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 ($R_{345}A$,$Y_{350}F$,$Y_{351}F$) and A2 ($R_{345}A$,ΔY_{350},ΔY_{351}), 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.

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 protoxin, 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 hemocele, and death results from bacterial septicemia. 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 Cry1Aa [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 500 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, R_{345}, Y_{350} and Y_{351}, 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

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Abbreviations: *B.t.*, *Bacillus thuringiensis*; BBMV, brush border membrane vesicles

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Wu and Dean [17]. We subcloned the *Bam*HI–*Hin*dIII 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 MutaGene 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. [32] 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 protease digestion of protoxins

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 protoxin was determined by Coomassie protein assay reagent (Pierce). Protoxin was activated with diluted *T. molitor* midgut juice with a juice protein/protoxin ratio of 1:20 (w/w) for 15 min at 37°C. The activated toxin was used immediately. Protoxin 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 (22.5% 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 (LC_{50}) and 95% fiducial limits (FL_{95}) 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 LC_{50} and slope data [36].

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 MacIntosh [37] and English and Readdy [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 $MgCl_2$ one time for *L. decemlineata* and twice more for *C. scripta*. The final pellet was resuspended 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. Iodination 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 iodination was more than 95% as determined by scanning densitometry. Iodination of toxins was carried out according to Wolfersberger [39], and specific activities of the wild-type Cry3A and mutant toxins ranged from 0.6 to 1.2 μCi/μg 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 ^{125}I-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, K_{com} [17], and binding site concentration, B_{max}, of bound ligand as a best fit of theoretical curves to the experimental data. The term K_{com} for the binding constant was derived from competition studies of Cry toxin with BBMV. This term is proportional to IC_{50} of classical kinetic analysis (median inhibition of binding by competitor).

2.7. Dissociation binding assays and calculation of k_{dis}

The ^{125}I-labeled wild-type or A1 mutant toxin was incubated with

Table 1
Construction of loop I mutants and toxicity assays to *T. molitor* larvae

Mutant	Construction	Expression	LD_{50}^{a}	$(FL_{95})^{a}$	Relative activity
Wild-type	Cry3A [17][b]	++	11.4	(8.5–14.9)	1.0
Single mutant	$R_{345}A$ [17][b]	++	9.0	(5.9–13.5)	1.3
Single mutant	$Y_{350}A$	−			
Single mutant	$Y_{351}A$	+	12.2	(7.5–16.3)	0.9
Single mutant	$Y_{350}F$	−			
Single mutant	$Y_{351}F$	−			
Single mutant	$N_{353}D$	++	16.7	(11.6–43.5)	0.7
Single mutant	$D_{354}E$	++	7.2	(4.5–11.0)	1.6
Single mutant	$D_{354}N$	++	17.2	(12.3–43.2)	0.7
Double mutant	$Y_{350}A,Y_{351}A$ [17][b]	−			
Double mutant	$N_{353}A,D_{354}A$ [17][b]	++	20.0[c]		<0.6
Double mutant	$Y_{350}F,Y_{351}F$	+[d]	ND[e]		
Double mutant	$\Delta Y_{350},\Delta Y_{351}$	+[d]	ND		
Triple mutant	$R_{345}A,Y_{350}A,Y_{351}A$ [17][b]	+	13.1	(9.1–23.4)	0.9
A1 mutant	$R_{345}A,Y_{350}F,Y_{351}F$	+	1.0	(0.6–1.4)	11.4
A2 mutant	$R_{345}A,\Delta Y_{350},\Delta Y_{351}$	+	4.2	(2.2–7.0)	2.7
Block mutant	$Y_{350}A,Y_{351}A,G_{352}A$ $N_{353}A,D_{354}A$				

[a]LD_{50} and FL_{95} are expressed as μg per larva.
[b]The bioassay data for wild-type Cry3A and mutants have been reported [17]. Those data are listed in this table for comparison.
[c]Double mutant $N_{353}A,D_{354}A$ was not toxic to *T. molitor* larvae even at the highest dose tested, 20 μg per larva.
[d]Double mutants $Y_{350}F,Y_{351}F$ and $\Delta Y_{350},\Delta Y_{351}$ were degraded soon after solubilization in 50 mM sodium carbonate buffer, pH 10.5.
[e]ND, not determined.

