CURRENT MICROBIOLOGY Vol. 48 (2004), pp. 1–9
DOI: 10.1007/s00284-003-4056-y

Current Microbiology
An International Journal

Interaction of Two *Bacillus thuringiensis* δ-Endotoxins with the Digestive System of *Lygus hesperus*

Sandra L. Brandt,[1]* Thomas A. Coudron,[1] Javad Habibi,[2] Gregory R. Brown,[3] Oliver M. Ilagan,[3] Renee M. Wagner,[1] Maureen K. Wright,[1] Elaine A. Backus,[2] Joseph E. Huesing[3]

[1]Biological Control of Insects Research Laboratory, U.S. Department of Agriculture, Agricultural Research Service, 1503 S. Providence Rd, Columbia, MO 65203
[2]Dept. of Entomology, University of Missouri, Columbia, MO 65211
[3]Monsanto Co., 700 Chesterfield Parkway North, Chesterfield, MO 63198

Received: 7 January 2003/Accepted: 18 April 2003

Abstract. The active-toxin form of Cry1Ac (65 kDa) or Cry2Ab was fed to a non-susceptible insect, *Lygus hesperus*, in an artificial diet. Biochemical and immunocytochemical methods were used to determine the distribution of ingested toxin. The toxins did not elicit a feeding deterrent response. Cry1Ac and Cry2Ab were ingested; small amounts were absorbed into the hemolymph as holoproteins, but most was excreted. SDS-PAGE analysis of Cry1Ac and Cry2Ab incubations with salivary gland homogenate showed a small decrease in the molecular weight of the active toxins. Proteolytic processing of the toxins also occurred in vivo, within the digestive system of *L. hesperus*. Excreted Cry1Ac and Cry2Ab retained activity toward lepidopteran larvae. Immunocytochemical in vivo localization studies showed negligible association of Cry1Ac with *L. hesperus* tissues. In contrast, strong extracellular association of Cry2Ab was observed with *L. hesperus* midgut brush border microvilli and basement membrane, as well as with cellular outlines within the hemolymph and fat body.

The soil bacterium *Bacillus thuringiensis* (Bt) produces various crystalline inclusions composed of insecticidal crystal proteins (ICPs) or δ-endotoxins. These ICPs have highly specific entomocidal activity against several orders of insects [26], increasing their attractiveness for use as biopesticides. Genetically engineered Bt plants have been commercially available for corn, cotton, and potato, with world-wide use increasing each year [24]. Consequently, information about the distribution and interactions of these toxins on target and non-target hosts is of value to the scientific community.

B. thuringiensis ICPs are classified into different groups, Cry and Cyt, based on amino acid sequence relationships [6, 7, 26, http://www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt]. Cry1Ac, produced as a 130-kDa protoxin, is solubilized and processed in the alkaline midgut of lepidopteran insects to create a 65-kDa activated toxin [17, 26]. Cry2Ab is produced as an estimated 71-kDa protein. The exact processing of this protoxin for insecticidal activity remains unknown [3, 10]. The model for Bt intoxication is similar for all Cry proteins, consisting of a series of events involving solubilization, activation, binding, and pore formation that eventually leads to the destruction of the midgut and death of the insect [13, 21–23]. The level of toxicity and insect specificity can be affected at each of these steps. The current study bypassed the solubilization and activation steps by feeding trypsin-activated Cry1Ac and solubilized Cry2Ab (lepidopteran active-toxin forms). This report focuses on the fate of these two Bt δ-endotoxins as they pass through the digestive system of a non-susceptible host, *Lygus hesperus* Knight (Heteroptera; Miridae).

The western tarnished plant bug, *L. hesperus*, is a phytophagous piercing-sucking insect that is reported to feed on 117 non-crop plants and over 25 cultivated plants [27], but is primarily known as a pest of cotton and seed alfalfa [18, 29]. Resistance to commonly used insecti-

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Correspondence to: T.A. Coudron; *email:* coudront@missouri.edu

cides is an increasing problem [9], and other means of controlling this pest are needed. The objectives of this study were to determine 1) whether *L. hesperus* ingested Bt δ-endotoxins when administered through diet, 2) whether any proteolytic processing of the active toxin occurred after ingestion by the insect, 3) the distribution of the toxins within the insect, and 4) the association of the toxins with insect tissues.

Materials and Methods

Toxin preparation. Purified and preparative (prep) forms of Cry1Ac and Cry2Ab were obtained from Monsanto Co. (Chesterfield, MO). For Cry1Ac, crystal proteins from recombinant Bt strain EG11771 were solubilized in 100 mM sodium carbonate, 10 mM DTT, pH 10.75, clarified (8000 *g*), treated with bovine pancreatic trypsin (Sigma-Aldrich, St. Louis, MO) after pH adjustment to 8.5, and dialyzed against 50 mM sodium chloride and 50 mM sodium carbonate, pH 10.5. This procedure yielded the Cry1Ac prep form. The Cry1Ac purified form was prepared by further purification of the prep form using anion-exchange chromatography (Sigma Q-Sepharose).

Cry2Ab was isolated from recombinant Bt strain EG7699 crystal proteins. For the prep form, proteins were purified by sucrose gradient separation (79%/70%/55% sucrose), solubilized in 100 mM sodium carbonate, 10 mM DTT, and 0.05 mM PMSF, pH 11.5, clarified (13,800 *g*), and dialyzed against 50 mM sodium carbonate, pH 10.5. For the Cry2Ab purified form, proteins were solubilized in 100 mM sodium carbonate, 10 mM DTT, and 0.05 mM PMSF, pH 11.5, clarified (13,800 *g*), dialyzed against 50 mM sodium carbonate, pH 10.5, and further purified by anion-exchange chromatography (Q-Sepharose).

Protein concentrations were determined by densitometry with bovine serum albumin as a standard. The in vitro studies were conducted with the purified form of the toxins. However, because more Cry protein was required in the diet delivery experiments, the in vivo studies were conducted with the prep form of the toxins.

