

Aromaticity of Tyr-202 in the α 4- α 5 Loop Is Essential for Toxicity of the *Bacillus thuringiensis* Cry4A Toxin

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The current model for the mechanism of action of the *Bacillus thuringiensis* Cry δ -endotoxins involves the penetration of the α 4- α 5 hairpin into the target midgut epithelial cell membranes, followed by pore formation. In this study, PCR-based mutagenesis was employed to identify a critical residue within the α 4- α 5 loop of the 130-kDa Cry4A mosquito-larvicidal protein. Alanine-substitutions of two charged (Asp-198 and Asp-200) and four polar (Asn-190, Asn-195, Tyr-201 and Tyr-202) residues in the α 4- α 5 loop were performed. Like the wild-type, all of the mutant toxins were over-expressed as inclusion bodies in *Escherichia coli*. When *E. coli* cells expressing each mutant toxin were bioassayed against *Aedes aegypti* larvae, larvicidal activity was completely abolished for the substitution of only Tyr-202, while replacements at the other positions still retained a high level of toxicity. Further replacement of Tyr-202 with an aromatic side chain, phenylalanine, did not affect the toxicity. These results revealed a crucial role in toxin activity for the conserved aromatic residue at the 202 position within the α 4- α 5 loop of the Cry4A toxin.

Keywords: Aromaticity, *Bacillus thuringiensis*, δ -Endotoxin, α 4- α 5 loop, Larvicidal activity, Mutagenesis

Introduction

Bacillus thuringiensis (*Bt*), a Gram-positive soil bacterium, produces crystalline inclusions during sporulation. These inclusions are composed of one or more proteins, known as δ -endotoxins, that are highly toxic to various insect larvae, and have been widely used as biological insecticides (Aronson *et al.*

et al., 1986; Höfte and Whiteley, 1989; Schnepf *et al.*, 1998). The *Bt* δ -endotoxins can be classified into the two families of Cry (crystal) and Cyt (cytolytic) toxins based on the similarity of their deduced amino acid sequences (Höfte and Whiteley, 1989; Crickmore *et al.*, 1998). For example, one of the four major larvicidal proteins that is produced by *Bt* subsp. *israelensis* (*Bti*) has been classified as Cry4A, which is highly toxic to mosquito-larvae of *Aedes* sp., *Culex* sp., and *Anopheles* sp. (Höfte and Whiteley, 1989; Schnepf *et al.*, 1998).

Upon ingestion by the susceptible insect larvae, the *Bt* crystalline inclusions are solubilized under the alkaline conditions of the larval midgut and are proteolytically activated by gut proteases to release the active toxins (Höfte and Whiteley, 1989). For several Cry toxins, the activated toxins bind to specific receptors that are located on the brush border membrane of midgut epithelial cells (Schnepf *et al.*, 1998; de Maagd *et al.*, 2001). Subsequently, the toxins insert into the membrane to form leakage pores that cause the cell to swell and lyse by colloid-osmotic lysis (Knowles and Ellar, 1987). However, the precise mechanism of action at the molecular level of *Bt* toxins is still not completely understood.

The X-ray crystal structures of four different Cry toxins, Cry1Aa (Grochulski *et al.*, 1995) Cry2Aa (Morse *et al.*, 2001), Cry3Aa (Li *et al.*, 1991), and Cry3Bb (Galitsky *et al.*, 2001) display a high degree of overall structural similarity and are composed of three distinct domains. The N-terminal domain I is a seven α -helix bundle in which the helix 5 is relatively hydrophobic and completely surrounded by six other amphipathic helices. A function for this domain in the membrane insertion and pore formation is supported by a number of studies that demonstrate the isolated helical fragments, α 1- α 7 of Cry3B2 and Cry1Ac (Walters *et al.*, 1993; Von Tersch *et al.*, 1994) or α 1- α 5 of Cry4B (Puntheeranurak *et al.*, 2001; 2004) that are responsible for the pore-formation and generation of ion-selective channels.

