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Involvement of Two Amino Acid Residues in the Loop Region of *Bacillus thuringiensis* Cry1Ab Toxin in Toxicity and Binding to *Lymantria dispar*

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Two amino acids, Gly and Ser, at positions 282 and 283 in the loop region of domain II of Cry1Ab2 toxin are substituted with Ala and Leu in the Cry1Ab9-033 toxin. Cry1Ab2 exhibited about a 10-fold increase in toxicity and a 9-fold increase in binding affinity to *Lymantria dispar* compared to Cry1Ab9-033. However, these toxins showed similar toxicity and binding affinity to *Manduca sexta* and *Spodoptera exigua.* Heterologous competition assays and brush border membrane vesicle (BBMV) ligand blotting experiments demonstrated that Cry1Ab2 and Cry1Ab9-033 toxins recognized the same 210-kDa *L. dispar* BBMV protein. No measurable differences in dissociation binding assays were observed between these two toxins. Digestion of these toxins with *L. dispar* gut enzymes and BBMV proteases indicated no differences in stability. Ala and Leu residues in Cry1Ab9-033 were substituted with Gly and Ser by site-directed mutagenesis to produce mutant Cry1Ab α8. This toxin exhibited full recovery of toxicity and binding affinity for *L. dispar*. These data suggested that the residues Gly and Ser in the loop region might be directly involved in receptor binding and toxicity in *L. dispar*.

Bacillus thuringiensis produces crystalline parasporal inclusions containing insecticidal crystal proteins during sporulation. These inclusions are solubilized in the insect midgut, where the protoxins are processed to active toxin by midgut proteases. The activated toxin binds to specific receptors on the brush border membrane of midgut epithelial cells. Binding of toxin to the receptor is believed to cause conformational changes in the toxin and enable it to integrate into the midgut membrane and to form pores or ion channels, resulting in insect death (1-6).

Interaction of the toxin with the receptor has been studied extensively with radiolabelled toxins and brush border membrane vesicles (BBMV) from lepidopteran insect midguts (3, 4, 7). The receptor binding step has been considered to be the most critical factor for toxicity and specificity in many studies, although several exceptions also have been reported (8, 9).

Recently, the crystal structure of Cry1Aa toxin has been obtained (10). Domain I (residues 33 to 253) is an α-helical structure containing eight α-helices. Domain II (265 to 461) consists of three anti-parallel β-sheets and two short α-helices. Domain III is a β-sandwich of two anti-parallel, highly twisted β-sheets and comprises residues 463 to 609 with an additional, outer strand in one of the β-sheets formed by residues 254 to 264. Domain I is predicted to participate in membrane insertion and ion channel formation (11, 12). Mutations in domain I result in the loss of toxicity with or without altering binding properties (13, 14). Previous studies with hybrid toxins and loop region mutants have demonstrated that domain II is essential for membrane receptor binding and toxicity (7, 15, 16). Domain III is believed to stabilize the toxin structure and may also participate in ion channel formation, receptor recognition, and insect specificity (17-21). These results suggest a degree of complexity in the functional role of each domain.

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Two residues, Gly and Ser, in the loop region (between α helix 8 and β sheet 2) of domain II of Cry1Ab2 toxin are replaced with Ala and Leu in the Cry1Ab9-033 toxin. In the present study, we investigated the functional role of these residues by performing bioassays and toxin-receptor binding assays to different insects. Cry1Ab2 exhibited higher toxicity (10 times) and binding affinity (9 times) than Cry1Ab9-033 to *L. dispar*. Ala and Leu residues in Cry1Ab9-033 were substituted with Gly and Ser residues by site directed mutagenesis (Cry1Ab α8). This toxin recovered toxicity and receptor binding to *L. dispar*. Toward *Manduca sexta* and *Spodoptera exigua*, however, only marginal or insignificant differences were observed in toxicity and binding affinity between Cry1Ab2 and Cry1Ab9-033. These results suggest that Gly and Ser residues in the loop region might be involved in toxicity and receptor recognition in *L. dispar*.

