Enhanced toxicity of *Bacillus thuringiensis* Cry3A δ-endotoxin in coleopterans by mutagenesis in a receptor binding loop

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Abstract We used site-directed mutagenesis to modify the *Bacillus thuringiensis* cry3A gene in amino acid residues 350–354. Two mutant toxins, A1 (R354A,Y359F,Y365F) and A2 (R354A,Y359D,Y365H), showed significantly improved toxicity against *Tenebrio molitor* (yellow mealworm). The mutant toxin A1 was also more potent against both *Leptinotarsa decemlineata* (Colorado potato beetle) and *Chrysomela scripta* (cottonwood leaf beetle), while A2 displayed enhanced toxicity only in *L. decemlineata*. Competitive binding assays of *L. decemlineata* brush border membrane vesicles (BBMV) revealed that binding affinities for the A1 and A2 mutant toxins were ca. 2.5-fold higher than for the wild-type Cry3 toxin. Similar binding assays with *C. scripta* BBMV revealed a ca. 5-fold lower dissociation rate for the A1 mutant as compared to that of Cry3A.

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Key words: *Bacillus thuringiensis*; Cry toxin; δ-Endotoxin; Coleopteran

1. Introduction

The naturally occurring crystal proteins of *Bacillus thuringiensis* (*B.t.*), known as δ-endotoxins or Cry toxins, are insecticidal agents considered safe for users, consumers, and the environment. These proteins are long established for conventional application throughout the world in agricultural, forested, and aquatic ecosystems. Today, insect-resistant plants, genetically engineered to express high concentrations of these proteinaceous toxins, are commercially available. Development of resistance to *B.t.* toxins by target insects, such as the diamondback moth, *Plutella xylostella*, after intensive management with conventionally applied *B.t.* [1], threatens the efficacy of high expression δ-endotoxins produced by plants unless tactics to manage resistance are developed and implemented [2,3]. Detailed structural and biochemical knowledge of *B.t.* δ-endotoxins permits the redesign of these insecticidal proteins to customize host range, alter activity, improve stability, and delay or overcome resistance.

Molecular mechanisms of intoxication by δ-endotoxins are the subject of intensive research, and a complex pathway is gradually emerging. [3–5]. After ingestion by a susceptible insect, crystalline δ-endotoxin is solubilized and proteolytically cleaved from an inactive prototoxin, to an active toxin form within the insect midgut. The activated toxin binds to receptors in the midgut and is believed to integrate into the lipid bilayer of the brush border membrane. Ion channels are formed, causing midgut cells to lose their membrane potential. If the rate of damage to the midgut exceeds the rate of repair, lesions form, bacteria invade the hemocoele, and death results from bacterial sepsisemia. The δ-endotoxins from *B.t.* comprise a group of over 100 related proteins [5], which were previously categorized by insecticidal activity [6] but currently by amino acid similarity [7]. The spectrum of toxicity for each toxin is relatively narrow, while the collective activity of this group of pesticidal toxins now spans seven orders of insect and several other invertebrate groups including nematodes, mites and protozoans [8].

The Cry3A toxin, produced by *B.t.* var. *tenebrionis* [9,10] and other strains [11,12], is toxic to coleopteran species including the yellow mealworm, *Tenebrio molitor* [9,11], the Colorado potato beetle, *Leptinotarsa decemlineata* [12,13] and the cottonwood leaf beetle, *Chrysomela scripta* [14]. The comparatively simple structure of the Cry3A toxin [15], which is remarkably similar to Cry1Aa1 [16], makes it a useful model for exploring the structure–function relationship between ligand and receptor. The three-dimensional structure of the Cry toxins consists of three functional domains: (I) a cluster of seven α-helices predicted to be involved in membrane interaction [15]; (II) three antiparallel β-sheets involved in receptor binding [15,17]; and (III) a β-sandwich implicated in receptor binding [18–21] and ion channel activity [22–24] in related Cry toxins. The use of oligonucleotide-directed mutagenesis to engineer or alter the three-dimensional atomic structure of this toxin is proving a powerful probe in the study of mechanisms of toxicity.

