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Structurally Conserved Aromaticity of Tyr^{249} and Phe^{264} in Helix 7 Is Important for Toxicity of the *Bacillus thuringiensis* Cry4Ba Toxin

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Functional elements of the conserved helix 7 in the pore-forming domain of the *Bacillus thuringiensis* Cry δ-endotoxins have not yet been clearly identified. Here, we initially performed alanine substitutions of four highly conserved aromatic residues, Trp^{243}, Phe^{246}, Tyr^{249} and Phe^{264}, in helix 7 of the Cry4Ba mosquito-larvicidal protein. All mutant toxins were overexpressed in *Escherichia coli* as 130-kDa protoxins at levels comparable to the wild-type. Bioassays against *Stegomyia aegypti* mosquito larvae revealed that only W243A, Y249A or F264A mutant toxins displayed a dramatic decrease in toxicity. Further mutagenic analysis showed that replacements with an aromatic residue particularly at Tyr^{249} and Phe^{264} still retained the high-level toxin activity. In addition, a nearly complete loss in larvicidal activity was found for Y249L/F264L or F264A/Y249A double mutants, confirming the involvement in toxicity of both aromatic residues which face towards the same direction. Furthermore, the Y249L/F264L mutant was found to be structurally stable upon toxin solubilisation and trypsin digestion, albeit a small change in the circular dichroism spectrum. Altogether, the present study provides for the first time an insight into the highly conserved aromaticity of Tyr^{249} and Phe^{264} within helix 7 playing an important role in larvicidal activity of the Cry4Ba toxin.

Keywords: *Bacillus thuringiensis*, Conserved aromaticity, δ-endotoxin, Larvicidal activity, Site-directed mutagenesis

Introduction

Bacillus thuringiensis produces highly specific insecticidal proteins (Cry and Cyt δ-endotoxins) in large quantities as parasporal crystalline inclusions during the sporulation phase (Schnepf *et al*., 1998). Of particular interest, the Cry δ-endotoxins have been shown to be specifically active against their target insect larvae including Diptera (mosquitoes and blackflies), Lepidoptera (moths and butterflies), Coleoptera (beetles) and Hymenoptera (wasps and bees) (Schnepf *et al*., 1998; De Maagd *et al*., 2001) and hence they are safe for human beings. For instance, the 130-kDa Cry4Ba toxin produced from *Bt* subsp. *israelensis* is specifically toxic to mosquito larvae of the genus *Stegomyia* (*Aedes*) and *Anopheles* which continue to be the most important vectors of such serious human diseases as dengue haemorrhagic fever and malaria (Becker *et al*., 1993).

Following the ingestion by susceptible insect larvae, the protoxin inclusions are solubilised in the midgut lumen which is highly alkaline for many dipteran and lepidopteran larvae (Schnepf *et al*., 1998). The liberated soluble protoxins are then processed by larval gut proteases to yield the active toxins. It has been shown for several Cry toxins that the activated toxins bind to specific receptors located on the apical brush-border membrane of midgut epithelium. Subsequent conformational changes allow the insertion of their pore-forming portions into the cell membranes to form ion-leakage pores which cause colloid-osmotic cell lysis, resulting in extensive damage to the midgut and eventual larval death (Knowles *et al*., 1994; Whalon *et al*., 2003). However, detailed understanding of the molecular mechanism of action of these toxins remains to be explored, especially the steps of toxin insertion and lytic pore-formation in the target cell membrane.

To date, *Bt* Cry toxin crystal structures have been solved in almost all the major specificity classes, including the lepidoteran-specific Cry1Aa (Grochulski *et al*., 1995) and Cry1Ac (Derbyshire *et al*., 2001), the lepidopteran, dipteran-dual

Abbreviations: *Bt* (*Bacillus thuringiensis*), CD (circular dichroism), Cry (crystal), Cyt (cytolytic), FPLC (fast-performance liquid chromatography), IPTG (isopropyl-β-D-thiogalactopyranoside), PCR (polymerase chain reaction), SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis)

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specific Cry2Aa (Morse *et al*., 2001), the coleopteran-specific Cry3Aa (Li *et al*., 1991), and more recently the dipteran-specific Cry4Ba (Boonserm *et al*., 2005) and Cry4Aa (Boonserm *et al*., 2006). They all share a high degree of overall structural similarity with three-distinct domain organisation. In particular, the N-terminal domain I that is an amphipathic α-helical bundle contains two highly conserved regions; block 1, which is α5; and block 2, which partly consists of α7 (Li *et al*., 1991). This domain has been shown to be responsible for membrane insertion, leading to formation of the ion-leakage pores (Puntheeranurak *et al*., 2004; Rausell *et al*., 2004). Even with the fact that the structures of the two closely-related mosquito-larvicidal proteins, Cry4Aa and Cry4Ba, bear a likeness to the other known Cry structures, the finer features are rather different (Li *et al*., 1991; Grochulski *et al*., 1995; Derbyshire *et al*., 2001; Morse *et al*., 2001; Boonserm *et al*., 2005; 2006). For instance, there is additional *in vitro* proteolysis by trypsin occurring in the loop connecting α5 and α6 of both the 65-kDa activated Cry4Aa and Cry4Ba toxins, thus generating two non-covalently associated fragments of ca. 47 and 20 kDa (Angsuthanasombat *et al*., 2004).

