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Susceptibility of *Manduca sexta* to Cry1Ab toxin of *Bacillus thuringiensis* correlates directly to developmental expression of the cadherin receptor BT-R_1

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ABSTRACT

The cadherin receptor BT-R_1, localized in the midgut epithelium of the tobacco hornworm, *Manduca sexta*, is coupled to programmed oncotic-like cell death, which is triggered by the univalent binding of the Cry1Ab toxin of *Bacillus thuringiensis* (Bt) to the receptor. Kinetic analysis of BT-R_1 expression during larval development reveals that the density of BT-R_1 on the midgut surface increases dramatically along with an equivalent rise in the concentration of Cry1Ab toxin molecules needed to kill each of the five larval stages of the insect. The increase in the number of BT-R_1 molecules per midgut surface area requires additional toxin molecules to kill older *versus* younger larvae, as evidenced by the corresponding LC_{50} values. Based on these observations, we developed a mathematical model to quantify both the expression of BT-R_1 and the susceptibility of *M. sexta* larvae to the Cry1Ab toxin. Interestingly, the toxin-receptor ratio remains constant during larval development regardless of larval size and mass. This ratio apparently is critical for insecticidal activity and the decrease in toxin effectiveness during larval development is due primarily to the number of effective toxins and available receptors in the larval midgut. Evidently, susceptibility of *M. sexta* to the Cry1Ab toxin of Bt correlates directly to the developmental expression of BT-R_1 in this insect.

1. Introduction

Cadherins, in general, mediate monotypic cell–cell adhesion and are fundamental to normal cell and tissue development. They act as both receptor and ligand and are responsible for internal cellular signaling and cell sorting, both of which are necessary for properly positioning different cell types during development. It is noteworthy that insect larval development depends on various mechanisms that regulate the development and behavior of different cell groups. Cell behavior involves processes such as cell growth, cell division and cell death, among others. The mechanisms controlling these activities include various signaling pathways, some of which are cadherin-related and are highly conserved (Lecuit and Lenne, 2007).

Previously, we reported on the developmental expression of BT-R_1, a midgut-specific cadherin of *Manduca sexta*, which apparently is essential to the organization and remodeling of midgut epithelium during rapid cell proliferation and differentiation as well as tissue growth (Midboe et al., 2003). Indeed, the dramatic tissue-specific increase in BT-R_1 along with accumulation of its mRNA most likely signifies the importance of the molecule in maintaining the structural integrity of the alimentary canal of this insect (Midboe et al., 2003). The cadherin BT-R_1 itself contains a highly conserved structural motif for the binding of Cry1Ab toxin which is characteristic of homologous lepidopteran cadherins (Griko et al., 2007). Binding of toxin to this motif triggers a signaling event that leads to oncotic-like cell death (Zhang et al., 2005, 2006). Furthermore, the 5′-UTR of the BT-R_1-encoding gene contains sequence motifs that apparently recruit specific transcription factors which determine posterior patterning and control intestinal cell proliferation, differentiation and identity during development (Midboe et al., 2003). Because cell death is a natural process that eliminates cells during normal insect development and is important to the appropriate structuring of organs and entire systems (Schwartz et al., 1990; Robinow et al., 1983; Zakeri and Lockshin, 1994; Steller, 1995), it is not surprising that there is such a dramatic proliferation in the number of BT-R_1 molecules which potentially could bind inborn death ligands and destroy the midgut epithelium, clearing the larva of excessive tissue before it enters the pupal stage. Any alien ligands such as the Cry toxins of *Bacillus thuringiensis* (Bt) could take advantage of such a situation and deliver a death blow to the insect.

In the present study, we investigated the susceptibility of *M. sexta* to the Cry1Ab toxin of Bt during larval development. The spectacular

Abbreviations: AC, adenylyl cyclase; BBMV, brush border membrane vesicles; BCA, bicinchoninic acid; BSA, bovine serum albumin; LC_{50}, the concentration of Cry1Ab toxin required to kill 50% of the larval population; PKA, protein kinase A; PMSF, phenylmethanesulphonylfluoride.

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growth and enlargement of the larva as it progresses through its five developmental stages (instars) reflects the rapid proliferation and differentiation of cells throughout the larva (Baldwin and Hakim, 1981; Midboe et al., 2003). We have quantified both the expression of BT-R_1 and the susceptibility of *M. sexta* to the Cry1Ab toxin. The increase (×50) we observed in the density of BT-R_1 molecules in the midgut surface of *M. sexta* during larval growth signifies the developmental importance of this protein, and the corresponding increase (×50) in the LC_{50} value for Cry1Ab toxin indicates that a critical ratio of toxin to receptor is required for larval death. We constructed a mathematical model to correlate susceptibility of *M. sexta* to the toxin and the developmental expression of its natural receptor in the insect. The model reveals a critical toxin-receptor ratio required for insecticidal activity.

2. Materials and methods

2.1. Preparation of midgut tissue and BBMV from M. sexta larvae

Eggs from *M. sexta* (Sphingidae) and artificial diet prepared especially for growing larvae were obtained from Carolina Biological Supply. Larvae were selected for analysis and midgut isolation as described by Midboe et al. (2003). Midguts were frozen immediately in liquid nitrogen and stored at −80 °C. Brush border membrane vesicles (BBMV) from the midgut of each larval instar of *M. sexta* were prepared according to Wolfersberger et al. (1987), as modified by Keeton et al. (1998). Proteins were extracted from BBMV according to the protocol of Wieczorek et al. (1986).

