Exploration of Receptor Binding of *Bacillus thuringiensis* Toxins

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Wild type and mutant toxins of Bacillus thuringiensis δ-*endotoxins were examined for their binding to midgut brush border membrane vesicles (BBMV). CryIAa, CryIAb, and CryIAc were examined for their binding to Gypsy moth (*Lymantria dispar*) BBMV. The binding of CryIAa and CryIAc was directly correlated with their toxicity, while CryIAb was observed to have lower binding than expected from its toxicity. The latter observation confirms the observation of Wolfersberger (1990). The "rule" of reciprocity of binding and toxicity is apparently obeyed by CryIAa and CryIAc, but broken by CryIAb on* L. dispar. *Alanine substitutions were made in several positions of the putative loops of CryIAa to test the hypothesis that the loops are intimately involved in binding to the receptor. The mutant toxins showed minor shifts in heterologous binding to* Bombyx mori *BBMV, but not enough to conclude that the residues chosen play critical roles in receptor binding.*

Key words: ion channel toxin - biological insecticide

What is known about the binding of *Bacillus thuringiensis* δ-endotoxins to their receptors? Research over the last five years has revealed four pieces of information about the binding of Cry toxins to their receptors:

Binding involves à reversible step and an irreversible step - This may be presented by the following equation:

$$R + T \underset{k-1}{\overset{k1}{\leftrightarrow}} R\text{-}T \overset{k2}{\rightarrow} RT$$

where R is the receptor, T is the toxin, R-T is the reversibly bound and RT is the irreversibly forms of the toxin. Despite the fact that both the reversible and irreversible steps were demonstrated early on (Hofmann et al. 1988a), previous theoretical evaluations of binding kinetics assume only the reversible step (Hofmann et al. 1988a; Van Rie et al. 1989, 1990). The existence of the irreversible binding step created a condition lacking true equilibrium; therefore, the binding constant should be referred to as the apparent dissociation constant, K_d app.

The operational hypothesis in reversible binding is by interaction of the loops of domain II of the toxin molecule with the receptor protein (Li et al. 1991). Irreversible binding is assumed to be due to the insertion of the toxin into the membrane (Ihara et al. 1993). The dissociation constant, K_d, has been reported as low as 0.2 nM (Van Rie et al. 1990a). This very tight binding may be affected by the irreversible step. Indeed, since the measurement of binding in this system is usually done by binding to brush border membrane vesicles (BBMV), the reversible and irreversible steps are not separated.

The specificity-determining region of a toxin is colinear with the receptor binding region - Binding of the toxin to the insect midgut has been, for the most part, considered a major determinant of the specificity of toxins (Hofmann et al. 1988a,b; Van Rie et al. 1990a,b; for review, see Milne et al. 1990). Hofmann et al. (1988b) first reported that specificity of *B. thuringiensis* δ-endotoxin is correlated with the presence of high-affinity binding sites on insect BBMV. The location of the insect specificity region of CryIAa for *B. mori* was first reported by Ge et al. (1989). The location of specificity regions of other toxins soon followed: CryIAc for *Trichoplusia* and *Heliothis* (Ge et al. 1991), and CryIIA to mosquito (Schnepf et al. 1990; Widner & Whiteley 1990; see Visser et al. 1993 for a review). These results point to a region extending from the center to the third quadrant (amino acid residues 283 to 450) or, in some cases, to the end of the toxin (ca. 620). The correlation of specificity region to receptor binding region has been shown for CryIAa to *B. mori* (Lee et al. 1992), CryIAc to *T. ni*, and CryIAc to *H. virescens* (MK Lee & DH Dean, unpub. observ.).

Correlation of the location of the binding region with the three-dimensional structure of CryIIIA has led to the proposal that domain II of the toxin is the binding domain (Li et al. 1991). Examination of the structure of domain II reveals

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obvious loops that may be the contact points between the toxin and its receptor. There is one paper that reports a mutation in domain I that has a dramatic effect on receptor binding (Wu & Aronson 1992). The mutation A92D is reported to virtually knock out binding and toxicity for *Manduca sexta*, but not for binding to *H. virescens*. The location of this mutation is at the bottom of domain I, on the same side of the molecule as the receptor binding region. This suggests that this mutation affects either reversible or irreversible binding and hence that domain I, in concert with domain II, plays a role in binding. Chen and Dean (in preparation) have repeated these mutations and performed binding studies with A92E and A92D of CryIAc and CryIAa, and we do not observe negative effects on reversable binding, but we do observe negative effects on irreversible binding. These mutations do, however, have dramatic knock-out effects on the toxicity of the mutant proteins.

