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## Binding of *Bacillus thuringiensis* subsp. *israelensis* Cry4Ba to Cyt1Aa has an important role in synergism

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### Abstract

*Bacillus thuringiensis* subs *israelensis* (Bti) produces at least four different crystal proteins that are specifically toxic to different mosquito species and that belong to two non-related family of toxins, Cry and Cyt named Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa. Cyt1Aa enhances the activity of Cry4Aa, Cry4Ba or Cry11Aa and overcomes resistance of *Culex quinquefasciatus* populations resistant to Cry11Aa, Cry4Aa or Cry4Ba. Cyt1Aa synergized Cry11Aa by their specific interaction since single point mutants on both Cyt1Aa and Cry11Aa that affected their binding interaction affected their synergistic insecticidal activity. In this work we show that Cyt1Aa loop  $\beta$ 6- $\alpha$ E K198A, E204A and  $\beta$ 7 K225A mutants affected binding and synergism with Cry4Ba. In addition, site directed mutagenesis showed that Cry4Ba domain II loop  $\alpha$ -8 is involved in binding and in synergism with Cyt1Aa since Cry4Ba SI303-304AA double mutant showed decreased binding and synergism with Cyt1Aa. These data suggest that similarly to the synergism between Cry11Aa and Cyt1Aa toxins, the Cyt1Aa also functions as a receptor for Cry4Ba explaining the mechanism of synergism between these two Bti toxins.

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## 1. Introduction

*Bacillus thuringiensis* subs *israelensis* (Bti) is a gram-positive bacterium that produces crystal toxins during its sporulation phase that are specifically toxic to different mosquito species as *Anopheles* sp, *Aedes aegypti* and *Culex* sp that are vectors of important human diseases as malaria, yellow and dengue fevers among others. Bti is used world wide in the control of these mosquito species, particularly in cases where resistance to chemical insecticides has developed. Despite the large use of Bti in mosquito control there are no reports of resistance to Bti in field conditions [3,19].

The lack of insect resistance development to Bti is mainly due to the fact that Bti produces at least four different crystal proteins that belong to two non-related family of toxins, Cry and Cyt named Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa. Interestingly the insecticidal activity of the isolated Bti toxins is magnitudes of order less toxic than the crystal inclusion containing all toxins [10,17,26]. This synergistic effect is mainly due to Cyt1Aa that enhances the activity of Cry4Aa, Cry4Ba or Cry11Aa [10,17,25]. Even more, it was shown that Cyt1Aa overcomes resistance of *C. quinquefasciatus* populations resistant to Cry11Aa, Cry4Aa or Cry4Ba [25].

Cry and Cyt toxins have a different three dimensional structures and mechanisms of action. Cry4Aa, Cry4Ba and Cry11Aa belong to the three-domain (3-D) family of Cry toxins that are composed of three discrete structural domains. Domain I is a seven  $\alpha$ -helix bundle that is involved in membrane insertion, oligomerization and pore-formation while domain II and domain III are  $\beta$ -sheet domains involved in receptor interaction [4,5]. In particular, the exposed loop regions in domain II are involved in receptor binding [4,5]. In contrast, Cyt proteins have a single  $\alpha$ - $\beta$ domain comprising of two outer layers of  $\alpha$ -helix hairpins wrapped around a  $\beta$ -sheet [18]. Both proteins are synthesized as protoxins that are solubilized in the gut of susceptible dipteran insects and proteolytically activated by midgut proteases leading to activated toxins. For exerting their toxic effect, 3-D Cry toxins rely on the sequential binding with at least two different midgut protein receptor molecules that in lepidopteran insects have been identified as a transmembrane cadherin and glycosyl-phosphatidil-inositol (GPI)-anchored proteins as aminopeptidase-N (APN) and alkaline phosphatase (ALP) [5,22]. Binding to cadherin facilitates further proteolytic cleavage of helix  $\alpha$ -1 and formation of an oligomer structure that gains affinity to GPI-anchored receptors leading to membrane insertion and pore formation [5,22]. Recently, cadherin, APN and ALP molecules have been identified as Cry11Aa or Cry4Ba binding molecules in different mosquito species suggesting a conserved mode of action of Cry toxins in dipteran insects [2,8,9,12,15,27]. In contrast, activated Cyt1Aa do not rely on midgut proteins to bind to the midgut epithelium and directly interacts with membrane lipids resulting in membrane insertion and pore-formation [7].

