An improved enzyme-linked immunosassay for the detection and quantification of the entomocidal parasporal crystal proteins of Bacillus thuringiensis subsp. kurstaki and israelensis


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Wee, S.I. Hammock, B.D. Gill, S.S. Grate, E. Andrews, R.E. Jr., Faust, R.M., Bulla, L.A. Jr., & Schaefer, C.H. 1984. An improved enzyme-linked immunosassay (ELISA) was developed for the detection and quantification of parasporal crystal toxins from Bacillus thuringiensis subsp. kurstaki. The improved procedure involved pretreatment of the polypeptide coating with glutaraldehyde before antibody coating. A direct comparison of treated and untreated coatings was provided. ELISAs were then used for the analysis of the entomocidal crystal proteins in commercial and experimental formulations of B. thuringiensis subsp. kurstaki and israelensis.

Although immunosassay has been widely used in numerous fields, its application to the analysis of pesticides has been very limited. Interest in the immunochromatography of synthetic pesticides is rapidly escalating (Hammock & Murmre 1980; Wee & Hammock 1982), and an obvious and immediate application of immunosassay is the analysis of the protein toxins from Bacillus thuringiensis. In a classic study Winkler et al. (1971) used immunoassay to optimize fermentation conditions while Andrews et al. (1980) significantly improved the speed of this assay. Although faster and more precise than bioassay, these methods are still somewhat slow and labor-intensive. Wee et al. (1982) first reported enzyme-linked immunosassay methods (ELISAS) for the detection and quantification of the entomocidal parasporal crystal proteins of B. thuringiensis subsp. kurstaki and israelensis while South & Ulrich (1983) demonstrated the reliability of an ELISA for analysis of fermentation materials from B. thuringiensis subsp. kurstaki. It is important to analyze these protein toxins because the toxicity of subsp. kurstaki is active on many lepidopterans and insects whereas that of subsp. israelensis is more lethal to certain dipteran insects such as mosquitoes and blackflies. Moreover, the toxic proteins of both subspecies are biochemically and immunologically distinct (Kryszewщи & Fast 1980; Tyrell et al. 1981; Wee et al. 1982). Work of Thomas & Ellar (1983) demonstrates that the toxicity of subsp. israelensis is toxic to several insects and vertebrates in cell lines and to Balb-c mice as well. Recent work from this laboratory confirms the vertebrate toxicity of the subspecies with an intraperitoneal lethal dose of <0.5 mg/kg in

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Swiss Webster mice. Moreover, the solubilized crystal proteins are toxic when injected into a wide variety of insect species (P. Y. K. Cheung, unpublished observations).

The potential of these B. thuringiensis toxins for controlling serious agricultural and medical pests with a minimum of environmental effects means that there is a great deal of interest in their direct commercialization and as targets for genetic engineering. Thus, for quality control, production optimization, efficacy evaluation, residue determination and genetic engineering as well as basic research, reliable, rapid analytical methods are needed for the toxic principles.

In this paper, significantly improved ELISAs for detecting and measuring insecticidal proteins of B. thuringiensis are described. The utility of these ELISAs is demonstrated by measuring insecticidal proteins in different commercial and experimental formulations and tank mixes of B. thuringiensis.

Materials and Methods

CULTURE CONDITIONS AND CRYSTAL PREPARATION

Bacillus thuringiensis subsp. kurstaki, israelensis, alecis, tolworthii and berliner were grown in a modified GYS medium containing (g/l) sodium citrate, 1.0; glucose, 1.0; MgSO4.7H2O, 0.15; (NH4)2SO4, 2 g; K2HPO4, 3H2O, 3.55; yeast extract, 2; final pH adjusted to 7.5 with conc. H2SO4. Following autoclaving a sterile solution of 0.08 g CaCl2 in 10 ml of water is added to bring the total volume to 1 liter. Paraflagellar crystals were then separated from spores and cellular debris on Renografin gradients as described elsewhere (Tyrrell et al. 1981).