2.2. Isolation of parasporal crystals and purification of Cry1Ab toxin

B. thuringiensis subsp. *berliner* 1715 was grown in liquid GYS medium at 30 °C for three days, allowing sporulated cells to lyse and release the spores and parasporal crystals. Separation of parasporal crystals from spores and purification of Cry1Ab toxin from the parasporal crystals were achieved as described by Vadlamudi et al. (1993). Purity and integrity of the active toxin was determined by SDS-PAGE and toxin concentration was quantified using the BCA protein assay with BSA fraction V as standard (Pierce).

2.3. Radio-iodination of Cry1Ab toxin

Cry1Ab toxin was radio-iodinated using the chloramine-T method (Hunter and Greenwood, 1962). Ten μg of Cry1Ab toxin were mixed with 100 μg of cloramine T and 5 μL of $Na^{125}I$ (0.5 mCi) in 100 μL of phosphate-buffered saline (PBS). The reaction mixture was shaken gently at room temperature for 20 s, and the reaction was stopped by the addition of 200 μg of $Na_2S_2O_5$. Free iodine was removed by gel-filtration on a Sephadex G-50 column equilibrated with PBS. Specific activities of the labeled toxin varied from 10–15 mCi/mg.

2.4. Biometrics and toxicity assays

Eggs were incubated at 27 °C on the artificial diet. During growth, larvae were selectively harvested according to their stage of development and weighed and measured. Larvae were designated as specific instars primarily according to their length: first instar, 5–8 mm; second instar, 9–18 mm; third instar, 19–30 mm; fourth instar, 31–45 mm; and fifth instar, 46–75 mm. Larvae selected at each developmental stage showed no signs of molting.

Toxicity assays were performed on all instars as described previously (Schesser et al., 1997). The assays were done in 38-cm^2 plant tissue culture vials filled with artificial insect diet to a depth of 1 cm. Various amounts of purified Cry1Ab toxin were diluted in water and 0.1 ml of each concentration was spread on the surface of the artificial diet and allowed to dry. First through fifth larval instars (50 larvae per treatment) were fed on a diet containing increasing concentrations of Cry1Ab toxin. Each bioassay was performed in triplicate. The larvae were monitored for 72 h and insect mortality was recorded. LC_{50} values (expressed as ng/cm^2) were based on the concentration of Cry1Ab toxin spread on the insect diet surface. All bioassays were repeated at least five times. Probit analysis was used to establish LC_{50} values and standard error (Finney, 1971).

2.5. Quantification of BT-R_1 expression

To determine the level of BT-R_1 expression during each stage of larval development, toxin binding assays (Hofman et al., 1988) were performed using BBMV. The final BBMV preparation was suspended in 150 mM NaCl, 2.7 mM KCl and 9.5 mM $NaPO_4$ buffer containing 0.1 mM PMSF. The BBMV were used either immediately or frozen in liquid nitrogen at −80 °C. The amount of BBMV protein was determined by the BCA protein assay. For competition binding assays, duplicate samples of BBMV (10 μg) were incubated with 0.3 nM ^{125}I-Cry1Ab toxin in the presence of varying amounts of unlabeled toxin. Labeled and unlabeled toxins were mixed together before adding them to BBMV. Radioactivity due to nonspecific binding (binding observed in the presence of 10 μM unlabeled Cry1Ab toxin) was

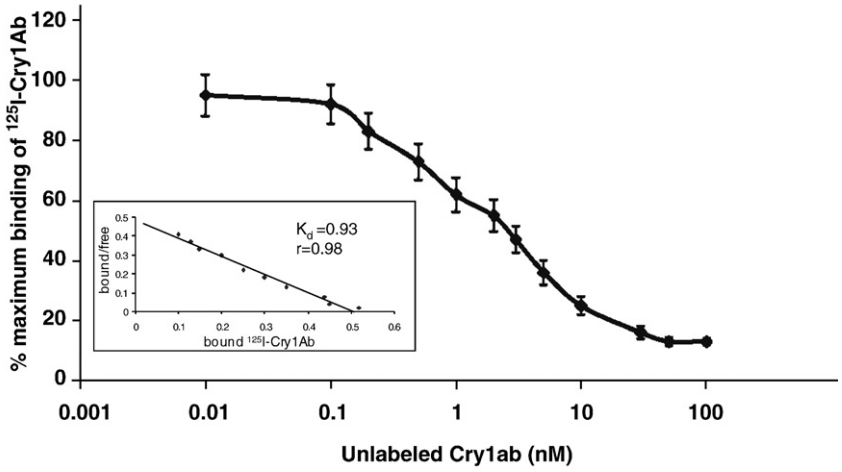

Fig. 1. Competition inhibition of CrylAb toxin binding to BBMV proteins of the third-instar larva of *M. sexta*. Specificity and affinity of Cry toxin binding to BBMV proteins were determined by competition inhibition analysis using ^{125}I-CrylAb binding to BBMV proteins in solution. Radiolabeled toxin binding was determined in the presence of increasing concentrations of unlabeled Cry1Ab toxin. The dissociation constant for toxin binding affinity (K_d=0.93 nM) and the binding site concentration (3.2 pmole/mg) were established by Scatchard analysis (Scatchard, 1949).