Thus far, the umbrella model seems to be the best description for the membrane-associated state of the Cry toxins, which involves an insertion of α4 and α5 into the lipid membrane as a helical hairpin whilst the remaining helices are rearranged to spread on the membrane surface (Knowles *et al*., 1994; Gazit *et al*., 1998). Several mutational studies have suggested that α4 is oriented to face the pore lumen and participates in ion permeation through the pore (Masson *et al*., 1999; Sramala *et al*., 2001), whilst the relatively hydrophobic helix-α5 appears to be in contact with the lipid membrane and plays a role in toxin-pore oligomerisation (Nunez-Valdez *et al*., 2001; Likitvivatanavong *et al*., 2006). More recently, we have strengthened this model by providing direct evidence for membrane-perturbing activity of the α4-loop-α5 hairpin isolated from the Cry4Ba toxin (Leetachewa *et al*., 2006). We have also shown that the structural rigidity of the α4-α5 loop of the Cry4Aa toxin is essential for larvicidal activity (Tapaneeyakorn *et al*., 2005). A refined model further suggested that α7 may serve as a binding sensor that could initiate the binding of the pore-forming domain to the lipid membrane, facilitating the membrane insertion of the α4-α5 hairpin (Gazit *et al*., 1998). Although helix 7 has also been implicated in ion-channel activity and receptor-binding affinity of Cry1A toxins (Chandra *et al*., 1999; Alcantara *et al*., 2001), or structural stability and crystallisation of Cry3Aa (Park *et al*., 2004), the functional importance for toxicity of the structurally conserved aromaticity in this helix has not been clearly highlighted. In the present study, we have identified functional elements by demonstrating for the first time that

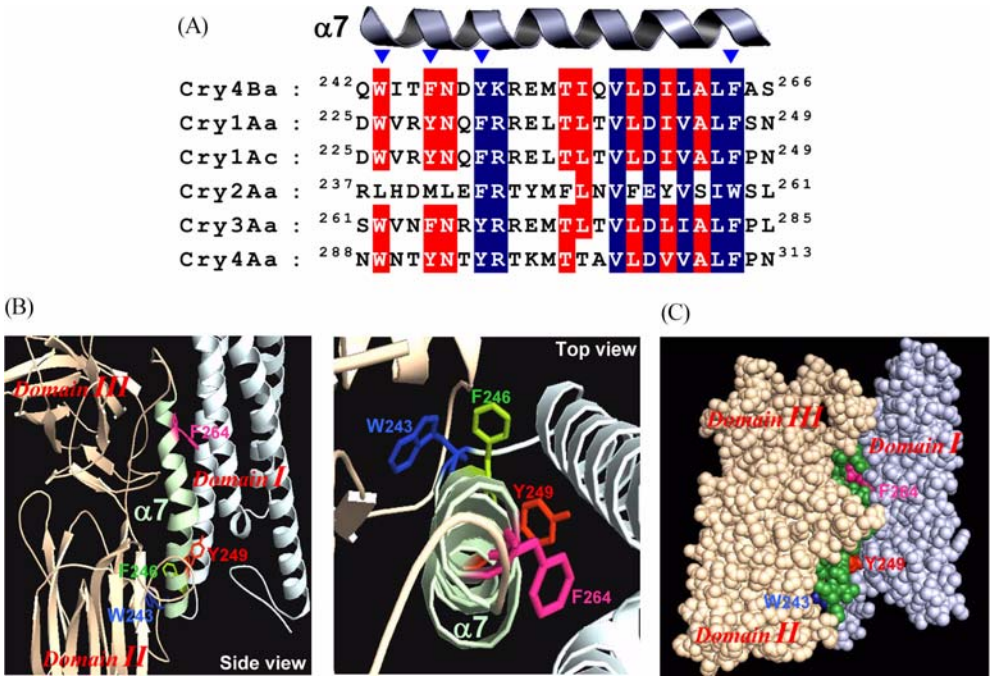

Fig. 1. (A) Multiple sequence alignment of helix 7 of Cry4Ba with that of other *Bt* crystal structures, Cry1Aa, Cry1Ac, Cry2Aa, Cry3Aa and Cry4Aa. Positions of the secondary structure of Cry4Ba-helix 7 are illustrated above the sequence. The mutated residues of Cry4Ba in this study are indicated by ▼. The degree of conservation among the six sequences is represented by background shading of the residues with red and blue shading for 83% and 100% sequence homology, respectively. (B) Part of the Cry4Ba crystal structure (side and top views) (Boonserm *et al*., 2005), showing the positions of four highly conserved aromatic residues in helix 7, as indicated by ▼ in (A) and illustrated in ball-and stick model. (C) Space-filling representation of the Cry4Ba structure showing locations of the two critical aromatic residues, Tyr^{249} (red) and Phe^{264} (pink) in helix 7 (green). The structures were generated by PyMOL program.

two highly conserved aromatic residues, Tyr^{249} and Phe^{264}, which are oriented on the same side of α7 (see Fig. 1) play an important role in larvicidal activity of the Cry4Ba toxin.

