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INVERTEBRATE MICROBIOLOGY

Enhancement of *Bacillus thuringiensis* Cry1Ab and Cry1Fa Toxicity to *Spodoptera frugiperda* by Domain III Mutations Indicates There Are Two Limiting Steps in Toxicity as Defined by Receptor Binding and Protein Stability

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ABSTRACT *Bacillus thuringiensis* Cry1Ab and Cry1Fa toxins are environmentally safe insecticides that control important insect pests. *Spodoptera frugiperda* is an important maize pest that shows low susceptibility to Cry1A toxins, in contrast to Cry1Fa, which is highly active against this pest and is used in transgenic maize for *S. frugiperda* control. The β16 region from domain III of Cry1Ab has been shown to be involved in interactions with receptors such as alkaline phosphatase (ALP) or aminopeptidase (APN) in different lepidopteran insects. Alanine-scanning mutagenesis of amino acids of Cry1Ab β16 (^{509}STLRVN^{514}) revealed that certain β16 mutations, such as N514A, resulted in increased toxicity of Cry1Ab for *S. frugiperda* without affecting the toxicity for other lepidopteran larvae, such as *Manduca sexta* larvae. Exhaustive mutagenesis of N514 was performed, showing that the Cry1Ab N514F, N514H, N514K, N514L, N514Q, and N514S mutations increased the toxicity toward *S. frugiperda*. A corresponding mutation was constructed in Cry1Fa (N507A). Toxicity assays of wild-type and mutant toxins (Cry1Ab, Cry1AbN514A, Cry1AbN514F, Cry1Fa, and Cry1FaN507A) against four *S. frugiperda* populations from Mexico and one from Brazil revealed that Cry1AbN514A and Cry1FaN507A consistently showed 3- to 18-fold increased toxicity against four of five *S. frugiperda* populations. In contrast, Cry1AbN514F showed increased toxicity in only two of the *S. frugiperda* populations analyzed. The mutants Cry1AbN514A and Cry1AbN514F showed greater stability to midgut protease treatment. In addition, binding analysis of the Cry1Ab mutants showed that the increased toxicity correlated with increased binding to brush border membrane vesicles and increased binding affinity for *S. frugiperda* ALP, APN, and cadherin receptors.

IMPORTANCE *Spodoptera frugiperda* is the main maize pest in South and North America and also is an invasive pest in different African countries. However, it is poorly controlled by *Bacillus thuringiensis* Cry1A toxins expressed in transgenic crops, which effectively control other lepidopteran pests. In contrast, maize expressing Cry1Fa is effective in the control of *S. frugiperda*, although its effectiveness is being lost due to resistance evolution. Some of the Cry1Ab domain III mutants characterized here show enhanced toxicity for *S. frugiperda* without loss of toxicity to *Man-*

Received 7 June 2018 **Accepted** 1 August 2018

Accepted manuscript posted online 10 August 2018

Citation Gómez I, Ocelotl J, Sánchez J, Lima C, Martins E, Rosales-Juárez A, Aguilar-Medel S, Abad A, Dong H, Monnerat R, Peña G, Zhang J, Nelson M, Wu G, Bravo A, Soberón M. 2018. Enhancement of *Bacillus thuringiensis* Cry1Ab and Cry1Fa toxicity to *Spodoptera frugiperda* by domain III mutations indicates there are two limiting steps in toxicity as defined by receptor binding and protein stability. Appl Environ Microbiol 84:e01393-18. https://doi.org/10.1128/AEM.01393-18.

Editor Janet L. Schottel, University of Minnesota

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duca sexta. Thus, these Cry1Ab mutants could provide useful engineered toxins that, along with other Cry toxins, would be useful for developing transgenic maize expressing stacked proteins for the effective control of *S. frugiperda* and other lepidopteran pests in the field.

KEYWORDS *Bacillus thuringiensis*, Cry toxins, receptor binding, *Spodoptera frugiperda*

Bacillus thuringiensis (Bt) Cry toxins are valuable proteins due to their insect pest control capacity. An important variety of Cry toxins with specific toxicity for different insect orders and other invertebrates have been described, and a few *cry* genes have been introduced into the genomes of important crops, producing transgenic plants that resist insect attack (1, 2). However, Cry toxins have pest control activity for a limited number of insects, and in several cases toxicity is not sufficient to control important insect pests (3).

Cry1Ab and Cry1Ac proteins are Bt toxins that kill some key Lepidoptera insects, such as the three main cotton pests, *Heliothis virescens*, *Pectinophora gossypiella*, and *Helicoverpa armigera*, as well as some maize insect pests, such as *Helicoverpa zea* and *Ostrinia nubilalis* (4). *Spodoptera frugiperda* is the main maize pest in South and North America but also is a polyphagous pest that feeds on a variety of crops besides maize (5, 6). In addition, *S. frugiperda* is a recent invasive pest in different African countries, spreading rapidly and jeopardizing maize, millet, and sorghum production (7). Cry1Ab and Cry1Ac toxins show low toxicity for *S. frugiperda* (5, 6), although *S. frugiperda* is susceptible to other Cry toxins, such as Cry1Fa, and maize plants expressing Cry1Fa have been shown to be effective in controlling this pest (6). However, *S. frugiperda* quite rapidly evolved resistance to Cry1Fa-maize under field conditions in Puerto Rico, Brazil, and the United States, endangering the use of transgenic plants to control this pest (7–11).

The toxicity of Cry1A for insect pests depends on its interaction with different types of larval midgut proteins, such as cadherin (CAD), aminopeptidase N (APN), or alkaline phosphatase (ALP), among others (12). It is proposed that Cry1A toxins bind first to the highly abundant APN or ALP proteins, to concentrate them in the midgut epithelium (13, 14). Then Cry1A toxins bind to CAD, which facilitates the removal of α-helix 1 from their amino-terminal regions and induces toxin oligomerization (15). Oligomers bind again to APN or ALP, resulting in toxin insertion into the midgut cell membrane and leading to osmotic shock and cellular death (13, 16). The tridimensional structure of protease-activated Cry1Aa toxin, which is closely related to Cry1Ab, was solved and shown to involve three structural domains (17). Domain I, composed of a bundle of seven α-helices, is involved in pore formation and oligomerization of the toxin. Domain II, composed of antiparallel β-sheets forming a β-prism structure with exposed loops, is involved in the interactions with midgut proteins of susceptible insects; domain III, an antiparallel β-sheet sandwich, also is involved in the binding interactions with midgut receptors (1, 2). Cry1Aa and Cry1Ab toxins interact with APN or ALP through domain III β16 and β22 regions (13, 14, 18, 19). In the case of Cry1Ab, a β16 (L511A) mutation affected ALP binding but not APN binding, and the mutant was not toxic to *Manduca sexta* larvae (14). Interestingly, resistance to Cry1Ac in *H. virescens* and *H. armigera*, as well as resistance to Cry1Fa in *S. frugiperda*, was shown to correlate with reduced levels of ALP expression (20). All of these data indicate that ALP binding is a limiting step for Cry1 toxicity in different insect species.

