APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1980, p. 897-900
0099-2240/80/11-0897/04$02.00/0

Vol. 40, No. 5

Rocket Immunoelectrophoresis of the Entomocidal Parasporal Crystal of *Bacillus thuringiensis* subsp. *kurstaki*†

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Rocket immunoelectrophoresis was used to quantitate the soluble parasporal crystal of *Bacillus thuringiensis* subsp. *kurstaki*. The method described is rapid, reliable, specific, and extremely accurate, and it can be used to measure crystal toxin in commercial microbial insecticides that contain a mixture of spores, vegetative cells, and carrier materials.

Bacillus thuringiensis is an entomocidal bacterium that is currently used to control insects that attack different crops (6). *B. thuringiensis* is formulated as an insecticide and sold in the United States under trade names such as Dipel and Thuricide. These two insecticides are formulated with strain HD-1 of *B. thuringiensis* subsp. *kurstaki*, which is highly toxic to lepidopteran larvae (4). The toxic activity associated with *B. thuringiensis* takes place primarily in the parasporal crystal formed within the mother cell during sporulation (3). This crystal is composed of a glycoprotein subunit (apparent molecular weight, 135,000) that is believed to be a protoxin which is converted to a toxin (apparent molecular weight, 68,000) by proteolytic activity (2).

In the commercial production of *B. thuringiensis*, it is critical that each fermentation be subject to quality control to insure a high level of insecticidal activity. Historically, such quality control has been based on the number of viable spores per unit weight or unit volume (4). This procedure now is considered unreliable because toxicities vary greatly in relation to spore counts among the different strains (5), and acrystalliferous mutants which are known to occur readily (10) are not detected.

Currently, the toxicity of all preparations is determined in the cabbage looper *Trichoplusia ni* and compared to an international standard designated E-61 prepared at the Institut Pasteur, Paris, France (4). Although this bioassay technique is efficacious, it is slow and time-consuming. We report here a modification of the rocket immunoelectrophoresis technique (7, 11) that provides a rapid, extremely accurate, and reliable alternative to insect bioassays. The utility of this technique is demonstrated with the commercial insecticide Dipel.

MATERIALS AND METHODS

Organism and crystal preparation. *B. thuringiensis* subsp. *kurstaki* was isolated from Dipel, supplied by Abbott Laboratories, North Chicago, Ill. Spores and crystals were grown in a liquid medium containing glucose, yeast extract, and salts (GYS medium) and separated on Renografin gradients as described elsewhere (9). The crystals were solubilized in alkali as specified by Bulla et al. (2) and dialyzed overnight against 20 mM NaH_2PO_4 at pH 7.5.

Preparation of antiserum. Antiserum was obtained by inoculating New Zealand white albino rabbits subcutaneously in four sites with a total of 1 mg of solubilized crystal toxin suspended in complete Freund adjuvant. After 4 weeks, the rabbits were reinoculated with another 1-mg dose of solubilized crystal toxin suspended in incomplete Freund adjuvant. The animals were bled at the end of 2 weeks and each week thereafter.

Insect toxicity. Toxicity was determined with neonate larvae of the tobacco hornworm *Manduca sexta*. Commercial Dipel powder (lot 77-565BJ) and purified crystals from *B. thuringiensis* subsp. *kurstaki*, prepared as described above, were diluted in water and then assayed as described by Schesser et al. (8).

Rocket immunoelectrophoresis. Rocket immunoelectrophoresis was carried out in 1% (wt/vol) agarose gels (10 by 10 cm) that were 1.5 mm thick as previously described (7). Both the gel and electrode buffers contained 0.089 M boric acid, 0.089 M Trizma base (Sigma Chemical Co., St. Louis, Mo.), and 0.001 M ethylenediaminetetraacetic acid. The agarose gel contained 2% polyethylene glycol 6000 to stabilize and enhance the immune precipitate. Low sensitivity gels contained 3.0% antiserum whereas high sensitivity gels contained 0.7% antiserum. The gels were electrophoresed at 250 V for 2.5 h at 15°C. The gels were pressed onto glass plates under 0.5 cm of filter paper and soaked in 0.1 M NaCl twice for 20 min each and then in running water once for 15 min. At the end of this time, the gels were again pressed onto a glass plate, dried with a portable hair dryer, stained with 0.25%

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Coomassie brilliant blue R250 for 15 min, and destained in methanol-acetic acid-water (25:7.5:67.5, vol/vol/vol). Peak heights were measured on a dry surface of the glass plate. Principles of the rocket immunoelectrophoresis technique are described elsewhere (11).