Insect rearing and dissections. A stock colony of western tarnished plant bug was reared continuously on an artificial diet (*L. hesperus* diet, Bioserv, Frenchtown, NJ) under 16L:8D photoperiod, 27°C, and 70% relative humidity [14]. Adult female *L. hesperus* that had been starved for 8 h were used in this study. Insects were dissected in HEPES wash buffer (HWB, 70 mM sodium chloride, 30 mM HEPES, 2 mM calcium chloride, pH 7.4). Hemolymph (containing some fat body and cellular material), salivary gland, and alimentary canal (hereafter referred to as gut) were isolated as described previously [15].

In vitro study of toxin susceptibility to insect proteolytic degradation. Insect tissues were pooled and homogenized; 0.5 insect equivalent (eq.) of each tissue was combined with 1.08 μg of the Cry1Ac purified form or 1.19 μg of the Cry2Ab purified form in 0.5 mL siliconized Eppendorf tubes and incubated at 37°C for 0 or 24 h. Samples were centrifuged at 12,000 *g* for 10 min, and the supernatants were immediately analyzed. Incubations of the tissue homogenates with FTC-casein were performed by using a protease assay kit (Calbiochem, San Diego, CA), following the manufacturer's directions.

In vivo study of toxin susceptibility to insect proteolytic degradation. For experimental purposes, diet and treatment substances were delivered in a sachet system [15] containing 40 μL of artificial diet (control) or 40 μL of artificial diet with 0.1% (1000 ppm) Cry1Ac or 0.05% (500 ppm) Cry2Ab (treatment) encapsulated between stretched Parafilm and Mylar sheets that were heat-sealed. Four insects were placed in a small petri dish containing two control or two treatment sachets. The insects were removed after 12 h and transferred to fresh control sachets for another 12 h, at which time the tissues were dissected. Tissues from four insects were pooled. Three groups of four insects were analyzed per study, with each study replicated a minimum of two times. Tissue samples were stored at −80°C. Sachets from the first 12-h period were weighed before and after feeding to determine the amount of diet that was ingested by each group of insects. Fecal material was extracted from each petri dish with 200 μL of purified water. Statistics were performed with SAS system software (1989–1996) [25]. The Shapiro-Wilk test ensured the normality of data.

Bioassay of *L. hesperus* fecal material. The biological activity of the Cry proteins in *L. hesperus* fecal material was evaluated by using a feeding bioassay with a susceptible host, the budworm, *Heliothis virescens*. Two or 20 μL of a 1:100 dilution of fecal material from treated insects (representing 1 or 10 ppm, respectively, of the Cry protein as estimated from Western blot densitometric analysis of the fecal material) or an identical dilution of fecal material from control insects was brought to a final volume of 50 μL and inoculated onto the surface of a 200-μL diet within each well of a 96-well assay tray. Eight to 12 budworm neonates were then placed on the diet and allowed to feed for 5–6 days, after which weights were recorded. A minimum of three replicates per treatment was performed.

SDS-PAGE analysis and Western blotting. Samples from both in vivo and in vitro studies were analyzed under denaturing conditions with 8% acrylamide sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by using the mini-Protean 3 electrophoresis system (Bio-Rad, Hercules, CA). Samples were mixed with loading buffer (0.042 M Tris pH 6.8, 4.2% glycerol, 4.2% 2-mercaptoethanol, 2% SDS, 0.002% bromophenol blue) and incubated at 100°C for 10 min. Sample volumes loaded were in vitro studies, 1–4 μL; Cry1Ac in vivo study, 0.7 eq. of hemolymph or gut homogenate, 1.3 eq. of salivary gland homogenate, or 25 μL fecal material; Cry2Ab in vivo study, 0.3 eq. of hemolymph or salivary gland homogenate, 0.07 eq. of gut homogenate, or 3 μL fecal material. Samples from control insects were prepared identically to the treatment samples. Samples were run at 120 volts for 1.5 h. For staining of the separated proteins, the gel was incubated for 10 min in staining solution [0.05% Coomassie Brilliant Blue R250 (Biorad), 50% methanol, 10% acetic acid] followed by overnight incubation in destaining solution (7% methanol, 5% acetic acid).

For Western blot analysis, proteins were transferred from the gel to a nitrocellulose membrane (Optitran BA-S83, Schleicher & Schuell, Keene, NH) by a semi-dry technique using the Trans-Blot SD semi-dry transfer cell (Bio-Rad). The membranes were blocked with 1% bovine serum albumin (Sigma Chemical Co., St. Louis MO) in Tris-buffered saline, probed with the appropriate rabbit polyclonal antisera (obtained from Monsanto Co.), diluted 1:1000 in PBST [phosphate buffered saline with 1% Tween 20 (Bio-Rad)], followed by an incubation with secondary antibodies: alkaline phosphatase-conjugated anti-rabbit immunoglobulin (Sigma) diluted 1:1000 in PBST for the Cry1Ac studies, or horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Pierce, Rockford, IL) diluted 1:20,000 in PBST for the Cry2Ab studies. The membranes were visualized colorimetrically with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) as a substrate for the Cry1Ac studies, or by chemiluminescence with a luminol-based substrate (SuperSignal, Pierce) and 1- to 10-s exposure to X-OMAT AR film (Eastman Kodak Co., Rochester, NY) for the Cry2Ab studies. Image and densitometric analyses of the Western blots and stained gels were performed with the Fluor-S MultiImager system with Quantity One software (Bio-Rad).

