were pooled as Cry4Ba-specific polyclonal antibodies (C4bPAbs). Specificity of antisera was determined by reaction of the wild type Cry4Ba protein on the membrane (blotted proteins) with antibodies C4bPAbs (1 : 5,000 dilution), and compared to anti-Cry4Ba domain III monoclonal antibody (mAb 2F1H2) (1 : 1,000 dilution) (26). Immunocomplexes were reacted with 1 : 6,000 dilution of horseradish peroxidase (HRP) labeled anti-mouse immunoglobulin (Igs) antibodies (DAKO, Glostrup, Sweden), and followed by visualization using enhanced chemiluminescence (Thermo Scientific). Detection sensitivity of the C4bPAbs was also determined by reaction of the C4bPAbs (1 : 250 diluted) with various amounts of the 65-kDa activated Cry4Ba. Dark blue-dots were developed by an hour-long reaction with 1 : 8,000 of biotin conjugated rabbit anti-mouse IgG (Thermo Scientific), 1 : 50,000 dilution of HRP labeled streptavidin (Thermo Scientific) and reaction with 3'-diaminobenzidine; DAB substrate kit (Vector Laboratories, Burlingame, CA).

Assays for *in vivo* toxin localization and midgut epithelial binding

A. aegypti larvae (a total of 100 larvae) were fed with 50 μg/ml of Cry4Ba inclusions for 1 h and from at least 3-independent feedings. Longitudinal-section slices (10 μm) were prepared from normal and toxin-treated larvae, according to Chayaratanasin *et al*. (27). After tissue blocking with 3% H_2O_2, non-specific binding of antibodies to the tissue section was blocked with 1 : 200 diluted normal rabbit serum. Tissue slides of normal larvae were reacted with 12.5 μg/ml of the purified 65 kDa of the activated Cry4Ba protein. Both tissue slides of normal and the toxin-treated larvae were probed with 1 : 250 dilution of antibodies C4bPAbs, followed by reaction with biotin conjugated anti-mouse IgG, and HRP conjugated streptavidin. A dark brown color representing a positive signal was developed for exactly 2 min/each reaction with DAB substrate kit (Vector Laboratories).

Western blot analysis of Cry4Ba associated with the PM and the gut

Larvae were fed with 50 μg/ml inclusions of the Cry4Ba wild type and mutants; R158A or Y170A for 1 h. PMs and guts were dissected and washed 3 times in deionized water containing 1 mM PMSF and 5 mM EDTA (pH 8.0). Proteins from the PM (10 pieces/lane) and gut (1 piece/lane) were resolved by 10% gel SDS-PAGE. Protein were transferred onto the membrane and reacted with 1 : 5,000 dilution of antibodies C4bPAbs, followed by 1 : 6,000 dilution of HRP labeled anti-mouse immunoglobulin antibodies, and followed by enhanced chemiluminescence.

PM permeability assay

PM-permeability of mosquito larvae to fluorescein isothiocyanate (FITC) labeled 2000-kDa dextrans (Sigma-Aldrich) upon Cry4Ba feeding was determined according to Edwards and Jacobs-Lorena (25). Briefly, 100 larvae were fed with 0.5 mg/ml of dextran-FITC alone or with inclusions (50 μg/ml) of Cry4Ba and mutants in 6-well tissue culture plates at room temperature for 1 h (25 larvae/well). The larvae were collected, rinsed with tap water, and fixed in 4% paraformaldehyde-PBS. Localization of fluorescent tracker was monitored under a fluorescent microscope (Carl Zeiss, Jena, Germany) equipped with a Canon digital imaging system. Images were analyzed by AxioVision LE Canon Module. LSM 700 confocal microscopy (Carl Zeiss) was used to assess the fluorescence inside the larval body. Serial optical sections were taken at 30-μm intervals throughout the thickness of the larval gut and analyzed under green fluorescence.

Analysis of Cry4Ba-high molecular weight oligomers

Cry4Ba oligomers were produced in carbonate buffer containing 150 mM NaCl and 0.2% Triton X-100 (submitted for publication) and separated by SDS-PAGE (10% gel, 0.1% cross link) in the presence and absence of dithiothreitol; a reducing agent.

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