Low sensitivity of some insect pests to the Cry1A toxins could be due to limited receptor binding and/or low stability against midgut proteases. In this work, we show that certain Cry1Ab or Cry1Fa domain III β16 amino acid substitutions increased their insecticidal activity against four of five *S. frugiperda* populations from two different countries. Analysis of binding in one of those *S. frugiperda* populations showed that the enhanced insecticidal activity of Cry1Ab domain III mutants correlated with their increased binding to *S. frugiperda* brush border membrane vesicles (BBMV) and to ALP, APN, or CAD recombinant proteins. Also, in the case of Cry1Ab β16 mutants, enhanced

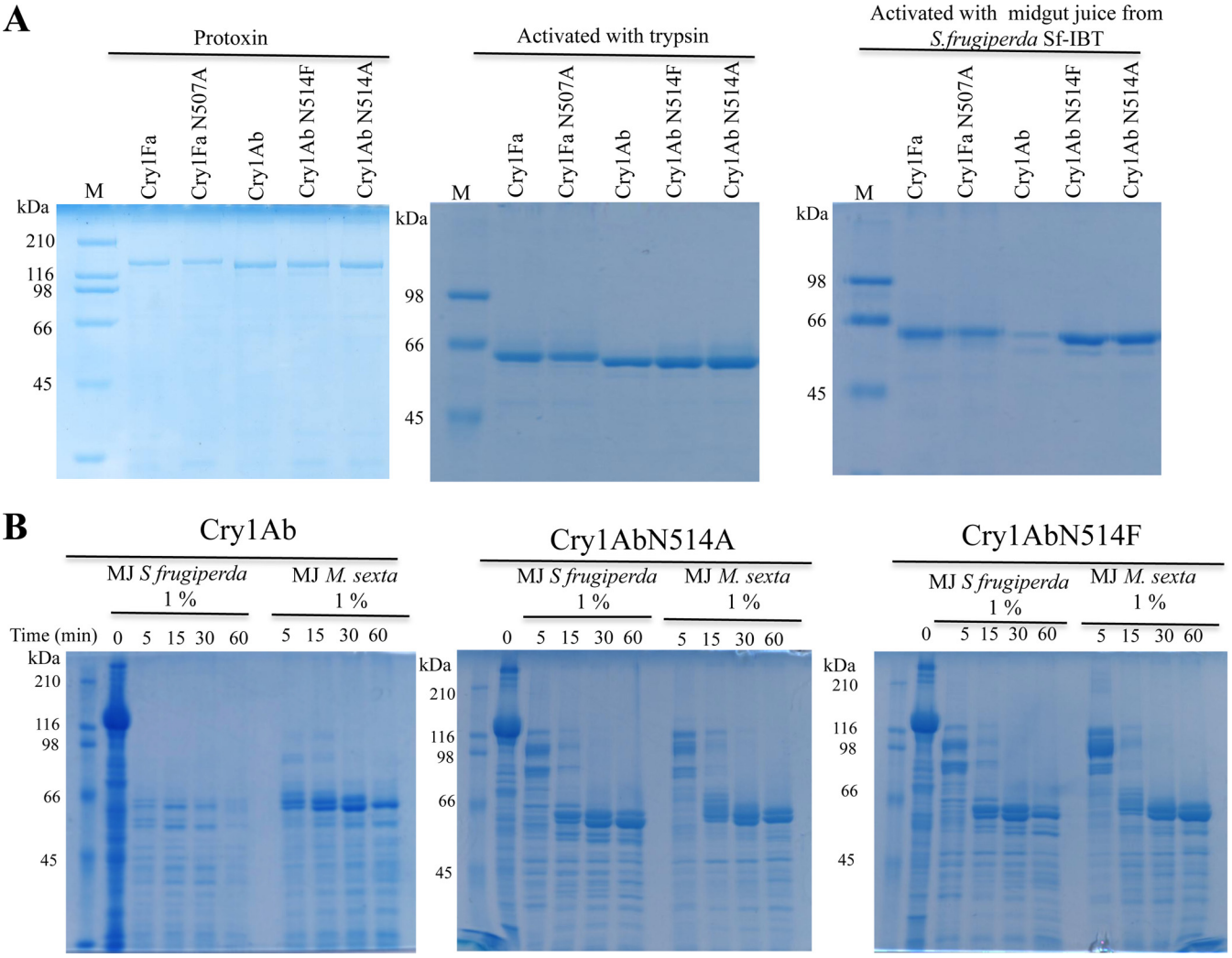

FIG 1 Enhanced toxicity of Cry1Ab domain III mutants correlating with enhanced stability to midgut juice treatment. (A) SDS-PAGE of protoxins and toxins activated with trypsin or with midgut juice from Sf-IBT. M, size markers. (B) Kinetic analysis of the treatment of Cry1Ab and domain III mutant protoxins with midgut juice (MJ) from *S. frugiperda* (Sf-IBT) or *M. sexta*.

stability to protease treatment was observed. These data suggest that two steps, one related to receptor binding and the other to protein stability, are limiting steps in Cry1Ab toxicity to *S. frugiperda*. Some of the Cry1Ab domain III mutants characterized here could provide useful engineered insecticidal Cry toxins for *S. frugiperda* control in the field.

RESULTS

Cry1Ab β16 alanine substitutions yield increased insecticidal activity against *S. frugiperda*. Cry1Ab β16 (^{509}STLRVN^{514}) has been shown to be involved in *M. sexta* APN and ALP binding (14, 19). Previously, we constructed and characterized a Cry1Ab β16 L511A mutant, which showed loss of toxicity to *M. sexta* larvae and reduced binding to *M. sexta* ALP (14). To determine the role in toxicity of different amino acid residues in the Cry1Ab β16 region, we mutated all residues of β16 (i.e., S509A, T510A, R512A, V513A, and N514A). All mutants were produced in Bt, and the production of the 130-kDa protoxin was analyzed by SDS-PAGE. All mutants produced the 130-kDa Cry1Ab protoxin and a 60-kDa protein activated with trypsin (Fig. 1A).

