

Small Scale Bioassay for the Determination of *Bacillus thuringiensis* Toxicity toward *Plodia interpunctella*

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A new bioassay procedure is described for determining the level of toxicity of *Bacillus thuringiensis* preparations toward the Indian meal moth, *Plodia interpunctella*. The method is rapid, accurate, and requires relatively small quantities of toxin protein. Probit dose-mortality results using the single larva bioassay technique compared favorably with results from conventional long-term diet bioassay procedures. Individual larvae could also be scored according to the approximate day of death, providing additional information regarding the kinetics of mortality. © 1991 Academic Press, Inc.

KEY WORDS: Bioassay; *Bacillus thuringiensis*; *Plodia interpunctella*; Indian meal moth.

INTRODUCTION

Many types of bioassay systems are available for measurement of *Bacillus thuringiensis* toxicity, based upon the behavior, feeding habits, and life cycle of the target insects (Burgess and Thomson, 1971). Assays using both artificial diets and plants or plant parts have been successful. Some involve ingestion of a known quantity of pathogen or toxin, while others relate dosage to surrounding media concentration. Still others are designed to utilize only small quantities of assay material, advantageous where toxin is scarce (Hughes et al., 1986). Most methods produce assay results that are both reliable and quantitative, but many cannot quantify actual toxin consumption by the target insect.

The Indian meal moth (*Plodia interpunctella* Hübner) is a common and serious pest of stored grain and milled cereal products. It is found in warehouses and storage bins throughout the world. Control of this insect is becoming increasingly more difficult due to imposed restrictions on the use of chemical insecticides and increasing resistance to common protectants such as malathion. The bacterial pathogen, *B. thuringiensis*, however, can effectively control Indian meal moth larvae (McGaughey, 1976, 1978, 1980). Yet, significant potential for resis-

tance development to *B. thuringiensis* in this insect has been demonstrated in the laboratory (McGaughey, 1985a; McGaughey and Beeman, 1988). Resistance appears to be highly selective and can be overcome by other *B. thuringiensis* strains that possess different toxin genes (McGaughey and Johnson, 1987). Because of this feature, the Indian meal moth has become a model system for resistance investigations and mode of action studies (Johnson et al., 1990; Van Rie et al., 1990). The normal bioassay procedure for this insect is a long-term diet exposure technique (hereafter referred to as the multiple larva cracked wheat bioassay), in which a known number of eggs are placed upon the treated cracked wheat diet and mortality is determined only after adults emerge some 40-45 days later (McGaughey, 1985a). It is not practical to assess mortality prior to adult emergence because larvae are typically very small prior to pupation and are difficult to locate amidst the cracked wheat diet. A large quantity of diet is normally used to ensure that the insects will have sufficient food to complete their development. Since all of this diet must be treated with toxin on a weight basis, the actual quantity used is far greater than that which is actually consumed.

Investigations of single gene toxicity from *B. thuringiensis* often involve limited quantities of protein. Consequently, we developed a faster, more conservative procedure to determine the toxicity of *B. thuringiensis* crystal protein using Indian meal moth larvae. We used multiwell tissue culture dishes to compartmentalize individual larvae and administered toxin by absorption onto a small cube of dried apple placed in each compartment. Thus, accurate dosage could be maintained using small quantities of toxin and the larvae could be inspected individually for complete uptake of the dose before additional food was provided. Because of the independent treatment of individual larvae, this procedure has been termed the single larva apple bioassay.

MATERIALS AND METHODS

Insects. The susceptible and resistant Indian meal moth strains used in this study were from colony 343 as described by McGaughey (1985a). The susceptible strain (343S) has been reared continuously on untreated diet and its susceptibility (LC_{50}) to Dipel (Abbott Laboratories, North Chicago, Illinois) is ca. 13 mg/kg. The resistant strain (343R) had been selected for *B. thuringiensis* resistance by continuous rearing on diet treated with Dipel at 62.5 mg/kg. The response (LC_{50}) of this strain to Dipel is ca. 1500 mg/kg.