For most cases, there is a direct correlation between binding and insecticidal specificity and activity (Hofmann et al. 1988b) - This is not as clear as originally believed, however. Perhaps the best comparison between binding and toxicity can be found in the results of Lee et al. (1992), which evaluate different mutants of two toxins against a single insect, *B. mori*.

Two types of exceptions to this correlation have been reported. One general exception is for toxins that bind with apparent high affinity, but are not toxic to the insect: CryIAc to *Spodoptera exigua* (Garczynski et al. 1991), CryIAc to *Lymantria dispa*r (Wolfersberger 1990), CryIC to *M. sexta* (Van Rie et al. 1990a), CryIAa to *H. virescens* (Van Rie et al. 1990a) and CryIAc to *S. frugipurda* (Garczynski et al. 1991). A second major category is for toxins that bind weakly, relative to other toxins, but have higher activity: CryIAb to *L. dispar* (Wolfersberger 1990). The mechanistic reasons for these exceptions have not been revealed. They may point to the importance of other toxin functions (Wolfersberger 1990), or to parameters that are not generally measured, such as the irreversible binding step (Ihara et al. 1993).

Receptor binding is necessary for full toxicity to insect larvae - Deletion of one or more toxin receptor binding regions causes great loss of toxicity; toxicity is not seen in insects which have no binding (Van Rie et al. 1990a,b). There is toxic activity to cultured cells, which have nonspecific receptors, but toxicity is very low (ca. 100x conc. of toxin is required). Recently, Lu et al. (1994) have shown that a deletion or block of substituting alanines in loop 2 of CryIAa will remove about 50% of binding and virtually all of the toxicity against *B. mori*. This provides support for the "loop hypothesis" of Li et al. (1991) that the loops of domain II are involved in binding to the receptor.

The present paper attempts to re-examine the finding of Wolfersberger (1990) concerning the lack of correlation between binding and toxicity of CryIAb and CryIAc on *L. dispar*. Further, it attempts to test the "loop hypothesis" by introducing alanine substitutions at certain positions in the three major loops of domain II of CryIAa.

MATERIALS AND METHODS

Preparation of BBMV and iodination of toxins - 5th instar larvae were dissected as described by Lee et al. (1992), and brush border membrane vesicles (BBMV) were prepared according to Wolfersberger et al. (1987). Toxins were iodinated using IODOBEADS (Pierce Chemical Co.) according to Wolfersberger et al. (1987).

Binding assay - BBMV were incubated with ^{125}I-labeled toxins in 100 µl of binding buffer (8 mM Na_2HPO_4, 2 mM KH_2PO_4, 150 m NaCl, pH 7.4, containing 0.1% bovine serum albumin (Van Rie et al. 1989). After 1 hr of incubation at room temperature, the sample was centrifuged in a Fisher microcentrifuge for 10 min at 13, 500 x g to separate bound from free toxin. The pellet containing the bound toxin was washed three times with binding buffer, and the resulting pellet was counted in a gamma counter (Beckman). Binding data were analyzed by using the LIGAND computer program (Munson & Rodbard 1980).

Site-directed mutagenesis - Site-directed mutagenesis was conducted by the method of Kunkel (1985) using the Bio-Rad MutaGene kit. Oligonucleotides were synthesized with an Applied Biosystems model 380 B DNA synthesizer at the Biochemical Instrumentation Center (Department of Biochemistry, The Ohio State University). Cloning and expression of the mutant genes is as described by Ge et al. (1991).

RESULTS

Re-examination of CrylA toxin binding to L. dispar *BBMV* - Our first experiment was to re-examine the unexpected results of Wolfersberger (1990) in which he observed that CryIAb bound more weakly than CryIAc to *L. dispar* BBMV, but was more toxic to *L. dispar* larvae. Fig. 1A shows homologous competition curves for CryIAa, CryIAb, and CryIAc. Fig. 1B through 1D show heterologous binding curves where the three toxins are competing against labeled CryIAa (Fig. 1B), labeled CryIAc (Fig. 1C), and labeled CryIAb (Fig. 1D). The summation of the binding constants from these data and the comparison to the data of Wolfersberger (1990) are shown in the Table.

