The toxicity of two *Bacillus thuringiensis* δ-endotoxins to gypsy moth larvae is inversely related to the affinity of binding sites on midgut brush border membranes for the toxins

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Summary. The δ-endotoxin from *Bacillus thuringiensis* subspecies *kurstaki* strain HD1-9 is almost 400 times more potent than the δ-endotoxin from strain HD-73 as a gypsy moth larvicide. The two δ-endotoxins compete for a high-affinity binding site on the brush border membrane of larval gypsy moth midguts. The affinity for the δ-endotoxin from strain HD-73 is much greater than the affinity for the δ-endotoxin from strain HD 1–9.
Key words. Membrane vesicles; *Lymantria dispar*.

During sporulation *Bacillus thuringiensis* produces parasporal inclusions with insecticidal activity. The parasporal inclusions produced by most subspecies of *B. thuringiensis* are active only against the larvae of a few lepidopteran insects. Lepidopteran-active parasporal inclusions are usually bipyramidal crystals consisting of one or more 130–140 kDa polypeptides. These crystal polypeptides are designated as protoxins. The complete insecticidal activity of each protoxin resides in a 55–70 kDa protease resistant toxin which results from solubilization and partial digestion of the crystals in the larval midgut[1].

The target of lepidopteran-active *B. thuringiensis* toxins is the apical membrane of larval lepidopteran midgut cells[2]. The high specific larvicidal activity and great specificity of *B. thuringiensis* toxins implies the involvement of specific membrane receptors in the insecticidal interactions between toxins and target cells. Convincing evidence for the existence of high affinity receptors for *B. thuringiensis* toxin in the brush border membrane of larval midgut cells was first obtained for *Pieris brassicae*[3]. Subsequently there have been reports of saturable high-affinity binding between several *B. thuringiensis* toxins and brush border membrane vesicles prepared from larval midguts of two other species of lepidopteran insects[4, 5]. In all of these studies a strong positive correlation between the binding and the toxicity of the various *B. thuringiensis* toxins was demonstrated.

The studies reported in this communication demonstrate the presence of a high-affinity binding site for two closely related *B. thuringiensis* toxins in the brush border membrane of larval *Lymantria dispar* midgut. They also show an inverse correlation between the binding and the toxicity of these two toxins.

Materials and methods

Insects. Lymantria dispar larvae were used in all experiments. *L. dispar* eggs were obtained from the USDA Forest Service Experiment Station (Hamden, CT). Larval diet was purchased from ICN Biochemicals (Cleveland, OH). The larvae were raised to experimental size at 25 °C with constant light.

Midgut isolation and vesicle preparation. Last instar larvae were chilled in crushed ice for 15–20 min. Chilled larvae were transected immediately behind the fourth pair of abdominal appendages and again immediately behind the first pair of thoracic appendages. The integument was cut open and spread apart exposing the midgut. The midgut was rinsed with ice-cold 0.25 M sucrose in 5 mM Tris-HCl, pH 8 (sucrose solution) and the tracheoles attaching it to the integument were severed. The midgut was cut longitudinally and opened to form a flat sheet. Midgut contents were rinsed away with ice-cold sucrose solution. The peritrophic membrane and the Malpighian tubules were removed using forceps. The isolated midgut was rinsed with ice-cold sucrose solution, gently blotted, and weighed. Isolated midguts were either used immediately for vesicle preparation or placed in a vial with a small amount of sucrose solution and frozen by immersing the vial in liquid nitrogen. Frozen midguts were stored at − 80 °C until use.

Brush border membrane vesicles (BBMV) were prepared from either fresh or frozen midguts by the differential magnesium precipitation method of Biber et al.[6] as modified and described by Wolfersberger et al.[7]. The protein concentration of BBMV preparations as well as toxin and protoxin solutions was determined by the method of Bradford[8] using a Bio-Rad (Richmond, CA) kit with bovine serum albumin as standard.

Bacterial strains. Bacillus thuringiensis subspecies *kurstaki* strain HD-73 was kindly provided by Dr Howard Dulmage (USDA Cotton Insects Research Unit, Brownsville, TX). *Bacillus thuringiensis* subspecies *kurstaki* strain HD 1–9 was kindly provided by Dr Bruce Carlton (Ecogen, Inc., Langhorne, PA). Each of these strains contains a single gene for a lepidopteran-active insecticidal crystal protein[9, 10]. The insecticidal crystal protein gene of strain HD 1–9 is classified as type *cryIA(b)* and encodes a 130.6 kDa polypeptide. The insecticidal crystal protein gene of strain HD-73 is classified as type *cryIA(c)* and encodes a 133.3 kDa polypeptide[11].

