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# Identification of Amino Acid Residues of *Bacillus thuringiensis* δ-Endotoxin CryIAa Associated with Membrane Binding and Toxicity to *Bombyx mori*

HONG LU,† FRANCIS RAJAMOHAN, AND DONALD H. DEAN*

*The Ohio State Biochemistry Program and Department of Biochemistry, The Ohio State University, Columbus, Ohio 43210*

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**Alanine substitution (A3) or deletion (D3) of residues 365 to 371 of *Bacillus thuringiensis* CryIAa insect toxin removed nearly all toxicity for *Bombyx mori* (>1,000-fold less active than the wild type). The loss of larvicidal activity in the mutants was not caused by increased sensitivity to larval gut enzymes but could be attributed to significantly reduced binding to *B. mori* brush border membrane vesicles. Some or all of the affected amino acid residues may interact directly or indirectly with the *B. mori* membrane receptor(s). Such receptor binding appears to be directly correlated with insect toxicity.**

*Bacillus thuringiensis*, a gram-positive bacterium, has been widely used as a biological pesticide in insect control for more than two decades. A major characteristic of *B. thuringiensis* is its ability to produce, during sporulation, crystalline inclusions of insecticidal proteins (ICPs, Cry proteins, or δ-endotoxins). Many ICPs, representing a spectrum of insect activity, have been identified. One class, the CryIA group, encodes protoxins, with molecular masses of between 130 and 140 kDa, that are active against *Lepidopteran* insect larvae. After ingestion by sensitive larvae, crystals are converted by larval midgut proteases into active toxin, a protease-resistant polypeptide of 55 to 70 kDa (1). The activated toxin binds to a specific receptor(s) on the luminal plasma membrane of susceptible larval midgut columnar epithelial cells (2, 7, 8, 16, 23), irreversibly inserts into the membrane (29), and alters the electrochemical potential gradient across the midgut (5) by forming ion channels (20–22). This destroys the osmotic balance of the cell membrane, which eventually leads the cell to swell and rupture (11). The crystal structure of CryIIIA toxin has recently been deduced by Li et al. (14). These authors have proposed that the Cry family might adopt the same protein-folding scheme, since the core of the CryIIIA molecule that holds the domain interfaces is built on five highly conserved sequence blocks. Receptor binding of ICPs has been studied for several years, using an in vitro binding assay system with brush border membrane vesicles (BBMV) isolated from an insect midgut epithelium (7). Binding studies have shown a positive correlation between toxicity and binding affinity or the number of binding sites for some insect species (7, 8, 13, 25, 26). A major exception to this is an inverse relationship between binding affinity and toxicity observed in the case of *Lymantria dispar* for the two toxins CryIAb and CryIAc (28). In addition, CryIAc toxin shows high-affinity binding to *Spodoptera frugiperda* membrane vesicles but does not kill the larvae (2). Therefore, toxicity and toxin binding may not always be directly correlated.

Recently, the lepidopteran and dipteran insect toxicity determining regions of several Cry toxins have been studied by exchanging fragments of different crystal proteins (3, 4, 10, 19, 27, 31). The receptor binding studies of Lee et al. (13) confirm that the region of CryIAa toxin responsible for binding to *B. mori* is located among the amino acids 332 to 450. By using site-directed mutagenesis techniques, we have either substituted with alanine or deleted 5 to 7 amino acids at various locations between amino acid residues 350 and 410 and investigated the structure-function relationship of the mutant toxins. We demonstrate that substitution or deletion of amino acids 365 to 371 (LYRRIIL) of CryIAa toxin significantly reduces the binding ability of the toxin to *B. mori* membrane vesicles and reduces its larvicidal potency by more than 1,000-fold.