Mutations in the loops - Li et al. (1991) proposed that *B. thuringiensis* toxins bind to their receptors by virtue of the loops of domain II.

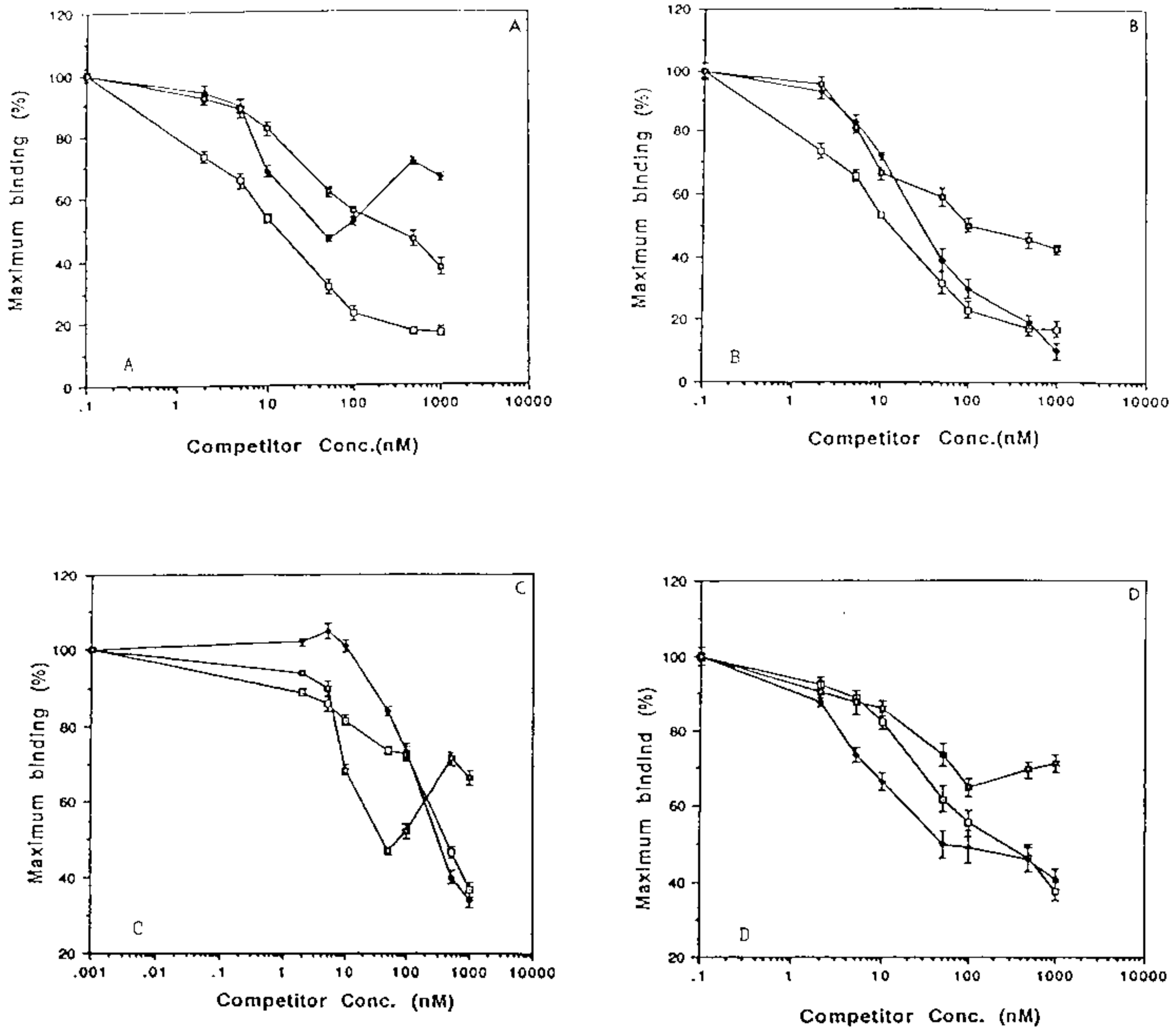

Fig. 1A: homologous competition binding assays of CryIAa (4101), CryIAb (4301), and CryIAc (4201) toxins with BBMV of *Lymantria dispar*. BBMV (300 µg/ml) were incubated with 2 nM of ^{125}I-labeled CryIAa, CryIAb, and CryIAc toxins in the presence of increasing concentration of the same type of unlabeled toxins 4101 ❑, 4201 ●, 4301 ❑ . Binding is expressed as a percentage of the amount of bound toxin. Each point is the mean of duplicate samples; B: heterologous binding of the three CryIA toxins to labeled CryIAa; C: heterologous binding of the three CryIA toxins to labeled CryIAb; D: heterologous binding of the three CryIA toxins to labeled CryIAc.