Materials and Methods

Construction of Cry4Ba-mutant plasmids. The pMU388 plasmid encoding the 130-kDa Cry4Ba toxin (Angsuthanasombat *et al*., 1987) was used as a template for the introduction of single mutations. Two resultant mutant plasmids, pY249L and pF264A, were used as templates for generating double mutations, Y249L/F264L and F264A/Y249A, respectively. Twenty one pairs of complementary mutagenic oligonucleotide primers (Table 1) designed according to the published *cry4Ba* gene sequence (Angsuthanasombat *et al*., 1987) were purchased from Sigma Proligo (Singapore). All mutant plasmids were generated by polymerase chain reaction using a high fidelity *Pfu* DNA polymerase, following the procedure of the QuikChange™ Mutagenesis Kit (Stratagene). Selected clones with the required mutations were first identified by restriction endonuclease digestion of the plasmids and then verified by automated DNA sequencing.

Toxin expression and inclusion purification. The Cry4Ba wild-type and its mutant toxins were expressed in *E. coli* strain JM 109 under control of the *lacZ* promoter. Cells were grown in a Luria-Bertani medium supplemented with ampicillin 100 μg/ml at 30°C. When the culture reached OD_{600} 0.3-0.4, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM, and incubation was continued for another 10 h. *E. coli* samples (10^7 cells) were analysed for toxin expression by using sodium dodecyl sulphate-(12% w/v) polyacrylamide gel electrophoresis.

E. coli cells, which express the toxins as inclusion bodies, were harvested by centrifugation and resuspended in cold distilled water. Cells were disrupted by using French Pressure Cell at 10,000 psi and the crude lysate was centrifuged at 7,000 × *g*, 4°C for 10 min. The pellets were washed three times in cold distilled water and resuspended by sonification. Protein concentrations of the purified inclusions were determined with the Bradford-based protein microassay (Bio-Rad), with bovine serum albumin fraction V (Sigma) as a standard.

Inclusion solubilisation and trypsin digestion. Protoxin inclusion (1 mg/ml) were solubilised in 50 mM Na_2CO_3, pH 10.0, and incubated at 37°C for 1 h as previously described (Uawithya *et al*., 1998). After centrifugation at 8,000 × *g* for 10 min, the supernatant and inclusion suspensions were analysed by SDS-PAGE. The solubilised protoxins were subsequently assessed for their proteolytic stability by digestion with tolysulfonyl phenylalanyl chloromethyl ketone-treated trypsin at a ratio of 1 : 20 (w/w) enzyme/toxin in 50 mM Na_2CO_3, pH 10.0 for 16 h. Prior to purification (see below), remaining trypsin activity was inhibited by adding phenylmethane sulfonyl fluoride to give a final concentration of 1 mM.

Purification of trypsin-digested toxins. After being examined by SDS-PAGE, the trypsin-digested fraction was further purified by a size-exclusion FPLC system (Superdex 200, Amersham Parmacia Biotech) with a linear gradient of 50 mM Na_2CO_3, pH 10.0, at a flow rate of 0.4 ml/min and monitored by UV absorption at 280 nm. Elution fractions across the 65-kDa protein peak were pooled and subjected to subsequent characterisation.

Circular dichroism spectroscopy. CD measurements of the FPLC-purified toxins were performed on a Jasco J-715 CD spectropolarimeter (Jasco Inc.) in the far UV region (190-280 nm) at 25°C using a rectangular quartz cuvette (0.2 mm optical path-length). Samples were prepared in 50 mM Na_2CO_3, pH 10.0, with protein concentrations of 0.40 mg/ml, as determined by far UV absorbance. CD spectra were recorded at a scanning rate of 50 nm/min with a spectral bandwidth of 2 nm and response times of 2 msec. Three accumulations were taken and the results were averaged. After background subtraction, all the CD data were converted from CD signal (mdegree) into mean residue ellipticity (deg cm^2/dmole).

Larvicidal activity assays. Bioassays were performed as previous described (Sramala *et al*., 2000), using 2-day-old *S. aegypti* mosquito larvae (hatched from eggs supplied by the mosquito-rearing facility of the Institute of Molecular Biology and Genetics, Mahidol University). Both the rearing of the larvae and bioassays were performed at room temperature (25°C). The assays were carried out in 1 ml of *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 *E. coli* expressing each type of the Cry4Ba toxin. *E. coli* cells containing the pUC12 vector were used as a negative control. Mortality was recorded after 24-h incubation period. Student's *t* test (Glover *et al*., 2002) was performed to determine significance levels between mutants and the wild type.

Results and Discussion

As was previously suggested in the "umbrella-like" model for the membrane-bound state of the *Bt* Cry δ-endotoxins, the conserved helix 7 could function as a membrane-binding sensor of the pore-forming domain (Gazit *et al*., 1998), although its functional elements have not yet been clearly identified. In our earlier studies, we have made single-proline substitutions in five helices (α3, α4, α5, α6 and α7) in the pore-forming domain of the Cry4Ba toxin, and found that the integrity of α7 conceivably plays a role in larvicidal activity similar to that was observed for α4 and α5 (Uawitya *et al*., 1998; Sramala *et al*., 2000). Recently, we have also revealed an important involvement in larvicidal activity for one highly conserved aromatic residue in the α4-α5 loop of both Cry4Aa (Tyr^{202}) and Cry4Ba (Tyr^{170}) (Kanintronkul *et al*., 2003; Pornwiroon *et al*., 2004). As shown in Fig. 1A, four aromatic residues, Trp^{243}, Phe^{246}, Tyr^{249} and Phe^{264} within α7 of the Cry4Ba toxin are structurally conserved among the known Cry toxin structures with the result that these highly conserved residues could also play a crucial role in toxicity. In the present study, we therefore initially generated four Cry4Ba mutant toxins in which all the four conserved aromatic residues within this helix were