MATERIALS AND METHODS

cry1Ab2 and cry1Ab9-033 genes and mutant construction. The *cry1Ab9-033* gene was kindly supplied by Dr. T. Yamamoto (Sandoz Agro Inc., Palo Alto, Calif). The *cry1Ab9-033* gene was constructed by changing two residues Gly282 and Ser283 of *cry1Ab9* gene to Ala and Leu, respectively. The *cry1Ab9* gene was originally cloned by Chak and Chen (22) from *B. thuringiensis* subsp. *kurstaki* HD-133. The *cry1Ab9-033* gene was cloned into pBluescript KS+ with a *cry1Ac* terminator and *cry1C* promoter and then the gene was expressed in *E. coli* MV 1190. We received the *cry1Ab2* gene from Dr. T. Pollock (23). The *cry1Ab2* gene was subcloned into the vector pKK 223-3 and expressed in *E. coli* JM 103. Two residues, Ala282 and Leu283, in the *cry1Ab9-033* were substituted to Gly and Ser (*cry1Ab α8*) by site-directed mutagenesis using the Bio-Rad Muta-Gene phagemid *in vitro* mutagenesis kit following the manufacturer's instructions. Single-stranded DNA sequencing was carried out by the method of Sanger *et al.* (24) following the manufacturer's instructions (United States Biochemical).

Toxicity assays. Crystal inclusion bodies from Cry1Ab2, Cry1Ab9-033, and Cry1Ab α8 toxins were purified and solubilized as described (7). The solubilized protoxin was digested with trypsin at a trypsin/protoxin ratio of 1 : 25 (by mass) at 37°C for 4 h. Protein concentration of protoxins and toxins was estimates by Coomassie Protein Assay Reagent (Pierce) and the purity was examined by SDS-10% PAGE (25).

Activities of toxins were determined with newly hatched *L. dispar* and *M. sexta* larvae by the surface contamination method as described (16). Mortality was recorded after 5 days and the effective dose estimates (LC_{50}, 50% lethal concentration of toxin) were calculated using PROBIT analysis (26). Force feeding bioassays were also performed with fourth instar larvae of *L. dispar* as described previously (27). The growth inhibition dosage (ID_{50}) values were calculated after 5 days using PROBIT analysis. The definition of growth inhibition is decreased or unchanged weight after toxin dose.

BBMV binding assays. BBMV was prepared from midguts of the last instar larvae of *L. dispar*, *M. sexta*, and *S. exigua* as described (28). Twenty five μg of each toxin was iodinated with 1mCi of $Na^{125}I$ (Amersham) and IODO-BEAD (Pierce) as described (7).

Homologous and heterologous competition binding assays were performed as described previously (7). Twenty μg of BBMV was incubated with 2 nM of ^{125}I-labeled toxins in 100 μl of 8 mM $NaHPO_4$, 2 mM KH_2PO_4, 150 mM NaCl, pH 7.4, containing 0.1 % BSA, for 1 h at room temperature in the presence of increasing amounts of corresponding unlabeled competitors (from 1 to 1000 nM). Bound toxins were separated from unbound toxin by centrifugation at 15,500 rpm for 10 min. The pellet containing the bound toxin was washed two times with binding buffer, and the radioactivity in the resulting pellet was counted in a gamma counter (Beckman). Binding affinities (Kcom) and binding site concentrations (Bmax) were calculated by the LIGAND computer program (29).

Dissociation binding assays were performed as described (30). 2 nM of ^{125}I-labeled Cry1Ab2, Cry1Ab9-033, and Cry1Ab α8 toxins were incubated with 20 μg of *L. dispar* BBMV for 1h. After association binding, the mixtures were diluted twofold in binding buffer containing 1 μM corresponding unlabeled toxin. The reaction was stopped at different time points (10 min to 120 min) by centrifugation. Nonspecific binding was subtracted from total binding.

Identification of cry1Ab toxin binding protein. BBMV ligand blotting was performed as described (19). Twenty five μg of BBMV proteins was separated onto SDS-7.5% PAGE, transferred to polyvinylidene difluoride (PVDF) membrane, and probed with 5 nM ^{125}I-labeled toxins.

