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Enzyme-Linked Immunosorbent Assays for Detection and Quantitation of the Entomocidal Parasporal Crystalline Protein of *Bacillus thuringiensis* subspp. *kurstaki* and *israelensis*†

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An enzyme-linked immunosorbent assay was used to detect and quantitate the parasporal crystal toxins of *Bacillus thuringiensis* subspp. *kurstaki* and *israelensis*. The assay method described is extremely sensitive, accurate, and highly specific. With this technique, crystalline insecticidal proteins from several subspecies of *B. thuringiensis* were compared. The dipteran crystal toxin produced by *B. thuringiensis* subsp. *israelensis* was shown to share few epitopes with the lepidopteran toxin from *B. thuringiensis* subspp. *kurstaki, tolworthi, berliner*, and *alesti*.

Insect bioassay (4) and rocket immunoelectrophoresis (2) are currently used to detect and measure the levels of crystal insect toxin produced by *Bacillus thuringiensis*. Because insect bioassays are time consuming and cumbersome and require relatively large amounts of material, they are impractical, especially for environmental applications. Although more sensitive than bioassay, rocket immunoelectrophoresis also requires a considerable amount of antigen (at least 10 μg/ml), so that the utility of the method is probably restricted to quality control for commercial fermentations and other industrial processes. In this paper, we describe two enzyme-linked immunosorbent assays (ELISAs) for the detection and quantitation of the entomocidal parasporal crystalline proteins of *B. thuringiensis* subspp. *kurstaki* and *israelensis*. The sensitivity of the assays should make them suitable for the detection of crystal proteins from these two organisms in environmental samples.

MATERIALS AND METHODS

Culture conditions and crystal preparation. *B. thuringiensis* subspp. *kurstaki, israelensis, alesti, tolworthi*, and *berliner* were grown in a liquid medium containing glucose, yeast extract, and salts. Insecticidal crystals were separated from spores and cellular debris on Renografin gradients as described elsewhere (5). Crystal proteins were solubilized in alkali as specified by Bulla et al. (3) and dialyzed overnight against 20 mM NaH_2PO_4 at pH 7.5.

Preparation of antisera. Antisera were obtained by inoculating New Zealand white albino rabbits with purified solubilized crystal toxin from *B. thuringiensis* subspp. *kurstaki* and *israelensis* as previously described (2). To prepare anti-*B. thuringiensis* crystal–alkaline phosphatase conjugates, antisera were precipitated with 40% saturated NH_4SO_4 and dialyzed against 0.15 M NaCl–0.1 M NaH_2PO_4 (phosphate-buffered saline) at pH 7.4. Alkaline phosphatase (Sigma Chemical Co., type VII) was then coupled to the immunoglobulins by the glutaraldehyde method described elsewhere (6).

Indirect ELISA. The ELISA was modified from the method described by Voller et al. (6). All reactions were carried out in Gilford cuvettes and read in a Gilford enzyme immunoassay reader at 405 nm. In all cases, crystals were dissolved overnight in 0.01 M borate buffer (pH 12.0). Solubilized crystal toxins were diluted into the assay buffer, which contained 0.05 M $NaCO_3$, 0.12 M $NaHCO_3$, and 0.01 M NaN_3. The carbonate buffer (300 μl), containing the appropriate amount of crystal protein, was placed in the cuvettes and held overnight at 4°C. Cuvettes were washed three times in phosphate-buffered saline which contained 0.05% Tween 20 and 3.2 mM NaN_3 (wash buffer). After the wash, 300 μl of antiserum (diluted 1:12,000) was added to the cuvettes and allowed to react for 2 h at 28°C. The cuvettes, containing adsorbed crystal protein and antiserum, were washed three times with wash buffer. Goat anti-rabbit immunoglobulin–alkaline phosphatase conjugate (Miles Laboratories, Inc.) was diluted 1:2500 in wash buffer, and 300 μl of this solution was added to the cuvettes and allowed to react for 2 h at 28°C. After

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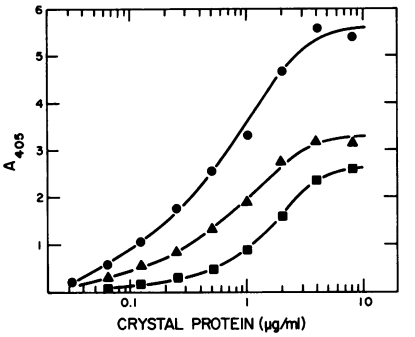

FIG. 1. Effect of anti-*B. thuringiensis* subsp. *kurstaki* crystal toxin serum concentration on ELISA antiserum dilutions: 1:3,000 (●), 1:6,000 (▲), and 1:12,000 (■).