Protein determination. The total amount of protein in the alkali-solubilized preparations was determined by absorbance at 280 nm, with an extinction coefficient of 1.1/1 mg of protein per ml, as reported earlier (3). Using this value, we measured the response of the crystal toxin to Coomassie brilliant blue with the commercial assay kit manufactured by Bio-Rad Laboratories, Richmond, Calif. The color response of the crystal protein was 88% as intense as that of Bio-Rad Protein Standard I bovine gamma globulin.

Alkali solubilization techniques. Commercial Dipel powder was weighed, suspended in water, and stirred while the pH of the water was raised to pH 12.0 with 1 N NaOH. For the time course experiments, 1 ml of sample was removed at the appropriate time and placed in a separate tube, and 50 μl of 1 M Tris-hydrochloride (pH 8.5) was added to render a final pH of approximately 8.5. These samples were subjected to rocket immunoelectrophoresis as described above, and peak heights were compared with standard curves obtained from pure alkali-solubilized crystals diluted to appropriate concentrations in phosphate buffer.

RESULTS

Standard curves were established at two levels of antiserum concentration. Figure 1 shows the activity of a gel containing 3% crystal antiserum that has reacted with various amounts (100 to 1,000 μg/ml) of soluble crystal protein in phosphate buffer. The precipitin rockets are discrete, sharply defined, and easy to measure. The antigen is a glycoprotein (3) and presumably elicits a high-affinity antibody that produces precipitin lines that are easily visualized in agarose gels. The peak height was proportional to the crystal toxin concentration in the range from 100 to 1,000 μg/ml (Fig. 2). Likewise, when a lower concentration of antiserum (0.7%) was used, the peak height was proportional to the crystal toxin concentration over a range of 10 to 100 μg/ml (Fig. 3). When the soluble crystal was diluted in GYS medium, the response, i.e., peak height, shape, and staining characteristics, was identical to that observed when phosphate buffer was the diluent.

The commercial insecticide Dipel consists of a mixture of spores, crystals, and vegetative cells carried in diatomaceous earth. We measured the total alkali-extractable protein in Dipel and found that it contained 165 mg of protein per g of dry powder. The amount of crystal protein, based on rocket immunoelectrophoresis, was 45 mg/g of Dipel (Fig. 4), or 27% of the total alkali-soluble protein. This experiment indicated that the rocket immunoelectrophoresis system can

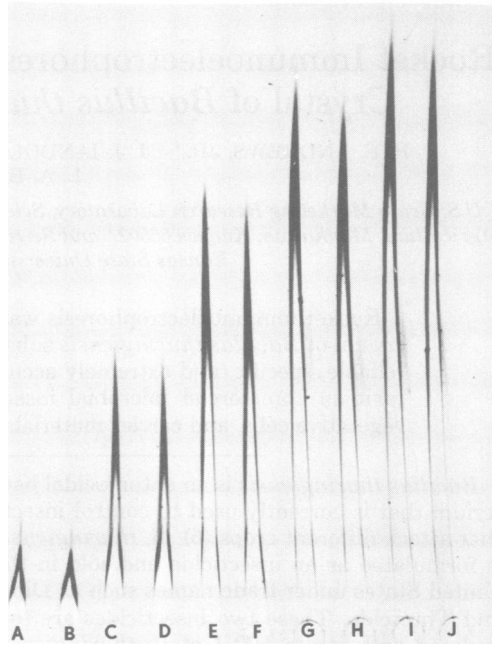

FIG. 1. *Rocket immunoelectrophoretogram of the B. thuringiensis parasporal crystal. The gel contained 3.0% crystal antiserum and 1.0% agarose. The wells contained the following concentrations (in micrograms per milliliter) of soluble crystal antigen: A and B, 100; C and D, 250; E and F, 500; G and H, 750; I and J, 1,000.*

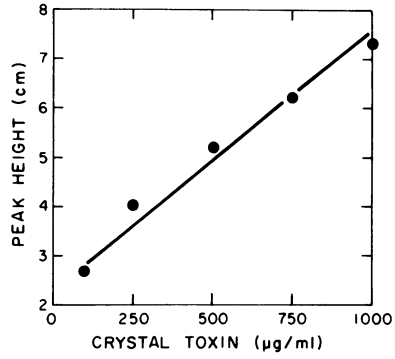

FIG. 2. *Relationship between the peak height and the concentration of crystal toxin in a rocket immunoelectrophoretic gel that contained 3.0% crystal antiserum.*

specifically measure the crystal toxin in a mixture of other proteins. Furthermore, it demonstrated that solubilization of the crystal can be accomplished very quickly; it is complete within 1 h. For routine analyses, a 5-h solubilization was used.