Understanding the molecular mechanism of synergism of Cyt1Aa and Cry4 and Cry11Aa toxins could have important implications to develop strategies for resistance management of Cry toxins in agriculture and in mosquito control [6].

The mechanism of synergism of Cyt1Aa and Cry11Aa was proposed to depend on the specific interaction between these toxins since single point mutations on Cyt1Aa affected its binding interaction with Cry11Aa and their synergism [20]. Also, it was shown that loop regions of domain II from Cry11Aa toxin that are involved in receptor binding were also involved in binding and synergism with Cyt1Aa [11,20]. These data led to the hypothesis that Cyt1Aa functions as a membrane bound binding protein of Cry11Aa [20]. Furthermore, recent data showed that binding of Cry11Aa to Cyt1Aa facilitates formation of Cry11Aa oligomers that were efficient in pore-formation indicating that Cyt1Aa at least fulfils the role

of the primary cadherin receptor that facilitates oligomer formation [21]. The mechanism of synergism of Cyt1Aa with Cry4Ba and Cry4Aa has not been analyzed. Although Cry4Ba and Cry11Aa share low amino acid sequence identity both bind to *A. aegypti* ALP receptor indicating that they share binding sites with at least this receptor [13]. In order to analyze if binding of Cry4Ba to Cyt1Aa was involved in their synergism, Cyt1Aa mutants that were previously shown to affect binding to Cry11Aa and in synergism were analyzed regarding to the binding of Cry4Ba and their effect on synergism against *A. aegypti* larvae was also analyzed. Even more, Cry4Ba domain II loop regions were subject to mutagenesis and Cry4Ba mutants analyzed regarding their binding and synergism with Cyt1Aa.

Here we show that some Cyt1Aa mutants affected in binding and synergism with Cry11Aa are also affected in binding and synergism with Cry4Ba. Also, that Cry4Ba domain II loop  $\alpha$ -8 is involved in its binding interaction and in synergism with Cyt1Aa. These data suggest that Cyt1Aa also functions as a specific binding protein for Cry4Ba explaining the mechanism of synergism between these two Bti toxins.

## 2. Methods

### 2.1 Growth of *Bacillus thuringiensis*, purification of Cry4Ba and Cyt1Aa crystal inclusions and toxin activation

For the production of Cry4Ba crystals and Cyt1A, acrySTALLIFEROUS Bti strain Q2-81 containing plasmid pHT618 or pWF45 respectively were cultured for 3 days at 29°C and 200 rpm in nutrient broth sporulation medium supplemented with 10 $\mu$ g erythromycin per ml. Spores and inclusions produced by the Bt strains were harvested and washed three times with 0.3 M NaCl, 0.01 M EDTA, pH 8.0. For Cry4Ba activation, the spores and crystals were solubilized in carbonate buffer (sodium bicarbonate/carbonate 0.1M, pH 9.5) supplemented with 0.2%  $\beta$ -mercaptoethanol [pH 10.5], and activated with 1:20 w/w trypsin (Sigma-Aldrich Co.), for 16 h at 37°C. Cyt1Aa inclusions were solubilized in 50 mM Na<sub>2</sub>CO<sub>3</sub>, 10 mM DTT, pH 10.5 and activated with 1:30 proteinase K (Sigma-Aldrich Co.) w/w for 1 h at 30 C.