Uracil-containing pOSM1313 (3), extracted from *Escherichia coli* CJ236 (Bio-Rad), was the template for site-directed mutagenesis, which followed the manufacturer's protocol (Muta-Gene M13 in vitro mutagenesis kit; Bio-Rad). DNA sequencing was carried out by the method of Sanger et al. (18) with Sequenase and by following the manufacturer's instructions (United States Biochemical). Mutant and wild-type proteins were expressed in *E. coli* JM103 and the δ-endotoxins were purified by a modification of the method of Höfte et al. (9). The purified crystal protein was solubilized in buffer (50 mM $Na_2CO_3$/$NaHCO_3$, pH 9.5, 10 mM dithiothreitol) at 37°C for 2 h. BBMV were prepared by the differential magnesium precipitation method as modified by Wolfersberger et al. (30). Trypsin-activated toxins were iodinated by using Iodo-beads (Pierce). Competition binding assays were performed as described by Lee et al. (13).

**Construction and expression of mutant toxins.** Amino acid alignment between CryIIIA and CryIAa toxin is shown in Fig. 1A, which is in agreement with the results of Hodgman and Ellar (6). Although the two toxins share only 37% identity over this region, conserved tracts of amino acids that lie on either side of these sequences helped to achieve the alignment. Deletions (D) by loop-out mutagenesis and block alanine substitutions (A) by oligonucleotide-directed mutagenesis were created at the positions shown in Fig. 1B. Alanine mutations were chosen because they may not be disruptive to the overall structure. Block alanine substitutions were preferred because removal of any single molecular contact by a

* Corresponding author. Phone: (614) 292-8829. Fax: (614) 292-3206.
† Present address: Oncogene Science, Uniondale, NY 11553-3649.

**A**

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                          Loop 2
                  ____ β6 ____    _____ β7 _____        __ β8 __
cryIIIA 392LEFNGEKVYRAVANTNLA----VWPSA--VYSGVTKVEFSQYNDQ-------TDEASTQTYDSK----RNVGAV448

cryIAa  350VSLTGLGIFRTLSSPLYRRIILGSGPNNQELFVLDGTEFSFASTTNL---------PSTIY-------RQRGTV407
```

**B**

**SUBSTITUTION MUTANTS:**