Several recent reports indicate that amino acid substitutions in the three surface-exposed loops at the molecular apex of domain II, from residue 291 to 300 of related Cry toxins, affect binding affinity and toxicity [17,18,22,25–29]. In Cry3A, alanine replacements in the three major loops of domain II result in disruption of receptor binding or structural instability [17]. The objectives of this study were to enhance toxicity of the Cry3A δ-endotoxin in beetle larvae by engineering the specific residues, R354, Y359 and Y365, of loop I in the binding domain.

2. Materials and methods

2.1. Construction of loop mutants

The construction of pOS4601 carrying the cry3Aa2 gene (hereafter referred to as cry3A) expressed in *Escherichia coli* was described by
Wu and Dean [17]. We subcloned the BomHI–HindIII fragment into M13mp18 RF DNA [30] to target the loop I region in domain II. Site-directed mutagenesis was carried out by the method of Kunkel [31] using the Bio-Rad M13 Mutagenesis kit; oligonucleotides were kindly provided by T. Yamamoto, Sandoz Agro Corporation (sequences available upon request). After mutagenesis was complete, sequencing of single-strand DNA was carried out by the method of Sanger et al. [23] following the manufacturer's instructions (United States Biochemical). The mutated fragment was then recloned into the vector, pOS4601. Other molecular cloning techniques were performed according to Sambrook et al. [33]. Restriction enzymes and fine chemicals were purchased from Boehringer Mannheim Biochemicals.

2.2. Purification and proteinase digestion of toxins

Wild-type and mutant genes were expressed in E. coli JM103. Cells were grown for 48 h in 500 ml of LB medium [33] containing 50 µg/ml ampicillin. Crystal extracts were prepared and solubilized in 50 mM sodium carbonate buffer (pH 10.5) as described [34]. The concentration of solubilized protein was determined by Coomassie protein assay reagent (Pierce). Proteinase was activated with diluted T. molitor midgut juice with a protein/nutrient ratio of 1:20 (w/v) for 15 min at 37°C. The activated toxin was used immediately. Proteinase and toxins were analyzed by sodium dodecyl sulfate–polyacrylamide gel (12.5%) electrophoresis (SDS–PAGE) according to Laemmli [35].

2.3. Toxicity bioassays

Bioassays with T. molitor larvae were used to screen the mutant toxins for shifts in toxicity when compared to the wild-type Cry3A. The force-feeding bioassay method and data analyses for each mutant toxin screened for toxicity in T. molitor are described by Wu and Dean [17]. Two of the mutant toxins, A1 and A2, showed enhanced toxicity in T. molitor, and were further assayed in C. scripta and L. decemlineata larvae. Cry3A-sensitive species in the family Chrysomelidae. The latter bioassays were done by applying serial dilutions of solubilized and activated toxin in a 1 µl droplet of sucrose solution (22.2% final concentration), onto 4 mm poplar or potato leaf disks placed on the surface of 2% agar (Gelcarin) in 24 well tissue culture plates. Second instar C. scripta or L. decemlineata larvae were placed individually in each well, allowed to feed for 24 h, and placed on fresh poplar or potato foliage, respectively. Mortality was recorded daily. Estimates of median lethal concentrations (LC50) and 95% fiducial limits (FL50) for the A1, A2 mutants, and wild-type Cry3A toxin in C. scripta and L. decemlineata were determined 96 h after treatment by probit analysis [36]. The lethal dose ratio test was used to determine if there were significant differences in toxicity between toxin pairs, Cry3A:A1, Cry3A:A2, and A1:A2. This test is used when probit lines are not parallel by analyzing the LC50 and slope data [36].