Stability of Cry1Ab2 and Cry1Ab9-033 toxins. 500 ng of each toxin was incubated with gut juice (collected from *L. dispar* larvae by gentle squeezing) at 37°C for 2 hr. After incubation, toxins were separated onto SDS-10 % PAGE and transferred onto PVDF membrane. The membrane was incubated with Cry1Ab2 antibody (1:2000 dilution) and probed with goat anti rabbit IgG conjugated with horse radish peroxidase (GAR-HRP, BioRad) and 3,3′-Diaminobenzidine Tetrahydrochloride (DAB) substrate (Sigma). Stability of labeled toxins by BBMV proteases was also examined. 2 nM ^{125}I-labeled toxins were incubated with 20 μg of BBMV for 1 hr. The bound toxins were separated on SDS-10 % PAGE. The gel was dried and exposed to Fuji-RX film for 1-2 days.

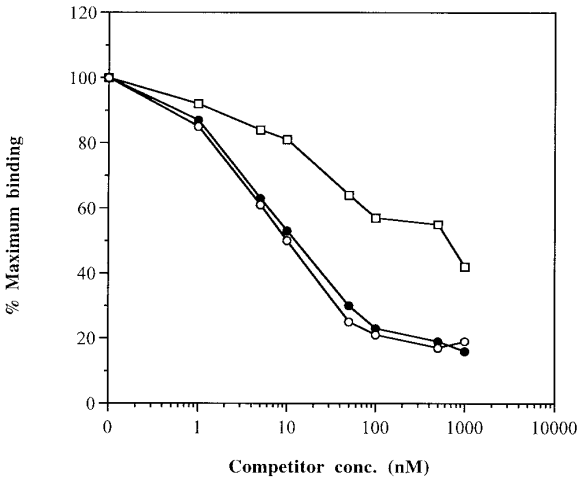

FIG. 1. Binding of ^{125}I-labeled Cry1Ab2, Cry1Ab9-033, and Cry1Ab α8 toxins to *L. dispar* BBMV. Each ^{125}I-labeled toxin (2 nM) was incubated with 20 μg of BBMV in the presence of increasing concentration of unlabeled Cry1Ab2 (○), Cry1Ab9-033 (□), and Cry1Ab α8 toxins (●). Binding is expressed as a percentage of the amount bound upon incubation with labeled toxin alone.

RESULTS AND DISCUSSION

Cry1Ab2 and Cry1Ab9-033 toxins. The Cry1Ab9-033 toxin contains Ala and Leu at positions 282 and 283, which are in the loop (α8 loop) between α helix 8 and β sheet 2 based on the crystal structure of Cry1Aa (10), while Gly and Ser residues are present in Cry1Ab2. Revisions of the *cry1Ab2* gene sequence (2) indicate that it also has the same DNA sequence as *cry1Ab3* (31). These sequences and gene names can be found on the Internet (http://www.sussex.ac.uk/Users/bafn6/bt/toxin) but the *cry1Ab2* gene sequence has not been corrected in the GenBank or the EMBL databases. Two residues, Ala282 and Leu283, in Cry1Ab9-033 were replaced with Gly and Ser, respectively, by site-directed mutagenesis. The mutated gene (*cry1Ab α8*) is basically the same as *cry1Ab2,* but cloned in pBluescript vector instead of pKK 223-3. The expression and yield of the Cry1Ab2, Cry1Ab9-033, Cry1Ab α8 toxins were comparable (data not shown).

Biological activity. Insecticidal activities of Cry1Ab2, Cry1Ab9-033, and Cry1Ab α8 toxins against *L. dispar* and *M. sexta* are reported in Table 1. Toward *L. dispar,* Cry1Ab2 showed about 10 times greater toxicity than Cry1Ab9-033 with first instar *L. dispar* larvae. Force feeding bioassays with fourth instar *L. dispar* larvae also demonstrated that Cry1Ab2 was about 11 fold more toxic than Cry1Ab9-033. The Cry1Ab α8 showed toxicity similar to Cry1Ab2 as expected. However, no such dramatic differences in toxicity between Cry1Ab2 and Cry1Ab9-033 toxins were observed to *M. sexta.* The Cry1Ab2 was observed to be about 2.5 fold more toxic than the Cry1Ab9-033. Toward *S. exigua,* toxicity was not observed at the concentration of 2000 ng/cm^2 surface of diet with either Cry1Ab2 and Cry1Ab9-033 (Dean and Rajamohan, unpublished observation).