```
cryIAa          VSLTGLGIFRTLSSPLYRRIILGSGPNNQELFVLDGTEFSFASTTNL---------PSTIY-------RQRGTV

A2 (357-363)    VSLTGLGAAAAAAAPLYRRIILGSGPNNQELFVLDGTEFSFASTTNL---------PSTIY-------RQRGTV

A3 (365-371)    VSLTGLGIFRTLSSPAAAAAAAGSGPNNQFLFVLDGTEFSFASTTNL---------PSTIY-------RQRGTV

A4 (378-384)    VSLTGLGIFRTLSSPLYRRIILGSGPNNAAAAAAAGTEFSFASTTNL---------PSTIY-------RQRGTV

A1 (385-389)    VSLTGLGIFRTLSSPLYRRIILGSGPNNQELFVLDAAAAAFASTTNL---------PSTIY-------RQRGTV

A5 (399-404)    VSLTGLGIFRTLSSPLYRRIILGSGPNNQELFVLDGTEFSFASTTNL---------PSAAA-------AAAGTV
```

**DELETION MUTANTS:**

```
cryIAa          VSLTGLGIFRTLSSPLYRRIILGSGPNNQELFVLDGTEFSFASTTNL---------PSTIY-------RQRGTV

D2 (357-363)    VSLTGLG-------PLYRRIILGSGPNNQELFVLDGTEFSFASTTNL---------PSTIY-------RQRGTV

D3 (365-371)    VSLTGLGIFRTLSSP-------GSGPNNQELFVLDGTEFSFASTTNL---------PSTIY-------RQRGTV

D4 (378-384)    VSLTGLGIFRTLSSPLYRRIILGSGPNN-------GTEFSFASTTNL---------PSTIY-------RQRGTV

D1 (385-389)    VSLTGLGIFRTLSSPLYRRIILGSGPNNQELFVLD-----FASTTNL---------PSTIY-------RQRGTV

D5 (399-404)    VSLTGLGIFRTLSSPLYRRIILGSGPNNQELFVLDGTEFSFASTTNL---------PS------------GTV
```

FIG. 1. (A) Alignment of the toxin protein sequences of CryIAa with that of a related beetle active toxin CryIIIA as given by Hodgman and Ellar (6). The positions of the secondary structural elements of CryIIIA are given above its sequence as described by Li et al. (14). The regions of amino acids considered here are shown. (B) Summary of the deletion (D) and alanine substitutions (A) constructed on CryIAa toxin. Numbers in parentheses indicate the corresponding amino acids deleted or substituted.

point mutation may cause a relatively small reduction in binding. Most of the mutants we created turned out to have significant structural alterations, as evidenced by their unusual sensitivity to trypsin and insolubility from inclusion bodies. Of 10 mutants constructed (Fig. 1B), only deletion or alanine substitution of amino acids 365 to 371 (D3 and A3) and alanine substitution of residues 385 to 389 (A1) produced proteins as stable as the wild-type CryIAa protein. Hence, only these proteins were considered for further study.

**Force feeding insect bioassay.** Biological activity of the toxins was assayed by delivering 5.0-μl drops of each toxin dilution into the midgut region of fourth-instar *B. mori* larvae with a model 1003 Microjector syringe drive and a model 1010 Microdoser (Houston Atlas, Houston, Tex.). Effective dose estimates were obtained by Probit analysis (17). Our toxicity assay data (Table 1) show a significant variation in the activities

TABLE 1. Insect toxicity and membrane vesicle binding of selected toxins to *B. mori*

| Toxin | $LD_{50}$ (ng of toxin/larva)[a] | $K_d$[b] |