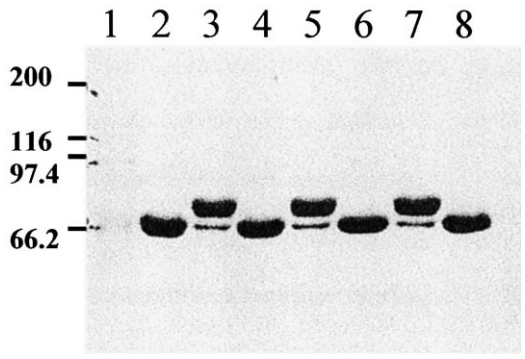

Fig. 1. Proteolytic digestion of wild-type Cry3A and loop I mutants. Coomassie blue-stained 7.5% SDS–PAGE gel is shown. Lane 1, protein standards (Bio-Rad). Positions of molecular weight markers (in kDa) are indicated on the left; lane 2, wild-type Cry3A toxin purified from *B.t.* var. *tenebrionis*; lane 3, Cry3A purified from *E. coli*; lane 4, Cry3A treated with *T. molitor* midgut juice; lane 5, A1 mutant, $R_{345}A,Y_{350}F,Y_{351}F$, purified from *E. coli*; lane 6, A1 mutant treated with *T. molitor* midgut juice; lane 7, A2 mutant, $R_{345}A,\Delta Y_{350},\Delta Y_{351}$, purified from *E. coli*; lane 8, A2 mutant treated with *T. molitor* midgut juice. Each lane contained 8–10 μg of protein.

BBMV to achieve saturation binding. The reaction mixture was then chased with corresponding unlabeled toxin. These dissociation binding assays were similar to the methods of Ihara et al. [42] with the following modification: 2.0 nM ^{125}I-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]:

$$y = \{\Delta T\}\{1-\exp(-k_{dis}x)\} + z \quad (1)$$

where y is fitted to the concentration of dissociated toxin, and ΔT is the maximum dissociated toxin; k_{dis} is the observed first-order dissociation rate constant; x is time; and z is the correction factor, which is near zero. 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 R_{345} (Table 1). Except for the single mutant $Y_{351}A$, mutagenesis of two surface-exposed Y_{350} and Y_{351} did not produce structurally stable protein (Table 1). Although the double mutant, $Y_{350}F,Y_{351}F$, and the double deletion, $\Delta Y_{350},\Delta Y_{351}$, 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 $R_{345}A$ was made as reported earlier [17]. Based on these results, the triple mutants A1 ($R_{345}A,Y_{350}F,Y_{351}F$) and A2 ($R_{345}A,\Delta Y_{350},\Delta Y_{351}$) were constructed to investigate the functional role of Y_{350} and Y_{351}. As a control, the single mutant, $R_{345}A$, 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 N_{353} and D_{354}. For comparison, $N_{353}D$, $D_{354}E$ and $D_{354}N$ 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, $N_{353}A,D_{354}A$ (Table 1).

Toxicity of the A1 and A2 mutant toxins compared to Cry3A in *C. scripta* and *L. decemlineata* revealed a trend toward lower LC_{50}s and steeper slopes for the mutants when compared to Cry3A (Tables 2 and 3).

Table 3
Median lethal concentration (LC_{50}) ratio test[a] on Cry3A, A1 and A2 mutant proteins for young second instar *C. scripta* and *L. decemlineata*

Toxin comparison	*C. scripta*		*L. decemlineata*	
	Ratio	CL_{95}	Ratio	CL_{95}
Cry3A:A1 mutant	2.56[b]	1.35–4.88	1.91[b]	1.28–2.85
Cry3A:A2 mutant	1.73	0.94–3.21	2.20[b]	1.44–3.36
A1 mutant:A2 mutant	0.68	0.38–1.20	1.15	0.77–1.73

[a]The lethal dose ratio test [36] was used to determine significance because the probit lines for the three toxins were not parallel.
[b]The LC_{50}s of each toxin comparison are not significantly different at the $P < 0.01$ level of significance if the CL_{95} interval for their ratio includes 1.

Table 2
Maximum likelihood estimates of median lethal concentrations (LC_{50}) of Cry3A, A1 and A2 mutant proteins for young second instar *C. scripta* and *L. decemlineata*

Protein[a]	*C. scripta*			*L. decemlineata*		
	LC_{50}^{b}	FL_{95}^{b}	Slope ± S.E.M.	LC_{50}	FL_{95}	Slope ± S.E.M.
Cry3A	1.70	0.85–2.97	1.65 ± 0.34	2.07	1.41–3.44	1.90 ± 0.27
A1 mutant	0.66	0.38–0.97	2.29 ± 0.47	1.08	0.64–1.63	2.35 ± 0.41
A2 mutant	0.98	0.63–1.42	2.14 ± 0.37	0.94	0.57–1.48	1.82 ± 0.26

[a]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 (poplar or potato) with 10–20 insects per concentration.
[b]LC_{50} and FL_{95} are expressed as ng/mm^2.