Bacterial toxins. *B. thuringiensis* subsp. *kurstaki* HD-1 and *aizawai* HD-133 were obtained from H. Dulmage (Brownsville, Texas) and grown on yeast extract (1.5%) with glucose (0.2%) and K_2HPO_4 (0.8%). Inclusion body purification by differential centrifugation in sodium bromide has been described previously (Ang and Nickerson, 1978). Purified inclusions from laboratory cultures of HD-1 were suspended in 0.05 M $NaHCO_3$, pH 9, before dilution and bioassay. For soluble toxin protein, purified inclusions were dissolved in 0.05 M $NaHCO_3$

and 10 mM dithiothreitol, pH 10, for 2 hr at room temperature. Dissolved protein was dialyzed overnight at 4°C against 0.05 M $NaHCO_3$ and 50 mM KCl, pH 9.0. Purified inclusions and soluble toxin were tested at dilutions of 2 mg/ml to 0.01 mg/ml (based either on dry weight [inclusions] or protein [solubilized inclusions]). Soluble protein content was determined using the bicinchoninic acid assay at room temperature (Smith et al., 1985). Dipel, a WP formulation of HD-1 containing 16,000 IU of potency per milligram of formulation, was suspended in deionized water for dilution in a graded series ranging from 10 to 0.05 mg/ml.

Larval bioassay. The assays were performed on larvae confined individually to compartments of a 24-well tissue culture plate. Small cubes (approximately 2 mm square and 8–10 mg in weight; 28.75% moisture content) were cut by hand from semidehydrated apple slices (Sun-Maid, Sun-Diamond Growers of California, Pleasanton, California) and placed singly into each well of the culture plate. Two microliters of toxin from each dilution of a series ranging from 10 to 0.01 mg/ml (eight doses) was applied to separate cubes and allowed to dry. A single third instar was placed into each well using a vacuum probe (Fig. 1). A glass cover was used to confine the larvae, and the plates were incubated at 25°C with 60% relative humidity. Larvae were inspected every 3–4 days, and those that consumed the toxin-coated apple cubes were fed a cracked wheat diet (McGaughey, 1985a) and maintained until death or pupation. Mortality was recorded every 4–5 days, and the duration of the bioassay was 20–25 days. Typically, 12 or more larvae were used per dose.

Comparative multiple larva bioassays were done using 30-g samples of cracked wheat diet each infested with 50 larvae (McGaughey and Johnson, 1987). Each sample was treated with one of a series of toxin dilutions at a rate of 10 ml/100 g of diet

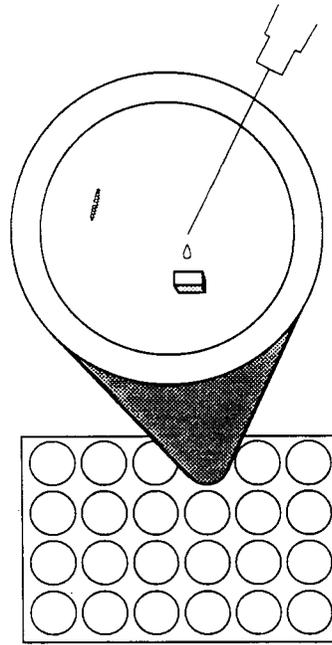


FIG. 1. Diagrammatic representation of the multi-well plate bioassay procedure. A small food cube (cut from semidehydrated apple) was placed in each well of a 24-well tissue culture plate. For each toxin dilution series, a 2- μ l volume of sample was applied to the surface of the food cube. After the sample was absorbed and the surface of the food cube was dry, a third instar *Plodia interpunctella* was added to the well. This process was repeated for each well of the dish, which was then covered with a glass lid and incubated.

to produce doses ranging from 1.95 to 1000 mg/kg of diet. Typically, 10 doses were used in this multiple larva bioassay.