|---|---|---|
| CryIAa | 28 (14–44) | 3.29 |
| A3 | >30,000[c] | 36.56 |
| D3 | >30,000[c] | 37.63 |
| A1 | 32 (18–51) | 3.01 |

[a] 95% confidence intervals are in parentheses. $LD_{50}$, 50% lethal dose.
[b] Expressed in nanomolar concentrations. Values are means of duplicate samples.
[c] Insufficient mortality to calculate $LD_{50}$.

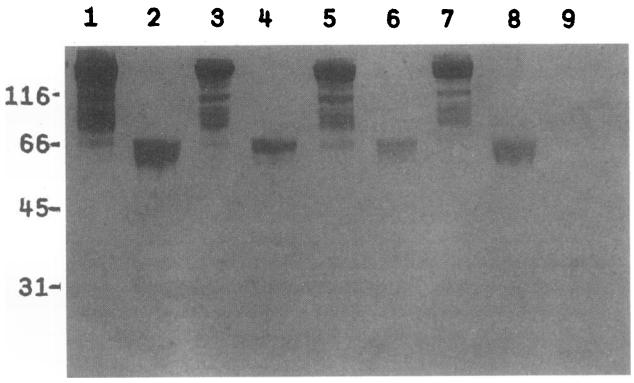


FIG. 2. Western blot analysis of the stability of wild-type and mutant proteins after digestion with *B. mori* gut enzymes. Lanes 1, 3, 5, and 7 protoxins of CryIAa, A3, D3, and A1, respectively; lanes 2, 4, 6, and 8, gut enzyme-activated toxins of CryIAa, A3, D3, and A1, respectively; lane 9, gut enzyme proteins. Molecular mass marker positions are indicated to the left in kilodaltons.

of mutant and wild-type proteins. CryIAa and A1 had the same level of activity (50% lethal doses of 28 and 32 ng per larva, respectively), whereas A3 and D3 gave only 10% mortality at the highest concentration tested (30 μg per larva). The mutant proteins A3 and D3 were about 1,000 times less toxic to *B. mori* than was the wild type. This suggests that the amino acid residues 365 to 371 (A3 or D3) might be important for the toxicity of CryIAa to *B. mori*. Alternatively, the mutant A3/D3 proteins might be unusually susceptible to degradation by larval midgut enzymes. To test this possibility we obtained *B. mori* gut juice, the vomited fluid collected by stimulating the mouthparts. This was incubated with 10.0 μg of protoxin (1:5 [vol/vol] ratio of gut juice to protoxin solution). After 2 h, the reaction was stopped by adding Laemmli sample buffer (12), resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane with a Bio-Rad Trans-Blot apparatus. The membrane was then incubated 1 h with anti-CryIAa polyclonal antiserum. The blot was developed as described in the Bio-Rad Alkaline Phosphatase Immunoblot Assay Kit. The Western blot (immunoblot) analysis showed that both mutant and wild-type protoxins were processed similarly into a 65-kDa active toxin without degraded product (Fig. 2). The mutant D3 protein was slightly more sensitive to gut enzymes than were the other proteins. These differences seem much too small to account for the 1,000-fold difference in toxicity, however. Moreover, Fig. 4A shows that the mutant protein A3 is as stable as the wild-type protein when subjected to higher amounts of trypsin (1:1 [wt/wt] ratio), whereas D3 is slightly less stable than the wild-type toxin. This protease digestion test also provides evidence that the mutant proteins have no gross structural alteration. These results indicate that the decreased toxicity of the mutant proteins is probably not caused by increased degradation of toxin by larval gut proteases but could be the result of reduced binding of the toxin to the membrane receptor.