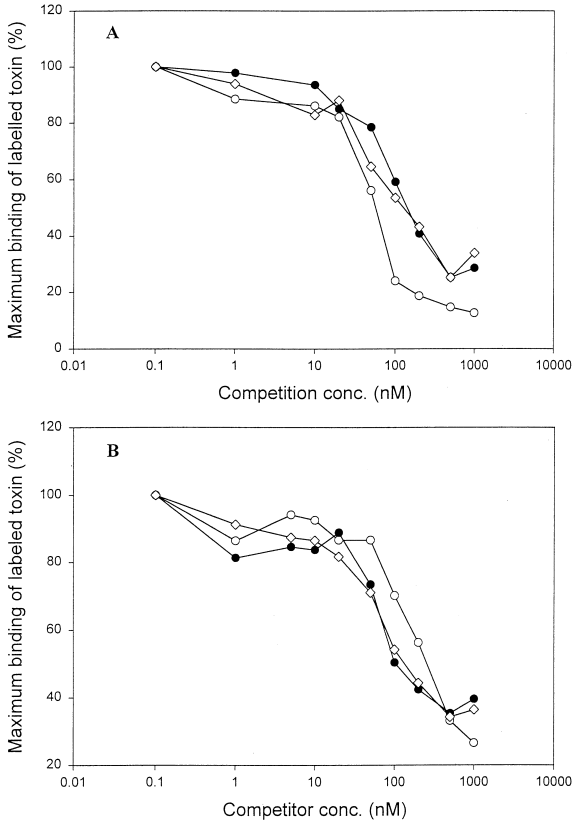

Fig. 2. Competition binding of ^{125}I-labeled Cry3A toxin to *C. scripta* (A) and *L. decemlineata* (B) BBMVs as a function of the concentration of unlabeled loop I mutant proteins. Vesicles (100 μg/ml) were incubated with labeled wild-type toxin (2.0 nM) in the presence of increasing concentrations of unlabeled competitors. Binding is expressed as percentage of the amount bound upon incubation with labeled toxin alone. A: ○, wild-type Cry3A toxin; ●, A1 mutant ($R_{345}A,Y_{350}F,Y_{351}F$); ◇, A2 mutant ($R_{345}A,\Delta Y_{350},\Delta Y_{351}$). B: ○, wild-type Cry3A toxin; ●, A1 mutant ($R_{345}A,Y_{350}F,Y_{351}F$); ◇, A2 mutant ($R_{345}A,\Delta Y_{350},\Delta Y_{351}$). Results are obtained from the average of three experiments. Each point represents the mean of duplicate samples.

3.3. Effects of mutations on membrane receptor binding

Competitive binding of ^{125}I-labeled Cry3A 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 ^{125}I-labeled wild-type Cry3A toxin, determined that *C. scripta* had a >3-fold higher binding affinity (K_{com}) than *L. decemlineata* (Table 4). In contrast, the binding site concentration (B_{max}) for *L. decemlineata* was >3-fold higher than *C. scripta* (Table 4).

Binding affinities for both the mutant toxins (A1 and A2) on *C. scripta* BBMV were similar and slightly lower than that for the wild-type Cry3A, although not significantly different (Table 4). The binding site concentration of *C. scripta* BBMV was significantly greater for the A1 mutant than that for either the wild-type Cry3A or the A2 mutant (Table 4). The binding assays with *L. decemlineata* BBMV revealed both greater affinity and a lower concentration of binding sites for the mutant toxins compared to the wild-type Cry3A (Table 4).

3.4. Dissociation analysis of A1 mutant on C. scripta BBMV

Since the A1 mutant toxin had minimal effect on K_{com} and B_{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 A1 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 A1 mutant, $k_{dis} = 0.05$ min^{-1}, was nearly five-fold lower when compared to the dissociation rate for the wild-type Cry3A, $k_{dis} = 0.24$ min^{-1} (Fig. 3B).

4. Discussion

Our results support that site-specific changes in loop I of the receptor binding domain of Cry3A δ-endotoxin altered the toxicity of this protein in coleopteran larvae. Of particular interest were two mutant toxins, A1 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 A1 phenylalanines were substituted for tyrosines at residues 350 and 351 ($R_{345}A,Y_{350}F,Y_{351}F$), and in A2 those tyrosines were deleted ($R_{345}A,\Delta Y_{350},\Delta Y_{351}$). Furthermore, receptor binding studies demonstrated that phenylalanine substitutions for Y_{350} and Y_{351} 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_{com}) to *C. scripta* BBMV was greater than that for *L. decemlineata* BBMV, whereas the Cry3A binding site concentration (B_{max}) was higher for *L. decemlineata* than that for *C. scripta* (Table 4).