Table 1

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Construction</th>
<th>Expression</th>
<th>LD50</th>
<th>(FL50)</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>Cry3A [17]</td>
<td>++</td>
<td>11.4</td>
<td>(8.5–14.9)</td>
<td>1.0</td>
</tr>
<tr>
<td>Single mutant</td>
<td>+B18A [17]</td>
<td>++</td>
<td>9.0</td>
<td>(5.9–13.5)</td>
<td>1.3</td>
</tr>
<tr>
<td>Single mutant</td>
<td>+Y35A</td>
<td>+</td>
<td>12.2</td>
<td>(7.5–16.3)</td>
<td>0.9</td>
</tr>
<tr>
<td>Single mutant</td>
<td>+Y35F</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Single mutant</td>
<td>+Y35F</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Single mutant</td>
<td>+Y35F</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Single mutant</td>
<td>+N35D</td>
<td>+</td>
<td>16.7</td>
<td>(11.6–43.5)</td>
<td>0.7</td>
</tr>
<tr>
<td>Single mutant</td>
<td>+D35E</td>
<td>++</td>
<td>7.2</td>
<td>(4.5–11.0)</td>
<td>1.6</td>
</tr>
<tr>
<td>Single mutant</td>
<td>+D35N</td>
<td>+</td>
<td>17.2</td>
<td>(12.3–43.2)</td>
<td>0.7</td>
</tr>
<tr>
<td>Double mutant</td>
<td>Y35A,V35A,18A</td>
<td>++</td>
<td>20.0F</td>
<td>&lt;0.6</td>
<td></td>
</tr>
<tr>
<td>Double mutant</td>
<td>Y35A,F35F,V35F</td>
<td>+d</td>
<td>ND*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Double mutant</td>
<td>Y35A,F35F,V35F</td>
<td>+d</td>
<td>ND*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triple mutant</td>
<td>+Y35F</td>
<td>+</td>
<td>13.1</td>
<td>(9.1–23.4)</td>
<td>0.9</td>
</tr>
<tr>
<td>A1 mutant</td>
<td>+N35A,D35A</td>
<td>+</td>
<td>18.7</td>
<td>(6.1–14)</td>
<td>11.4</td>
</tr>
<tr>
<td>A2 mutant</td>
<td>+N35A,D35A</td>
<td>+</td>
<td>4.2</td>
<td>(2.2–7.0)</td>
<td>2.7</td>
</tr>
<tr>
<td>Block mutant</td>
<td>+N35A,D35A</td>
<td>+</td>
<td>4.2</td>
<td>(2.2–7.0)</td>
<td>2.7</td>
</tr>
</tbody>
</table>

*LD50 and FL50 are expressed as µg per larva.
*The bioassay data for wild-type Cry3A and mutants have been reported [17]. These data are listed in this table for comparison.
*Double mutant +N35A,D35A was not toxic to T. molitor larvae even at the highest dose tested, 20 µg per larva.
*Double mutants Y35A,F35F and Y35A,D35F were degraded soon after solubilization in 50 mM sodium carbonate buffer, pH 10.5.
*ND, not determined.

2.4. Preparation of brush border membrane vesicles

Brush border membrane vesicles (BBMVs) were prepared from whole, third instar C. scripta larvae or midguts of fourth instar L. decemlineata larvae. The methods used for making BBMVs were described by Machtoosh [37] and English and Readly [38], respectively, with some modifications. Homogenization buffer (50 mM sucrose and 4 mM Tris at pH 7.5) included a protease inhibitor cocktail with a final concentration in the buffer of 1.0 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 5 µg/ml each of pepstatin A, leupeptin, and antipain, and 1 µg/ml E-64. The buffer was made fresh and added to each stage of purification. The volume of ice-cold homogenization buffer for use throughout the first precipitation procedure was equal to nine times the weight of tissue to be processed. The initial BBMV pellet was resuspended and reprecipitated with MgCl2 one time for L. decemlineata and twice more for C. scripta. The final pellet was suspended in 0.32 M sucrose, frozen in liquid nitrogen, and stored at –80°C until use. Protein concentration was determined by the BCA protein assay reagent (Pierce). Enrichment, determined by leucine aminopeptidase activity (Sigma assay), was 15–40 times greater than the initial homogenate.