**Binding assays.** Heterologous competition experiments between one labeled toxin and another unlabeled toxin were performed to investigate whether the binding sites for different toxins were related. CryIAa and A1 displayed high-affinity binding to *B. mori* vesicles, whereas A3 and D3 curves shifted to the right by a factor of approximately 25 compared with that of CryIAa or A1 (Fig. 3). Also, toxins A3 and D3 displaced only 50% of the bound $^{125}$I-labeled CryIAa or A1 (Fig. 3). This confirms the lower affinity of A3 and D3 toxin for *B. mori* vesicles. The dissociation constants ($K_d$) for these toxins, calculated by the LIGAND computer program (15), are given in Table 1. The $K_d$ values of the wild-type CryIAa and mutant A1 were similar (3.29 and 3.01 nM, respectively), whereas the binding affinities of mutants A3 and D3 were approximately 10 times lower ($K_d$ = 36.56 and 37.63 nM, respectively) than that of the wild-type toxin. These correlate with our bioassay data that toxins with higher activity (CryIAa and A1) exhibit strong binding, whereas toxins with no or marginal activity show weak binding. This does not exclude the possibility that the toxin binds to two different receptor sites, one with a high affinity ($K_d$ = 3.2 nM) and one with a low affinity ($K_d$ = 36 nM). If so, the A3 and D3 mutations must disrupt high-affinity binding but leave the low-affinity interaction intact.

It is also possible that instability of the mutant proteins A3 and D3, during the incubation period with the BBMV proteins, could cause the lower binding. The structural stability of $^{125}$I-labeled mutant and wild-type proteins during incubation with *B. mori* vesicles was analyzed by incubating 2 nM of labeled toxins with 50 μg of BBMV (final volume, 20 μl) at room temperature for 1 h. The reaction was stopped by adding Laemmli sample buffer (12) and subjected to SDS-PAGE. We observed a single 65-kDa peptide both on wild type and on mutant proteins, showing that the reduced binding of the mutant A3 and D3 proteins were not due to any possible degradation by BBMV vesicle proteins. The lower intensity of the labeled D3 mutant protein when compared with that of other proteins is due to the low specific activity of the toxin and not the result of proteolytic degradation (Fig. 4B).

From our experimental results, we conclude that some or all of the amino acid residues 365 to 371 of CryIAa toxin are directly or indirectly involved in receptor binding to *B. mori*. The involvement of other predicted amino acids of loops 1 and 3 in membrane binding is under investigation. Schnepf et al. (19) showed that seven substitutions in the amino acids 434 to 441 in CryIAc toxin considerably reduced toxicity to *Manduca sexta* but had only a moderate effect on toxicity to *Heliothis virescens*.