The "umbrella model" has been proposed to describe the toxicity mechanism of the *Bt* Cry toxins that involves an

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insertion of helices 4 and 5 into the membrane as a helical hairpin structure, with the remaining helices lying on the membrane surface (Gazit *et al.*, 1998). This model is supported by a number of studies that demonstrate the crucial role of α 4 and α 5 in the toxicity of different Cry toxins. The results of *in vitro* studies suggest that α 4 lines the lumen of the pore and participates in ion-channel formation, while the hydrophobic α 5 interacts with the lipid membranes and participates in toxin oligomerisation (Schwartz *et al.*, 1997; Masson *et al.*, 1999; Nunez-Valdez *et al.*, 2001). Furthermore, membrane permeation studies with Cry1Ac have revealed that the α 4-loop- α 5 segment is more active in membrane permeation than either the isolated helices or their mixtures, suggesting that the presence of the loop-structure is a requirement for efficient insertion of the toxin into the membrane (Gerber and Shai, 2000).

In previous studies, we demonstrated that α 4 and α 5 of the 130-kDa *Bti* Cry4B toxin are important determinants of toxicity, likely being involved in membrane insertion and pore formation (Uawithya *et al.*, 1998; Sramala *et al.*, 2000). In addition, the specific structure for the positively-charged side-chain of Arg-158 in α 4 was found to play a pivotal role in the Cry4B larvicidal activity (Sramala *et al.*, 2001). We further demonstrated that the polar residue at position-166 and the aromatic residue at position-170 within the α 4- α 5 loop are important structural determinations for the toxicity of this toxin (Kanintronkul *et al.*, 2003). In this report, we showed that an analogous effect on mosquito-larvicidal activity of the closely-related 130-kDa *Bti* toxin, Cry4A, was observed when charged or polar residues in the α 4- α 5 loop were altered. The results reveal that Tyr-202, which is very conserved among the Cry toxins, is critically involved in the Cry4A toxicity, supporting the notion that an aromatic structure of the highly-conserved tyrosine residue within the α 4- α 5 loop is an essential prerequisite for the toxic action of the Cry δ -endotoxins.

Materials and Methods

Construction of mutant toxins The recombinant plasmid pMEx-B4A encoding the 130-kDa *Bt* Cry4A larvicidal protein, which has been reconstructed in the pMEx8 expression vector (Buttcher *et al.*, 1990) under control of the *tac* promoter together with the *cry4B* promoter (Angsuthanasombat *et al.*, 1987), was used as a template for the site-directed mutagenesis. Mutant plasmids were generated by a polymerase chain reaction (PCR) using a pair of mutagenic primers (Table 1) that were purchased from Prologo Inc. (Singapore) and *Pfu* DNA polymerase, following the procedure of the QuickChange™ Mutagenesis Kit (Stratagene, La Jolla, USA). The mutant clones with the required mutation were first identified by restriction endonuclease digestion of the plasmids, and verified by DNA sequencing, using a BigDye™ Terminator Cycle Sequencing Kit (Perkin-Elmer, Norwalk, USA).

Expression and solubilisation of toxin inclusions Each *E. coli*

JM109 clone that harbored the wild-type plasmid or its mutants was grown at 30°C in a Luria-Bertani medium containing 100 μ g mL⁻¹ ampicillin until OD₆₀₀ of the culture reached 0.3-0.5. Toxin expression was induced with isopropyl- β -D-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM for 10 hrs, and subsequently analyzed by sodium dodecyl sulfate-(10% w/v) polyacrylamide gel electrophoresis (SDS-PAGE). The cells were harvested by centrifugation and resuspended in cold distilled water. The cell suspension was lysed in a French Pressure Cell at 10,000 psi. The cell lysate was centrifuged at 8,000 \times g, 4°C for 15 min. The pellets were washed 3 times in cold distilled water and suspended by sonication. Protein concentrations of the partial-purified inclusions were determined by using a protein microassay reagent (Bio-Rad, Hercules, USA) with bovine serum albumin fraction V (Sigma, St. Louis, USA) as the standard. Protoxin inclusions were solubilized in 50 mM Na₂CO₃, pH 10.0, and incubated at 37°C for 1 hr, as previously described (Uawithya *et al.*, 1998). After centrifugation at 8,000 \times g for 10 min, the supernatants were analyzed by SDS-PAGE and compared with the inclusion suspension.