Competition binding and dissociation binding. To determine factors affecting toxicity to *L. dispar,* competition binding assays were performed. Homologous competition binding data showed that Cry1Ab2 bound to *L. dispar* BBMV with high binding affinity (Kcom) of 3.21 nM. Cry1Ab9-033 showed about 9 fold less binding affinity, while Cry1Ab α8 showed high binding affinity similar to Cry1Ab2 (Fig.1, Table 1). In this study, we used the term Kcom rather than Kd, as suggested by Wu and Dean (31). The definition of Kcom is the dissociation constant calculated from homologous competition assays with toxin and BBMV, so Kcom is

TABLE 1
Toxicity and Binding Parameters of Cry1Ab2, Cry1Ab9-033, and Cry1Abα8 Toxins to *L. dispar* and *M. sexta* Larvae

Insects	Toxins	Toxicity		Kcom[a] (nM)	Bmax[b] (pmoles/mg)
		LC_{50}[c] (ng/cm_2)	ID_{50}[d] (mg/larvae)		
L. dispar	Cry1Ab9-033	375 (280–435)	2358 (1758–2780)	30.56 ± 3.65	7.35 ± 1.23
	Cry1Ab2	37 (28–45)	205 (157–267)	63.21 ± 1.15	5.98 ± 0.97
	Cry1Abα8	53 (43–62)	358 (250–398)	3.45 ± 1.12	6.24 ± 1.01
		LC_{50}[c] (ng/cm_2)			
M. sexta	Cry1Ab9-033	19.5 (15–27)		2.98 ± 1.03	4.78 ± 1.31
	Cry1Ab2	7.5 (4.5–10)		3.12 ± 1.14	4.98 ± 1.29

[a] Kcom, dissociation constant calculated from homologous competition binding assays. Each value is the mean of three experiments.

[b] Bmax, binding site concentration calculated from homologous competition binding assays. Each values is the mean of three experiments.

[c] LC_{50}, 50% lethal concentration of toxin to neonate larvea. 95% confidence limits are in parentheses.

[d] ID_{50}, dose of toxins that causes growth inhibition in 50% of fourth instar *L. dispar* larvae assayed. 95% confidence limits are in parentheses.

equivalent to the term Kd used in previous studies (3, 4, 7, 9, 13, 15, 16, 18). Binding site concentrations for these toxins were comparable (Table 1). These competition binding data are well correlated with toxicity assay data. The higher toxicity of Cry1Ab2 to *L. dispar* might be due to the higher binding affinity compared to Cry1Ab9-033.

Heterologous competition assays were also performed to investigate whether Cry1Ab2 and Cry1Ab9-033 recognize same binding sites (Fig. 2). Unlabeled Cry1Ab9-033 and Cry1Ab α8 competed for the binding of labeled Cry1Ab2 suggesting that these toxins share same binding

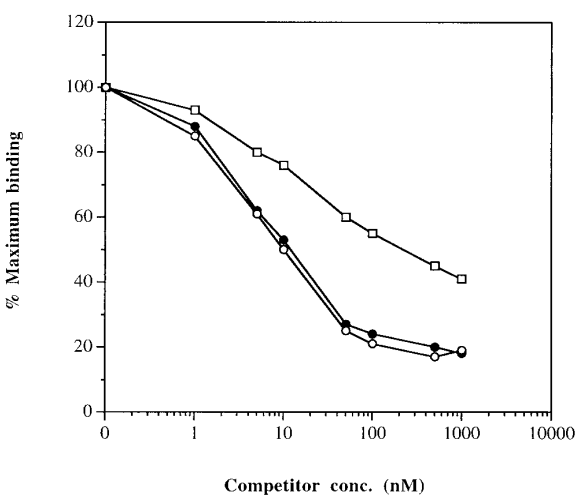

FIG. 2. Competitive binding of ^{125}I-labeled Cry1Ab2 toxin to *L. dispar* BBMV as a function of the concentration of unlabeled Cry1Ab2, Cry1Ab9-033, and Cry1Ab α8 toxins. ^{125}I-labeled Cry1Ab2 (2 nM) was incubated with 20 μg of BBMV in the presence of increasing concentration of unlabeled Cry1Ab2 (○), Cry1Ab9-033 (□), and Cry1Ab α8 toxins (●). Binding is expressed as a percentage of the amount bound upon incubation with labeled toxin alone.