Table 1. Complementary mutagenic primers for the generation of Cry4Ba mutant toxins

Primer	Sequence[a]	Restriction site
W243A-f:	5'-ATGGACAG**GCC**ATTACGTTTAATG-3'	*Hae*III
W243A-r:	3'-TAGATTACCTGTC**CGG**TAATGCAA-5'	
F246A-f:	5'-GATTACG**GCC**AATGATTATAAAAG-3'	*Hae*III
F246A-r:	3'-TTACCTAATGC**CGG**TTACTAATA-5'	
Y249A-f:	5'-TTTAATGAT**GC**TAAG**C**G**C**GAGATGACTATTC-3'	*Hha*I
Y249A-r:	3'-TAATGCAAATTACTA**CG**ATT**C**G**C**G**C**TCTACT-5'	
F264A-f:	5'-CGCTCTT**GC**TGCCTCATATGATCCACG-3'	*Nde*I
F264A-r:	3'-ATGAGCGAGAA**CG**ACGGAGTATACTAGG-5'	
W243K-f:	5'-CTAATGG**C**CA**AA**AGATTACGTTTAAT-3'	*Hae*III
W243K-r:	3'-TATTTAGATTACC**G**GT**TT**TCTAATGC-5'	
W243L-f:	5'-TCTAATGG**C**CAGT**T**GATTACGTTTAAT-3'	*HaeIII*
W243L-r:	3'-TATTTAGATTACC**G**GTTA**A**CTAATGCA-5'	
W243F-f:	5'-CTAATGG**C**CAAT**TT**ATTACGTTTAATG-3'	*Hae*III
W243F-r:	3'-TATTTAGATTACC**G**GTTA**AA**TAATGCAA-5'	
Y249E-f:	5'-TTTAATGAT**G**AA**A**AA**GC**G**C**GAGATGACTATTC-3'	*Hha*I
Y249E-r:	3'-TAATGCAAATTACTA**C**TT**T**TT**CG**C**G**CTCTACT-5'	
Y249R-f:	5'-TTTAATGAT**CG**TAA**GC**G**C**GAGATGACTATTC-3'	*Hha*I
Y249R-r:	3'-AATGCAAATTACTA**GC**ATT**CG**C**G**CTCTACT-5'	
Y249H-f:	5'-TTTAATGAT**C**ATAA**GC**G**C**GAGATGACTATTC-3'	*Hha*I
Y249H-r:	3'-TAATGCAAATTACTA**G**TATT**CG**C**G**CTCTACT-5'	
Y249F-f:	5'-TTAATGATT**T**TAA**GC**G**C**GAGATGACTATTC-3'	*Hha*I
Y249F-r:	3'-AATGCAAATTACTAA**A**ATT**CG**C**G**CTCTACT-5'	
Y249W-f:	5'-TTAATGATT**GG**AA**GC**G**C**GAGATGACTATTC-3'	*Hha*I
Y249W-r:	3'-AATGCAAATTACTAA**CC**TT**CG**C**G**CTCTACT-5'	
Y249L-f:	5'-TTTAATGAT**CT**TAA**GC**G**C**GAGATGACTATTC-3'	*Hha*I
Y249L-r:	3'-TAATGCAAATTACTA**GA**ATT**CG**C**G**CTCTACT-5'	
F264E-f:	5'-CGCTCT**AGAG**GCCAGTTATG-3'	*Xba*I
F264E-r:	3'-ATGAGCGAGA**TCTC**CGGTCA-5'	
F264R-f:	5'-TCGCTCTT**CG**TGCC**TCA**TATGATCCACG-3'	*Nde*I
F264R-r:	3'-TATGAGCGAGAA**GC**ACGGA**GT**ATACTAGG-5'	
F264H-f:	5'-GTATTAGATAT**C**CTCGCTCTT**CA**TGCCAGTTATGA-3'	*EcoR*V
F264H-r:	3'-GTTCATAATCTATA**G**GAGCGAGAA**GT**ACGGTCAAT-5'	
F264Y-f:	5'-CGCTCTTT**A**TGCC**TCA**TATGATCCACG-3'	*Nde*I
F264Y-r:	3'-ATGAGCGAGAAA**T**ACGG**AGT**ATACTAGGA-5'	
F264W-f:	5'-CGCTCTTT**GG**GCCAGTTATGAT-3'	*Sau*96I
F264W-r:	3'-ATGAGCGAGAAA**CC**CGGTCAAT-5'	
F264L-f:	5'-TAGATATACT**GGCC**CTTTT**A**GCCAGTTATGAT-3'	*Sau*96I
F264L-r:	3'-CATAATCTATATGA**CCGG**GAAAA**T**CGGTCAAT-5'	
Y249L/F264L-f:	5'-AGATATACT**GGCC**CTT**C**TTGCCAGTTATGAT-3'	*Sau*96I
Y249L/F264L-r:	3'-TTCATAATCTATATGA**CCGG**GAA**G**AACGGTC-5'	
F264A/Y249A-f:	5'-GTTTAATGAT**GC**TAA**GC**G**C**GAGATGACTATTC-3'	*Hha*I
F264A/Y249A-r:	3'-TAATGCAAATTACTA**CG**ATT**CG**C**G**CTCTACTG-5'	