The insecticidal activity of domain III mutants was analyzed against *M. sexta* and one *S. frugiperda* population that had been shown previously not to be sensitive to Cry1Ab (Sf-IBT) (5). Table 1 shows that only the L511A substitution characterized previously

TABLE 1 Insecticidal activity of Cry1Ab and domain III mutant toxins

Toxin	LC_{50} (fiducial limits) (ng/cm²) for[a]:	
	Manduca sexta	*Spodoptera frugiperda* (Sf-IBT)
Cry1Ab	3.4 (2.6–4.3)	>5,000
Cry1AbS509A	5.7 (4.6–7.2)	526 (381–751)
Cry1AbT510A	31.9 (14.7–202)	>5,000
Cry1AbL511A	>1,000	>5,000
Cry1AbR512A	6.5 (5–9)	>5,000
Cry1AbV513A	45 (26–105)	393 (296–523)
Cry1AbN514A	5.7 (4.5–7.3)	98 (54–148)

[a]Values are means of at least 3 repetitions; fiducial limits are 95% confidence intervals.

affected the toxicity toward *M. sexta* significantly, reducing its activity at least 100-fold in comparison to the wild-type Cry1Ab (14). Some Cry1Ab domain III substitutions, such as T510A and V513A, moderately reduced the insecticidal toxicity (8- to 10-fold), while other mutants (S509A, R512A, and N514A) showed similar or slightly lower toxicity (3- to 4-fold) toward *M. sexta* larvae, compared to Cry1Ab. Regarding their toxicity toward *S. frugiperda* Sf-IBT, we found that the S509A, V513A, and N514A mutants showed significantly greater toxicity than the Cry1Ab toxin, which was nontoxic at the highest dosage used (5,000 ng/cm²). The β16 N514A mutant showed the largest increment in toxicity toward *S. frugiperda* (Table 1).

Since the N514A mutant showed a significant increase in toxicity for Sf-IBT, with toxicity similar to that of *M. sexta*, we decided to mutagenize to saturation the N514 residue. Nine additional N514 substitutions were recovered and characterized. Insecticidal activity against Sf-IBT showed that the N514I and N514M mutants were not toxic to Sf-IBT, while N514 mutants with F, H, K, L, Q, S, and V substitutions showed significant toxicity; the N514F, N514Q, and N514S mutants showed the highest toxicity (Table 2). The Cry1AbN514A and Cry1AbN514F mutants were further characterized as representative mutants with high toxicity against *S. frugiperda* (Tables 1 and 2).

To further characterize the toxicity of the Cry1AbN514A and Cry1AbN514F mutants for *S. frugiperda*, the toxicity of these mutant proteins and of Cry1Ab was analyzed in three other *S. frugiperda* populations isolated from different geographical regions in Mexico. Table 3 shows that Cry1Ab showed low toxicity for the three *S. frugiperda* populations, with 50% lethal concentration (LC_{50}) values that ranged between 482 and 1,590 ng/cm² against these three *S. frugiperda* populations (Sf-Valle del Fuerte, Sf-La Laguna, and SfS-Mex). Cry1AbN514A showed 3-fold higher toxicity than Cry1Ab against the Sf-Valle del Fuerte population, 10-fold higher toxicity than Cry1Ab against the SfS-Mex population, and toxicity similar to that of Cry1Ab against the Sf-La Laguna population (Table 3). Cry1AbN514F showed an apparent trend of higher toxicity against the Sf-Valle del Fuerte population, compared to Cry1Ab, but this difference was not statistically significant since their fiducial limits overlapped (Table 3). The Cry1AbN514F mutant showed apparently lower toxicity than Cry1Ab against the Sf-La Laguna pop-

TABLE 2 Insecticidal activity of different Cry1AbN514 mutants

Toxin	LC_{50} (fiducial limits) (ng/cm²) for[a]:	
	Manduca sexta	*Spodoptera frugiperda* (Sf-IBT)
Cry1Ab	4 (2.6–4.6)	>2,000
Cry1AbN514F	4 (2.2–4.6)	45 (20–70)
Cry1AbN514H	2 (0.9–2.7)	125 (93–167)
Cry1AbN514I	3 (2.1–5.1)	>2,000
Cry1AbN514K	4 (2.4–6.5)	282 (186–520)
Cry1AbN514L	4 (2.2–5.5)	222 (160–325)
Cry1AbN514M	2 (0.8–2.4)	>2,000
Cry1AbN514Q	3 (1.7–5.3)	77 (51–110)
Cry1AbN514S	2 (0.5–1.9)	86 (48–122)
Cry1AbN514V	2 (0.7–2.3)	226 (120–700)

[a]Values are means of at least 3 repetitions; fiducial limits are 95% confidence intervals.

TABLE 3 Toxicity of Cry1Ab and Cry1Fa and domain III mutants against different *S. frugiperda* populations from Mexico and Brazil

	LC_{50} (fiducial limits) (ng/cm^2) for[a]:				
Population	**Cry1Ab**	**Cry1AbN514A**	**Cry1AbN514F**	**Cry1Fa**	**Cry1FaN507A**
Sf-Valle del Fuerte	1,420 (752–3,730)	446 (329–636)	393 (233–755)	171 (104–347)	28.7 (24.6–33.5)
Sf-La Laguna	482 (326–802)	355 (275–474)	1,250 (694–3,040)	81.4 (62.8–115)	50.7 (39.1–68.9)
SfS-Mex	1,590 (744–5,480)	152 (125–189)	221 (160–324)	135 (69.6–428)	24.5 (16.9–35.5)
SfLab-Brazil	468 (330–763)	26.4 (0.8–80.5)	374 (158–1,170)	149 (81.6–243)	36.1 (2.4–97.9)

[a]Values are means of at least 3 repetitions; fiducial limits are 95% confidence intervals.

ulation, but again this difference was not statistically significant since their fiducial limits overlapped. In the case of the SfS-Mex population only, the Cry1AbN514F protein showed 7-fold higher toxicity than Cry1Ab (Table 3).