Mosquito-larvicidal activity assays Bioassays were performed as previously described (Sramala *et al.*, 2001), using 2-day old *Aedes aegypti* larvae that was reared from eggs that were obtained from the mosquito-rearing facility of the Institute of Molecular Biology and Genetics, Mahidol University, Thailand. Both the rearing and bioassays were performed at room temperature (25°C). Assays were carried out in 1 mL of *E. coli* suspension (10⁸ cells suspended in distilled water) in a 48-well microtitre plate (11.3-mm

Table 1. Complementary primers for substituting a code residue with different amino acids

Primer	Sequence ^a	Restriction site
	E L V A S C P	
N190A-f:	5' CAGAGCTTGTGGCCTCTTGTCTCTC 3'	HaeIII
N190A-r:	5' GAGGACAAGAGGCCACAAAGCTCTG 3'	
	C P P A P S D	
N195A-f:	5' CTTGTCTCTCCGGCCCTAGTGATTG 3'	NarI
N195A-r:	5' CAATCACTAGGCGCCGAGGACAAG 3'	
	N P S A C D Y Y N	
D198A-f:	5' CTAATCCTAGTGCATGCGATTACTATAAC 3'	SphI
D198A-r:	5' GTTATAGTAATCGCATGCACTAGGATTAG 3'	
	S D C A Y Y N I	
D200A-f:	5' CTAGTGATTGCGCATACTATAACATAC 3'	HhaI
D200A-r:	5' GTATGTTATAGTATGCGCACTACTAG 3'	
	D C D A Y N I L V	
Y201A-f:	5' GTGATTGCGATGCGTACAACATACTAGTA 3'	RsaI
Y201A-r:	5' TACTAGTAGTGTGACGCATCGCAATCAC 3'	
	D C D Y A N I L V L S	
Y202A-f:	5' GATTGCGATTACGCAACATCCTGGTATTATCTAG 3'	BstNI
Y202A-r:	5' CTAGATAATACAGGATGTTGCGTAATCGCAATC 3'	
	D C D Y C N I L V L S S	
Y202C-f:	5' GATTGCGATTACTGCAACATACTAGTACTATCTAGTTA 3'	ScaI
Y202C-r:	5' TAACCTAGATAGTACTAGTAGTGTGCGAATCGCAATC 3'	
	D C D Y F N I L V L S S Y	
Y202F-f:	5' GATTGCGATTACTTTAACATACTAGTACTCTCGAGTTATG 3'	XhoI
Y202F-r:	5' CATAACTCGAGAGTACTAGTAGTGTAAAGTAATCGCAATC 3'	

^aIntroduced restriction enzyme recognition sites are underlined. Mutated nucleotide residues are shown in boldface. Deduced amino acid sequences are shown above of each pair of oligonucleotide primers.

well diameter, Costar, USA) with 10 larvae per well and a total of 100 larvae for each type of *E. coli* samples. *E. coli* cells containing the pMEx8 vector were used as a negative control. Mortality was recorded after a 24-hr incubation period.

Results and Discussion

Based on multiple sequence alignments of the known *Bt* Cry toxin structures and the homology-based Cry4 models (Angsuthanasombat *et al.*, 2004), the interhelical loop connecting $\alpha 4$ and $\alpha 5$ of the Cry4A toxin is composed of sixteen amino acids with the majority being polar and charged residues (Fig. 1a). Previously, we demonstrated that polarity and aromaticity for Asn-166 and Tyr-170, respectively, in the $\alpha 4$ - $\alpha 5$ loop are critically involved in the larvicidal activity of the Cry4B toxin (Kanintronkul *et al.*, 2003). In the present study, we constructed several mutants in the $\alpha 4$ - $\alpha 5$ loop region of Cry4A in order to determine the residue that is responsible for the toxin activity. Two negatively-charged (Asp-198 and Asp-200) and four polar (Asn-190, Asn-195, Tyr-201 and Tyr-202) residues were initially selected for the substitution with alanine *via* PCR-based directed mutagenesis. The loop mutant toxins were expressed in *E. coli* under inducible control of the *tac* promoter. Upon addition of IPTG

to the mid-exponential phase cultures, all of the mutant Cry4A protoxins were predominantly produced in the form of sedimentable inclusion bodies. When the *E. coli* lysates were analyzed by SDS-PAGE, the protein expression levels for all of the mutants were comparable to those of the wild-type (data not shown).