2.5. Ligation of toxins

Protoxins were activated by midgut juice from T. molitor and analyzed for proteolysis with silver-stained SDS–PAGE gel. The purity of the activated Cry3A toxin used in the codominant was more than 95% as determined by scanning densitometry. Ligation of toxins was carried out according to Woltersberger [39], and specific activities of the wild-type Cry3A and mutant toxins ranged from 0.6 to 1.2 µCi/mg protein.

2.6. Competition binding assays

Homologous and heterologous competition binding assays were performed as described by Lee et al. [40] with the following modifications: BBMV (100 µg/ml) were incubated with 125I-labeled toxins (2.0 nM) in 100 µl of binding buffer (50 mM Tris, 0.9% NaCl, pH 8.0, containing 0.5% bovine serum albumin and 0.15% Tween 20). Samples were assayed in duplicate for each data point, and binding data were analyzed by the LIGAND program [41]. This program calculates the relative binding affinity based on homologous competition, Kdass [17], and binding site concentration, Bmax, of bound ligand as a best fit of theoretical curves to the experimental data. The term Kdass for the binding constant was derived from competition studies of Cry toxin with BBMV. This term is proportional to IC50 of classical kinetic analysis (median inhibition of binding by competitor).

2.7. Dissociation binding assays and calculation of Kd

The 125I-labeled wild-type or A1 mutant toxin was incubated with...
1 2 3 4 5 6 7 8

200 -

116 -

97.4 -

66.2 -

Fig. 1. Proteolytic digestion of wild-type Cry3A and loop I mutants. Coomassie blue-stained 7.5% SDS-PAGE gel is shown. Lane 1, protein standards (Bio-Rad). Positions of molecular weight markers (in kDa) are indicated on the left; lane 2, wild-type Cry3A toxin purified from Bt var. tenetiviscus lane 3, Cry3A purified from E. coli; lane 4, Cry3A treated with T. molitor midgut juice; lane 5, A1 mutant, R353A, Y356F, Y359F purified from E. coli; lane 6, A1 mutant treated with T. molitor midgut juice; lane 7, A2 mutant, R353A, Y356A, Y359A purified from E. coli; lane 8, A2 mutant treated with T. molitor midgut juice. Each lane contained 5-10 μg of protein.

BBMV to achieve saturation binding. The reaction mixture was then chased with corresponding unlabeled toxin. The dissociation binding assays were similar to the methods of Imura et al. [42] with the following modification: 2.0 nM [125I]-labeled toxin (wild-type or mutant toxin) was incubated with 100 μg/ml BBMV at room temperature for 60 min to achieve association binding. After association binding, the mixtures were diluted two-fold in binding buffer containing 0.5 μM corresponding unlabeled toxin. The reaction was stopped at different time points (5-80 min) by centrifugation. The pellet was washed three times with binding buffer and the final pellet was counted in a gamma counter (Beckman). Non-specific binding was subtracted from total binding.

The post-incubation time course of these dissociation reactions was transformed to fit the single exponential equation [43]:

\[ y = \frac{[AT]}{[1 - \exp(-k_{obs}t)]} + z \]  

(1)

where \( y \) is fitted to the concentration of dissociated toxin, and \( k_{obs} \) is the maximum dissociation rate constant; \( k_{obs} \) is the observed first-order dissociation rate constant; \( x \) is time; and \( z \) is the correction factor, which is zero near. Dissociation rate constants were determined using Sigma Plot software (Jandel Scientific Co.) to solve the rate equation. Eq. 1.