We also analyzed the toxicity of the Cry1Ab mutants to a susceptible population from Brazil (SfLab-Brazil). Table 3 shows that SfLab-Brazil was moderately susceptible to Cry1Ab, showing an LC_{50} of 468 ng/cm^2. The Cry1AbN514A mutant showed almost 18-fold higher toxicity than Cry1Ab against this population from Brazil (Table 3). In the case of Cry1AbN514F, no difference in toxicity was observed (Table 3).

Cry1Fa toxin is used in transgenic maize for the control of *S. frugiperda*. Domain III residue N514 of Cry1Ab is conserved in Cry1Fa (corresponding to residue N507 of Cry1Fa). To determine the effect of N507A mutations in Cry1Fa, we analyzed the toxicity of the Cry1FaN507A mutant against the three Mexican *S. frugiperda* populations that were maintained in the Universidad Autónoma del Estado de México (see Materials and Methods). Table 3 shows that wild-type Cry1Fa was more toxic to the three *S. frugiperda* populations than Cry1Ab, showing 8-fold higher toxicity against the Sf-Valle del Fuerte population, 6-fold higher toxicity against the Sf-La Laguna population, and 11-fold higher toxicity against the SfS-Mex population. The Cry1FaN507A mutant showed 6-fold higher toxicity than the Cry1Fa toxin against the Sf-Valle del Fuerte population, toxicity similar to that of Cry1Fa against the Sf-La Laguna population, and 5.5-fold higher toxicity than Cry1Fa against the SfS-Mex population (Table 3). Finally, the Cry1Fa domain III mutant was also analyzed against the SfLab-Brazil population. Table 3 shows that the Cry1FaN507A mutant showed 4-fold higher toxicity than Cry1Fa; however, this was not a significant difference since the fiducial limits overlapped.

Increased toxicity of Cry1Ab and β16 mutants for *S. frugiperda* correlates with increased stability to *S. frugiperda* gut protease treatment. To determine the effect of domain III mutations on the stability of the mutant toxins to protease treatment, protoxins of Cry1Ab, Cry1AbN514A, and Cry1AbN514F were treated with trypsin or with midgut juice from *S. frugiperda* (Sf-IBT). Figure 1A shows that all proteins yielded a 60-kDa protein after treatment with trypsin. However, after treatment with midgut juice from Sf-IBT, Cry1Ab was barely observed, while Cry1AbN514A and Cry1Ab514F yielded 60-kDa bands, suggesting that these β16 mutants were more stable to the *S. frugiperda* midgut proteases. In the case of Cry1Fa and Cry1Fa507A, no differences were observed between these two proteins, since they yielded similar 60-kDa proteins after treatment with midgut juice from Sf-IBT (Fig. 1A). To better correlate the protein stability of Cry1Ab domain III mutants during treatment with midgut juice with their insecticidal activity, a kinetic analysis of the treatment of the Cry1Ab and domain III protoxins with the midgut juices from *S. frugiperda* (Sf-IBT) or *M. sexta* was performed. Figure 1B shows that, upon treatment with *S. frugiperda* midgut juice, the Cry1Ab protoxin of 130 kDa was rapidly processed into a 60-kDa band that was barely observed after 60 min of treatment. In contrast, upon treatment of the Cry1Ab protoxin with *M. sexta* midgut juice, Cry1Ab showed greater stability, since the 60-kDa band was clearly observed after 60 min (Fig. 1B). When both Cry1Ab domain III mutant protoxins, Cry1AbN514A and Cry1AbN514F, were assayed, they showed greater stability to the treatment with *S. frugiperda* midgut juice, since the 60-kDa band was clearly observed after 60 min and bands of greater molecular size were also observed at shorter times with this treatment (Fig. 1B). In the case of the N514F mutant, however, greater amounts of the 60-kDa