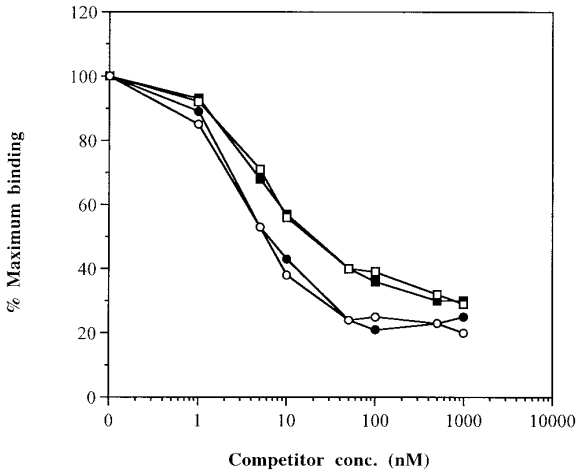

FIG. 3. Binding of ^{125}I-labeled Cry1Ab2 and Cry1Ab9-033 toxins to *M. sexta* and *S. exigua* BBMV. 2 nM ^{125}I-labeled Cry1Ab2 and Cry1Ab9-033 toxins was incubated with 20 μg of BBMV in the presence of increasing concentration of unlabeled Cry1Ab2 (○, *M. sexta;* □, *S. exigua*) and Cry1Ab9-033 (●, *M. sexta;* ■, *S. exigua*). Binding is expressed as a percentage of the amount bound upon incubation with labeled toxin alone.

sites. Heterologous competition assays with labeled Cry1Ab9-033 and unlabeled Cry1Ab2 or Cry1Ab α8 also showed cross competition among these toxins (data not shown).

Toward *M. sexta,* no significant difference was observed in binding affinity between Cry1Ab2 and Cry1Ab9-033, although there was a 2.5 fold difference in toxicity (Fig. 3, Table 1). The Cry1Ab2 and Cry1Ab9-033 bound to *S. exigua* BBMV specifically with similar binding affinities although these toxins do not show high toxicity to this insect (Fig. 3). These data suggest that Gly and Ser residues in the α8 loop region might be important in receptor binding and subsequent toxicity in *L. dispar,* but not in *M. sexta* or *S. exigua.* Possibly, *M. sexta* and *S. exigua* midgut receptors recognize a region of Cry1Ab distinct from the *L. dispar* receptor binding region.

Dissociation binding assays were performed to examine whether different ability of irreversible binding (insertion of the toxins into the BBMV membrane) is the factor for the different toxicity. It was observed that about 10-15% of Cry1Ab2, Cry1Ab9-033, and Cry1Ab α8 toxins were dissociated from the BBMV (Fig. 4). A previous study with *M. sexta* BBMV demonstrated that about 20-25 % of bound Cry1Ab (our Cry1Ab9-033) was dissociated from the binding site (16). No measurable differences among Cry1Ab2, Cry1Ab9-033, and Cry1Ab α8 were observed in dissociation binding assays, suggesting that the irreversible binding step might not be altered in Cry1Ab9-033. Therefore, we can eliminate the possibility that differences in the irreversible binding property might determine the toxicity to *L. dispar.*

Identification of toxin binding proteins. To identify toxin binding proteins in *L. dispar* BBMV, BBMV ligand blotting experiments were performed. The results showed that both Cry1Ab2 and Cry1Ab9-033 recognized a 210 kDa BBMV molecule (Fig. 5). These data suggest that substitutions of Gly and Ser residues with Ala and Leu residues do not affect receptor recognition. Although alterations of these residues significantly reduce the binding affinity (Table 1), these differences may not be enough to be detected in the ligand blotting. Combined with heterologous competition assay data, the possibility that Cry1Ab2 and Cry1Ab9-033 toxins might recognize different receptors can be eliminated.