Immunocytochemical in vivo localization studies. Insects were fed

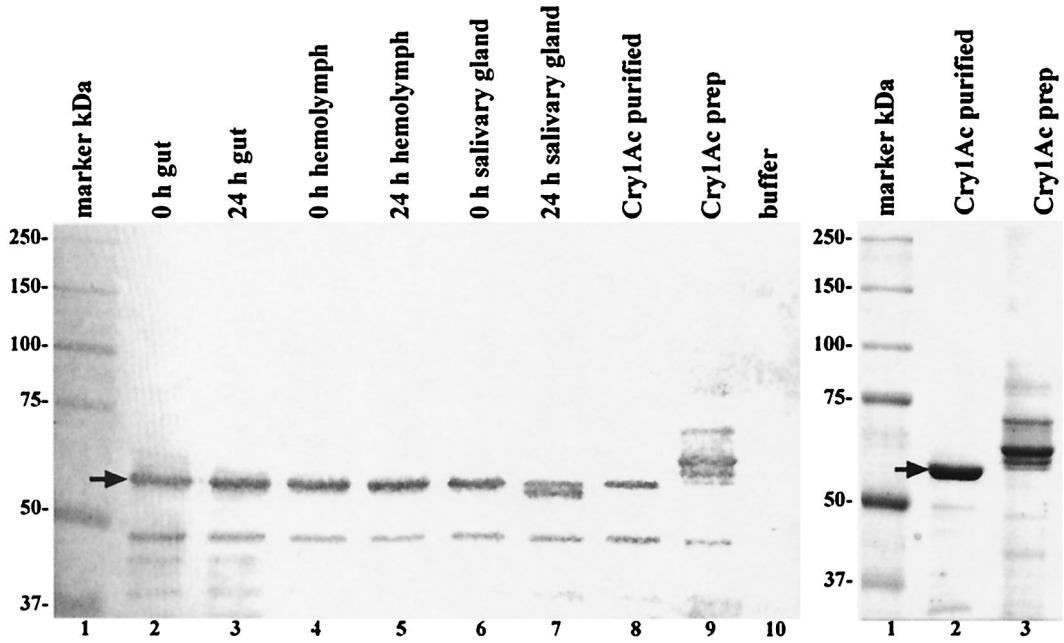

Fig. 1. Left panel: Western blot analysis under denaturing conditions of purified Cry1Ac incubated with *L. hesperus* tissues for 0 or 24 h. Lane 1, precision pre-stained molecular weight markers (Bio-Rad) with size indicated in the left margin. Lanes 2 and 3, gut homogenate. Lanes 4 and 5, hemolymph. Lanes 6 and 7, salivary gland homogenate. Lane 8, purified form of Cry1Ac. Lane 9, prep form of Cry1Ac. Lane 10, incubation buffer. The arrow indicates the region of the active form of the toxin at ca. 65 kDa. Right panel: Coomassie-stained SDS-PAGE analysis of Cry1Ac. Lane 1, Precision pre-stained molecular weight markers (Bio-Rad) with size indicated in the left margin. Lane 2, 2.5 μg of the purified form of Cry1Ac. Lane 3, 2.5 μg of the prep form of Cry1Ac. The arrow indicates the region of the active form of the toxin at ca. 65 kDa.

0.1% (1000 ppm) Cry1Ab for 16 h or 0.05% (500 ppm) Cry2Ab for 12 h, and whole insects were immediately fixed, embedded, and sectioned as described previously [15]. In addition, insects were fed 0.05% Cry2Ab for 2 h and then moved to control diet sachets. Insects were prepared immediately and at 2, 6, and 14 h after placement on control diet. The 10-μm sections were placed on saline-coated slides and incubated with 3% bovine serum albumin (BSA, Bio-Rad) and 1% non-fat dry milk (Bio-Rad) in HWB (Cry1Ac sections) or 1% donkey sera (Sigma), 3% BSA and 0.2% TritonX-100 (Sigma) in HWB (Cry2Ab sections). Sections were then incubated with polyclonal anti-Cry1Ac diluted 1:300 in HWB or with polyclonal anti-Cry2Ab diluted 1:600 in HWB, followed by incubation with donkey anti-rabbit immunoglobulin labeled with pentamethine cyanine dye, Cy™5 (Jackson ImmunoResearch Laboratories, West Grove, PA), diluted 1:300 in HWB. The slides were viewed on a confocal laser scanning microscope (Bio-Rad) with images captured using Laser Sharp 2000 software (Bio-Rad). The specificity of the signal was confirmed by incubating tissue sections from control and toxin-fed insects with a series of immunocytochemical labeling steps, in which each of the various steps of the regular labeling process was omitted, each of which resulted in the absence of signal.

Results

Toxin electrophoretic analysis. A prominent band at ca. 65 kDa (arrow) is observed in the Cry1Ac purified form in the Western blot analysis and Coomassie-stained gel (Fig. 1, left panel, lane 8; right panel, lane 2). Cry1Ac prep form has the band at ca. 65 kDa as a minor component, with several other bands at higher molecular weights (ca. 65–70 kDa) (Fig. 1, left panel, lane 9; right panel, lane 3). In the case of Cry2Ab, a band at ca. 65 kDa is more prominent in both forms with Coomassie staining. In the Western blot analysis, the band at ca. 71 kDa is more prominent than the band at ca. 65 kDa in the purified form (Fig. 2, arrow; left panel, lane 7), and the band at ca. 65 kDa is more prominent than the band at 71 kDa in the prep form of the Cry2Ab samples (Fig. 2, left panel, lane 8). Several bands of high molecular weights (ca. 150 and >250 kDa), thought to be oligomers of the protoxin, are also present in both forms.

In vitro studies of toxin susceptibility to insect proteolytic degradation. Degradation of FTC-casein over the 24-h incubation period confirmed the presence of endogenous protease activity within tissue homogenates (data not shown). Western blot analysis of the protein banding pattern of Cry1Ac purified form incubated with gut homogenate or hemolymph appears unchanged after 24 h (Fig. 1, left panel, lanes 2–5). Cry1Ac purified form incubated with salivary gland homogenate for 24 h showed a doublet banding pattern, with the presence of an additional band at ca. 62 kDa, not seen in the purified form at 0 h (Fig. 1, left panel, lanes 6 and 7). Western blot analysis of the protein banding pattern of Cry2Ab