### 2.2 Site directed mutagenesis of Cry4Ba

Plasmid pHT618 was used as template for site directed loop  $\alpha$ -8 mutagenesis using QuikChange®Multi following the manufacturer's instructions (Stratagene). In this site directed mutagenesis system synthetic primers with the mutated sequence are annealed to previously thermal-denatured plasmid template and amplified with PfuTurbo DNA polymerase to obtain single stranded mutated DNA. Non-mutated DNA template is digested with *DpnI* restriction enzyme that digests methylated DNA, prior to transformation of XL10-Gold *E. coli* cells. The following oligonucleotides were used for site directed mutagenesis of *cry4Ba* gene:  $\alpha$ 8M1

5'CAGCTTTAGTAGAATCTCCTGCTGCTGCATCTATAGCAGCACTGGAGGC3';  
 $\alpha$ 8M2

5'GTAGAATCTCCTTCTAGTAAAGCTGCAGCAGCACTGGAGGCAGCACTTAC3'; $\alpha$ 8M35'CTAGTAAATCTATAGCAGCAGCGGCGGCAGCACTTACACGAGATGTTTC3'.

Mutants were sequenced and transformed into acrySTALLIFEROUS Bt strain 407 as reported [11].

### 2.3 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA plates, 96-wells, were incubated 12 h at 4 C with 250 ng/well of Cyt1Aa in 50 mM NaHCO<sub>3</sub> pH 9.6, followed by five washes with PBS, 0.2% Tween 20. The plates were then incubated with PBS, 2% BSA (Sigma-Aldrich Co.) 0.2%/ Tween 20, for 1 h at 37 C and washed five times with buffer A (PBS, 0.1% Tween 20). The ELISA plates were incubated with different concentrations of Cry4Ba or Cry4Ba mutants for 2 h at 37 C and washed three

times with buffer A. The Cry4Ba proteins bound to Cyt1Aa were detected with anti-Cry4Ba antibody (1:10000) 2 h at 37 C, followed by a secondary goat-anti-rabbit- antibody coupled to horseradish peroxidase (HRP) 1 h at 37 C (1:10000). The HRP enzymatic activity was revealed with a freshly prepared substrate 840 mg of *o*- phenylenediamine, 18 ml of H<sub>2</sub>O<sub>2</sub> in 100 ml of 100 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 5.09. The enzymatic reaction was stopped with 6 N HCl and the absorbance read at 490 nm with a Pharmacia LKB Ultraspec II.

## 2.4 Ligand blot assays

Three µg of Cry4Ba or Cry4Ba mutant proteins were separated in 10% SDS-PAGE and electrotransferred to Hybond-ECL membranes. After blocking with PBS-M (PBS, 5% skim milk), the membranes were incubated for 2 h with 5 nM of Cyt1Aa, and the bound protein was revealed with anti-Cyt1Aa antibody (1:70000) 2 h at 37 C, followed by a secondary goat-anti-rabbit-HRP antibody 1 h at 37 C (1:10000) and SuperSignal chemiluminescent substrate (Pierce). 2.5 Bioassays and synergism factors (SF). Twenty 4<sup>th</sup>-instar *A. aegypti* larvae in 100 ml of dechlorinated water were exposed to different concentrations of Cyt1Aa and Cry4Ba toxins tested at different protein ratios (1:1, 0.5:1 and 0.2:1). Positive (Bti) and negative controls [dechlorinated water] were included in the bioassay, and larvae examined 24 h after treatment. The mean lethal concentration (LC<sub>50</sub>) was estimated by Probit analysis using statistical parameters [14] after four independent assays (Polo-PC LeOra Software). The theoretical toxicity of each ratio mixture was evaluated according to Tabashnik's equation [23], assuming a simple additive effect. The theoretical LC<sub>50</sub> value is the mean of the intrinsic LC<sub>50</sub> values of each component weighted by the ratio used in the mixture:

$$LC_{50}(\text{Cyt1Aa}+\text{Cry4BaA})=\left(\frac{r\text{Cyt1A}}{LC_{50}[\text{Cyt1A}]}+\frac{r\text{Cry4Ba}}{LC_{50}[\text{Cry4Ba}]}\right)^{-1}$$

where *r*Cyt1A and *r*Cry4Ba are the Cyt1A and Cry4Ba protein proportions used in the final ratio of the mixture. LC<sub>50</sub> (Cyt1A) and LC<sub>50</sub> (Cry4Ba) are the LC<sub>50</sub> values for each individual toxin. The SF was calculated by dividing the theoretical toxicity by the observed toxicity of the mixture in bioassays. SF values greater than 1 indicate synergism.