Stability of toxins. To eliminate the possibility that stability differences might account for differences in toxicity and receptor binding, stability of Cry1Ab2 and Cry1Ab9-033 toxins by

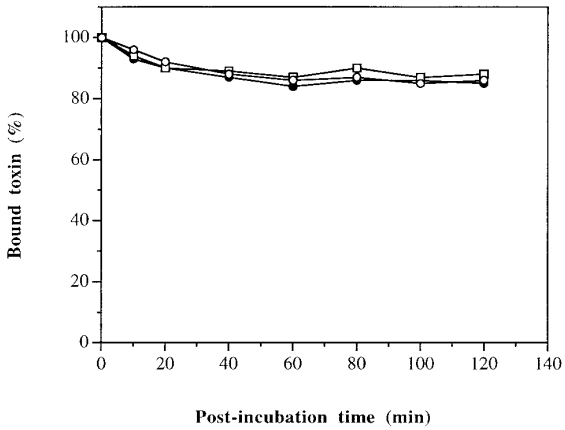

FIG. 4. Dissociation of bound ^{125}I-labeled toxins from *L. dispar* BBMV. 2 nM ^{125}I-labeled Cry1Ab2 (○), Cry1Ab9-033 (□), and Cry1Ab α8 toxins (●) was incubated with 20 μg of BBMV for 1h. After incubation, the mixtures were diluted twofold in binding buffer containing 1 μM corresponding unlabeled toxins. The reaction was stopped at different time points by centrifugation. Nonspecific binding was subtracted from total binding.

proteases from gut juice extract was examined. As seen in Figure 6A, Cry1Ab9-033 was as stable under digestion with gut juice as was Cry1Ab2. However, about a 45 kDa peptide was detected in both toxins after digestion with gut juice. Stability of ^{125}I-labeled Cry1Ab2 and Cry1Ab9-033 toxins during incubation with BBMV proteins, containing various proteases, was also examined (Fig. 6B). BBMV bound ^{125}I-labeled Cry1Ab2 and Cry1Ab9-033 showed no degradation in the 65 kDa toxin band. Therefore, stability differences can not explain the reduced toxicity and binding of Cry1Ab9-033.

Previously, we observed that Cry1Aa recognized a 210 kDa *L. dispar* BBMV protein, while Cry1Ac toxin bound to the 120 kDa aminopeptidase N (19, 32, 33). Hybrid toxins which have domain III of either Cry1Aa or Cry1Ac bound to the 210 kDa or the 120 kDa BBMV proteins, respectively (19). These data suggest that domain III of Cry1Aa and Cry1Ac might be the determining factor for receptor recognition in *L. dispar*. Cry1Ab is a natural hybrid toxin between Cry1Aa and Cry1Ac, sharing a highly similar domain I and II with Cry1Ac but a similar domain III with Cry1Aa. Since Cry1Aa and Cry1Ab are more toxic than Cry1Ac to *L. dispar* (27) and bind to the same 210 kDa BBMV protein (32), which is distinct from 120

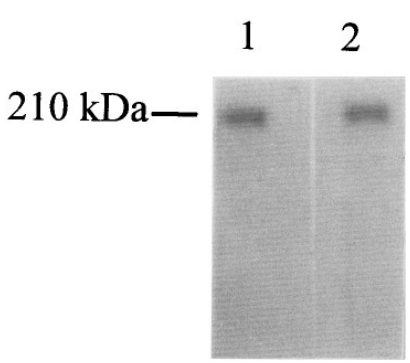

FIG. 5. Binding of ^{125}I-labeled Cry1Ab2 (lane 1) and Cry1Ab9-033 (lane 2) toxins to protein blots of *L. dispar* BBMV proteins. A total of 25 μg of BBMV protein was separated onto SDS-7.5% PAGE, transferred to PVDF membrane, and probed with 5 nM ^{125}I-labeled toxins.