For both types of bioassays, mortalities were calculated from the proportion of larvae that pupated and were corrected for mortality in water or buffer-treated controls (Abbott, 1925). Log-probit estimates of the LC_{50} for each toxin were calculated according to the method of Finney (1971) using a probit analysis program written by G. A. Milliken, Kansas State University, Manhattan, Kansas. Statistical analysis was performed using the Statistical Analysis System (SAS Institute, 1982). Precision of the two techniques was evaluated by calculating the standard deviation and error of

the LC_{50} and slope, including the coefficient of variation (C.V.) of the LC_{50} .

RESULTS AND DISCUSSION

A comparison of results from a series of single larva apple bioassays and multiple larva cracked wheat bioassays using the commercial formulation Dipel is shown in Table 1. The mean LC_{50} for the single larva apple method using susceptible larvae was 1.23 μ g/larva vs 12.85 mg/kg diet for the multiple larva cracked wheat procedure. Depending on the individual experiment, there was an approximate 10-fold difference between the absolute LC_{50} values for the two procedures. The average slopes of the combined dose-mortality regressions from each technique were nearly identical (1.7 ± 0.5 and 1.8 ± 0.5 , respectively). The standard errors for the individual slopes indicated good uniformity between data points. Also, 95% fiducial limits for the LC_{50} dose were quite narrow for separate trials of both techniques. A plot of the best-fit regression lines for all of the individual bioassays is shown in Figure 2. Individual plots from the two methods displayed similar slopes, but tended to group together into two separate families of lines that clearly revealed an approximate 10-fold difference in LC_{50} . The coefficient of variation of the multiple larva cracked wheat bioassays was 19% vs 33% for the single larva apple bioassays.

Differences in toxicity data using the multiple larva cracked wheat bioassay and the single larva apple bioassay were expected. Both assay methods exposed the larvae to similar toxin concentrations (0.002 to 1 μ g toxin/mg diet for the cracked wheat method; 0.002 to 2 μ g toxin/mg apple for the single larva apple method). However, exposure was constant in the cracked wheat method for the duration of the assay, whereas toxin exposure in the single larva apple bioassay was limited to the duration of the apple cube. The result was a roughly

TABLE I
COMPARISON OF TWO METHODS FOR DETERMINATION OF THE DOSE-RESPONSE OF *Plodia interpunctella* LARVAE TO *Bacillus thuringiensis*^a

Method	N ^b	LC ₅₀ (95% FL)	Slope
Multiple larvae	1200	14.51 (10.1–19.8) ^c	1.9 ± 0.2
Cracked wheat	1200	16.63 (6.7–32.3)	1.6 ± 0.4
Bioassay	1200	9.43 (5.6–13.9)	1.1 ± 0.1
	1200	11.83 (10.3–13.5)	1.5 ± 0.7
	1200	12.08 (10.8–13.5)	2.1 ± 0.9
	1400	12.59 (11.5–13.7)	2.4 ± 0.1
Mean		12.85	1.8
SD		2.47	0.5
SE		1.01	0.2
± C.V. ^d		19.2	
Single larva	72	1.22 (0.8–2.9) ^e	1.8 ± 0.1
Apple bioassay	192	1.73 (1.1–3.1)	1.1 ± 0.2
	92	0.72 (0.4–1.6)	1.1 ± 0.2
	21	0.99 (0.4–4.8)	2.3 ± 1.1
	28	1.02 (0.5–2.2)	2.2 ± 0.7
	44	1.70 (1.0–11.8)	1.9 ± 0.7
Mean		1.23	1.7
SD		0.41	0.5
SE		0.17	0.2
% C.V.		33	

^a Dipel (Abbott Laboratories, N. Chicago, Illinois).

^b Number of insects.

^c Milligram per kilogram diet.

^d Coefficient of variation.

^e Microgram/larva.

10-fold difference in actual LC₅₀ between the two methods, 0.0129 µg toxin/mg cracked wheat diet vs 0.122 µg toxin/mg apple.