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          304--loop1---317 364---loop2---378 429-----loop3-------448
           *               *   *               *   *               *
CryIAa    TIYTDVHRGFNYWS...LYURRIILGSGPNNQ...RLSHVTM-LSQAAGAVYTL-RA
CryIAb    TIYTDAHRGEYYWS...LYRRP--FNIGINNQ...RLSHVSMFRSGFSNSSVSIIRA
CryIAc    TIYTDAHRCYYYWS...LYRRP--FNIGINNQ...RLSHVSMFRSGFSNSSVSIIRA
                   ^^^             ^^                  ^^
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Fig. 2: comparison of the residues predicted to be at and around the loops of CryIAa, CryIAb, and CryIAc (Hodgman & Ellar 1990). Underlined residues are in the predicted loops. The symbol ^ indicates the position where alanine substitutions were made in CryIAa toxins.

Alignment of the sequences of the loop regions of CryIAa, CryIAb, and CryIAc is shown in Fig. 2. From this, one can observe that there are minor differences among the three toxins in loop 1. For loops 2 and 3, CryIAa is unique, and CryIAb and CryIAc are the same. This is not consistent with the results of Fig. 1A-D, nor with the finding that CryIAb and CryIAc usually do not bind with the same affinity to any particular insect (Ihara et al. 1993,Van Rie et al. 1990b). To test the effect of mutations in these loops, alanine substitution mutations were made in three amino acids in loop1 (F313A, N314A, and Y315A), two amino acids in loop2 (N376A and N377A), and two amino acids in loop3 (Y445A and T446A) in the CryIAa gene. Various combinations of these loop mutations were also constructed. Heterologous competition studies are shown in Fig. 3.