To assess the solubility of the mutant protoxin inclusions in comparison with that of the wild-type, experiments were conducted using carbonate buffer, pH 10.0. The amount of the 130-kDa Cry4A soluble proteins in the supernatant were compared with those of the proteins that were initially used in order to determine the percentage of toxin solubilisation. Figure 2 shows that the toxin inclusions of the N190A, D198A, D200A, and Y201A mutants were soluble in this buffer, giving approximately 60-70% solubility. This is comparable to the solubility of the wild-type inclusions under similar conditions. On the other hand, a nearly complete loss of the inclusion solubility was observed for the two remaining mutants, N195A and Y202A. However, toxin inclusions of the two closely-related loop-Cry4B mutants, N166A and Y170A as mentioned previously, were found to be relatively soluble in this buffer (Kanintronkul *et al.*, 2003). At this stage, the reason for this difference in solubility between the two loop-mutants of Cry4A and Cry4B is unclear. It does, however, lead to the interesting possibility that single-alanine

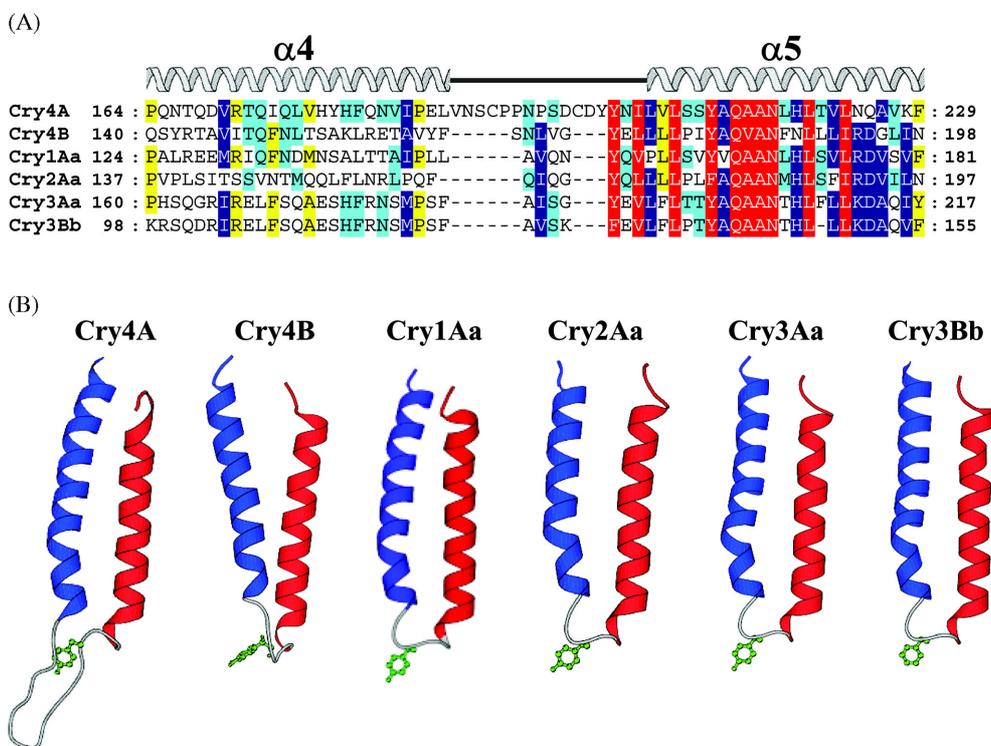


Fig. 1. (A) Multiple sequence alignment of the transmembrane $\alpha 4$ -loop- $\alpha 5$ fragment of Cry4A with those of the crystal structures of Cry1Aa, Cry2Aa, Cry3Aa, and Cry3Bb toxins and the homology-based Cry4B model. The sequences were aligned using the Clustal X Program. The corresponding $\alpha 4$ -loop- $\alpha 5$ is shown above the sequences. (B) Side views of the $\alpha 4$ -loop- $\alpha 5$ helical hairpins of the homology-based Cry4A and Cry4B models, and the known structures of Cry1Aa, Cry2Aa, Cry3Aa, and Cry3Bb. Blue and red ribbons represent $\alpha 4$ and $\alpha 5$, respectively. The conserved loop-residue, Tyr, is shown in ball-and-stick in all six helical hairpins.

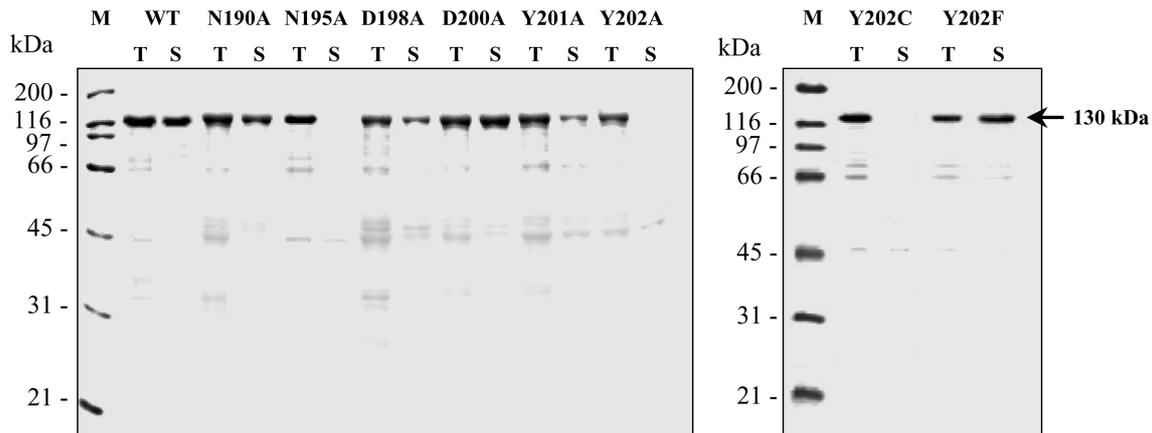