3. Results

3.1. Expression of loop I mutant proteins

A total of 15 novel mutant toxins were produced by site-directed mutagenesis in the loop I region of domain II and at nearby R545 (Table 1). Except for the single mutant Y359A, mutagenesis of two surface-exposed Y359 and Y350 did not produce structurally stable protein (Table 1). Although the double mutant, Y359F, Y351F, and the double deletion, ΔY359, ΔY351, expressed proteins in E. coli, these mutant proteins were degraded soon after solubilization in 50 mM sodium carbonate buffer, pH 10.5. To suppress the unstable mutants, a nearby mutation R353A was made as reported earlier [17]. Based on these results, the triple mutants A1 (R353A, Y356F, Y359F) and A2 (R353A,ΔY359,ΔY351) were constructed to investigate the functional role of Y359 and Y351. As a control, the single mutant, R353A, was constructed for comparison with triple mutants and wild-type Cry3A δ-endotoxin. These triple mutants produce structurally stable proteins, with a yield of about 5 mg from 500 ml LB medium (Table 1).

To complete the analysis of loop I residues, alanine replacements were generated for N353 and D354. For comparison, N353D, D354E and D354W were constructed. These mutants produced stable δ-endotoxins with a yield similar to wild-type Cry3A (Table 1). Protein stabilities of wild-type A1 and A2 mutants were determined by incubation with T. molitor midgut juice, followed by analysis of the products by SDS-PAGE (Fig. 1).

3.2. Toxicity assays of loop I mutants in coleopterans

Both triple mutants A1 and A2 were significantly more toxic to larvae of T. molitor than the wild-type Cry3A (Table 1). The A1 mutant, with phenylalanine substitutions of the two tyrosine residues in loop I, was ca. 10-fold more toxic than the wild-type Cry3A. The A2 mutant, with deletions of the two tyrosine residues in loop I, was ca. three-fold more toxic. Mutations at positions 353 and 354 had no significant effect on toxicity in T. molitor, except for a reduction in toxicity for the double mutant, N353D, D354A (Table 1).

Toxicity of the A1 and A2 mutant toxins compared to Cry3A in C. scripta and L. decemlineata revealed a trend toward lower LC50s and steeper slopes for the mutants when compared to Cry3A (Tables 2 and 3).

Table 1

<table>
<thead>
<tr>
<th>Protein</th>
<th>C. scripta</th>
<th>L. decemlineata</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LC50 g</td>
<td>FL50 %</td>
</tr>
<tr>
<td>Cry3A</td>
<td>1.70</td>
<td>0.82±0.97</td>
</tr>
<tr>
<td>A1 mutant</td>
<td>0.66</td>
<td>0.38±0.97</td>
</tr>
<tr>
<td>A2 mutant</td>
<td>0.83</td>
<td>0.63±1.42</td>
</tr>
</tbody>
</table>

Each protein assay was replicated three times. Each replicate assay included four to six protein concentrations and a control applied to the appropriate leaf disks (peapod or potato) with 10-20 insects per concentration.

LC50 and FL50 are expressed as mg/ml.
3.3. Effects of mutations on membrane receptor binding

Competitive binding of radio-labeled Cry3A toxin in the presence of unlabeled wild-type Cry3A was reduced to 13% for C. scripta (Fig. 2A) and 27% for L. decemlineata (Fig. 2B). Homologous competition binding assays, using radio-labeled wild-type Cry3A toxin, determined that C. scripta had a > 3-fold higher binding affinity ($K_{\text{com}}$) than L. decemlineata (Table 4). In contrast, the binding site concentration ($B_{\text{max}}$) for L. decemlineata was > 3-fold higher than C. scripta (Table 4).