## 3. Results

### 3.1 Cyt1Aa single point mutations affect Cry4Ba binding and synergism

Previously, we isolated and characterized Cyt1Aa single point mutations in loop β6-αE or in β7 that are affected in their binding-interaction with Cry11Aa and their synergistic activity against *A. aegypti* insect larvae. In addition, Cyt1Aa loop β6-αE mutant K198A showed enhanced synergism with Cry11Aa [20]. Table 1 shows the mean lethal concentration (LC<sub>50</sub>) of Cyt1Aa and three Cyt1Aa mutants (K198A, E204A, K225A) that were in the range of the insecticidal activities previously reported [20]. To determine if the same Cyt1Aa amino acid regions involved in Cry11Aa binding and synergism were also involved on Cry4Ba synergism, we analyzed the synergism of Cyt1Aa mutants with Cry4Ba. Figure 1A shows a 3D model of the Cyt1Aa structure that was obtained by comparison to that of Cyt2Ab and the location of the Cyt1Aa residues K198A, E204E and K225A. The synergism factor (SF) of different ratio mixtures of wild type Cyt1Aa and Cry4Ba toxins was determined by bioassays against *A. aegypti* fourth instar larvae as described in Methods. For the two wild type proteins the highest SF value of 9 was found at the 0.2:1 Cyt1Aa:Cry4Ba ratio (Table 2). At 0.2:1 mixture ratio the Cyt1Aa mutants K198A, E204A and K225A showed a reduced SF value of 3.5, 2.6 and 1.8 respectively, indicating that these mutations affected the synergistic activity between Cyt1Aa and Cry4Ba.

To determine if the effects of Cyt1Aa mutations on Cry4Ba synergism correlated with a decrease in their binding interaction, ELISA binding assays of Cry4Ba to Cyt1Aa mutants were performed. First, a saturation binding curve of Cyt1Aa and Cry4Ba was obtained, supporting the interaction between these two toxins and revealing an apparent binding affinity ( $K_d$ ) of 47 nM (Fig. 2A). Figure 2B shows that Cyt1Aa mutants K198A, E204A and K225A showed a reduced binding with Cry4Ba in comparison with Cyt1Aa. These results show that synergism of Cry4Ba and Cyt1Aa correlate with their binding interaction.

### 3.2 Cry4Ba mutations in loop $\alpha$ -8 affect binding and synergism with Cyt1Aa

Domain II loops of Cry toxins have been recognized as important receptor binding regions in different Cry toxins [5,11,16,24]. In the case of Cry11Aa, it was shown that Domain II loop  $\alpha$ -8 was an important binding region with the ALP GPI-anchored receptor [11]. Also, this amino acid region in Cry11Aa was shown to be involved on Cyt1Aa binding and in synergism suggesting that Cyt1Aa functions as a Cry11Aa receptor [20]. To determine if Domain II loop regions of Cry4Ba are involved on Cyt1Aa binding and in synergism, we performed site directed mutagenesis of Cry4Ba Domain II loop  $\alpha$ -8 since a synthetic peptide corresponding to this amino acid region competed the binding with ALP1 protein in contrast with synthetic peptides corresponding to the other Domain II loop regions (Reyes E. Z. and Soberón M. unpublished data). Figure 1B shows the 3D structure of Cry4Ba and the location of Domain II loop  $\alpha$ -8. Three mutants of loop  $\alpha$ -8 region ( $^{300}$ SSKSIAALE $^{308}$ ) were constructed, mutant  $\alpha$ 8M1 (SSK300-302AAA), mutant  $\alpha$ 8M2 (SI303-304AA) and mutant  $\alpha$ 8M3 (LE307-308AA). The three Cry4Ba mutants were transformed to the Bt 407 acrycristiferous strain and cultured until sporulation. Crystals from the three mutants were solubilized and activated with trypsin. From the solubilization step it was clear that  $\alpha$ 8M3 mutant produced lower yields of the 130 kDa Cry4Ba protoxin (data not shown). Figure 3 shows that  $\alpha$ 8M1 and  $\alpha$ 8M2 produced two bands of 48 kDa and 18 kDa as wild type Cry4Ba toxin after trypsin treatment suggesting that these two proteins have no structural constraints. In contrast,  $\alpha$ 8M3 mutations affected protein stability since it produce very low levels of the expected bands after trypsin treatment and therefore  $\alpha$ 8M3 was not further analyzed. The toxicity of Cry4Ba and loop  $\alpha$ 8 mutants was analyzed in *A. aegypti* fourth instar larvae. Table 1 shows that  $\alpha$ 8M2 had a slight reduction in toxicity but with an overlap in the confidential limits with Cry4Ba toxicity while  $\alpha$ 8M1 showed a similar toxicity to *A. aegypti* larvae as Cry4Ba toxin.