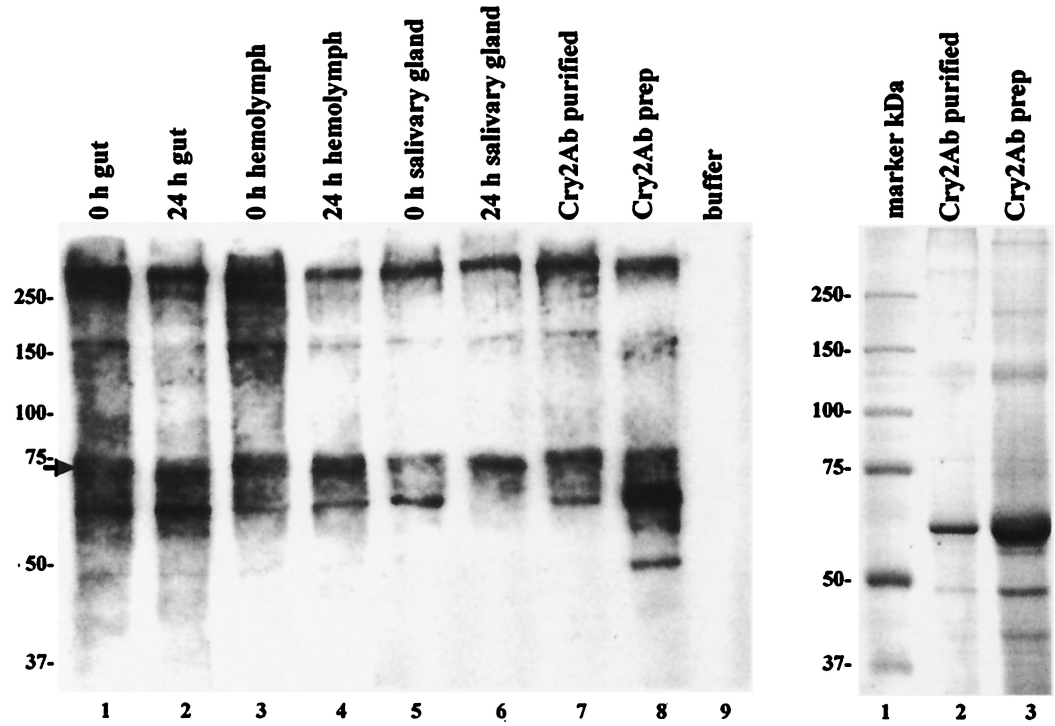

Fig. 2. Left panel: Western blot analysis under denaturing conditions of purified Cry2Ab incubated with *L. hesperus* tissues for 0 or 24 h. The location of molecular weight markers is indicated in the left margin. Lanes 1 and 2, gut homogenate. Lanes 3 and 4, hemolymph. Lanes 5 and 6, salivary gland homogenate. Lane 7, purified form of Cry2Ab. Lane 8, prep form of Cry2Ab. Lane 9, incubation buffer. The arrow indicates the region of the protoxin at ca. 71 kDa. Right panel: Coomassie-stained SDS-PAGE analysis of Cry2Ab. Lane 1, Precision pre-stained molecular weight markers (BioRad) with size indicated in the left margin. Lane 2, 2.5 μg of the purified form of Cry2Ab. Lane 3, 2.5 μg of the prep form of Cry2Ab.

purified form incubated with gut homogenate or hemolymph appears unchanged from the 0 h to 24 h time points (Fig. 2, left panel, lanes 1–4). However, comparison of the 0 h salivary gland homogenate with the 24-h time point showed a band of slightly lower molecular weight than the original band at ca. 71 kDa, and the disappearance of the band at ca. 65 kDa after a 24 h incubation (Fig. 2, left panel, lanes 5 and 6).

Diet consumption. Over a 12-h period, each group of four insects consumed an average of 24.66 mg of control diet versus 28.4 mg of Cry1Ac diet or 27.2 mg of Cry2Ab diet (Table 1). This corresponds to an average of 28.4 μg Cry1Ac or 13.6 μg Cry2Ab ingested per group of four insects over a 12-h period.

Table 1. Diet ingestion (mg/group of four insects)

Time fed (h)	Control diet[a] (n=20)	Treatment Cry1Ac[b] (n=10)	Treatment Cry2Ab[b] (n=19)
12	24.66 ± 0.96A	28.40 ± 1.36A [0.0284][c]	27.20 ± 0.99A [0.0136][c]

[a] *Lygus* diet diluted 2:1 with 50 mM sodium bicarbonate buffer, pH 10.2, to achieve a concentration similar to treatment diet.
[b] Cry1Ac and Cry2Ab prep forms were formulated in the diet at 1000 and 500 ppm, respectively.
[c] [mg Cry protein ingested] was calculated from the value of ingested diet. Means followed by the same letter are not significantly different at $P > 0.05$ and are reported as mean ± SD with significance determined by ANOVA analysis.

In vivo studies of toxin susceptibility to insect proteolytic degradation. No death was observed when insects were continuously fed 0.1% Cry1Ac or 0.05% Cry1Ac for 3 days (data not shown). Western blot analysis of tissues dissected from Cry1Ac-fed insects (Fig. 3) showed uniform and low background signal in the control tissues (lanes 2–5). Very little signal was detected above 65 kDa in the insect samples (Fig. 3, lanes 6, 7, 9, and 10). A band at ca. 65 kDa (arrow) and several bands in the 50–65 kDa range were seen in the gut (Fig. 3, lane 6). Two less intense bands at ca. 65 and ca. 60 kDa were seen in the hemolymph (Fig. 3, lane 7). No signal was visible in the 50–65 kDa region in the salivary gland