[a]Recognition sites introduced for restriction enzyme analysis are underlined. Bold letters indicate mutated nucleotide residues; f and r represent forward and reversed primers, respectively.

mutated individually to alanine, using PCR-based directed mutagenesis. When each mutant toxin was expressed in *E. coli* upon IPTG induction, all were produced as inclusion bodies, and the expression level of the 130-kDa mutant protoxins was approximately the same as that of the wild-type toxin (Fig. 2A). However, although these mutations did not result in low levels of protein expression, the possibility cannot be excluded whether the mutations would affect the folding of the mutant molecules, as protein misfolding can lead to the formation of an insoluble inclusion (see below).

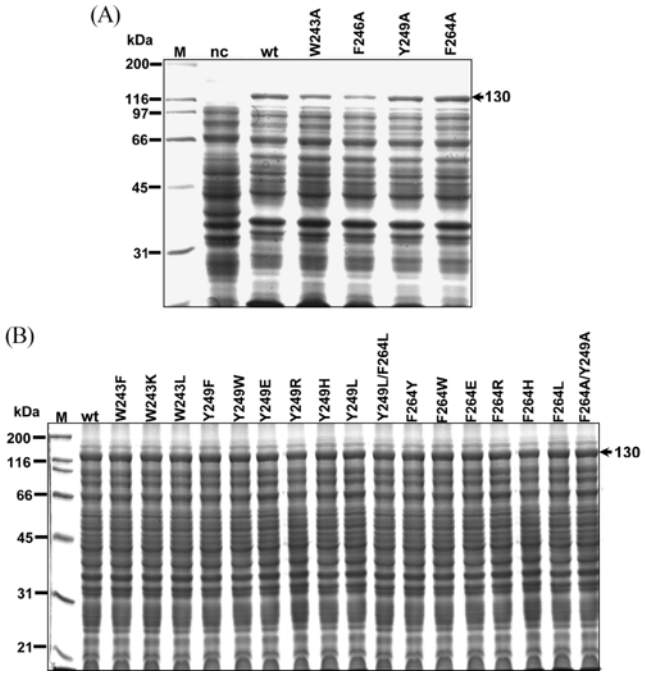

Fig. 2. (A & B) Toxin expression of the 130-kDa Cry4Ba wild-type and its mutant protoxins. SDS-PAGE (Coomassie brilliant blue-stained 12% gel) analysis of lysates extracted from *E. coli* (10^7 cells) expressing 130-kDa protoxins of Cry4Ba wild-type (wt) or its mutants (W243A, F246A, Y249A, F264A, W243F, W243K, W243L, Y249F, Y249W, Y249E, Y249R, Y249H, Y249L, Y249L/F264L, F264Y, F264W, F264E, F264R, F264H, F264L and F264A/Y249A). *E. coli* cells harbouring the pUC12 vector were used as a negative control (nc). M represents the molecular mass standards.

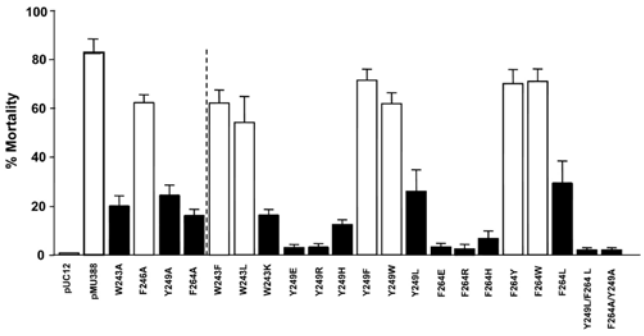

Fig. 3. Mosquito-larvicidal activity of *E. coli* cells expressing the Cry4Ba wild-type toxin (pMU388) or its mutants (W243A, F246A, Y249A, F264A, W243F, W243L, W243K, Y249E, Y249R, Y249H, Y249F, Y249W, Y249L, F264E, F264R, F264H, F264Y, F264W, F264L, Y249L/F264L and F264A/Y249A) against 2-day old *S. aegypti* mosquito larvae at concentrations of 10^8 cells/ml. *E. coli* cells harbouring the pUC12 vector were used as a negative control. Error bars indicated standard errors of the mean from three independent experiments. Shaded boxes represent the larvicidal activity of the mutants that are significantly different (p values < 0.001) from that of the wild type.

To determine an effect of these single-alanine substitutions on toxicity, we tested *E. coli* cells expressing each mutant toxin for their relative biological activity against *S. aegypti* mosquito larvae. All bioassays were conducted in 10 replicas for each sample, and were repeated at least three times. The mortality data recorded after 24-h incubation revealed that the W243A, Y249A and F264A mutants exhibited a drastic decrease in larvicidal activity, whereas the F246A mutant showed only a small reduction in toxicity when compared to the wild-type toxin (Fig. 3). These results suggested that Trp^{243}, Tyr^{249} and Phe^{264} in α7 play a role in larvicidal activity of the Cry4Ba toxin.