Initial concerns that the preservative contained in the dried apple (usually so-

dium benzoate) would affect larval development were unfounded. Control larvae developed normally while feeding on apple cubes containing only buffer or deionized water, and the food cube was usually consumed in 7 to 10 days. Cracked wheat diet is also prepared with preservatives consisting of 0.33% sorbic acid and 0.33% methyl-*p*-hydroxybenzoate (McGaughey and Beman, 1988). These preservatives present no ill effects toward developing Indian meal moth larvae.

We used this method to determine the toxicity of several *B. thuringiensis* preparations. The particulate nature of Dipel and laboratory preparations containing spores and/or inclusions made accurate sample dispensing difficult. Precautions were taken to ensure that all samples were sufficiently dispersed prior to application onto the apple cube. These steps produced uniform results between particulate and soluble prep-

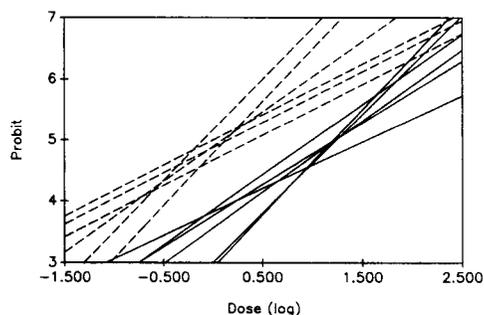


FIG. 2. Dose-mortality response of *Plodia interpunctella* 343S to Dipel, measured by either multiple larvae cracked wheat bioassay (—) (100 µg Dipel/kg diet) or single larva apple bioassay (---) (100 µg Dipel/larva).

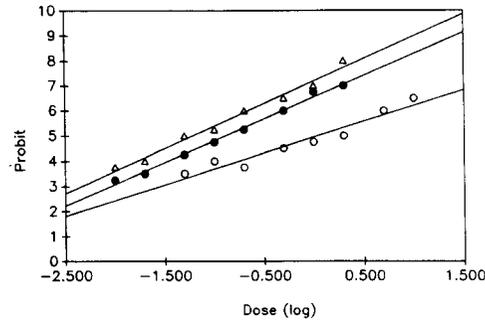


FIG. 3. Single larva apple dose-mortality response of *Plodia interpunctella* 343S to three different spore/inclusion preparations from *Bacillus thuringiensis* subsp. *kurstaki* HD-1. Spores + inclusions (○), purified inclusions (●), and soluble inclusion protein (△). Dose: μg toxin (dry weight)/larva.

arations. This consistency is shown in Figure 3, where the dose-mortality for Indian meal moth larvae of a spore/inclusion mixture, purified inclusions, and soluble inclusion preparations from *B. thuringiensis* subsp. *kurstaki* HD-1 are compared. The LC_{50} and 95% fiducial limit was 1.38 (1.07–1.87) $\mu\text{g}/\text{larva}$ for spores and inclusions in combination, 0.20 (0.13–0.31) $\mu\text{g}/\text{larva}$ for inclusions alone, and 0.07 (0.06–0.09) $\mu\text{g}/\text{larva}$ for soluble inclusion protein. The slopes of the probit regression plots of the inclusion body preparations (1.7 ± 0.2 and 1.6 ± 0.2) were significantly different ($P > 0.01$) from the slope of the spore/inclusion probit regression (1.2 ± 0.1) and may reflect the contribution of spores to toxicity. Although slopes of probit regression plots can vary widely, the higher slopes are indicative in general of greater toxin purity (McGaughey and Johnson, 1987; Mohd-Salleh et al., 1980).

In addition to routine LC_{50} determinations, the method has revealed an interesting relationship between susceptible and resistant Indian meal moth larvae when comparing dose and time of death. Although resistant larvae exhibit a reduced sensitivity to *B. thuringiensis* subsp. *kurstaki*, they remain susceptible to insecticidal crystal protein from certain other *B. thuringiensis* strains, including *B. thuringiensis* subsp.