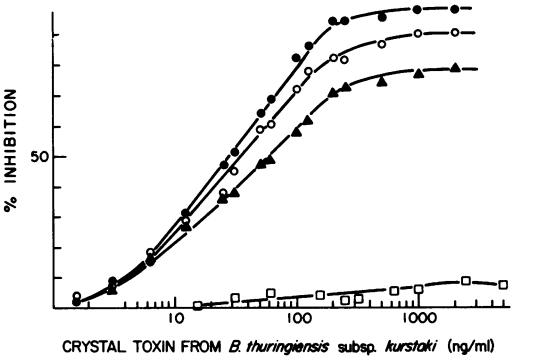

FIG. 2. Inhibition of binding of *B. thuringiensis* subsp. *kurstaki* antibody by heterologous crystal toxins: *B. thuringiensis* subsp. *berliner* (●), *B. thuringiensis* subsp. *tolworthi* (○), *B. thuringiensis* subsp. *alesti* (▲), and *B. thuringiensis* subsp. *israelensis* (□).

three more washes in wash buffer, 300 μl of a 1-mg/ml solution of *p*-nitrophenyl phosphate (Sigma) in 10% diethanolamine buffer (pH 9.8) was added to each cuvette. Reactions were stopped 30 to 40 min later by the addition of 75 μl of 3 M NaOH. The color intensity of each reaction was measured in the immunoassay reader.

One-step ELISA. Solubilized crystal toxins (0.75 μg/ml) were adsorbed to the cuvettes and washed as described above. A combination of rabbit anti-*B. thuringiensis* subsp. *kurstaki* crystal and alkaline phosphatase conjugates (300 μl, 2.5 μg/ml) was added to the cuvettes and incubated for 2 h at 28°C, and the cuvettes were washed three more times. Next, 300 μl of *p*-nitrophenyl phosphate in 10% diethanolamine buffer was added and allowed to react for 120 min. The reactions were stopped by the addition of 3 M NaOH as stated above, and absorbance was read at 405 nm.

Inhibition studies. By direct and indirect ELISA, *B. thuringiensis* crystal proteins were tested for their ability to inhibit the binding of antibody to the *B. thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *israelensis* proteins that had been adsorbed to the titer wells. Four heterologous toxins were incubated at room temperature overnight with a standard dilution of anti-*B. thuringiensis* subsp. *kurstaki* or anti-*B. thuringiensis* subsp. *israelensis* crystal serum. This antiserum (300 μl) was added to cuvettes which had been previously coated with the appropriate homologous crystal toxin, and the cuvettes were examined for binding inhibition by the ELISA methods described above.

Ouchterlony assays. Ouchterlony gels were prepared in 1% agarose–2% polyethylene glycol 6000–0.089 M boric acid–0.089 M Tris base–0.001 M EDTA (pH 8.3). Antigen and antiserum were placed into the gels and allowed to incubate overnight at room temperature. Precipitin bands were visible within 10 to 12 h.

RESULTS AND DISCUSSION

An indirect ELISA was developed to detect and quantitate crystal protein with commercially prepared anti-rabbit–enzyme conjugate. The effect of antiserum concentration on color intensity was studied with various concentrations of the *B. thuringiensis* subsp. *kurstaki* protein toxin which was adsorbed to the solid phase (Fig. 1). Color intensity (i.e., absorbance at 405 nm) was a function of crystal toxin concentration over a range of 0.03 to 3 μg/ml (30 to 3,000 ng/ml). The lepidopteran toxins produced by *B. thuringiensis* subspp. *berliner, alesti*, and *tolworthi* also reacted with the antiserum in a concentration-dependent manner. However, the mosquito toxin of *B. thuringiensis* subsp. *israelensis* did not react efficiently with antiserum prepared against the moth toxin of *B. thuringiensis* subsp. *kurstaki* (data not shown). Similarly, the negative control keyhole limpet hemocyanin (5 μg/ml) exhibited no reaction, even at antibody dilutions as low as 1:400.

It has previously been shown (5) that the lepidopteran crystal toxins of *B. thuringiensis* subspp. *kurstaki, berliner, alesti*, and *tolworthi* are biochemically similar. Because no antigenic cross-reactivity was observed by indirect ELISA between the lepidopteran toxins and the dipteran toxin of *B. thuringiensis* subsp. *israelensis*, the relationship of these proteins was further investigated. To determine how closely related these toxins are immunologically, two types of binding inhibition studies were performed. The ability of solubilized heterologous crystal toxins to inhibit the binding of anti-*B. thuringiensis* subsp. *kurstaki* antibody to *B. thuringiensis* subsp. *kurstaki* crystal toxin adsorbed to the solid phase was studied (Fig. 2). Crystal toxins from *B. thuringiensis* subspp. *kurstaki* and *berliner* were found to completely inhibit the binding reaction. Crystals from *B. thuringiensis* subspp. *tolworthi* and *alesti* also showed significant inhibition (91 and 79%, respectively), but neither completely inhibited binding. *B. thuringiensis* subsp. *israelensis* crystal toxin inhibited the binding less than 10% at