As can be inferred from the Cry4Ba crystal structure (Fig. 1B, top view), both Tyr^{249} and Phe^{264} are oriented such that their aromatic side chains face towards in the same direction. Together with the toxicity data, this configuration might imply a functional and/or structural role for these two conserved residues in the membrane-binding step of the toxin. It has been shown by numerous studies that the binding of membrane-inserting proteins to the lipid membrane surface *via* an aromatic cluster is a prerequisite for their membrane insertion and pore formation (Braun *et al*., 1999; Malovrh *et al*., 2003; Drechsler *et al*., 2006). In this regard, we therefore performed further mutagenic analysis, using more refined substitutions at these two critical residues (Tyr^{249} and Phe^{264}). It was found that only *E. coli* cells expressing the mutants that were replaced with an aromatic residue, Y249F or Y249W and F264Y or F264W, still retained relatively high levels of larvicidal activity (see Fig. 3). On the other hand, single substitutions of either Tyr^{249} or Phe^{264} with a hydrophobic residue (i.e. Leu) showed an adverse effect on larvicidal activity comparable to the single-alanine substitutions (Y249A and F264A), whilst replacements with charged (i.e. Glu or Arg) or non-charged polar (i.e. His) residues almost completely abolished the larvicidal activity (Fig. 3). In addition, two double mutants, Y249L/F264L and F264A/Y249A, were also constructed. As expected, the Y249L/F264L and F264A/Y249A mutations revealed a drastically higher loss in mosquito-larvicidal activity when compared to their corresponding single mutants (Fig. 3). These results further revealed a crucial role in toxin activity for the structurally conserved aromatic side chain of both Tyr^{249} and Phe^{264} in α7 of the Cry4Ba toxin. It should be noted that all mutant toxins were also highly produced as 130-kDa protoxins at levels comparable to the wild-type (see Fig. 2B). The larvae tested in the bioassays were therefore reckoned to receive a similar amount of the protoxin doses.

Trp^{243} that is buried within the domain I-II interface and located opposite to Tyr^{249} and Phe^{264} (see Fig. 1B), was also mutated to Lys, Leu or Phe. SDS-PAGE analysis of these three mutants showed that all the mutant protoxins were also expressed at wild-type levels (see Fig. 2B). Interestingly, while *E. coli* cells expressing the W243K mutant showed a severe loss in larvicidal activity, the cells expressing either W243L or W243F still exerted high toxicity apparently at the

same level (approximately 60% of the wild-type control value) (see Fig. 3). This might imply that the existence of a large hydrophobic side chain, *e.g.* Leu and Phe, at this position (Trp^{243}) is important for a structural role of helix 7 in Cry4Ba toxicity. This is in agreement with the recent study for the lepidoteran-specific Cry1Ab toxin that replacement by a hydrophobic residue (*i.e.* Phe) at Trp^{226}, which situates at the same position as Trp^{243} of Cry4Ba, did not affect larvicidal activity of the toxin (Padilla *et al*., 2006). The same authors also suggested that hydrophobicity at this position play an essential role in Cry1Ab toxin-crystal formation since the W226C mutant could not produce inclusion bodies in *Bt* transformants (Padilla *et al*., 2006). However, the latter was contrary to our observation that the W243K mutation did not affect Cry4Ba toxin-inclusion formation in *E. coli* (see below). At this stage, the exact role of Trp^{243} in the structure and function of the Cry4Ba toxin remains to be clearly elucidated.

Experiments were further conducted to assess the solubility *in vitro* of the mutant protein inclusions in comparison with that of the wild-type. For determining the percentage of toxin solubility in carbonate buffer, pH 10.0, the amounts of the 130-kDa soluble proteins in the supernatant were compared with those of the total protein inclusions that were initially used. All the toxin inclusions of the larvicidal-active mutants that were substituted with an aromatic residue (W243F, Y249F, Y249W, F264Y and F264W) were found to be soluble in the carbonate buffer, giving at least 70-80% solubility which is comparable to that of the wild-type and the F246A larvicidal-active mutant under similar conditions. In addition, toxin inclusions of the W243K, W243L, Y249L, F264L and Y249L/F264L mutants were also observed to be relatively soluble in this buffer (approximately 60% solubility), although they all with exception for the W243L mutant showed drastically reduced larvicidal activity. On the other hand, a complete loss of the inclusion solubility was found for all the remaining mutants (W243A, Y249A, Y249E, Y249R, Y249H, F264A, F264E, F264R, F264H and F264A/Y249A), which all exhibited a severe loss in toxicity. This indicate that specific substitutions, particularly with alanine or polar non-charged or charged residues at the two critical conserved aromatic residues (Tyr^{249} and Phe^{264}) in α7 could disturb the structural characteristics of the Cry4Ba toxin that would result in improper folding of the mutant proteins, leading to the formation of an insoluble aggregate *in vivo*. In this context, the packing interactions of both Tyr^{249} and Phe^{264} with their neighbouring residues were therefore analysed in comparison with that of mutated residues. As illustrated in Fig. 4, both critical aromatic residues are located inside packing areas made up of several hydrophobic residues within 4Å. The packing interactions of the substituted Leu at each critical position showed much the same appearance as that observed in the wild-type and its conservative mutants (Y249F, Y249W, F264Y and F264W). However, there were diminished contacts or cavities (see Fig. 4, arrowed) introduced inside each