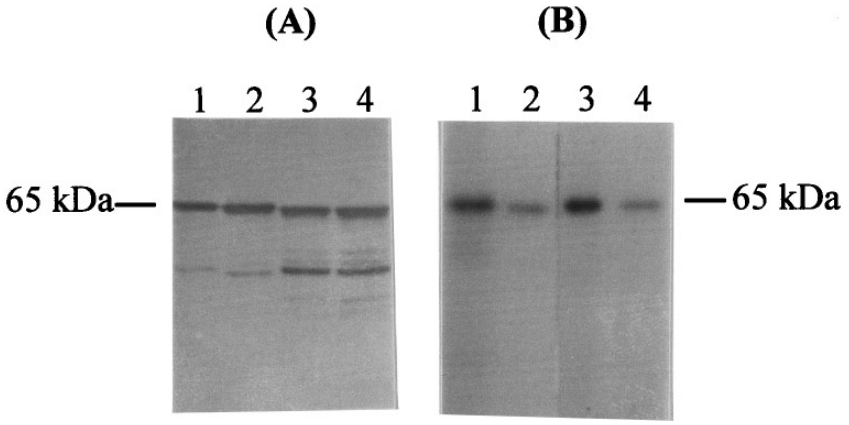

FIG. 6. (A) Stability of Cry1Ab2 and Cry1Ab9-033 toxins by digestion with *L. dispar* gut juice. 500 ng of Cry1Ab2 (lane 3) and Cry1Ab9-033 (lane 4) toxins was digested with 2 μl of gut juice and incubated at 37°C for 2 hr. Lanes 1 and 2 represent 500 ng of Cry1Ab2 and Cry1Ab9-033 toxins alone. Reaction mixtures were separated onto SDS-10% PAGE, transferred to PVDF membrane, and incubated with Cry1Ab2 antibody for 1 hr. Toxin bands were visualized as described under Materials and Methods. (B) Stability of ^{125}I-labeled Cry1Ab2 and Cry1Ab9-033 toxins by incubation with *L. dispar* BBMV. Lanes 1 and 3 represent 2 nM ^{125}I-labeled Cry1Ab2 and Cry1Ab9-033 toxins. 20 μg BBMV protein was incubated with 2 nM ^{125}I-labeled Cry1Ab2 (lane 2) and Cry1Ab9-033 (lane 4) toxins and bound toxins were separated onto SDS-10% PAGE. The gel was dried and exposed to Fuji-RX film for 1-2 days.

kDa Cry1Ac receptor, it is plausible to predict that domain III might be more important than other domains in determining binding and subsequent toxicity to *L. dispar*. However, in this study, we have observed that amino acid residues 282 and 283 in the α8 loop of domain II are important in the initial binding of toxin to receptor, since alteration of these residues significantly reduces binding affinity to the *L. dispar* brush border membrane.

Earlier studies have shown that the loop 2 region in domain II of Cry1Aa is involved in initial binding to the receptor on *B. mori* midgut membrane (15). Similarly, mutations in the loop 1 and 3 regions in domain II of Cry3A affect toxicity to *Tenebrio molitor* by altering initial binding to the receptor or membrane insertion (30). Mutations in the loop 2 residues in domain II of Cry1Ab toxin likewise reduce toxicity dramatically by reducing either binding affinities or irreversible binding to *M. sexta* and *H. virescens* (16). Mutations in domain III of Cry1Ac toxin alter the binding property to *M. sexta* larvae (21). Also domain III switch experiments have demonstrated that domain III determines toxicity or receptor recognition against target insects (17, 19). Therefore, receptor binding regions on *B. thuringiensis* toxin might be broad and extend into several regions of domain II and III.

In this study, we examined the role of two residues (Gly282 and Ser283) in the α8 loop region of domain II of Cry1Ab2 toxin in receptor binding and toxicity to *L. dispar*. It was observed that these residues are involved in high affinity binding and subsequent high potency. Other experimental data from heterologous competition, ligand blotting, dissociation binding, and protease stability assays eliminate the possibilities that other factors besides binding affinity might account for differences in toxicity. Further studies of mutations in other residues in this loop and mutations in other toxins will be examined with different insects to extend our knowledge about its functional role.

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