The crystals were dissolved in alkali as described by Bulla et al. (1979, 1981) and dialysed overnight against 200 ml 0.1 M NaH2PO4 at pH 7.5.

PREPARATION OF ANTIBERA

Antiserum were obtained by inoculating New Zealand white albino rabbits with a solution of purified crystal toxin from B. thuringiensis subsp. kurstaki and israelensis as described earlier (Andrews et al. 1980). Crude immunoglobulin fractions were obtained by precipitating 2 ml of antiserum with 40% saturated (NH4)2SO4 at room temperature for 30 min and dialysing against 7 mol/l phosphate buffer containing 0.15 mol/l NaCl and 0.05% NaN3 (pH 7.7).

PREPARATION OF ALKALINE-PHOSPHATASE-B. thuringiensis SUBSP. BERTERI CONJUGATES

Lyophilized protein toxin from B. thuringiensis subsp. berliner was stirred overnight with 2 ml of 0.2% Na2CO3 containing 0.02% NaN3, at pH 12.0. It was then dialysed for 2 days against 0.1 mol/l phosphate buffer containing 0.15 mol/l NaCl, pH 7.4 with 2 changes per day. The toxic protein, 4.57 mg, was then coupled to 5 mg alkaline phosphatase in 2 ml of buffer by adding 15 µl of 25% glutaraldehyde. The mixture was stirred for 70 min at room temperature and then dialysed at pH 7.7. Following dialysis 0.4 ml of BSA solution (30 µg/ml) was added to stabilize the conjugate (Voller et al. 1978).

ENZYME-LINKED IMMUNOSORBENT ASSAY

Direct ELISA was carried out according to the method of Voller et al. (1980) with slight modifications and was used in this study for the detection of B. thuringiensis subsp. israelensis toxin. The toxin was diluted in 0.1 mol/l sodium carbonate buffer (pH 9.5) containing 0.02% NaN3, and 250 µl was then added to each cuvette. The cuvettes were kept overnight at 4°C and washed three times with phosphate-buffered saline containing 0.05% Tween 20 and 0.02% NaN3 (PBS-Tween). The assay procedure entailed two parts. First, 1 ml of the appropriately diluted rabbit antiserum was transferred to 13 x 100 mm culture tubes. To these antibody solutions were added inhibitor solutions of increasing concentration and they were incubated at 28°C overnight. The second step in the assay involved adding 250 µl of the above solutions to the B. thuringiensis subsp. israelensis toxin coated cuvettes which were incubated for 2 h at room temperature. After these washes with PBS-Tween, goat anti-rabbit IgG-alkaline phosphatase conjugate diluted in PBS-Tween (1/2500) was added and incubated at room temperature for 2 h. After another three washes, phosphatase activity was measured by adding 250 µl of p-nitrophenyl phosphate (1 mg/ml) in 10% dis-
Immunogen for B. thuringiensis

ThIOIamine buffer, pH 9.8. The reaction was stopped 30-40 min later by adding 3 mol/L NaOH and the colour intensity was measured at 405 nm in a Gilford EIA reader. Maximum binding as well as background binding were assessed by adding solvent only or no antibody, respectively, to the system.

For the direct ELISA, Gilford cuvettes were first treated with 0.2% glutaraldehyde in H₂O and incubated at 28°C. After 30 min the cuvettes were washed with distilled water and 150 μl of rabbit anti-B. thuringiensis subsp. kurstaki serum of different dilutions were added to the cuvettes, and they were then kept in a moist- ened chamber at 28°C overnight. The coated cuvettes then were washed three times with PBS-Tween. The B. thuringiensis subsp. hetlerov- alkaline phosphatase in PBS-Tween was then added to the cuvettes which were incubated for an additional 2 h at 20°C. For the preparation of standard inhibition curves, the inhibitors were added before the addition of toxin-enzyme conjugate. After three more washes, 250 μl of 1 mg/ml solution of p-nitrophenyl phosphate in 10% diethanolamine buffer, pH 9.8, were then added to the cuvettes. The reactions were stopped 50-60 min later by addition of 50 μl of 0.5 mol/L NaOH and the absorbance at 405 nm was monitored on a Gilford EIA reader.