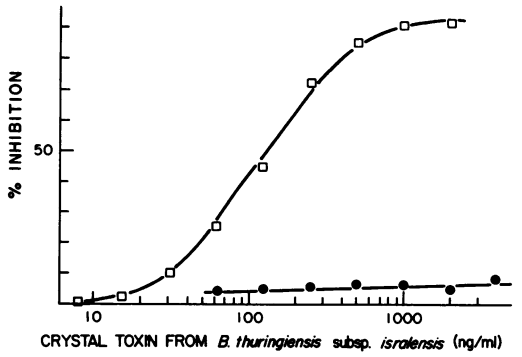

FIG. 3. Inhibition of binding of *B. thuringiensis* subsp. *israelensis* antibody by heterologous crystal toxins. *B. thuringiensis* subsp. *israelensis* (□), *B. thuringiensis* subspp. *kurstaki, berliner, tolworthi*, and *alesti* (●).

TABLE 1. Immunological comparison of the crystal toxins from selected subspecies of *B. thuringiensis*[a]

Subsp.	Immunoprecipitation[b]	% Binding inhibition (maximum)
B. thuringiensis subsp. *kurstaki*	+	100
B. thuringiensis subsp. *berliner*	+	100
B. thuringiensis subsp. *tolworthi*	+	91
B. thuringiensis subsp. *alesti*	+	79
B. thuringiensis subsp. *israelensis*	−	<10

[a] Antiserum was prepared against *B. thuringiensis* subsp. *kurstaki* crystal protein.

[b] Based on Ouchterlony double-diffusion analysis (see reference 5).

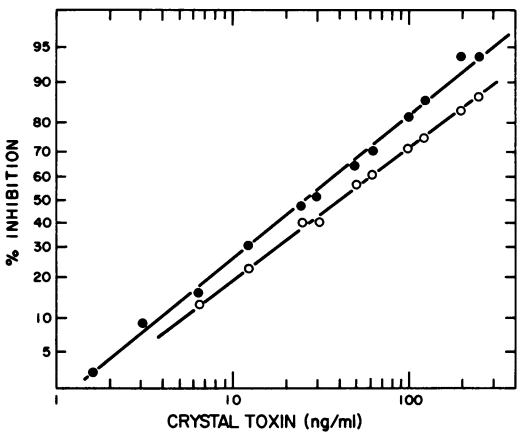

FIG. 4. Comparison of the indirect (●) and one-step (○) ELISA methods for determination of *B. thuringiensis* subsp. *kurstaki* crystal toxin.

concentrations as high as 1,400 ng/ml. In the reciprocal experiment, it was found that none of the lepidopteran crystal toxins was an effective inhibitor (maximum inhibition ≈ 10%), including bovine serum albumin (data not shown), in the *B. thuringiensis* subsp. *israelensis* system (Fig. 3).

To simplify the ELISA for quantitation of crystal toxin, alkaline phosphatase was coupled directly to rabbit anti-*B. thuringiensis* subsp. *kurstaki* antibody, eliminating one antibody-antigen reaction step in the ELISA procedure. An enzyme-to-immunoglobulin ratio of 1:2 was used in the preparation of this conjugate, and a concentration of 0.25 μg/ml of immunoglobulin was sufficient for a routine ELISA. The sensitivities of the ELISA methods were similar (Fig. 4), and inhibition was linear over a wide range of concentrations (2 to 200 ng/ml).

Although it has been reported (5) that the crystal proteins from the four lepidopteran-toxic subspecies of *B. thuringiensis* are closely related, evidence indicates that the crystal of *B. thuringiensis* subsp. *israelensis* is biochemically different. The results of the Ouchterlony double-diffusion gel analyses and the ELISA (Table 1) showed that the crystal protein toxin from *B. thuringiensis* subsp. *israelensis* also differs immunologically. Antiserum prepared against *B. thuringiensis* subsp. *kurstaki* crystal protein reacted with solubilized crystals to yield an immunoprecipitate with protein from the four lepidopteran subspecies. Crystals from *B. thuringiensis* subsp. *israelensis*, however, showed no cross-reactivity with anti-*B. thuringiensis* subsp. *kurstaki* crystal antiserum, indicating that the toxin of *B. thuringiensis* subsp. *israelensis* shares few epitopes with the toxins from the other four subspecies.

The ELISA methods described here provide fast and efficient techniques for quantitation and differentiation of crystal toxins from *B. thuringiensis* subspp. *kurstaki* and *israelensis*. The assays are inexpensive, the immunoreagents are highly stable, and the methods do not require radioactive compounds. The extreme sensitivity of these ELISAs should make them useful for field studies. Previously, we showed that the measurement of antigen with antibodies prepared as described (see above) correlates very well with insect toxicity (1, 2). The safety of the ELISAs and the relatively simple equipment required should provide investigators with methods to determine the dispersal of insecticides formulated with *B. thuringiensis* and their persistence after application. Such information may facilitate the development of more efficient and cost-effective insecticides that have the crystal toxin of *B. thuringiensis* as their active agent.

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