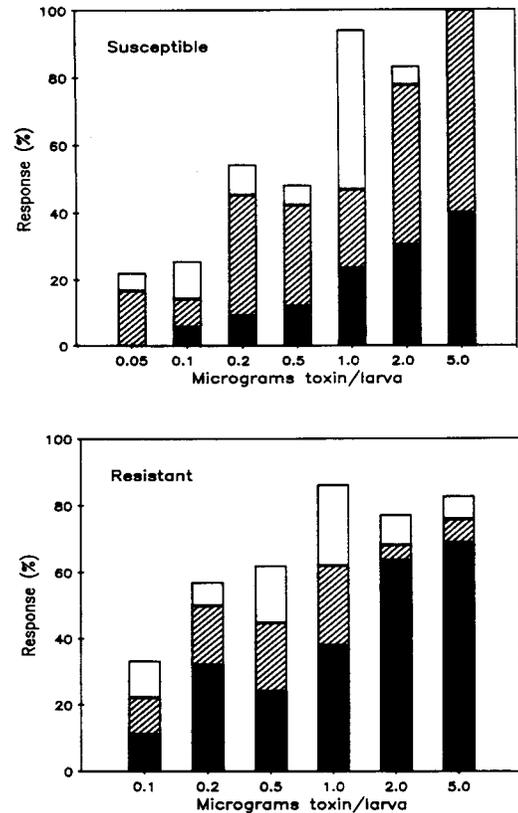


FIG. 4. Response rate between susceptible and resistant Indian meal moth larvae to *Bacillus thuringiensis* subsp. *aizawai* HD-133 insecticidal crystal protein. Measurements were taken at 7 (■), 17 (▨), and 28 (□) days of incubation following exposure to toxin. Response values are cumulative, based upon the percentage of larvae dead during the sampling interval/total larvae per treatment.

aizawai (McGaughey and Johnson, 1987). Surprisingly, the response of the resistant insects to the *aizawai* toxin is faster than that of the susceptible insects. If mortality data for *B. thuringiensis* subsp. *aizawai* is plotted based upon the day of measurement (7, 17, or 28 days), a change is evident in the response time between susceptible and resistant larvae (Fig. 4). However, the slopes of the dose-response curves were essentially the same at each sampling time for either susceptible or resistant larvae. Not all toxin samples showed this effect; the effect was most pronounced for preparations demonstrating greater activity toward resis-

tant larvae. The time of larval death for susceptible and resistant larvae exposed to toxin protein from *B. thuringiensis* subsp. *kurstaki* was similar, even though much greater quantities of toxin were needed to kill resistant insects. Apparently, resistant Indian meal moth larvae respond to specific proteins from *B. thuringiensis* subsp. *aiizawai* inclusions differently than they do to the proteins present in inclusions from *B. thuringiensis* subsp. *kurstaki*. This response to a specific type or types of toxin protein exposed to the midgut of susceptible larvae may be associated with the number or affinity of specific receptors located on the midgut membrane (Van Rie et al., 1990). This finding is currently under investigation.

This technique is especially useful for bioassays involving scarce material, since smaller quantities of toxin are needed than with a conventional diet bioassay. Commonly, a 10 mg/ml stock solution is used for dilutions, and only 2 μ l/larva/dilution is needed for the actual bioassay. Thus, relatively small quantities of toxin are required for a complete systematic bioassay. In a poorly responding toxin-host system such as with resistant Indian meal moth larvae, it may be difficult to suspend an adequate dosage in 2 μ l. In these instances, it is necessary to make multiple applications of 2- μ l aliquots each upon the apple cube, with sufficient time between applications to allow for adequate absorption of sample.

Besides requiring less time and material to conduct an experiment, the single larva apple bioassay is carried out in such a manner that the resulting data more nearly satisfy the assumptions for using the probit analysis (Finney, 1971) than does the multiple larva cracked wheat bioassay. The analysis assumes that all experimental units (larvae) are treated independently. By using the multiwell plate, each larva is treated independently of other larva, whereas the multiple larva cracked wheat bioassay treats a large number of insects in a single

container and thus they are not independent.

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