To insure that toxicity correlates with crystal antigen measured by rocket immunoelectropho-

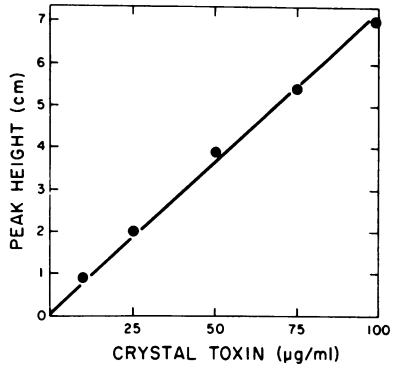

FIG. 3. *Relationship between the peak height and the concentration of crystal toxin in a rocket immunoelectrophoretic gel that contained 0.7% crystal antiserum.*

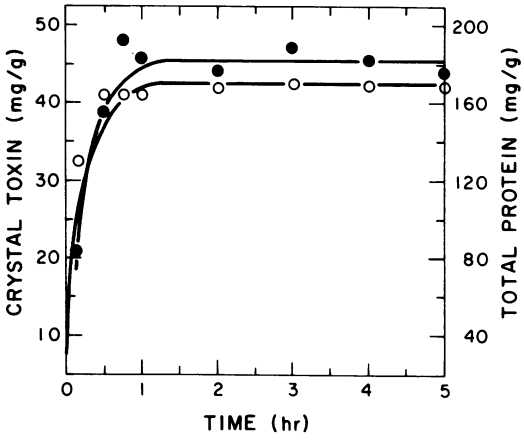

FIG. 4. *Total alkali-soluble protein* (○) *and crystal toxin* (●) *in Dipel. Preparations were titrated to pH 12.0 with 1 N NaOH. Samples were removed at various times and buffered with Tris-hydrochloride to pH 8.5. The total amount of protein was measured by Coomassie brilliant blue binding, and crystal toxin was measured by rocket immunoelectrophoresis.*

resis, we bioassayed both Dipel and Renografin-purified crystals against the tobacco hornworm *M. sexta*. The 50% lethal concentration (LC_{50}) values for Dipel and pure crystals were 45 and 2.7 ng/cm^2, respectively (Table 1). When the LC_{50} of Dipel was recalculated with 45 mg/g of crystal toxin, the LC_{50} was 2.0 ng/cm^2. Based on appropriate confidence intervals, the LC_{50} values for both preparations are statistically the same.

DISCUSSION

Rocket immunoelectrophoresis is an efficient means for determining the crystal toxin of *B. thuringiensis* in a fermentation medium. The entire procedure can be carried out in as little as 4 h, requiring only about 3 man-hours of labor. Insect bioassays, on the other hand, routinely require 4 to 11 days (1, 8) and as many as 30 man-hours. There is no elaborate purification scheme required for samples to be electrophoresed because the method described is specific for crystal toxin in the presence of medium components, vegetative cells, and other protein(s) such as those found in Dipel. Commercial fermentation of *B. thuringiensis* involves very large volumes of culture medium (routinely over 100,000 liters per fermentation vessel; M. C. Bartlett, Abbott Laboratories, personal communication).

Because crystal toxin can be measured directly in a fermentation liquor, the storage and handling of large fermentation batches that may prove to have insufficient insecticidal activity are precluded. Furthermore, very small quantities of material are required for measurement of crystal toxin compared with that quantity needed in an insect bioassay. As shown in Fig. 3, the lower limit of antigen detection is 10 µg/ml. Based on the application of 5 µl of soluble crystal toxin per well, the system is sensitive to 50 ng of antigen. This quantity is the approximate amount of crystal toxin provided to a single insect larva at the LC_{50} (Table 1).

In addition to its potential industrial use, rocket immunoelectrophoresis should provide a convenient method by which to study the biosynthesis of toxic protein that is contained in both the parasporal crystal and the spore of *B. thuringiensis*. We are currently investigating the temporal relationship of such synthesis to vegetative growth and sporulation.

TABLE 1. *Toxicity of crystal toxin and Dipel for the tobacco hornworm*

Toxin source	LC_{50}[a]	95% Confidence limit[b]	
		Lower[a]	Upper[a]
Dipel[c]	45	15	76
Dipel toxin[d]	2.0	0.7	3.4
Purified crystal	2.7	1.4	4.1

[a] In nanograms per square centimeter; 1.0 ml of solution was applied to 14.6 cm^2 of diet surface.
[b] Calculated by probit analysis.
[c] Crude powder.
[d] Calculated from crude powder, using a crystal toxin content of 45 mg/g.

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