Sample Preparation

Because the crystal toxins were soluble in basic buffer at pH 12.0 (Bolla et al. 1981), a 0.2% Na₂CO₃ solution containing 90% NaCl was used to dissolved them. For the commercial formulatıons a total weight of 100 mg was suspended in 10 ml of Na₂CO₃ buffer and incubated at 28°C with vigorous shaking. Incubation to lysis cells was not required for optimal detection of toxin in either freshly harvested or formulated materials. Very little difference in solubility was noted when the samples were incubated from 4 to 16 h. Following incubation the samples were centrifuged at 13,000 g for 15 min. and the supernatant fractions were used to prepare serial dilutions for the ELISA.

Commercial formulations were generously provided by Sandusky Inc. (Homestead, Florida) and Abbott Laboratories (North Chicago, Illinois). Levels of toxins were also determined in standard unknown samples provided by the World Health Organization (WHO unknowns) of B. thuringiensis subsp. kurstaki. These unknowns were sent to numerous laboratories throughout the world in an attempt to standardize bioassay. Antigenicity of pure crystal preparations and toxin levels in tank mixes were determined by dissolving a known weight of crystal, respectively, in carbonate buffer. Tank mixes were prepared in the field by the US Forest Service for actual spray operations in the Lake States. Small aliquots of these tank mixes and the formulated materials used to prepare the tank mixes were numbered, frozen in dry ice and shipped to U.C. Davis for blind analysis.

Reagents

Alkaline phosphatase conjugated goat anti-rabbit IgG was obtained from Miles Laboratory (Elkhart, IN.). Normal rabbit immunoglobulin was purchased from Antibodies Incorporated (Davis, CA). Alkaline phosphatase (1000 units/ml) type VII from bovine intestine and glutaraldehyde were purchased from Sigma Chemical Company (St. Louis, MO.).

Insect bioassay

The concentration needed to kill 50% (LD₅₀) of the early fourth instar mosquito larvae of Aedes aegypti (strain Bora-Bora from Polynesia) was determined by World Health Organization approved methods. The bioassays were standardized using the Institut Pasteur Standard 1978 (85% 94) which has an arbitrarily defined potency of 1000 IU/mg (International Units). De Burjac & Large (1979).

