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Bioassay for Homogeneous Parasporal Crystal of *Bacillus thuringiensis* Using the Tobacco Hornworm, *Manduca sexta*

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A method for determining the toxicity of *Bacillus thuringiensis* subsp. *kurstaki* parasporal crystal to the tobacco hornworm, *Manduca sexta*, is described. The use of both mortality and weight loss data have provided a highly sensitive and reproducible bioassay that can be used to compare relative toxicities of crystals from other subspecies as well as toxic components contained therein.

It has been well documented that *Bacillus thuringiensis* produces an intracellular proteinaceous crystal that is toxic to insects, primarily lepidopteran larvae (3-5). In our laboratory we are examining both the insecticidal properties and biochemical mode of action of the parasporal crystal. As a first step, we have developed a sensitive and reproducible bioassay for homogeneous parasporal crystal, using neonate larvae of the tobacco hornworm, *Manduca sexta* (L.). Most bioassays for the crystal have employed formulations containing spores (1, 5a) that are also toxic (8).

MATERIALS AND METHODS

Test organisms. *M. sexta* larvae were hatched from eggs received from J. P. Reinecke, Metabolism and Radiation Research Laboratory, U.S. Department of Agriculture, Fargo, N. Dak. Neonate larvae were used in all tests. The diet used was that of Yamamoto (10) as modified by Bell and Joachim (2). It contained formalin and streptomycin to restrict the growth of microorganisms.

The strain of *B. thuringiensis* used in these studies was HD-1, isolated from a commercial insecticidal formulation called Dipel (Abbott Laboratories, North Chicago, Ill.), and has been identified as *B. thuringiensis* subsp. *kurstaki* by H. de Barjac, Institut Pasteur, Paris, France. Stock cultures of HD-1 were maintained on modified GYS (6) agar slants. Cells for experimental use were cultured in modified liquid GYS medium at 28°C in 2-liter Erlenmeyer flasks and aerated by rotary agitation at 250 rpm. The cultures were held for additional time after sporulation to allow individual cells to lyse and release spores and parasporal crystals. Spores and crystals were removed from the culture medium by centrifugation (10,000 rpm for 20 min). The crystals were subsequently separated from spores and cellular debris by buoyant density centrifugation in Renografin gradients (7). Crystals isolated in this manner were washed at least three times in water and lyophilized to constant weight. The purity was checked by visual examination of an aqueous suspension with a Petroff-Hausser bacteria counter. The population of crystals was 6.17×10^9 per mg of dry weight and contained less than 0.01% other cellular material.

Experimental treatments. Two methods of introducing the crystal to the test insect were attempted. First, the material was incorporated into the agar-based diet while still in the liquid stage in a manner similar to that of Splittstoesser and McEwen (9). Second, we used the method of Ignoffo et al. (5a), whereby a known amount of material was layered onto the surface of the solidified artificial diet. Preliminary results indicated that more reproducible data could be obtained by the latter method. Subsequently, a series of studies was undertaken in triplicate in the following manner. Freshly prepared artificial diet was poured into disposable tri-pour beakers to the 30-ml level and, after it had congealed, disposable cardboard lids were placed on the beakers. Dilutions of the crystalline material were made by homogenizing 560 μg of the purified parasporal crystal in 7 ml of distilled water. Dilutions to give 1.0, 0.8, 0.6, 0.4, 0.2, 0.1, 0.08, and 0.04 μg of crystals per ml of solution were prepared, and 1 ml of each was applied uniformly to the diet surface (16 cm^2) and allowed to air-dry. The applications resulted in surface treatments ranging from 2.5 ng/cm^2 (0.04 μg/ml) to 62.8 ng/cm^2 (1 μg/ml).

One neonate larva of *M. sexta* was placed on the treated surface in each container. After introduction of the larvae to the treated diet surface, they were held at a constant temperature (27°C) and humidity (60%) until the observation period ended. A mortality count was made after 7 days of exposure to the treated surface. All larvae surviving on day 10 were pooled according to treatment and weighed, and the average weight per larva was recorded. Larvae were characterized according to instar at 7 days if deceased or at 10 days if alive by measuring the size of the head capsule under a light microscope with a micrometer eyepiece. All of the head sizes were averaged, and the average measurement was converted to instar or stadia of growth. The bioassay was repeated several times to check reproducibility.