Fig. 2. SDS-PAGE (Coomassie brilliant blue-stained 10% gel) analysis of the partially-purified 130-kDa protein inclusions that were extracted from *E. coli* expressing the wild-type (WT) and Cry4A mutant toxins and solubilized in a carbonate buffer, pH 10.0. (T) and (S) represent total fractions and an equivalent volume of the supernatants after centrifugation, respectively. (M) represents the molecular mass standards.

substitutions at Asn-195 and Tyr-202 of the Cry4A toxin could disturb the structural characteristics that consequently affect the toxin-inclusion formation, as shown by a drastic decrease in solubility.

To determine the effect of the α 4- α 5 loop mutations on the Cry4A bioactivity, *E. coli* cells expressing each mutant toxin were tested for their biological activity towards *A. aegypti* mosquito-larvae. Replacement at only Tyr-202 with alanine almost completely abolished larvicidal activity, whereas alanine-substitutions at the other positions (Asn-190, Asn-195, Asp-198, Asp-200 and Tyr-201) did not affect the Cry4A toxicity. Further analysis *via* specific mutations revealed that the conversion of this critical tyrosine residue to cysteine resulted in a drastic loss of toxicity, but replacement with the aromatic residue (*i.e.* phenylalanine) retained the high level of larvicidal activity (Fig. 3). The level of the protein expression of both the Y202C and Y202F mutant toxins was approximately the same as that of the wild-type. These results, together with the highly structural conserved level of the tyrosine residue at this position among the Cry toxins (Fig. 1b), suggest the essential feature of an aromatic structure at this critical position for the toxin activity. The data further support our previous findings that Tyr-170 in the α 4- α 5 loop plays an important role in the larvicidal activity of the 130-kDa Cry4B toxin, since substitutions with only the aromatic residues (*i.e.* Phe or Trp) were shown to restore bioactivity towards mosquito-larvae (Kanintronkul *et al.*, 2003).