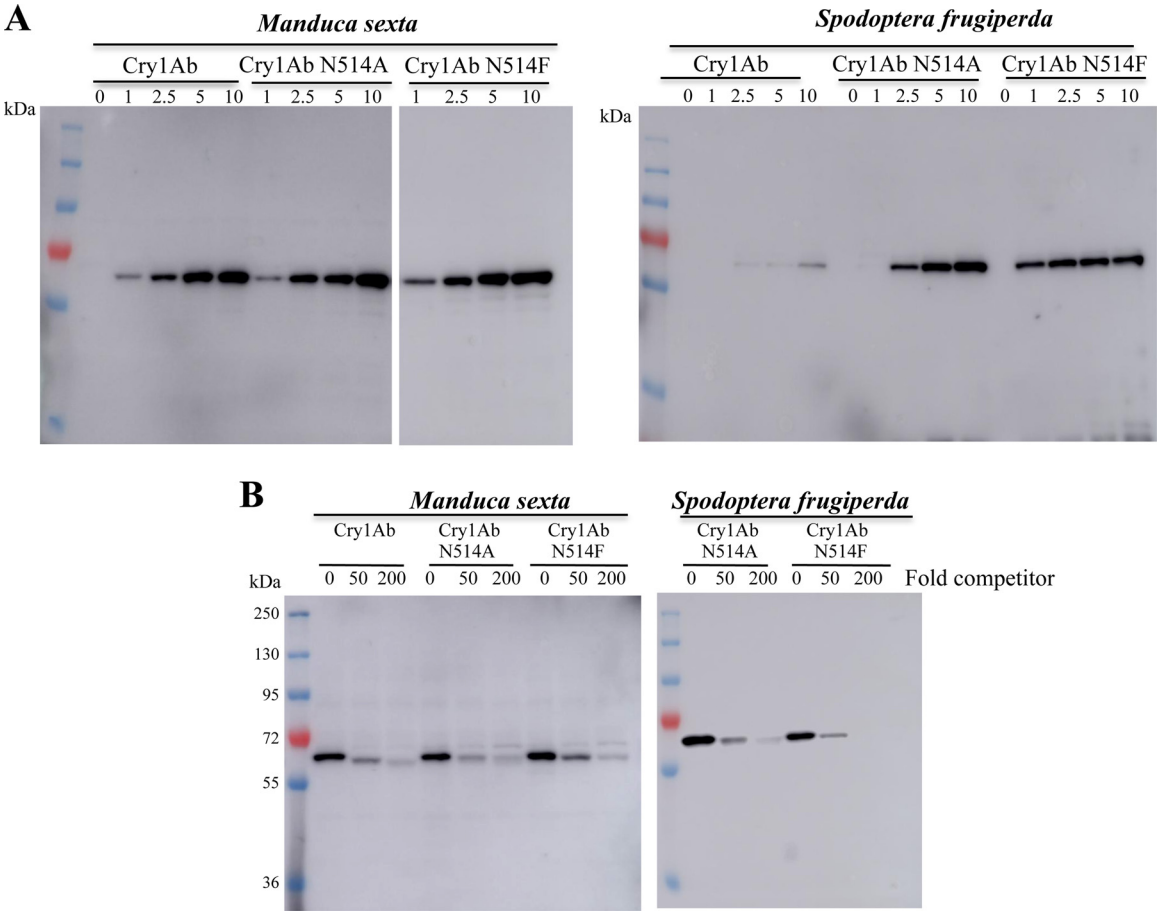

FIG 2 Enhanced toxicity of Cry1Ab domain III mutants correlating with enhanced binding to *S. frugiperda* BBMV. (A) Binding of Cry1Ab toxins to BBMV from *S. frugiperda* Sf-IBT or to BBMV from *M. sexta*. Ten micrograms of BBMV protein was incubated with increasing concentrations (in nanomolar) of biotin-labeled Cry1Ab or domain III-activated toxins, and BBMV were recovered by centrifugation. Unbound toxin was washed out, and the toxin bound to the BBMV was separated by SDS-PAGE (10% acrylamide), electrotransferred to a PVDF membrane, and finally detected by using streptavidin-horseradish peroxidase. (B) Homologous binding competition. Biotin-labeled Cry1Ab or domain III mutants (5 nM) were incubated with BBMV in the presence of a 50-fold or 200-fold excess of the corresponding unlabeled toxin. Results shown are representative of at least three replicates.

band were observed after 30 min, compared to 60 min, of treatment with *S. frugiperda* midgut juice. This is likely due to further protein degradation by the midgut juice. The treatment with *M. sexta* midgut juice also suggested greater stability of both Cry1Ab domain III mutants, since the 130-kDa protoxin band was observed with shorter times of treatment, in contrast to the Cry1Ab protoxin, which was rapidly digested (Fig. 1B). These data indicate that protoxin stability to *S. frugiperda* midgut juice correlates with the increased toxicity of Cry1Ab domain III mutants.

Increased toxicity of Cry1Ab β16 mutants for *S. frugiperda* correlates with increased binding to BBMV. To analyze the effects of Cry1Ab β16 mutations on binding, we performed qualitative assays of the binding of Cry1Ab and the β16 N514A and N514F mutants to BBMV isolated from *M. sexta* and *S. frugiperda* (Sf-IBT strain) larvae. We selected to work with this *S. frugiperda* population since it showed the lowest susceptibility to Cry1Ab. Figure 2A shows that Cry1Ab did not bind to Sf-IBT BBMV, correlating with the lack of toxicity of Cry1Ab against this *S. frugiperda* colony. Interestingly, the Cry1Ab β16 N514A and N514F mutants bound to Sf-IBT BBMV (Fig. 2A). Figure 2 also shows that the three proteins (Cry1Ab, Cry1AbN514A, and Cry1AbN514F) bound to BBMV isolated from *M. sexta* larvae. Homologous competition binding assays showed that these interactions were specific, since the binding of Cry1Ab and the β16 N514A and N514F mutants to BBMV from *M. sexta* and the binding

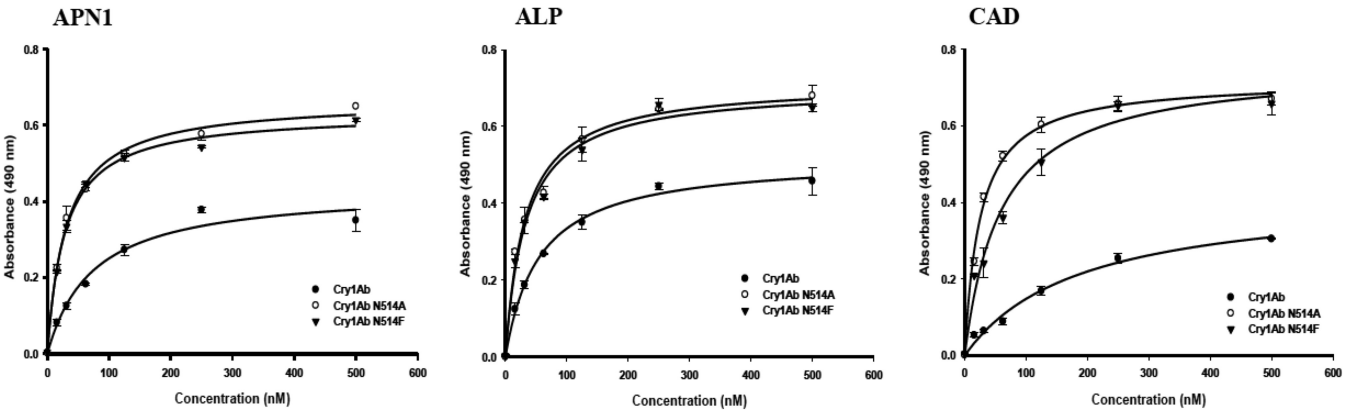

FIG 3 ELISA analysis of binding of Cry1Ab toxins to recombinant APN1, ALP, and CAD receptors. Enhanced toxicity of Cry1Ab domain III mutants correlated with enhanced binding to *S. frugiperda* CAD, ALP, and APN proteins. A total of 2.5 μg of each protein was fixed to ELISA plates, the plates were incubated with different concentrations of Cry1Ab or Cry1Ab domain III mutants, and bound toxins were revealed with anti-Cry1Ab antibody and anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase. Results are means of three repetitions.

of the N514A and N514F mutants to BBMV from *S. frugiperda* were competed with an excess of the corresponding unlabeled toxin (Fig. 2B).