Binding of Cry4Ba and loop  $\alpha$ 8 mutants to Cyt1Aa was analyzed by ligand blot. Cry4Ba and the two loop  $\alpha$ -8 mutants were subject to SDS-PAGE, blotted to a membrane and detected with Cyt1Aa followed by anti-Cyt antibody. Figure 4A shows a significant reduction in binding of  $\alpha$ 8M2 to Cyt1Aa in contrast with  $\alpha$ 8M1 that bound Cyt1Aa as wild type Cry4Ba. ELISA binding assays confirmed that  $\alpha$ 8M2 had a reduced binding to Cyt1Aa in comparison with  $\alpha$ 8M1 and Cry4Ba (Fig. 2B). Figure 2B also shows that Cry4Ba  $\alpha$ 8M2 showed very weak binding to the mutant Cyt1Aa E204A indicating that both Cyt1Aa and Cry4Ba mutations had an additive effect on the binding interaction of these proteins. Bioassays were performed to determine the SF factor of Cyt1Aa and  $\alpha$ 8M2 and also of the two mutants Cyt1Aa E204A and  $\alpha$ 8M2. Table 2 shows that Cyt1Aa- $\alpha$ 8M2 showed a SF value of 5.7, while Cyt1AaE204A and  $\alpha$ 8M2 had a SF value of 2, indicating a direct correlation between Cry4Ba binding to Cyt1Aa and synergism.

## 4. Discussion

Previous work identified specific amino acids of Cyt1Aa located in loop  $\beta$ 6- $\alpha$ Eor in  $\beta$ 7 that are affected in binding to Cry11Aa and synergism. In this work we analyzed the effect of three Cyt1Aa mutants in these structural regions regarding to its binding and synergism with Cry4Ba. The three Cyt1Aa mutants analyzed, K198A, E204A and K225A showed a



reduction on synergism and a correlative reduction on Cry4Ba binding indicating that the same Cyt1Aa regions are involved on Cry11Aa and Cry4Ba binding interaction. However, the Cyt1Aa K198A mutant phenotype was different regarding its effect on Cry11Aa or Cry4Ba. In the case of Cry11Aa, Cyt1Aa K198A showed an enhancement on synergism and correlated with higher binding affinity to Cry11Aa [20], while in the case of Cry4Ba, this Cyt1Aa mutation reduced the binding interaction and synergism [Fig. 1]. This data shows that specific residues of Cyt1Aa binding epitope have a differential effect on its binding to different Cry toxins.

Regarding the Cry4Ba regions involved in Cyt1Aa interaction, we show that a Domain II loop  $\alpha$ -8 mutation affected Cyt1Aa binding and synergism between these toxins. As mentioned earlier Domain II exposed loops have been shown to be important for receptor interaction in different Cry toxins [5,11,16,24]. In the case of Cry4Ba it has been shown that Domain II exposed loops 1, 2 and 3 are involved in binding to *A. aegypti* BBMV and in toxicity [16,24]. This is the first report of the mutagenesis of Cry4Ba loop  $\alpha$ -8. The mutants isolated showed a non-significant marginal effect on toxicity to *A. aegypti* larvae. However, one double alanine mutant,  $\alpha$ 8M2 [SI303-304AA], showed reduced binding to Cyt1Aa and a correlative reduction in synergism. The fact that Domain II loops are involved in both receptor and Cyt1Aa binding is a clear indication that Cyt1Aa functions as a specific binding protein of Cry4Ba mediating its toxicity.