TABLE 1. *Mortality of M. sexta after 7 days on a diet surface treated with B. thuringiensis endotoxin and average weight per larva of survivors at 10 days*

Dose		Mortality after 7 days		Surviving larvae at 10 days		
μg/ml	ng/cm²	%	Instar	Avg wt/larvae (mg)	wt reduction (%)	Instar
Control	0	0		814.0		4 and 5
0.04	2.5	0		86.0	89.0	3
0.08	5.0	33	2	27.0	97.0	2
0.10	6.2	60	2	19.0	98.0	2
0.20	12.5	80	2	8.0	99.0	2
0.40	25.1	90	2	6.5	99.2	2
0.60	37.7	93	2	5.6	99.3	2
0.80	50.3	96	2	4.3	99.5	2
1.00	62.8	100	2			

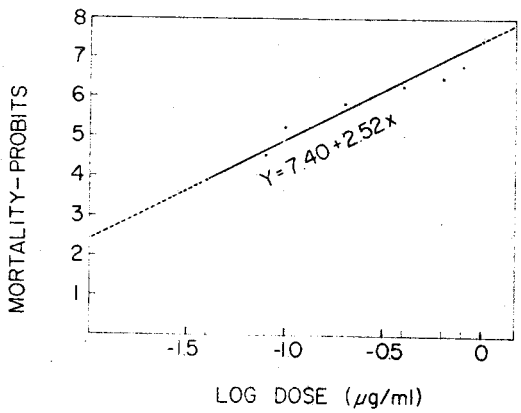

FIG. 1. *Dosage mortality curve for larvae of the tobacco hornworm, M. sexta, fed on various dosages of parasporal crystal of B. thuringiensis.*

TABLE 2. *Lethal doses and confidence limits of B. thuringiensis parasporal crystal for M. sexta*

Lethal dosage[a]			95% Confidence limts	
End point	μg/ml per 16 cm²	ng/cm²	Upper (ng/cm)[a]	Lower (ng/cm²)
50	0.11	6.97	8.53	5.52
95	0.50	31.45	48.47	23.46
99	0.94	58.70	107.25	39.67
99.9	1.89	118.11	263.83	70.75

[a] Slope = 2.52; intercept = 7.40.

RESULTS AND DISCUSSION

The effect of a homogeneous preparation of parasporal crystal of *B. thuringiensis* on the growth of the tobacco hornworm is given in Table 1. Although the larvae were able to molt to the second instar at all concentrations tested, only two-thirds of the larvae survived after 7 days at a surface application of 5 ng/cm², and none survived at 62.8 ng/cm². When the probit mortality is plotted against the logarithm of the dose of crystal used (Fig. 1), the lethal dose values and 95% confidence limits shown in Table 2 are obtained. The parasporal crystal is an extremely effective insecticide, with a mean lethal dose value for neonate *M. sexta* larvae of only 6×10^{-9} g/cm².

The effect of the microbial insecticide on surviving larvae after 10 days of incubation is also shown in Table 1. As expected, all control animals had attained either the fourth or fifth instar at that time, with the majority in the fourth. All other larvae, however, hardly grew and displayed a dramatic reduction in weight. At the lowest dose tested (2.5 ng/cm²), the insects had molted to the third instar, but weighed only 11% of that of the control animals. Higher doses prevented growth beyond even the second instar and caused weight reductions of 97% or greater (Fig. 2). These grossly underweight larvae died soon thereafter. Preliminary results revealed that a dose as low as 0.6 ng/cm² brings about a significant weight reduction (67%) after the 10-day incubation period. No lower levels were tested. These results indicate that the crystal causes either a drastic reduction in the amount of diet consumed or a severe interference with movement of food through the gut wall. From the known physical effects of the endotoxin (8), it seems likely that the feeding inhibition mechanism is correct.

Before the mode of action of the parasporal crystal of *B. thuringiensis* can be understood in molecular and cellular terms, the physiological properties of the alimentary canal of the lepidopteran larval host as well as the biochemical features of the toxin must be more fully understood. One of the first steps in such a study is the development of a technique for bioassaying small quantities of the paraspore. The bioassay

FIG. 2. *Comparison of the sizes of 8-day-old larvae of M. sexta with and without exposure to B. thuringiensis parasporal crystal on a diet surface. (A) Control (fourth instar); (B) dose of 5 ng/cm² of crystal (second instar).*

described in this report utilizes a highly susceptible experimental insect, *M. sexta*, that responds to a crystal dose of ≤ 0.6 ng/cm² of insect diet surface. To our knowledge, this concentration is the lowest effective dosage reported for purified *B. thuringiensis* crystalline endotoxin.

Now that a reliable bioassay is available, a more detailed investigation into the biochemical nature of the crystalline toxin and the physiological response by a susceptible host to its invasion will be facilitated. Earlier work in our laboratory (L. A. Bulla, Jr., K. J. Kramer, D. B. Bechtel, and L. I. Davidson, Microbiology—1976, p. 534–539, American Society for Microbiology, Washington, D.C., 1976) has shown that the toxic moiety of the crystal is associated with a polypeptide whose molecular weight has not been established. When the cysteine residues of the activated protoxin are alkylated with iodoacetate, the derivatized material is not functional in the *Manduca* bioassay described in this report, even at concentrations 160 times the minimal effective dose for the native crystal. Apparently, sulfhydryl groups in the crystal are essential for toxicity.

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