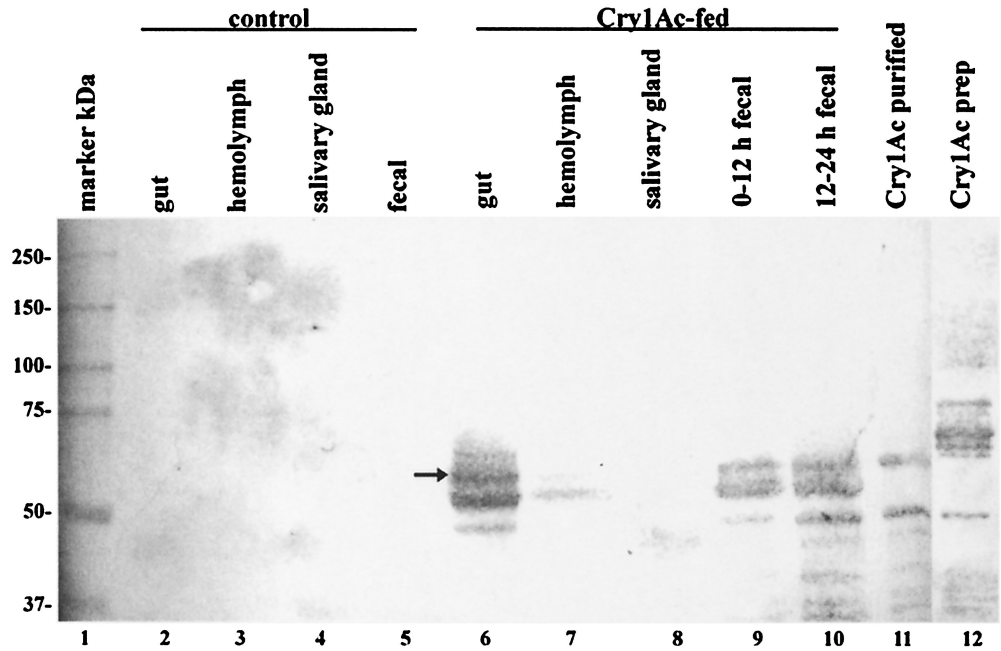

Fig. 3. Western blot analysis under denaturing conditions of the Cry1Ac in vivo study. Lane 1, precision pre-stained molecular weight markers with size indicated at the left margin. Lanes 2–5, tissues from control insects. Lanes 6–10, tissues from insects fed 0.1% Cry1Ac for 12 h and dissected after an additional 12 h. Lane 11, purified form of Cry1Ac. Lane 12, prep form of Cry1Ac. Arrow indicates the region at ca. 65 kDa.

(Fig. 3, lane 8). A band at ca. 65 kDa and several intense bands in the range of 50–65 kDa were seen in the 0–12 h and 12–24 h fecal material (Fig. 3, lanes 9 and 10). Western blot analysis of tissues dissected from Cry2Ab-fed insects (Fig. 4) showed low background signal in the control tissues and fecal material (lanes 1–3). Negligible signal was found in the salivary gland (Fig. 4, lane 6), similar to what was observed for the control salivary

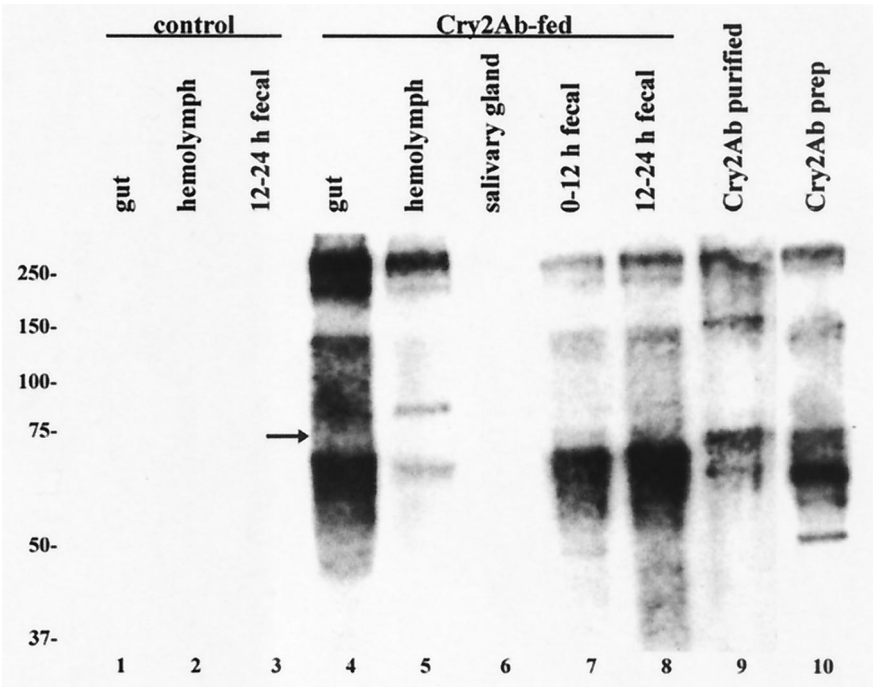

Fig. 4. Western blot analysis under denaturing conditions of the Cry2Ab in vivo study. The location of molecular weight markers is indicated in the left margin. Lanes 1–3, tissues from control insects. Lanes 4–8, tissues from insects fed 0.05% Cry2Ab for 12 h and dissected after an additional 12 h. Lane 9, purified form of Cry2Ab. Lane 10, prep form of Cry2Ab. Arrow indicates the region of ca. 71 kDa.

Table 2. Response of neonate budworm fed fecal material (FM) collected from control and Cry1Ac-fed (A) or Cry2Ab-fed (B) *Lygus hesperus*

A. Five-day feeding study of Cry1Ac activity	
Treatment[a]	Weight[b] (mg)
Untreated	5.97 ± 1.03A
Control FM (Ed 1)[c]	7.23 ± 0.74A
Control FM (Ed 10)[c]	5.60 ± 0.39A
Cry1Ac-fed FM 1 ppm	0.30 ± 0.09B
Cry1Ac-fed FM 10 ppm	0.13 ± 0.04B
B. Six-day feeding study of Cry2Ab activity	
Treatment[a]	Weight[b] (mg)
Untreated	22.41 ± 4.93A
Control FM (Ed 1)[c]	22.34 ± 8.49A
Control FM (Ed 10)[c]	30.63 ± 8.92A
Cry2Ab-fed FM 1 ppm	19.93 ± 1.84A
Cry2Ab-fed FM 10 ppm	4.32 ± 1.24B

[a] Treatment inoculated onto the surface of diet.
[b] Weights followed by the same letter within each table are not significantly different with Levene's test and Dunnett's method with significance level 0.05 and are reported as mean ± SD.
[c] Control fecal material was applied at 1 or 10 Equivalent dilution (Ed) to equal the treatment fecal material 1 ppm or 10 ppm, respectively, dilution.

gland (data not shown). Signal was observed at ca. 250, 140, 80, and just below ca. 71 kDa in the gut, hemolymph, and fecal material (Fig. 4, lanes 4, 5, 7, and 8, respectively).