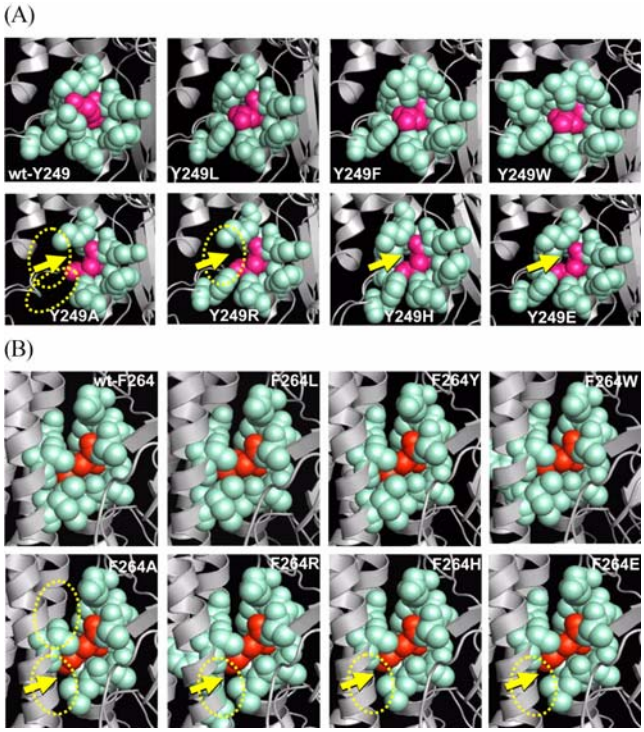

Fig. 4. Close-up views of part of the Cry4Ba crystal structure (Boonserm *et al*., 2005), illustrating the packing interactions (as shown in space-filling form) between residues at the two critical positions in α7, 249 (A) and 264 (B) and neighbouring hydrophobic residues within 4 Å, as analysed by Swiss-PdbViewer v.3.7. The arrows indicate cavities introduced inside the packing interactions of the mutant proteins. Positions of certain interacting residues that are absent from the packing clusters are encircled. The structures were generated by PyMOL program.

packing cluster of the other mutant proteins when substituted with Ala, Arg, His or Glu. These are due to large conformational rearrangements of certain residues to be away from the packing interactions (see Fig. 4, encircled). Hence, a disruption of this hydrophobic packing would result in serious structural consequences, and thus prevent the correct folding of the molecule, affecting the toxin-inclusion formation as shown by a loss in protein solubility.

As previously demonstrated, the 130-kDa Cry4Ba toxin was cleaved by trypsin into two protease-resistant fragments of approximately 47 and 20 kDa, in addition to the removal of the C-terminal half of the protoxin (Angsuthanasombat *et al*., 2004). In the present study, we further examined for trypsin digestion susceptibility of the mutant protoxins which could be solubilised in the carbonate buffer. All the 130-kDa soluble toxins were found to produce two major trypsin-resistant fragments of ca. 47 and 20 kDa that are identical to the products obtained with the wild-type protoxin (Fig. 5), indicating that all these mutants were produced as structurally stable protoxins and that the mutations had no apparent effect on proteolytic processing of the solubilised mutant toxins. Therefore, the

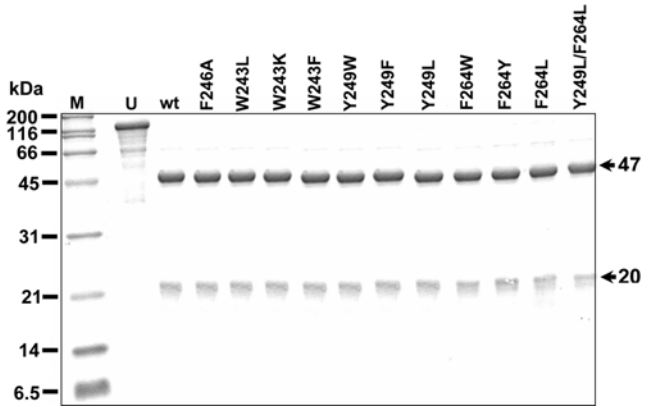

Fig. 5. Proteolytic processing of Cry4Ba solubilised mutant protoxins. SDS-PAGE (Coomassie brilliant blue-stained 15% gel) analysis of trypsin-treated products of 130-kDa protoxins solubilised from toxin inclusions of *E. coli* cells expressing the Cry4Ba wild-type or its mutant toxins (F246A, W243L, W243K, W243F, Y249W, Y249F, Y249L, F264W, F264Y, F264L and Y249L/F264L). M, molecular mass standards. U, the 130-kDa trypsin-untreated product of the Cry4Ba wild-type.