For *in vitro* solubility, like the Y202A mutation, the substitution of Tyr-202 with Cys reduced the solubility *in vitro* of toxin inclusions, while a conversion to Phe still exhibited the same solubility characteristics as that of the wild-type (Fig. 2). Although insolubility of the toxin inclusions and the loss of toxicity are seemingly correlated for both the Y202A and Y202C mutants, the inclusion solubility *in vitro* may not necessarily reflect toxin activity *in vivo*, as observed for the N195A mutant which was insoluble in the carbonate buffer,

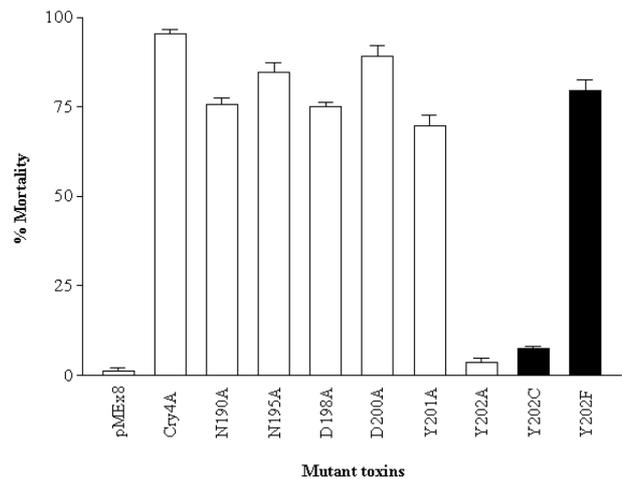


Fig. 3. Mosquito-larvicidal activities of *E. coli* cells expressing the Cry4A wild-type or its mutant toxins (N190A, N195A, D198A, D200A, Y201A, Y202A, Y202C, and Y202F) against *A. aegypti* larvae. Error bars indicate standard errors of the mean from three independent experiments.

but still bioactive (Figs. 2 and 3). It has been demonstrated that the single-proline substitution in α 6 of Cry4B dramatically perturbed the inclusion dissolvability, but did not affect its larvicidal activity (Sramala *et al.*, 2000). Also, the detected difference in solubilisation *in vitro* for the cloned Cry4A toxin inclusions, which were purified from two different *Bt* recipient strains, is not a factor for toxicity *in vivo* (Angsuthanasonbat *et al.*, 1992). Presumably, the larval gut proteases *in vivo* might facilitate the dissolution of the ingested toxin inclusions that would negate the differences between the observed larvicidal activities of the bioactive N195A and non-active Y202A or Y200C mutants.

Studies with several membrane proteins have indicated that the aromatic residues are predominantly found at or near the

lipid-water interface (Ulmschneider and Sansom, 2001). These aromatic residues have been proposed to function in anchoring the proteins into the membrane through interactions of their aromatic rings with phospholipid head groups (Yau *et al.*, 1998; Killian and Von Heijne, 2000), maintaining rigidity in the periphery of the transmembrane segments (Tsang and Saier, 1996), allowing vertical mobility of the transmembrane helical region with respect to the membranes (Schiffer and Deber, 1990), facilitating translocation of the periplasmic portion of proteins through the membrane, thereby acting as determinants of protein orientation (Schiffer *et al.*, 1992). Taken together, a function for Tyr-202 in the $\alpha 4$ - $\alpha 5$ loop of the Cry4A toxin may conceivably be an interaction with the phospholipid head groups for stabilizing the oligomeric pore structure.

In conclusion, this study demonstrates that the aromaticity of Tyr-202 in the $\alpha 4$ - $\alpha 5$ loop plays a crucial role in the Cry4A toxicity that further supports the notion that the aromatic structure of the highly-conserved tyrosine residue within the loop connecting the two transmembrane helices, $\alpha 4$ and $\alpha 5$, is essential for toxic action of the *Bt* Cry δ -endotoxins. However, further studies are still required to elucidate the exact role of this critical residue in toxin function.

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