Bioassay of *L. hesperus* fecal material. In the Cry1Ac study (Table 2A), insects fed control fecal material attained a mass of ca. 5.6–7.2 mg at 5 days. However, reduced weights of ca. 0.3 and ca. 0.13 mg were observed at 5 days for insects fed 1 or 10 ppm, respectively, of fecal material from Cry1Ac-fed *L. hesperus.* In the Cry2Ab study (Table 2B), insects fed control fecal material attained a mass of ca. 22.3–30.6 mg at 6 days. No change was observed in the weight of insects fed 1 ppm of fecal material from Cry2Ab-fed *L. hesperus.* In contrast, at the 10 ppm dose, a reduced weight of ca. 4.32 mg at 6 days was observed.

Immunocytochemical in vivo localization studies. Immunofluorescent light micrographs of Cry2Ab insect sections detected evenly distributed signals associated with the brush border microvilli of the midgut epithelial cells (EC) and basement membrane (BM) (Fig. 5, panel C), as well as with cellular outlines within the hemolymph (Fig. 5, panel F). The observed signals were similar for all time points for Cry2Ab-fed insects (data not shown). Negligible signals were detected in the immunofluorescent light micrographs of the control sections (Fig. 5, panels B and E). Immunofluorescent light micrographs of hemolymph and cross sections of gut from Cry1Ac-fed insects, probed with polyclonal anti-Cry1Ac, did not have signal above that of the sections from control insects (data not shown).

Discussion

The active forms of Cry1Ac and Cry2Ab were ingested by *L. hesperus* when administered through an artificial diet. No reduction or cessation in feeding was observed, in contrast to what has been found when Cry toxins are fed to sensitive lepidopteran and coleopteran hosts [5, 10]. The toxins were found within the gut 12 h after the insects were removed from the treatment diet. In this time frame, inert FITC-dextran has been shown to move completely through the digestive system of *L. hesperus* [15], implying that in our studies some digestive system association or an inhibition of gut motility resulted in a delayed passage. Some of each ingested toxin was absorbed as a holoprotein into the hemolymph, similar to the results observed for GFP and FITC-casein ingested by *L. hesperus* [15]. Densitometric analysis of the Western blot of the Cry1Ac in vivo study showed that within the region of ca. 65 kDa, less than 10% of the ingested Cry1Ac prep form was found in the hemolymph. Most of the ingested toxin was excreted. In the case of Cry1Ac, the active 65-kDa protein and degradation products were found in the fecal material. Fecal material from Cry2Ab-fed insects contained a protein slightly smaller than the 71-kDa protoxin.

Some proteolytic processing of the toxins occurred within the digestive system of *L. hesperus,* although trypsinized Cry1Ac has been found to be resistant to further proteolytic cleavage [21]. The in vitro study of insect tissues incubated with Cry1Ac showed a decrease of ca. 3 kDa in the molecular weight, but only in the salivary gland homogenate. A similar small decrease in the molecular weight of Cry1Ac with protease K incubation has been reported [2]. The differences in the proteolytic processing observed in the in vitro and in vivo Cry1Ac studies could be explained by the possibility that the salivary gland proteolytic activity is necessary before the gut enzymes can further degrade Cry1Ac. In any event, at the 1000-ppm dose tested, in vivo degradation was not sufficient to eliminate toxin activity against lepidopteran larvae.

Cry2Ab in vitro incubations with insect tissues showed a very slight reduction in molecular weight of the 71-kDa protein and total degradation of the 65-kDa protein, occurring only in the salivary gland homogenate. In a related in vitro study, the 63-kDa Cry2Aa1 protoxin