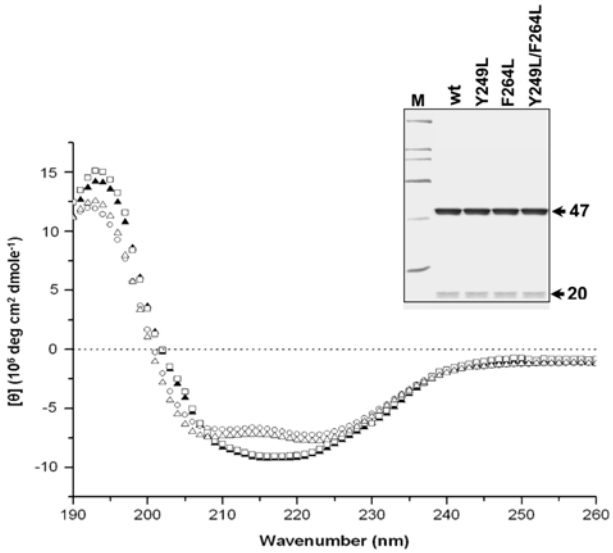

Fig. 6. CD measurements of Cry4Ba and its mutant toxins**.** CD spectra of the 65-kDa purified Cry4Ba toxin (▲) in comparison with that of the mutant toxins, Y249L (○), F264L (□) and Y249L/F264L (△). Inset, SDS-PAGE analysis of the purified trypsin-treated Cry4Ba toxins (wild-type, Y249L, F264L and Y249L/F264L) that are composed of 47 and 20-kDa fragments. M represents molecular mass standards.

reduced larvicidal-activity observed in particular for the Y249L, F264L and Y249L/F264L mutants is not caused by incomplete solubilisation or proteolytic activation.

To further determine if the leucine substitutions would induce structural changes in these three mutants, the sum of the secondary structure components of the FPLC-purified trypsin-treated mutants, Y249L, F264L and Y249L/F264L, were studied by far-UV CD spectroscopy in comparison with that of the 65-kDa purified wild-type toxin. It is worth noting that the 47- and 20-kDa trypsin-resistant fragments of each mutant (see Fig. 6, inset) were found non-covalently associated, forming a 65-kDa protein complex similar to the wild-type under non-denaturing conditions *via* size-exclusion FPLC purification. As shown in Fig. 6, all the CD profiles are basically similar. Particularly, the spectrum of F264L is identical to that of the wild type, confirming that no dramatic structural changes had occurred as a result of these three mutations. Nonetheless, both Y249L and Y249L/F264L, whose CD spectra were much the same, had small reduced CD intensity in the region between 210 and 222 nm compared to the wild-type and F264L mutant, suggesting that there is a slight rearrangement in protein conformation of these two mutants. However, trypsin digestion assays as demonstrated earlier (see Fig. 5) revealed that such little conformational changes did not cause misfolding of the Y249L and Y249L/F264L mutant proteins. Thus far, these results also confirmed that the detrimental effects on toxicity of seen for the Y249L, F264L and Y249L/F264L mutants are least likely to be caused by midfolding of the proteins after mutations.

As was noted in several studies, the aromatic residues could play several different roles in membrane-protein interactions, depending on their location and side-chain orientation (Sumandea *et al*., 1999; Arbuzova *et al*., 2000; Haga *et al*., 2003; Bemporad *et al*., 2006). For example, alanine substitutions of aromatic side chains (Trp^{19}, Trp^{61}, Phe^{64}) of phospholipase A_2 from the Chinese cobra venom (*Naja naja atra*) were shown to influence interfacial binding (Sumandea *et al*., 1999). Also, replacements of all the five Phe residues with Ala in the MARCKS peptide, one of the major substrates for protein kinase C, revealed a significant decrease in binding of the peptide to membrane vesicles (Arbuzova *et al*., 2000). Notwithstanding the lack of membrane interaction and insertion studies, the two critical aromatic residues, Tyr^{249} and Phe^{264}, whose aromaticity was shown to be important for Cry4Ba toxicity, may indeed be the essential functional elements of helix 7, which could serve as a membrane-binding sensor to trigger the structural rearrangement of the pore-forming domain prior to membrane insertion. However, the resolved Cry4Ba crystal structure reveals an obstacle to the proposed role of these two aromatic residues as the basis for membrane interaction. As illustrated in Fig. 1C, both Tyr^{249} and Phe^{264} are found partially exposed to the solvent, and therefore if both or either of them interact with the lipid membrane, major conformational changes must occur to ensure that these critical residues come in close contact with the membrane surface. It has been shown that the binding of the Cry1Ab toxin to its receptor resulted in a conformational change in the toxin molecule consequently promoting complete proteolytic activation and pre-pore formation (Gómez *et al*., 2002). Also, one study *via* disulphide bond-cross linking within the Cry1Aa toxin demonstrated that its ion-channel forming

activity required the helical domain to swing away from the rest of the toxin molecule thus exposing the conserved helix 7 for the initial interaction with the membrane prior to the insertion of the transmembrane region (Schwartz *et al*., 1997).

In conclusion, the data presented here revealed for the first time that the structurally conserved aromaticity of Tyr^{249} and Phe^{264} in α7, plays an important role in larvicidal activity of the Cry4Ba toxin. Conceivably, these two critical aromatic residues, which both are oriented on the same side of the helix, may function to attach the pore-forming domain to the membrane interface that would be needed for an efficient insertion of the α4-loop-α5 transmembrane hairpin into lipid bilayers to form a lytic pore. However, additional experiments are required to verify definitively the functional role of these two conserved aromatic residues, particularly in the steps of membrane-protein interactions. Recently, we have demonstrated *via* Langmuir-Blodgett technique that the 65-kDa activated Cry4Ba toxin was capable of inserting itself into the lipid monolayers (Kanintronkul *et al*., 2005). Further studies of the membrane-insertion behavior of these α7-mutant toxins are therefore of great interest, since this would shed light on the sequestration mechanism that underlie membrane-interfacial binding of this mosquito-larvicidal protein.

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