Increased toxicity of Cry1Ab β16 mutants for *S. frugiperda* correlates with increased receptor binding. As mentioned previously, The β16 Cry1AbL511A mutant is affected in ALP binding and toxicity against *M. sexta* (14). To determine whether the β16 mutants that gained toxicity to *S. frugiperda* increased their binding to *S. frugiperda* receptors, we cloned APN1 and ALP receptors and a CAD fragment containing the CAD repeats (CRs) CR7 to CR12 from the Sf-IBT strain, as described in Materials and Methods. These recombinant proteins were produced in *Escherichia coli* cells and used to perform binding assays. Figure 3 shows enzyme-linked immunosorbent assay (ELISA) results for Cry1Ab, Cry1AbN514A, and Cry1AbN514F binding to APN1, ALP, or the CR7-CR12 CAD fragment. Both Cry1AbN514A and Cry1AbN514F showed increased binding affinity for the three *S. frugiperda* proteins, showing 4- to 7-fold increased apparent binding affinity for the three receptor molecules, in comparison with Cry1Ab (Fig. 3). In the case of APN1 binding, Cry1Ab showed an apparent binding affinity (K_d) of 147 nM, while the Cry1AbN514A and Cry1AbN514F mutants showed K_d values of 37 and 24 nM, respectively. In the case of ALP binding, Cry1Ab showed a K_d of 90 nM, while the Cry1AbN514A and Cry1AbN514F mutants showed K_d values of 24 nM and 15 nM, respectively. In the case of CAD binding, Cry1Ab showed a K_d of 147 nM, while the Cry1AbN514A and Cry1AbN514F mutants showed K_d values of 21 nM and 29 nM, respectively.

DISCUSSION

S. frugiperda is a pest that shows variable activity against host crops, and different populations vary in their susceptibility to different Cry1 toxins (21). Here we analyzed four *S. frugiperda* populations from Mexico and one population from Brazil regarding their susceptibility to Cry1Ab and Cry1Fa toxins and to several domain III mutants. Our data showed that all *S. frugiperda* populations analyzed were more sensitive to Cry1Fa than to Cry1Ab toxin; however, the sensitivity to Cry1Ab varied significantly among the populations. One *S. frugiperda* population (Sf-IBT) was not sensitive to Cry1Ab, as reported previously (5), while other Mexican populations (Sf-Valle del Fuerte and SfS-Mex) showed low sensitivity to this toxin, with LC_{50}s around 1,500 ng/cm^2. One of the Mexican populations (Sf-La Laguna) and the Brazilian population (SfLab-Brazil) showed moderate sensitivity to Cry1Ab, with LC_{50}s around 500 ng/cm^2 (Table 3). Nevertheless, taking into account the fiducial limits, none of these four populations had significantly different LC_{50}s for Cry1Ab. In the case of Cry1Fa, the four populations were 3- to 11-fold more susceptible than to Cry1Ab, showing statistically significant values, since the fiducial limits of the Cry1Fa toxicity did not overlap those of the Cry1Ab

toxicity. However, the differences in Cry1Fa susceptibility among these four populations were not significant.

Domain III has been shown to be important for receptor interaction. Domain-swapping experiments showed previously that the low toxicity of Cry1Ab for *Spodoptera exigua* mapped to domain III, since a chimeric protein containing domains I and II from Cry1Ab and domain III from Cry1Ca showed 6-fold higher insecticidal activity than Cry1Ca toxin (22). In this work, we characterized Cry1Ab domain III β16 mutants in order to determine the role of this region in binding and toxicity. The Sf-IBT population is relatively insensitive to Cry1Ab toxin. Surprisingly, some of the Cry1Ab β16 mutants showed significant insecticidal activity against Sf-IBT (Table 2). Among the β16 residues analyzed, mutations in N514 yielded more significant increases in Cry1Ab toxicity for all *S. frugiperda* populations analyzed. Therefore, we analyzed multiple amino acid substitutions at N514. Five different amino acid substitutions at N514 showed greater increases in toxicity. These substitutions include two uncharged polar residues (Q and S), two hydrophobic residues (F and A), and one basic polar residue (H) (Table 2). We analyzed the toxicity of Cry1Ab, Cry1AbN514A, and Cry1AbN514F for three other *S. frugiperda* populations from Mexico and one from Brazil. In the case of the two populations that showed low sensitivity to Cry1Ab (Sf-Valle del Fuerte and SfS-Mex), Cry1AbN514A was 3- to 10-fold more toxic than Cry1Ab. This enhancement in toxicity was not observed in the Sf-La Laguna population, which was more sensitive to the native Cry1Ab toxin than were the other two populations (Table 1). Interestingly, the SfLab-Brazil population showed 13-fold greater sensitivity to Cry1AbN514A than to Cry1Ab, while Cry1AbN514F showed toxicity similar to that of Cry1Ab. These results indicate that the Cry1AbN514A mutant showed consistently greater toxicity for most of the *S. frugiperda* populations analyzed. A similar trend was observed when the Cry1Fa and Cry1FaN507A toxins were compared, since the Cry1FaN507A mutant toxin showed increased toxicity for the Sf-Valle del Fuerte and SfS-Mex populations and toxicity similar to that of Cry1Fa for the Sf-La Laguna population (Table 3). Our data show that domain III mutations increased the toxicity of Cry1Ab or Cry1Fa for most *S. frugiperda* populations analyzed.

In order to identify the limiting steps for Cry1Ab toxicity in *S. frugiperda*, we analyzed the stability of these mutants to midgut protease treatment and their binding interactions with BBMV and Cry toxin receptors cloned from *S. frugiperda* larvae. The increased insecticidal activity of Cry1Ab β16 mutants correlated with enhanced stability of these protoxins to *S. frugiperda* midgut juice treatment (Fig. 1). The Cry1Fa protein showed susceptibility to protease treatment similar to that of Cry1FaN510A, suggesting that enhanced stability of Cry1Fa to *S. frugiperda* midgut proteases could explain in part its greater toxicity, compared with Cry1Ab, for this insect pest. Previous work suggested that Cry1Ab was more rapidly degraded in the presence of midgut juice from *S. frugiperda* than midgut juice from *M. sexta* (23), supporting our findings that Cry1Ab was more sensitive to midgut juice proteases from *S. frugiperda* and the Cry1Ab domain III mutations N514A and N514F resulted in greater stability to treatment with *S. frugiperda* midgut proteases.