Culture conditions, parasporal crystal, and toxin isolation. The growth of cultures, isolation of parasporal crystals, and preparation of crystal protein solutions were as de-

scribed by Jaquet et al.[12]. Preparation and purification of toxins was as described by Hofmann and Lüthy[13]. The purity and molecular size of crystal protein and toxin preparations was routinely monitored by SDS-PAGE[14].

Iodination of toxins. Both toxins were iodinated using IODO-BEADS according to the recommendations of the manufacturer (Pierce, Rockford, IL). 0.5 mCi of carrier-free $Na^{125}I$ (Dupont-NEN, Boston, MA), was added to a vial which contained one IODO-BEAD and 0.1 mg of toxin in 0.1 ml of 50-mM sodium carbonate buffer, pH 9.5. After a 15-min incubation at room temperature, reaction was stopped by the addition of 0.1 ml of 50-mM sodium metabisulfite. The reaction mixture was loaded onto a 2-ml EXCULOSE column (Pierce, Rockford, IL) and iodinated toxin was eluted with 1.5 ml of carbonate buffer. The specific activity of the ^{125}I-labeled HD-73 toxin was 1.5 mCi/mg. The specific activity of the ^{125}I-labeled HD 1–9 toxin was 1.7 mCi/mg. Crystalline bovine serum albumin was added to the iodinated toxin solutions to a final concentration of 1 mg/ml and the resulting solutions were stored at 4 °C.

Binding assays. Preparation of incubation mixtures, 30-min incubations, separation of bound from free toxin, washing, and final resuspension of the membrane vesicles were as described by Hofmann et al.[3]. The washed vesicle resuspensions were mixed with 10 ml of liquid scintillation cocktail (Scinti-Verse BD, Fisher Scientific, Pittsburgh, PA) and counted in a liquid scintillation spectrometer (Model 2000CA, Packard Instrument, Downers Grove, IL). All assays were replicated at least three times. Incubation mixtures (0.1 ml) typically contained 1 nM ^{125}I-labeled HD-73 toxin and 18 µg of BBMV or 2 nM ^{125}I-labeled HD 1–9 toxin and 36 µg of BBMV plus unlabeled toxins as indicated in the figure legend. BBMV were always the last component added to the incubation mixtures. Dissociation constants and binding site concentrations were determined by the method of Scatchard[15].

Bioassays. The larvicidal activities of δ-endotoxins were determined by ad libitum feeding assays. Larval diet was dispenced into 24-well tissue culture plates (Falcon 3047, Becton Dickinson, Lincoln Park, NJ). When the diet had solidified, 20 µl of solution containing between 0 and 10 µg of δ-endotoxin was pipetted onto the diet in each well. After the solvent had evaporated, a first instar larva was placed in each well and the plates were covered with their lids. The plates were incubated at 25 °C for four days. At least 20 larvae were challenged with each of five different amounts of each toxin. The results of the assays were evaluated by probit analysis[16]. The concentrations required for 50% mortality within four days were determined by linear regression.

Results

The results of bioassays to determine the *L. dispar* larvicidal activity of toxins from *B. thuringiensis* strains HD 1–9 and HD-73 are reported in table 1. The toxin from strain HD 1–9 was approximately 400 times more potent than the toxin from strain HD-73. Studies with other lepidopteran insect larvae have shown strong positive correlations between the larvicidal activity of *B. thuringiensis* toxins and binding between the toxins and brush border membrane vesicles prepared from larval midguts[4,5]. Therefore, it was of interest to study the binding between HD-73 and HD 1–9 toxins and larval *L. dispar* BBMV.

Table 1. Gypsy moth toxicity of two *Bacillus thuringiensis* delta-endotoxins

Toxin	LC_{50}	FL_{95}
HD 1-9	1.08	0.35 – 3.41
HD-73	425	144 – 1253

50% lethal concentrations (LC_{50}) and 95% fiducial limits (FL_{95}) are expressed in ng of toxin per cm^2 of larval diet.

Table 2. Concentration of binding sites and equilibrium dissociation constants for two *Bacillus thuringiensis* toxins on brush border membrane vesicles of larval gypsy moth midgut