Results and Discussion

Development of a practical direct ELISA for toxins from Bacillus thuringiensis requires the optimization of conditions for coating the anti-toxin antibody to the solid phase (substrate). Initial studies using untreated cuvettes coated with a fixed concentration (1/100) of anti-B. thuringiensis subsp. kurstaki serum showed that antibody-coated cuvettes can be used to develop an ELISA for the toxins (Table 1). The absorbance at 405 nm was directly proportional to the concentration of B. thuringiensis subsp. hetlerov-alkaline phosphatase used in the assay. Analogous experiments using nonimmune (normal)
rabbit serum and either treated or untreated cuvettes showed that little if any of the enzyme-toxin conjugates adhered to the cuvettes. Since it was reported that polysaccharide plates that were treated with BSA and subsequently fixed with glutaraldehyde facilitated the binding of antibody to the solid phase (Penna et al., 1981), such experiments were then carried out as described and some of the results using anti-B. thun-
igeriensis subsp. kurstaki antiserum are shown in Table 1. Although comparable absorbance was obtained for normal BSA and glutaraldehyde-treated cuvettes at the higher antigen-enzyme concentrations, a significantly lower absorbance was found for BSA-glu-
araldehyde-treated cuvettes at lower antigen-enzyme concentrations. It appears that BSA treatment in our system did not offer an advantage in facilitating the antibody-antigen interaction. On the other hand, cuvettes treated with glutaraldehyde alone gave a higher absorbance in all the antigen-enzyme combinations used in the direct ELISA which in turn increased the sensitivity of the assay and con-
served toxin-coupled alkaline phosphatase. When different dilutions of anti-B. thun-
gigeriensis subsp. kurstaki serum and a fixed concentration of B. thun
gigeriensis subsp. berliner-alkaline phos-
pitatase conjugate were used in the ELISA, glutaraldehyde-treated cuvettes gave a > two-
fold increase in the absorbance at serum dilu-
tions of 1:1600-1:2000 as compared with the untreated cuvettes (Fig. 1). When cuvettes were coated with the antibody first and then treated with 0.2 mg/ml solution of BSA, slightly lower absorbances were observed with all antibody concentrations used. Thus, in this study sub-
sequent blocking of active sites on the plate only slightly decreased assay background while causing a substantial decrease in assay sensi-
tivity. As mentioned earlier, non-specific binding of the enzyme-toxin to the cuvette is unlikely, and this notion is reinforced by the observation that the absorbance decreased to the back-
ground level with the increasing dilution of the antiserum. The specificity of the assay was also shown by the fact that there was no detectable enzymatic activity in glutaraldehyde-treated cuvettes when rabbit anti-B. thun
gigeriensis subsp. kurstaki serum was used in place of the anti-subspecies kurstaki antibodies. This experi-
ment also confirms our previous observation that no antigenic cross-reaction in ELISA assays was found between the B. thun
gigeriensis subsp. iracinalis and the other B. thun
gigeriensis toxins including those from subsp. kurstaki, obt-
usus, and berliner. In a separate experiment, cuvettes were treated with 0.02% glutaraldehyde and incu-
batated at room temperature for various time intervals (5 min to 2 h) before washing. The antiserum was used with a 1:2500 dilution of rabbit anti-B. thun
gigeriensis subsp. kurstaki and the enzyme was a 1:2000 dilution of B. thun
gigeriensis subsp. berliner-alkaline phosphatase conjugate. The results indicate that with this system the length of incubation with glutaraldehyde is not critical since no significant difference in absorb-

The Gifford cuvettes were coated with 150 ml of a 1:100 dilution of rabbit anti-B. thun
gigeriensis subsp. kurstaki serum for 2 h at 37°C to Materials and Methods. After washing with PBS-Twice, serial dilutions of B. thun
gigeriensis subsp. berliner-alkaline phosphatase were added and incubated for 2 h. After subsequent washes, 1 mg/ml of p-nitrophenyl phosphatase was added and the reading at 405 nm was taken 60 min later.

Cuvettes were first treated with 150 ml of 0.2 mg/ml BSA and subsequently fixed with 0.2% of glutaraldehyde.

* Cuvettes treated with 150 ml of 0.2% glutaraldehyde only.

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<th>Dilution of toxin coupled alkaline phosphatase</th>
<th>Absorbance at 405 nm</th>
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<td>Normal plates</td>
<td>BSA and Glutaraldehyde treated</td>
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<tr>
<td>1000</td>
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<td>2000</td>
<td>1.25</td>
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Table 1. Direct enzyme-linked immunosorbent assay using rabbit anti-B. thun
gigeriensis subsp. kurstaki serum and subpecies berliner-alkaline phosphatase conjugates *
The effect of glutaraldehyde pre-treatment of polystyrene cuvettes in direct ELISA. The extent of binding of the alkaline phosphatase-coupled B. thuringiensis subsp. berneri toxin to the ELISA cuvettes is indicated by the absorbance at 405 nm assayed at multiple serum dilutions. □, Cuvettes treated with 0.01% glutaraldehyde and coated with the anti-subspecies kurstaki antibody; ▲, non-treatment cuvettes coated with anti-subspecies kurstaki antibody; ■, 0.01% glutaraldehyde treated cuvettes coated with anti-subspecies israelensis antibodies.

ance was observed. Overnight incubation with glutaraldehyde yielded a slight increase in absorbance. This result further simplifies the use of the glutaraldehyde method since the timing of glutaraldehyde pre-treatment of the plates is not especially critical. In other studies (results not shown) antibody binding to the glutaraldehyde-treated plates was shown to occur rapidly and then very slowly increase when the coating antigen was held for several days in the plates at 5°C. The glutaraldehyde treatment appears useful with a variety of solid phases including Falcon plates and polystyrene balls. In all cases tested, glutaraldehyde pretreatment increased the effective titre of the antibody used for coating the plates as well as the sensitivity of the resulting ELISA. This effect may be due, in part, to the glutaraldehyde treated plates favouring an effective orientation of the coating antibody on the plate surface. It is not universally applicable however to proteins other than antibodies. In some systems it conserved coating antigen while in others glutaraldehyde pretreatment had little if any effect on indirect ELISAs.