Based on the molecular mechanism proposed for the synergistic effect of Cyt1Aa on Cry11Aa, it was proposed that Bti is the first example of an insect pathogenic bacterium that carries a toxin and also its functional receptor, promoting toxin binding to target membranes and toxicity [20]. As shown in this manuscript, this mechanism also applies for Cry4Ba. This molecular mechanism of synergism could be important to develop tools for countering insect resistance [6]. It would be interesting to identify Cyt proteins active against lepidopteran or coleopteran insects and that could synergize other Cry toxins. Alternatively, an attractive strategy to select Cyt1Aa toxins that show synergism with other Cry toxins, such as lepidopteran or coleopteran specific Cry toxins, could be the engineering of the binding regions in Cyt1Aa, thus providing additional binding sites in Cyt1Aa that could promote synergism with other Cry toxins. This would be a promising development to counteract resistant insects or to control insects with low susceptibility to the known Cry toxins.

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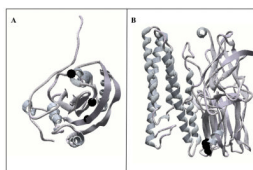
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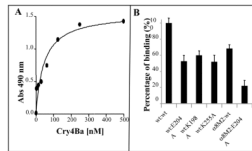
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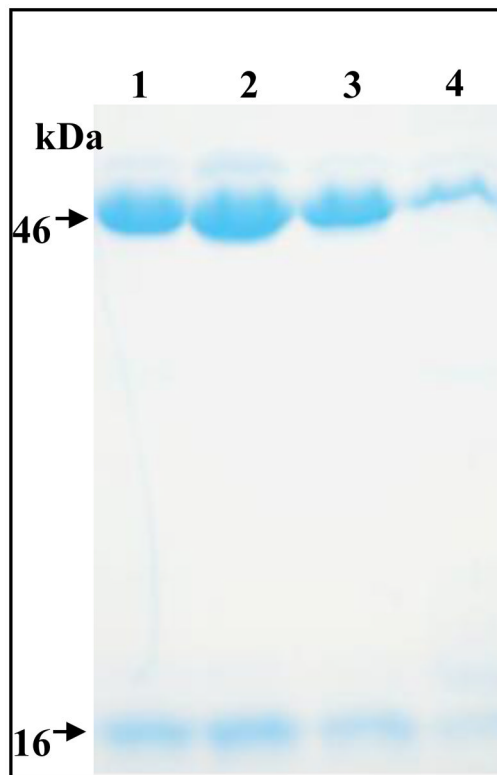


**Figure 1.** Three dimensional structures of insecticidal toxins produced by *Bacillus thuringiensis* subs. *israelensis*. A. Top view of the three dimensional structure model of Cyt1Aa obtained by comparison to the three dimensional structure of Cyt2Ab. Residues K198, E204 and K225 are highlighted by space filling black figures. B. Three dimensional structure of Cry4Ba . Domain II loop a-8 <sup>303</sup>SI<sup>304</sup> residues are highlighted by space filling black figures.