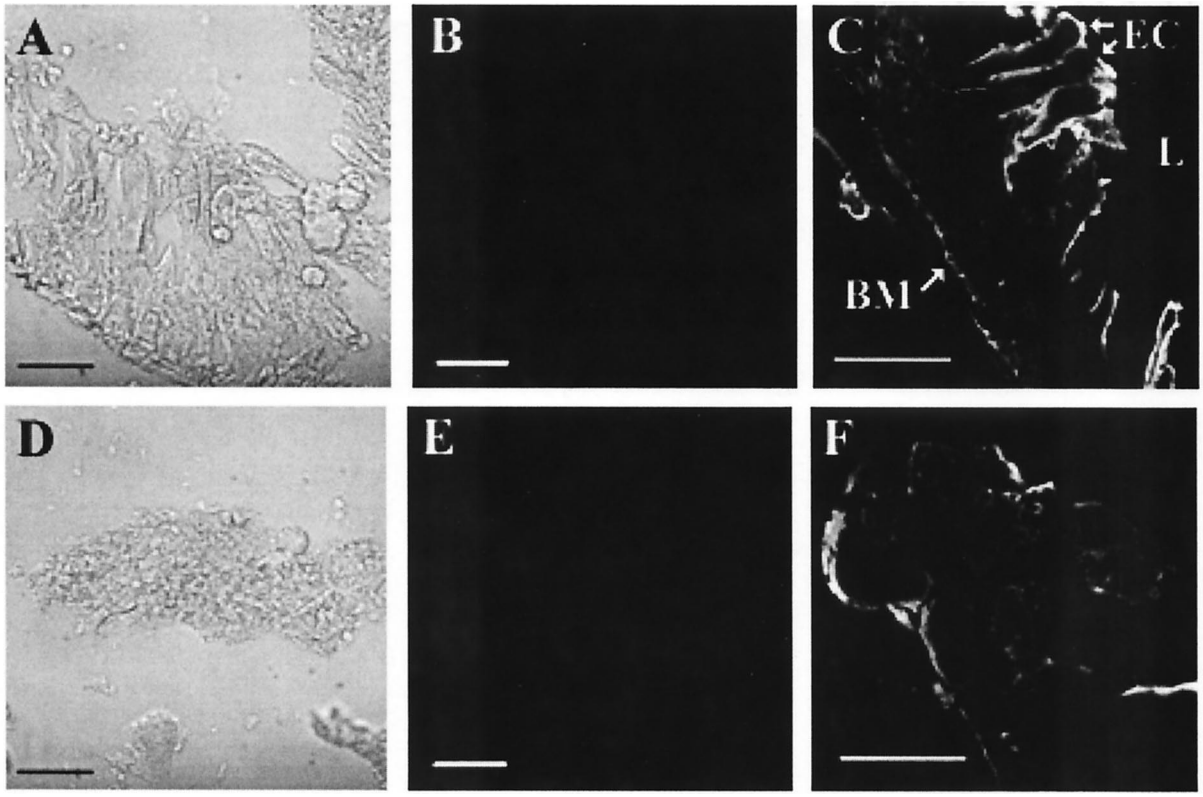

Fig. 5. Transmitted and immunofluorescent light micrographs of 10-μm cross sections from control and Cry2Ab-fed insects. Cross sections of midgut (top panels) and sections of hemolymph (bottom panels) were probed with polyclonal anti-Cry2Ab and Cy™5-conjugated secondary antibody, with visualization by confocal microscopy under 60X magnification, 2.5 iris, and 20% laser. Control sections were visualized with transmitted light (A and D) or immunofluorescence (B and E) of the identical field. Cross-sections from insects fed 0.05% Cry2Ab for 12 h were visualized by immunofluorescence (C and F). Scale bar = 40 μm. L = midgut lumen. EC = epithelial cell. BM = basement membrane.

was cleaved by the "midgut juice" of *Lymantria dispar* larvae into a 58-kDa toxic fragment and a 49-kDa nontoxic fragment [3]. Our in vivo Cry2Ab studies, which detected a protein slightly smaller than the original 71 kDa protein and the absence of the 65 kDa protein in the gut, hemolymph, and fecal material, were in agreement with the degradation observed in our in vitro salivary gland incubations. Furthermore, while the prep form of Cry2Ab fed to *L. hesperus* consisted primarily of the 65-kDa protein, a protein slightly smaller than ca. 71 kDa was the major component of Cry2Ab found in the gut, hemolymph, and fecal material samples from the in vivo study. It is possible that some of the high-molecular-weight proteins (ca. 150 and >250 kDa) observed in the Cry2Ab prep form and in the in vivo gut, hemolymph, and fecal material samples were degraded in the digestive system of *L. hesperus* to a protein slightly less than 71 kDa. However, similar to the results for ingested Cry1Ac, the degradation of Cry2Ab in vivo was not sufficient to eliminate toxin activity toward lepidopteran larvae.

Cry1Ac and Cry2Ab associated differently with *L. hesperus* tissues. Immunocytochemical localization studies of Cry1Ac-fed insects showed no detectable association of Cry1Ac with the insect tissues, indicating a lack of specific midgut binding receptors for the trypsinized form of Cry1Ac. Similar analysis of Cry2Ab-fed insects showed extensive, evenly distributed signals associated with the brush border microvilli and basement membrane of midgut epithelial cells, as well as outlining cellular structures within the hemolymph and fat body. A review of the recent literature found no documentation of the movement of an ingested Cry protein into the hemolymph of any other insects. Nevertheless, this association did not lead to toxicity.

The insect specificity of the Cry toxins is thought to be related to the binding of the toxins to specific midgut receptors [16, 26, 28]. Indeed, an in vivo immunocytochemical localization study of Bt ICPs fed to *Manduca sexta, Plutella xylostella*, and *Leptinotarsa decemlineata* larva by Bravo et al. [4] found that only toxic ICPs bound to the apical microvilli of the midgut epithelial cells. English et al. [10] found that Cry2Aa bound non-saturably to *Helicoverpa zea* midgut brush border, producing channels with variable ionic selectivity in planar lipid

bilayers, and suggested that Cry2Aa might bind to an unlimited number of relatively low affinity sites—"a unique mode of action among the δ-endotoxins."

Although in most cases toxicity is correlated with receptor binding, some reports have demonstrated binding in non-susceptible or resistant insects. High-affinity binding with low toxicity of Cry1Ac has been documented for *Lymantria dispar* and *Spodoptera frugiperda* using isolated brush border membrane vesicles [12, 30]. In addition, Aranda et al. [1] found that ICPs interact with the microvilli of epithelial cells of *S. frugiperda* in two different ways. Highly toxic proteins were found to have saturable and specific interactions, while nontoxic proteins interacted nonspecifically. Other studies with mutated toxins suggest that specific binding involves two steps, one that is reversible and the other irreversible, with toxicity correlated with the irreversible binding step [8, 11, 19, 20, 23].

This study adds further evidence that binding alone may not be sufficient for toxicity, and that the mode of action of Bt δ-endotoxins is complex. Additional Cry2Ab studies are needed to further delineate the midgut binding affinity and specificity, as well as to determine whether membrane channel formation occurs.

ACKNOWLEDGMENTS

We thank Floyd Shockeley and Philip Lake for the assistance they provided in rearing *L. hesperus*, Yangjin Kim for her assistance with statistical analysis, and Marcia Loeb, Alexandria Bravo, Owain Edwards, and Algimantas Valaitis for their critical review of this manuscript. This work was funded in part by a Cooperative Research and Development Agreement between ARS and Monsanto Co. (#58-3K95-9-708) and a Specific Cooperative Agreement between ARS and the University of Missouri (#58-3622-9-114).