Table 4

<table>
<thead>
<tr>
<th>Protein</th>
<th>C. scripta</th>
<th>L. decemlineata</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_{\text{com}}$ (nM)</td>
<td>$B_{\text{max}}$</td>
</tr>
<tr>
<td>Cry3A</td>
<td>35.9 ± 4.2 a</td>
<td>23.3 ± 3.3 b</td>
</tr>
<tr>
<td>AI mutant</td>
<td>49.7 ± 5.6 a</td>
<td>32.7 ± 2.1 b</td>
</tr>
<tr>
<td>A2 mutant</td>
<td>46.2 ± 5.1 a</td>
<td>25.6 ± 1.5 b</td>
</tr>
</tbody>
</table>

Values for $K_{\text{com}}$ or $B_{\text{max}}$ within one species and followed by the same letter are not significantly different using Student’s t-test with paired comparisons.

$^a$The binding constants ($K_{\text{com}}$) and binding site concentrations ($B_{\text{max}}$) represent the calculated mean ± S.E.M. of three competition binding experiments.

$^b$The $B_{\text{max}}$ values are expressed as pmol/mg of BBMV protein.

3.4. Dissociation analysis of AI mutant on C. scripta BBMV

Since the AI mutant toxin had minimal effect on $K_{\text{com}}$ and $B_{\text{max}}$, but substantially increased toxicity to C. scripta larvae, dissociation assays were performed to further examine the kinetic aspects of membrane receptor binding. We observed that about 44% of wild-type Cry3A could be dissociated from the BBMV, reaching equilibrium after 10–20 min (Fig. 3A). In contrast, only 24% of AI mutant toxin was dissociated from its binding site in C. scripta by the addition of excess unlabeled ligand, reaching equilibrium after 30 min (Fig. 3A). The dissociation rate calculated for AI mutant, $k_{\text{diss}} = 0.05$ min⁻¹, was nearly five-fold lower when compared to the dissociation rate for the wild-type Cry3A, $k_{\text{diss}} = 0.24$ min⁻¹ (Fig. 3B).

4. Discussion

Our results support that site-specific changes in loop I of the receptor binding domain of Cry3A 6-endotoxin altered the toxicity of this protein in coleopteran larvae. Of particular interest were two mutant toxins, AI and A2, which were more toxic than the wild-type Cry3A in bioassays of three coleopteran species (Tables 1 and 2). These two mutants differed only slightly, in AI phenylalanines were substituted for tyrosines at residues 350 and 351 (R345A,Y350F,Y351F), and in A2 those tyrosines were deleted (R345A,A,Y350F,Y351F). Furthermore, receptor binding studies demonstrated that phenylalanine substitutions for Y350 and Y351 mostly affect dissociation, apparently accounting for the enhancement of toxicity. Ultimately, we hope to understand the structural role of loop I residues in insect toxicity and parameters of binding to insect midgut receptors.

The similar toxicity of wild-type Cry3A in C. scripta and L. decemlineata, both within the same family of Chrysomelidae, was achieved through quite different receptor binding parameters. The wild-type Cry3A binding affinity ($K_{\text{com}}$) to C. scripta BBMV was greater than that for L. decemlineata BBMV, whereas the Cry3A binding site concentration ($B_{\text{max}}$) was higher for L. decemlineata than that for C. scripta (Table 4).
The mutant toxins, A1 and A2, also displayed different receptor binding parameters in the two chrysomelid species. In BBMV prepared from *L. decemlineata*, both mutant strains showed enhanced binding affinity and fewer binding sites (Table 4). The differences in toxicity between the two mutants were similar to differences in binding site concentration. In contrast, receptor binding parameters measured in *C. scripta* BBMV revealed slightly lower binding affinities for both mutants, and a significantly greater number of binding sites for the more toxic mutant A1 (Table 4).