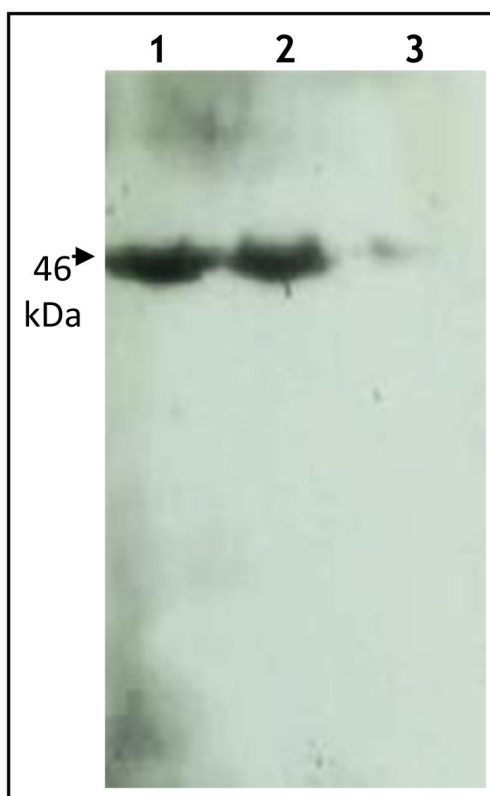


**Figure 2.**

ELISA binding assays of Cry4Ba to Cyt1Aa. A. Saturation binding curve of Cry4Ba to Cyt1Aa. ELISA plates were coated with Cyt1Aa (250 ng/well) and revealed with different Cry4Ba concentrations followed by anti-Cry4Ba antibody and by a secondary goat-anti-rabbit- antibody coupled to horseradish peroxidase. B. ELISA plates were coated with Cyt1Aa or Cyt1Aa mutants K198A, E204A and K225A (250 ng/well) and revealed with 75 nM of Cry4Ba or loop  $\alpha$ 8M2 mutant as described above. Data of three replicates with standard deviations.



**Figure 3.** Proteolytic activation of Cry4Ba and Domain II loop  $\alpha$ -8 mutants. Different Cry4Ba proteins (5  $\mu$ g) were solubilized, treated with trypsin (1:20 W/W) and separated by 10% SDS-PAGE electrophoresis. Lane 1 Cry4Ba, lane 2  $\alpha$ 8M1, lane 3  $\alpha$ 8M2, lane 4  $\alpha$ 8M3.



**Figure 4.** Cry4Ba Domain II loop  $\alpha$ -8 is involved on Cyt1Aa binding. Different Cry4Ba proteins (three  $\mu$ g) were activated by trypsin treatment separated by 10% SDS-PAGE electrophoresis and blotted into nylon membranes. Blots were revealed with 10 nM Cyt1Aa followed with anti-Cyt1Aa and by a secondary goat-anti-rabbit- antibody. Lane 1 Cry4Ba, lane 2  $\alpha$ 8M1 and lane 3  $\alpha$ 8M2.

**Table 1**

Toxicity of Cyt1Aa, Cry4Ba and toxin mutants

Toxin	LC <sub>50</sub> <sup>a</sup> (ng/ml)
Cyt1Aa	952.5 (602.5–1824.3)
K198A	601.8 (466.3–792.7)
E204A	103.9 (80.3–134.9)
K225A	858.6 (548.5–1527.6)
Cry4Ba	79.4 (61–102)
α8M1	117.2 (93.9–146.6)
α8M2	182.2 (77.9–358.8)

<sup>a</sup> Bioassays were performed with 10 early fourth-instar *Ae. aegypti* larvae in 100 ml H<sub>2</sub>O per dose. Three independent experiments of ten different doses each were done. Mean lethal 95% confidence limits are given in concentration (LC<sub>50</sub>) was estimated by Probit analysis, parentheses.

**Table 2**

Synergism factor and binding affinities of mixtures of proteins at 0.2:1 ratio.

Toxins Cyt1Aa:Cry4Ba	Predicted LC <sub>50</sub> (ng/ml)	Experimental LC <sub>50</sub> (ng/ml)	SF <sup>a</sup>
wt:wt	75.2	8.5 (6.5–10.8) <sup>b</sup>	8.8
K198A:wt	74.5	21.0 (10.1–30.1)	3.5
E204A:wt	66.7	25.2 (20.1–32.3)	2.6
K225A:wt	75.1	41.4 (31.1–52.3)	1.8
wt:α8M2	218.9	38.4 (32.7–44.7)	5.7
E204:α8M2	158.3	77.2 (67.1–88.9)	2

<sup>a</sup> Synergism factor (Predicted LC<sub>50</sub>/Experimental LC<sub>50</sub>)<sup>b</sup> 95% fiducial limits.