Toxin	K_d	B_{max}
HD 1-9	19.8 ± 6.1	2.70 ± 0.69
HD-73	2.03 ± 0.82	3.69 ± 1.23

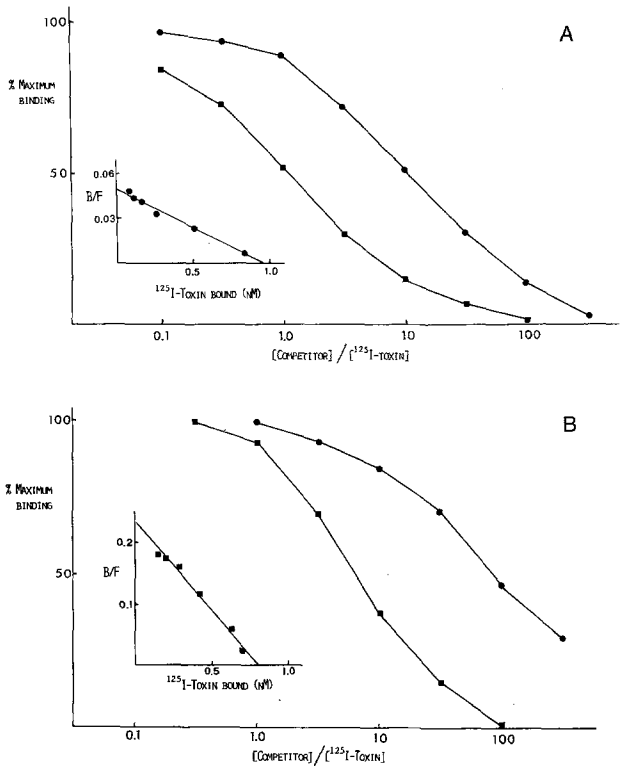
Dissociation constants (K_d) are expressed in nM. Binding site concentrations (B_{max}) are expressed in pmol per mg of vesicle protein. Dissociation constants and binding site concentrations are the mean of 4 (HD 1–9) or 6 (HD-73) determinations ± 1 SD.

The results of these binding studies are summarized in table 2. The dissociation constant for binding between HD 1–9 toxin and larval *L. dispar* midgut BBMV was approximately 10 times greater than that for binding between the BBMV and HD-73 toxin. The correlation between the *L. dispar* larvicidal activity of these two toxins and the affinity of binding sites on brush border membrane vesicles prepared from larval *L. dispar* midguts was clearly inverse. However, there was no difference in the concentration of high affinity binding sites for the two toxins. Therefore, it was of interest to determine whether the two toxins compete for the same site.

The results of a typical competition study are shown in the figure. In the absence of competitor approximately 5% of the ^{125}I-labeled HD 1–9 toxin was bound to *L. dispar* midgut BBMV. This binding was saturable with unlabeled HD 1–9 toxin in the range of 2–200 nM. HD-73 toxin, in the range of 0.2–20 nM, competed for the binding of ^{125}I-labeled HD 1–9 toxin to the vesicles. In the absence of competitor 20–25% of the ^{125}I-labeled HD-73 toxin was bound to *L. dispar* midgut BBMV. This binding was saturable with unlabeled HD-73 toxin in the range of 1–100 nM. HD 1–9 toxin, in the range of 10–1000 nM, competed for the binding of ^{125}I-labeled HD-73 toxin to the vesicles.

Discussion

Insecticidal toxins from *B. thuringiensis* subsp. *kurstaki* strains HD 1–9 and HD-73 each bind to brush border



A Binding of ^{125}I-labeled HD 1–9 toxin to *L. dispar* brush border membrane vesicles. *B* Binding of ^{125}I-labeled HD-73 toxin to *L. dispar* brush border membrane vesicles. Vesicles were incubated with labeled toxin plus the indicated concentrations of unlabeled HD 1–9 toxin (●) or HD-73 toxin (■). Binding is expressed as percent of labeled toxin bound in the absence of unlabeled toxin. (Insets) Data from competition between labeled toxin and the same unlabeled toxin plotted in Scatchard coordinates. The linear regression correlation coefficient of both lines is greater than 0.97.

membrane vesicles prepared from larval *L. dispar* midguts in a manner consistent with the presence of a single high-affinity binding site. The measured binding site concentrations as well as the results of competition experiments are consistent with both toxins binding to the same site with the dissociation constants reported in table 2. The finding of an inverse correlation between toxicity and binding of these two toxins is a novel result of the studies reported herein.

Previous studies of interactions between *B. thuringiensis* toxins and larval lepidopteran midgut BBMV have shown that toxin binding is not a simple reversible process[3,5]. Initial reversible binding is followed rapidly by an irreversible interaction between the toxin and the membrane. This irreversible step is thought to involve insertion of at least a portion of the toxin molecule into the membrane, initiating formation of a pore[17]. This membrane pore is the primary lesion in the cytolytic mode of action of insecticidal *B. thuringiensis* toxins[2,18]. HD-73 toxin is clearly able to compete very effectively with HD 1–9 toxin in the initial membrane receptor binding step of this mechanism of action. However, HD-73 toxin is a much less potent larvicide than HD 1–9 toxin (table 1). One possible explanation for this inverse corrolation between receptor affinity and toxicity is that formation of pores in the brush border membrane of larval gypsy moth midgut by the HD 1–9 toxin-receptor complexes proceeds much more readily than pore formation by the HD-73 toxin-receptor complexes.

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