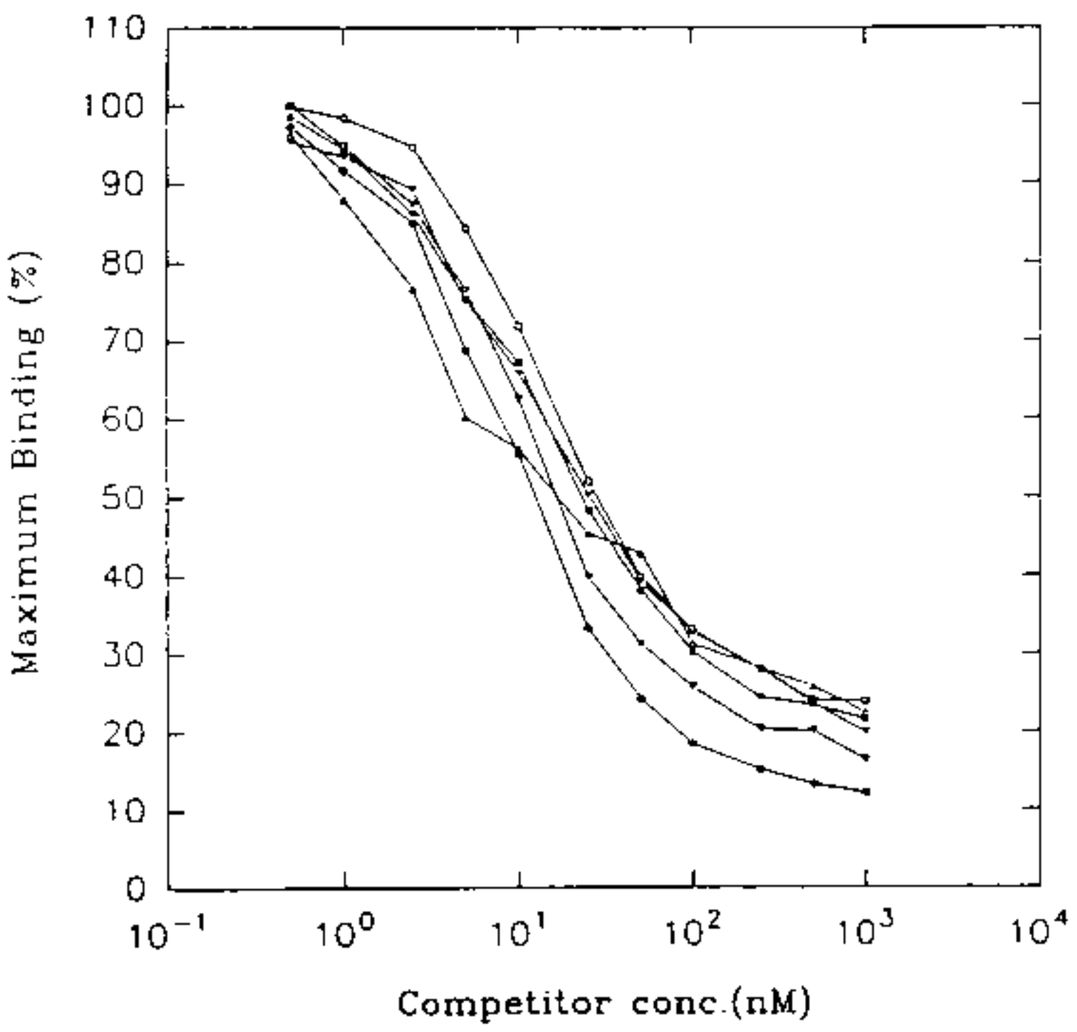

Fig. 3: heterologous competition binding of ^{125}I-labeled CryIAa toxin with increasing amounts of unlabeled CryIAa toxin and other mutants in loop regions. 1 nM labeled toxin with increasing amounts (0-1000nM) of competitor toxins were incubated with 40 µg *Bombix mori* BBMV in 100 µl binding buffer at room temperature for 1 hr. Maximum binding was obtained as described by Lee et al. (1992). Competitor toxins are CryIAa (●), loop1 mutation (▽), loop2 mutation (▼), loop3 mutation (❑), loop1,2 double mutation (■), loop1,3 double mutation (Δ).

DISCUSSION AND CONCLUSIONS

Binding studies with the three CryIA toxins against Gypsy moth (Fig. 1, Table) showed that the relative binding affinities were CryIAa CryIAc CryIAb, while the toxicities of these toxins are CryIAa CryIAb CryIAc (NR Dubois, pers. commun.). Our results agree with Wolfersberger (1990) with respect to the comparison between binding and toxicity for CryIAb and CryIAc.

TABLE

The concentration of binding sites and equilibrium dissociation constants for CryIA toxins on brush border membrane vesicles of larval Gypsy moth (*Lymantria dispar*)

Toxin	K_d	Bmax	Ref
CrylAa(4101)	0.44	9.26	Kwak (1992)
CrylAc (4201)	1.25	5.72	Kwak (1992)
CrylAb (4301)	3.65	3.33	Kwak (1992)
CrylAb (HD1-9)	19.8	2.70	Wolfersberg (1990)
CrylAc (HD-73)	2.03	3.69	Wolfersberger (1990)

Comparison between CryIAa and CryIAc, however, reveals that a direct correlation exists between binding and toxicity. Therefore, it is CryIAb that shows unusually lower binding than expected for its toxicity. This phenomenon remains unexplained.

Alanine-scanning mutations were made in the loop regions to test the hypothesis that loops are involved in binding (Li et al. 1991). These results seem inconclusive because only minor alterations in the heterologous competition binding curves can be observed in Fig. 3. Further experiments testing other properties of these mutations need to be performed before the full effects of these mutations can be evaluated. For example, measurements of the saturation binding and dissociation rates would allow a better understanding of the role of the particular amino acids in binding and insertion.

Binding of *B. thuringiensis* δ-endotoxin to its receptors is a subject still in its infancy. What we think we know about this binding is still more a matter of speculation than of demonstrated fact. We will be better in viewing this "knowledge" as hypotheses to be tested. This is even more evident in the current state of knowledge about the ion channel function of the δ-endotoxin. Further experimentation on these functions is needed at every level: entomology, biochemistry, genetics, and electrophysiology. As we apply reductionist experimentation, we seem to be led into greater confusion and questions about how the toxin functions. But endeavor we must until one day, soon we hope, enough data will be collected to allow a clearer picture of the mechanism of action of *B. thuringiensis* δ-endotoxin.

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