In addition, our results show that enhanced toxicity of Cry1AbN514A and Cry1AbN514F correlated with increased binding to *S. frugiperda* BBMV. Cry1Ab showed reduced binding to *S. frugiperda* BBMV, in contrast to Cry1AbN514A and Cry1AbN514F mutants, which were able to bind to *S. frugiperda* BBMV (Fig. 2A). These results show that the low toxicity of Cry1Ab is also due to its reduced binding affinity for *S. frugiperda* BBMV. Also, we show here that Cry1AbN514A and Cry1AbN514F showed enhanced binding to purified preparations of *S. frugiperda* ALP, APN1, or CAD fragment (Fig. 3), which explains their increased binding to BBMV and increased toxicity. Previous work showed that a nontoxic Cry1Ab β16 L511A mutation affected *M. sexta* ALP binding of the monomeric toxin while oligomer binding to ALP was not affected, indicating that monomer binding to ALP through domain III β16 residues is a limiting step in toxicity (14). Also, β16 has been implicated in binding to APN1, since single-chain variable fragment (scFv) molecules that bind this domain III region blocked APN binding but did

TABLE 4 Oligonucleotides used for site-directed mutagenesis

Mutant	Primer sequence
Cry1AbS509A	5′-TCACCTGGCCAGATTGCGACCTTAAGAGTAAAT-3′
Cry1AbT510A	5′-TCACCTGGCCAGATTTCAGCGTTAAGAGTAAATATTACT-3′
Cry1AbR512A	5′-GGCCAGATTTCAACCTTAGCGGTAAATATTACTGCACCA-3′
Cry1AbV513A	5′-CAGATTTCAACCTTAAGACGCAATATTACTGCACCATTATCA-3′
Cry1AbN514A	5′-TCAACCTTAAGAGTAGCGATTACTGCACCATTATCA-3′
Cry1AbN514X	5′-TCACCTGGCCAGATTNNSACCTTAAGAGTAAAT-3′
Cry1FaN507A	5′-TTTGCTTATACTATTGTTGCGATAAATGGGCAATTA-3′

not affect Cry1Ab binding to CAD (19). It was shown previously that domain II loop regions are involved in binding to CAD receptors (13, 19, 24, 25). Thus, we did not expect the Cry1AbN514A or Cry1AbN514F toxins to display increased binding to CAD. Since domain III mutants showed enhanced tolerance to protease treatment, it is possible that subtle structural changes in the toxin caused by the β16 mutations resulted in protease stability but also enhanced binding to CAD. However, we cannot rule out the possibility that domain III is also involved in CAD binding; this remains to be analyzed in the future.

Most of the Cry1Ab N514 mutants with increased toxicity toward *S. frugiperda* showed toxicity similar to that of Cry1Ab toward *M. sexta*, indicating that the N514 mutations did not affect toxicity at least to one other lepidopteran species (Tables 1 and 2). These data suggest that these Cry1Ab mutants could provide useful insecticidal engineered Cry1Ab toxins for control of *S. frugiperda* and other lepidopteran pests that could be pyramided with other insecticidal toxins. In addition, *S. frugiperda* populations that showed high resistance to Cry1Fa toxin showed moderate 4-fold cross-resistance to Cry1Ab (11). Thus, increasing the toxicity of Cry1Ab with domain III mutations could be a useful strategy for managing Cry1Fa-resistant insects in the field; this remains to be analyzed.

MATERIALS AND METHODS

***Spodoptera frugiperda* populations.** A *S. frugiperda* population characterized previously (5) was maintained in the Instituto de Biotecnología, Universidad Nacional Autónoma de Mexico, without exposure to any Cry toxin and here is called Sf-IBT. Three other *S. frugiperda* populations were maintained in the Centro Universitario Universidad Autónoma del Estado de México, i.e., a susceptible *S. frugiperda* colony that has been maintained for 3 years without exposure to Bt toxins (SfS-Mex) and two recently established *S. frugiperda* colonies that were seeded from individuals collected in July 2016 from La Laguna in the state of Coahuila, Mexico, from different maize fields (Sf-La Laguna) or from individuals collected in August 2016 from Valle del Fuerte in the state of Sinaloa, Mexico, also from different maize fields (Sf-Valle del Fuerte). Finally, a *S. frugiperda* population from Brazil that has been maintained in Embrapa (Brasilia, Brazil) since 1988 without exposure to Bt toxins was named SfLab-Brazil (11). All colonies were maintained at 25°C, with relative humidity of 75% and a 13-h light/11-h dark cycle.

Site-directed mutagenesis. Plasmids pHT315-Cry1Ab (26) and pHT315-Cry1Fa were used as the templates for site-directed alanine mutagenesis using a QuikChange Multi site-directed mutagenesis kit (Stratagene), following the manufacturer's instructions. The oligonucleotides used for mutant construction are shown in Table 4. Saturation mutagenesis of the Cry1Ab N514 residue was performed with the primers described in Table 4. Candidate mutated plasmids were purified from *E. coli* cells and verified by DNA sequencing in the facilities of the Instituto de Biotecnología, Universidad Nacional Autónoma de Mexico. Plasmids were transformed into *E. coli* SCS110 (*dam dcm* mutant strain) cells, and purified plasmids from those cells were used for Bt transformation of the Bt 407 Cry$^-$ strain (27) by electroporation, as reported previously (28).

Expression, purification, and proteolytic activation of Cry1Ab, Cry1Fa, and β16 mutants. The acrystalliferous 407 Cry$^-$ strain transformed with wild-type or domain III mutant genes was grown for 72 h at 30°C in nutrient sporulation medium (29) with 10 μg/ml erythromycin, with shaking at 250 rpm, until sporulation and crystal production were completed. For binding assays, the spore/crystal suspensions were solubilized for 2 h at 37°C in 50 mM Na_2CO_3/$NaHCO_3$ buffer containing 0.02% mercaptoethanol (pH 10.5) and then were proteolytically activated with trypsin (Sigma) (trypsin/protoxin ratio of 1:50) for 2 h at 37°C. Protein concentrations of protoxins and toxins were determined by the Bradford method, using bovine serum albumin (BSA) as a standard. For treatment of Cry1Ab and domain III mutants with *S. frugiperda* midgut juice, 100 μg of Cry protein was incubated for 5, 15, 30, or 60 min at 37°C with a dilution of midgut juice (1%) obtained from third instar larvae, in a final volume of 100 μl of solubilization buffer. The reaction was stopped with 1 mM phenylmethylsulfonyl fluoride (PMSF), the reaction mixtures were centrifuged at 14,000 rpm for 10 min, and the supernatants were recovered and analyzed by SDS-PAGE with 10% acrylamide.

Preparation of BBMV. Third instar *M. sexta* or *S. frugiperda* (Sf-IBT) larvae were dissected to obtain midgut tissue, which was used to prepare BBMV by the differential precipitation method using $MgCl_2$, as reported previously (30). BBMV were stored at −70°C until use.