Table 4
Binding properties of Cry3A and mutant proteins incubated with BBMVs of *C. scripta* and *L. decemlineata*

Protein	*C. scripta*		*L. decemlineata*	
	K_{com} (nM)[a]	$B_{max}^{a,b}$	K_{com} (nM)[a]	$B_{max}^{a,b}$
Cry3A	37.9 ± 4.2 a	23.3 ± 3.3 b	135.9 ± 11.8 a	79.3 ± 6.4 a
A1 mutant	49.7 ± 5.6 a	32.7 ± 2.1 a	54.4 ± 6.8 b	50.3 ± 2.9 c
A2 mutant	46.3 ± 5.1 a	25.6 ± 1.5 b	53.8 ± 6.5 b	63.2 ± 1.9 b

Values for K_{com} or B_{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_{com}) and binding site concentrations (B_{max}) represent the calculated mean ± S.E.M. of three competition binding experiments.
[b]The B_{max} values are expressed as pmol/mg of BBMV protein.

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 I 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 N_{353} and D_{354} residues contribute to receptor binding and toxicity, but previous results indicated that alanine substitutions for N_{353} and D_{354} caused the loss of binding affinity and toxicity [17]. In addition, toxicity of the $D_{354}E$ mutant was similar to that of the wild-type Cry3A (Table 1). Perhaps, the deletions of Y_{350} and Y_{351} allow the aliphatic side chains of N_{353} and D_{354} 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 I interact with the receptor. Hence, the fact that phenylalanine can effectively substitute for Y_{350} and Y_{351} 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 Y_{350} and Y_{351} resulted in increased binding affinity, a ca. 2.5-fold lower K_{com} 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 Y_{350} and Y_{351} 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

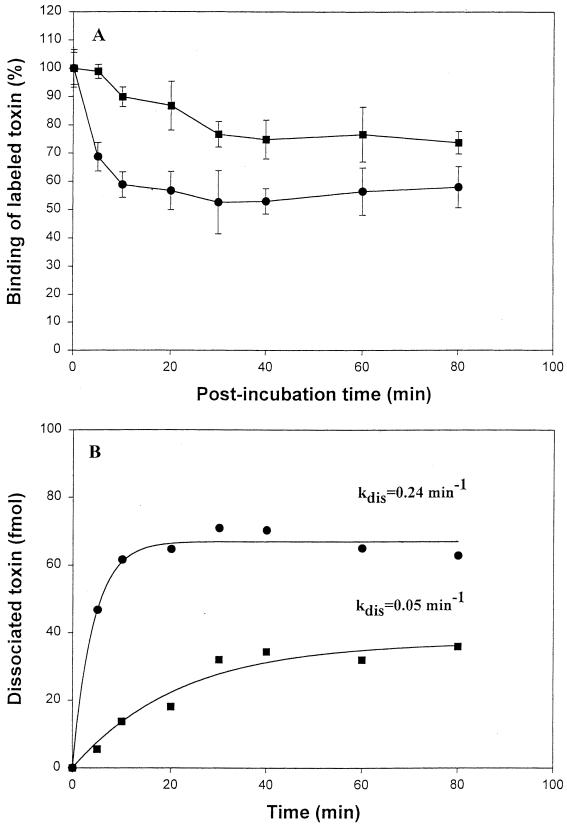

Fig. 3. Dissociation of ^{125}I-labeled Cry3A wild-type and A1 mutant toxins from *C. scripta* BBMV. A: 2.0 mM ^{125}I-labeled toxin (wild-type or A1 mutant) was incubated with 100 μg/ml vesicles at room temperature for 60 min. After incubation, the mixtures were diluted two-fold in binding buffer containing 0.5 μM corresponding unlabeled toxins. Non-specific binding was subtracted. ●, wild-type Cry3A toxin; ■, A1 mutant ($R_{345}A,Y_{350}F,Y_{351}F$). Each point represents the mean ± S.D. of triplicate experiments. B: Single exponential fits for dissociation binding curves give dissociation rate constants, k_{dis}, for wild-type and mutant toxins. ●, wild-type Cry3A toxin; ■, A1 mutant ($R_{345}A,Y_{350}F,Y_{351}F$).

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, F_{371}, 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_{com}) 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 I region in domain II, $F_{313}NYW_{316}$ of Cry1Aa, $E_{313}YYW_{316}$ of Cry1Ab and $Y_{313}YYW_{316}$ of Cry1Ac toxins, may be identified as the homologue of $Y_{350}YGND_{354}$ 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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