The structural alignment of CryIA toxins with that of CryIIIA toxin (6) shows that the genetically identified specificity determination regions of CryIA toxins fall mainly in domain II, the most likely apical region to bind the membrane receptor (14). For instance, the specificity of CryIAa to *B. mori* determined by Lee et al. (13) corresponds roughly to the outer strands β4 and β5 of sheet 1 and the whole of sheet 2, including the loop entering β10 in sheet 3. According to our current observation, residues of CryIAa essential for binding to *B. mori* (365 to 371) align more closely to loop 2 of sheet 2, which is a potential surface region for membrane interaction. We are currently exploring the binding and toxicity of A3, D3, and A1 mutants for other insect species.

Our results may aid in the construction of a safer biopesticide. For example, CryIA proteins have a wide range of insect specificity (24), including *B. mori* (silk worm), an insect highly valued by the natural silk industry. This toxicity restricts the use of CryIA toxins in areas where silk moth is being grown. The judicious modification of the *B. mori* specificity region from CryIA toxins (as defined by Ge et al. [3]), without altering the structural ability and activity of the toxin to other insects, would reduce concerns over the use of this toxin. Simultaneously, the identification of insect specificity residues would help in construction of new bacterial strains capable of targeting a diverse array of insects.

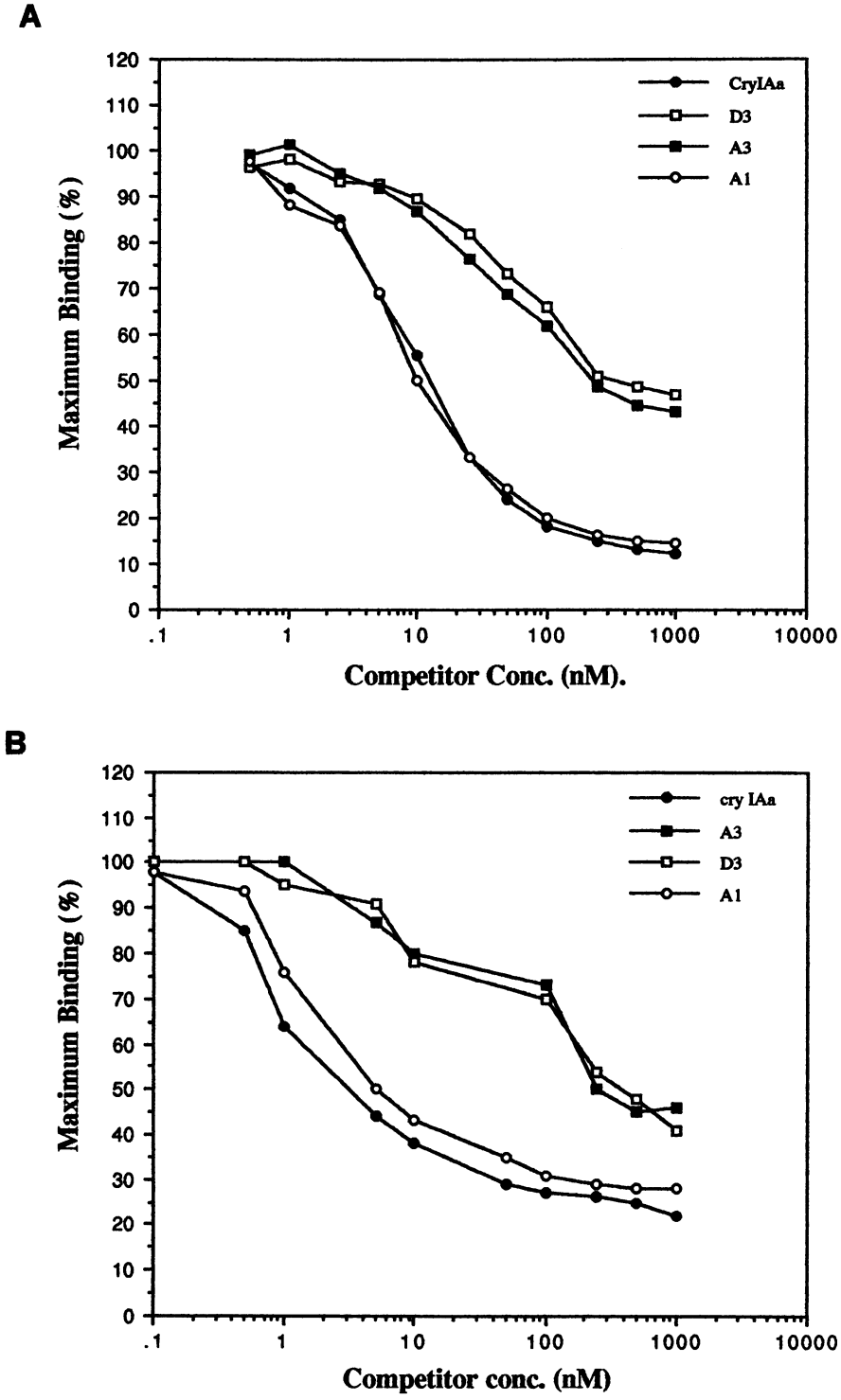


FIG. 3. Competition binding assay of $^{125}$I-labeled CryIAa (A) and A1 (B) toxin to *B. mori* BBMV. A total of 100 μg of vesicle proteins per ml were incubated with 1 nM $^{125}$I-labeled toxin in the presence of increasing concentrations of unlabeled CryIAa, A3, D3, and A1 competitor toxin. The maximum binding is expressed as percentage of the amount bound upon incubation with labeled toxin alone.

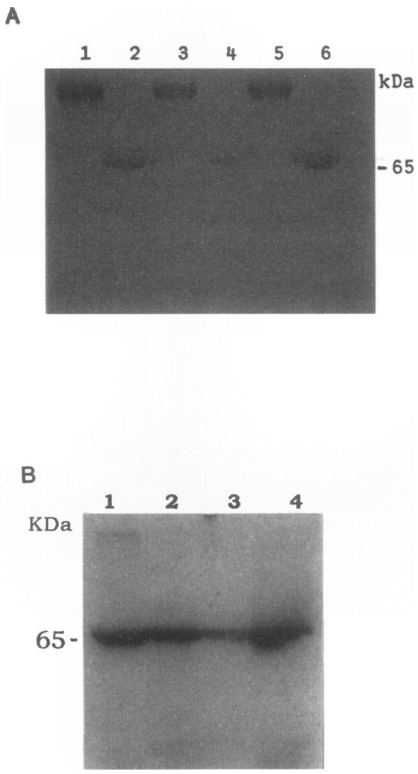


FIG. 4. (A) Coomassie blue-stained SDS–9% PAGE of protoxins treated with excess amounts of trypsin. Lanes 1, 3, and 5, A3, D3, and CryIAa protoxins, respectively; lanes 2, 4, and 6, A3, D3, and CryIAa trypsin-treated proteins. (B) Determination of stability of $^{125}$I-labeled toxins after incubation with *B. mori* BBMV proteins. The dried gel was exposed to Fuji RX film for 2 to 3 days. The specific activities of $^{125}$I-labeled CryIAa, A3, D3, and A1 toxins were 2.3, 2.1, 1.0, and 2.5 mCi/mg, respectively.

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