Binding assay of Cry1Ab toxins with BBMV. Ten micrograms of BBMV protein was incubated in binding buffer (phosphate-buffered saline [PBS] with 0.1% BSA and 0.1% Tween 20) with 5 nM Cry1Ab or domain III mutants labeled with biotin. Activated Cry1Ab or domain III mutants were biotinylated using biotinyl-*N*-hydroxysuccinimide ester (Amersham Biosciences, Waltham MA), according to the manufacturer's instructions. Binding of biotinylated Cry1Ab was performed as described previously (13). Samples were centrifuged (10 min at 14,000 rpm) to remove unbound toxin. BBMV were washed three times with binding buffer and suspended in 10 μl PBS with 3 μl of Laemmli buffer. Samples were boiled as described above, separated by SDS-PAGE (10% acrylamide), and electrotransferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was visualized using streptavidin coupled to horseradish peroxidase (1:50,000; Millipore), followed by incubation with Super Signal chemiluminescence substrate (Pierce), according to the manufacturer's instructions. For homologous competition experiments, BBMV were incubated with 5 nM Cry1Ab or domain III mutants in the presence of a 50- or 200-fold excess of the corresponding nonlabeled toxin. After 1 h, samples were processed as described above.

Insect bioassay. Bioassays were performed by the surface contamination method with *M. sexta* or *S. frugiperda* neonate larvae (5). Different doses of crystals/spores (from 0.1 to 1,000 ng cm^{-2}) were applied to the diet surface contained in 24-well polystyrene plates (Cell Wells; Corning Glass Works, Corning, NY). A total of 24 larvae per plaque (1 larva per well) were fed with different doses of crystals/spores. The plates were incubated at 28°C, with 65% ± 5% relative humidity and a 16-h light/8-h dark cycle. For bioassays with the *S. frugiperda* populations maintained at the Centro Universitario Tenancingo, Universidad Autónoma del Estado de México, different doses of crystals/spores (from 0.1 to 1,000 ng cm^{-2}) were applied to the diet surface contained in 128-well polystyrene plates (Bio-BA-128 bioassay trays; C-D International, Inc.). One larva per well was seeded, fed with different doses of crystals/spore proteins, and incubated at 25°C ± 2°C with 75% ± 5% humidity and a 13-h light/11-h dark cycle. Four repetitions were performed on different days, deaths were recorded after 7 days, and LC_{50} values were analyzed with Probit software. Larvae were considered dead if no movement was apparent after the larvae were molested or if the larvae remained the size of neonates.

Cloning and expression of ALP, APN1, and CAD from *S. frugiperda*. Total RNA was prepared from dissected midguts of third instar *S. frugiperda* larvae using the RNeasy minikit (Qiagen), and cDNA was synthesized using oligo(dT) (Sigma). As the templates, cDNA samples were used with specific oligonucleotides to amplify full-length APN1 (2,900 bp) with NcoI and BamHI restriction sites (reverse, 5′-ATG ACCATGGCGAATCGCTGGTTTAGCCTC; forward, 5′-ATTAGGATCCTTAACCATGTTGATGATAACTGTGAT) or a CAD fragment containing CAD repeats CR7 to CR12 (2,132 bp) with NdeI and HindIII restriction sites (reverse, 5′-GTGCGGCCGCAAGCTTGTTGGCGAACGTTTGTGC; forward, 5′-GTGCGGCCGCAAGCTTGTTGGCG AACGTTTGTGC). Both PCR products were digested with the corresponding restriction enzymes, cloned into expression vector pET22b, and transformed into *E. coli* strain BL21(DE3). Gene sequences were confirmed by sequencing in the facilities of the Instituto de Biotecnología, Universidad Nacional Autónoma de Mexico. For ALP cloning, sequencing of a *S. frugiperda* midgut expressed sequence tag (EST) library revealed one ALP sequence (DuPont Pioneer) (M. Nelson, unpublished data). The ALP open reading frame was cloned into the expression vector pET28a by using the restriction enzymes EcoRI and HindIII and was transformed into *E. coli* strain BL21(DE3).

For protein production and purification, *E. coli* cells were grown overnight at 37°C, with constant agitation, in 5 ml of LB broth containing 50 μg/ml ampicillin or 50 μg/ml kanamycin (for ALP). One hundred microliters of culture was used to inoculate 100 ml of 2× tryptone yeast (TY) broth supplemented with 100 μg/ml ampicillin or 100 μg/ml kanamycin, in a 250-ml flask. This culture was incubated for 2 h at 37°C, with constant agitation, until the optical density at 600 nm (OD_{600}) reached 0.6, and expression of ALP, APN1, or CAD fragment was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) for 5 h at 30°C. Cells were collected by centrifugation (5,000 rpm for 15 min at 4°C), and the pellet was suspended in STE buffer (10 mM Tris–HCl, 1 mM EDTA, 8 M urea [pH 8]). After sonication for 5 min on ice, cell debris was eliminated by centrifugation (70,000 rpm for 30 min at 15°C), and the supernatant was subjected to affinity purification using Ni-agarose beads (Qiagen). After washing with 35 mM imidazole in PBS (pH 7.5), the recombinant proteins were eluted with 250 mM imidazole and gradually dialyzed against PBS. The purified proteins were separated by SDS-PAGE with 10% acrylamide.

ELISA analysis of binding of Cry1Ab or domain III mutants to ALP, APN1, and CAD proteins. ELISA plates (Nunc) were coated with 1 μg of recombinant proteins. Different concentrations of activated Cry1Ab or Cry1Ab domain III mutants were incubated with the coated ELISA plates, and bound toxins were detected using rabbit anti-Cry1Ab antibody (1:20,000) and anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase. Finally, 0.5 mg/ml *o*-phenylenediamine (Sigma) and 0.0075% H_2O_2 were used as the substrates for peroxidase activity detection. Reactions were stopped by adding 50 μl of 1 M H_2SO_4, and the reaction product was measured at 490 nm using an ELISA microplate reader. All experiments were performed in triplicate. Differences in binding data were analyzed by *t* test using GraphPad Prism 7 (version 5.0b), and relative binding affinities (K_d) were obtained by Scatchard analysis with SigmaPlot (Systat Software).

ACKNOWLEDGMENTS

Thanks go to Mario Trejo, Martín Patiño, Lizbeth Cabrera, Ricardo Grande, and Blanca-Ines Garcia-Gomez for technical assistance.

This research was supported in part by DGAPA/UNAM grants IN209011 and IN201016, CONACyT Fronteras de la Ciencia grant 008, and Pioneer Hi Bred.

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