Bioassays for the A2 mutant indicated that deletion of the two tyrosines in loop 1 showed greater toxicity in both *T. molitor* and *L. decemlineata* (2.7- and 2.2-fold, respectively), but only a minimal effect on toxicity in *C. scripta* (Tables 1 and 3). It is not clear how the adjacent N353 and D354 residues contribute to receptor binding and toxicity, but previous results indicated that alanine substitutions for N353 and D354 caused the loss of binding affinity and toxicity [17]. In addition, toxicity of the D354E mutant was similar to that of the wild-type Cry3A (Table 1). Perhaps, the deletions of Y359 and Y351 allow the aliphatic side chains of N353 and D354 to make better contact with the receptor, enhancing toxicity.

The results of our mutational, bioassay and receptor binding studies suggest that hydrophobic side chains for the residues in loop 1 interact with the receptor. Hence, the fact that phenylalanine can effectively substitute for Y359 and Y351 in toxicity enhancement suggests that it is not the phenolic hydroxyl group of tyrosine at these positions, but rather its aromatic phenol ring that is important for toxicity (Tables 1 and 2). This observation implies that the hydrophobic interaction of aromatic residues with the contact interface on the receptor side may be necessary for tight binding affinity. The well-packed hydrophobic contact residues may play an important role in the insecticidal activity and binding affinity.

The involvement of hydrophobic interactions between receptors and ligands is well documented. Mutagenesis and crystallographic studies have revealed that aromatic amino acids, tyrosine, phenylalanine and tryptophan, were the major determinants of binding affinity in human lysozyme [44], diphtheria toxin [45], human growth hormone and the extracellular binding domain of its receptor [46,47], and other insecticidal Cry toxins [48]. In our study, the binding analysis of A1 and A2 mutants to *C. scripta* and *L. decemlineata* BBMV verified the correlation between hydrophobic interaction in binding affinity and insecticidal activity. In *L. decemlineata* larvae, competition binding analysis indicated that both the deletions and phenylalanine substitutions for Y359 and Y351 resulted in increased binding affinity, a ca. 2.5-fold lower K_d value than wild-type toxin (Fig. 2B and Table 4). These results suggest that the more hydrophobic phenylalanine rings or removal of the phenolic hydroxyl groups at residues 350 and 351 can lead to higher binding affinity and higher toxicity.

Interestingly, there was no significant difference in the competition analysis between wild-type, A1 and A2 mutant toxins to *C. scripta* (Fig. 2A and Table 4) or *T. molitor* (unpublished data), despite enhanced toxicity of the A1 mutant toxin in *T. molitor* and *C. scripta* larvae (ca. 11.4- and 2.6-fold, respectively). Our investigations of the dissociation of the A1 mutant toxin suggested that the hydrophobic phenylalanines substituted for Y359 and Y351 reduced the dissociation rate by nearly five-fold, as compared with wild-type Cry3A (Fig. 3B). This slower dissociation rate may account for its greatly enhanced toxicity in *C. scripta*. This result may have direct relevance to the enhancement of insecticidal activity. Our results are consistent with the tight binding affinity of Cry1Ab toxin to *Manduca sexta* BBMV, also caused by the hydrophobic phenylalanine, F351, in the surface-exposed loop [29].

Overall binding affinity can be considered a dynamic balance of association and dissociation reactions. Since the affinity constant (K_on) is determined by competition binding experiments, it reflects all binding events. Distinction of association and dissociation will be necessary to explain the subtle quantitative differences in the toxicity of δ-endotoxins in their hosts.

The implications of our findings are important for rational design of δ-endotoxins that overcome insect resistance to these toxins and for altered or improved insecticidal activity, as has been achieved in other Cry toxins [4,27]. For instance, the hydrophobic residues around the loop 1 region in domain II, F351NYW356 of Cry1Aa, E311YYW316 of Cry1Ab and Y311YYW316 of Cry1Ac toxins, may be identified as the homologue of Y359YGN354 of Cry3A by functional mapping and sequence alignment [49]. Identification of these hydrophobic contact residues provides the potential for constructing
genetically enhanced and durable toxins for use as biological pesticides.

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