Literature Cited

1. Aranda E, Sanchez J, Peferoen M, Güereca L, Bravo A (1996) Interactions of *Bacillus thuringiensis* crystal proteins with the midgut epithelial cells of *Spodoptera frugiperda* (Lepidoptera: Noctuidae). J Invert Pathol 68:203–212
2. Aronson AI, Geng C, Wu L (1999) Aggregation of *Bacillus thuringiensis* Cry1A toxins upon binding to target insect larval midgut vesicles. Appl Environ Microbiol 65:2503–2507
3. Audtho M, Valaitis AP, Alzate O, Dean DH (1999) Production of chymotrypsin-resistant *Bacillus thuringiensis* Cry2Aal δ-endotoxin by protein engineering. Appl Environ Microbiol 65:4601–4605
4. Bravo A, Jansens S, Peferoen M (1992) Immunocytochemical localization of *Bacillus thuringiensis* insecticidal crystal proteins in intoxicated insects. J Invert Pathol 60:237–246
5. Costa SD, Barbercheck ME, Kennedy GG (2000) Sublethal acute and chronic exposure of Colorado potato beetle (Coleoptera: Chrysomelidae) to the δ-endotoxin of *Bacillus thuringiensis.* J Econ Entomol 93:680–689
6. Crickmore N (2000) The diversity of *Bacillus thuringiensis* δ-endotoxins. In: Charles J-F, Delécluse A, Nielsen-LeRoux C (eds), Entomopathogenic bacteria: from laboratory to field application. Dordrecht; Boston: Kluwer Academic Publishers, pp 65–79
7. Crickmore N, Zeigler DR, Feitelson J, Schnepf E, van Rie J, Lereclus D, Baum J, Dean DH (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol Mol Biol Rev 62:807–813
8. de Maagd RA, Bakker PL, Masson L, Adang MJ, Sangadala S, Stiekema W, Bosch D (1999) Domain III of the *Bacillus thuringiensis* δ-endotoxin Cry1Ac is involved in binding to *Manduca sexta* brush border membranes and to its purified aminopeptidase N. Mol Microbiol 31:463–471
9. Dennehy TJ, Williams L (1997) Management of resistance in *Bemisia* in Arizona cotton. Pestic Sci 51:398–406
10. English L, Robbins HL, Von Tersch MA, Kulesze CA, Ave D, Coyle D, Jany CS, Slatin SL (1994) Mode of action of CryIIA: a *Bacillus thuringiensis* delta-endotoxin. Insect Biochem Mol Biol 24:1025–1035
11. Flores H, Soberón X, Sánchez J, Bravo A (1997) δ-Endotoxin binds to lepidopteran midgut membranes. FEBS Lett 414:313–318
12. Garczynski SF, Crim JW, Adang MJ (1991) Identification of putative insect brush border membrane-binding molecules specific to *Bacillus thuringiensis* δ-endotoxin by protein blot analysis. Appl Environ Microbiol 57:2816–2820
13. Gill SS, Cowles EA, Pietrantonio PV (1992) The mode of action of *Bacillus thuringiensis* endotoxins. Annu Rev Entomol 37:615–636
14. Habibi J, Backus EA, Huesing JE (2000) Effects of phytohemagglutinin (PHA) on the structure of midgut epithelial cells and localization of its binding sites in western tarnished plant bug, *Lygus hesperus* Knight. J Insect Physiol 46:611–619
15. Habibi J, Brandt SL, Coudron TA, Wagner RM, Wright MK, Backus EA, Huesing JE (2002) Uptake, flow, and digestion of casein and green fluorescent protein in the digestive system of *Lygus hesperus* Knight. Arch Insect Biochem Physiol 50:62–74
16. Hofmann C, Vanderbruggen H, Höfte H, Van Rie J, Jansens S, Val Mellaert H (1988) Specificity of *Bacillus thuringiensis* δ-endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. Proc Natl Acad Sci USA 85:7844–7848
17. Höfte H, Whiteley HR (1989) Insecticidal crystal proteins of *Bacillus thuringlensis.* Microbiol Rev 53:242–255
18. Jones WA, Jackson CG (1990) Mass production of *Anaphes iole* for augmentation against *Lygus hesperus:* effects of food on fecundity and longevity. Southwest Entomol 15:463–468
19. Lee MK, You TH, Gould FL, Dean DH (1999) Identification of residues in domain III of *Bacillus thuringiensis* Cry1Ac toxin that affect binding and toxicity. Appl Environ Microbiol 65:4513–4520
20. Liang Y, Patel SS, Dean DH (1995) Irreversible binding kinetics of *Bacillus thuringiensis* CryIA δ-endotoxins to gypsy moth brush border membrane vesicles is directly correlated to toxicity. J Biol Chem 270:24719–24724
21. Milne R, Ge AZ, Rivers D, Dean DH (1990) Specificity of insecticidal crystal proteins. In: Hickle LA, Fitch WL (eds), Analytical chemistry of *Bacillus thuringiensis.* American Chemical Society Symposium series 432, Washington, DC, pp 22–35
22. Pietrantonio PV, Gill SS (1996) *Bacillus thuringiensis* endotoxins: action on the midgut. In: Lehane MJ and Billingsley PF (eds), Biology of the insect midgut. London: Chapman and Hall, pp 345–372
23. Rajamohan F, Lee MK, Dean DH (1998) *Bacillus thuringiensis* insecticidal proteins: molecular mode of action. Prog Nucleic Acid Res Mol Biol 60:1–27
24. Sanchis V (2000) Biotechnological improvement of *Bacillus thuringiensis* for agricultural control of insect pests: benefits and ecological implications. In: Charles J-F, Delécluse A, Nielsen-LeRoux C (eds), Entomopathogenic bacteria: from laboratory to

field application. Dordrecht; Boston: Kluwer Academic Publishers, pp 441–459
25. SAS Institute (1990) SAS/STAT User's Guide, vols. 1 and 2, Version 6, 4th ed., Cary, NC
26. Schnepf E, Crickmore N, van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 62:775–806
27. Schwartz MD, Foottit RG (1998) Revision of the Nearctic species of the genus *Lygus* Hahn, with a review of the Palearctic Species (Heteroptera: Miridae). Gainesville, FL: Associated Publishers
28. Van Rie J, Jansens S, Höfte H, Degheele D, Van Mellaert H (1990) Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis* delta-endotoxins. Appl Environ Microbiol 56:1378–1385
29. Williams MR (2000) Cotton insect loss estimates-1999. 2000 Proceedings, Beltwide Cotton Conferences. Vol 2:884–885
30. Wolfersberger MG (1990) 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. Experientia 46:475–477