The bell-shaped curve shown in Fig. 1 may explain why some workers failed to see a significant improvement in their immunosassay when using glutaraldehyde. If only high serum concentrations had been evaluated in this study, glutaraldehyde treatment would not have demonstrated an advantage. The reasons for the shape of the curve in Fig. 1 are not clear. Possibly at high antibody concentrations the glutaraldehyde is able to introduce cross links between individual antibody molecules and thereby prevent them from subsequent effective
Fig. 2. Inhibition of binding of B. thuringiensis subsp. berliner-alkaline phosphatase by crystal toxins from several subspecies of B. thuringiensis. (a) The direct ELISA employed glutaraddehyde-precipitated rabbit anti-B. thuringiensis subsp. kurstaki sera; B. thuringiensis subsp. kurstaki; C, B. thuringiensis subsp. kurstaki; D, B. thuringiensis subsp. Berliner; E, B. thuringiensis subsp. kurstaki. (b) Inhibition of binding of B. thuringiensis subsp. berliner-alkaline phosphatase to the anti-B. thuringiensis subsp. kurstaki antibody coated cuvettes. Results shown in Fig. 2 are expressed as the percent inhibition of the absorbance observed using a standard amount of toxin-enzyme conjugate plotted against a variable concentration of toxins. Two criteria were used to compare the cross-reactivity of various toxins. First, the concentration of solubilized crystal which reduced absorbance by 50% indicated the concentration of added toxin which caused a 50% displacement of the alkaline phosphatase coupled toxin from the antibody coated cuvettes (Ksh). This Ksh is related to the affinity of the toxin bound for the antibody. Second, the maximum displacement which could be obtained indicates the antigenic determinants which the various toxins have in common. As expected, the B. thuringiensis subsp. kurstaki toxin gave the strongest displacement with an Ksh of 105 ng/ml and a maximum displacement of 94%. The toxin from B. thuringiensis subsp. berliner also gave significant displacement with an Ksh of 220 ng/ml and a 94% maximum displacement. Toxin from B. thuringiensis subsp. kurstaki and other were found to contain fewer antigenic determinants than the toxin from subsp. kurstaki and therefore could not completely displace the toxin-enzyme conjugate. On the other hand, the toxin from B. thuringiensis subsp. kurstaki appears to be unique since it did not appear to cross-react with the other toxins (Fig. 2). Similar results were also obtained in the indirect ELISA as described in the previous study (Wie et al. 1982).

It should be recalled, however, that ELISAs are competitive assays. Thus, the B. thuringiensis subsp. kurstaki toxin could have some antigenic determinants in common with the B. thuringiensis subsp. kurstaki toxin, but if the average Ksh with the antibody population was signi-
The direct ELISA was then used to determine the possible contamination of *B. thuringiensis* subsp. *kurstaki* in several *B. thuringiensis* subsp. *israelensis* experimental and commercial formulations. Toxins from *B. thuringiensis* subsp. *israelensis* were determined by an indirect ELISA developed in this laboratory using specific rabbit anti-*B. thuringiensis* subsp. *israelensis* antisera which do not recognize antigenic determinants of toxins derived from subspecies *kurstaki* and its related strains (Wun et al. 1982). As shown in Table 2, WHO IPS 78, Sandoz T15-2A and Abbott ARG 6108D had similar amounts of *B. thuringiensis* subsp. *israelensis* toxin, whereas only negligible amounts of *B. thuringiensis* subsp. *kurstaki* determinants were found in both WHO IPS 78 and Sandoz T15-2A. The formulation from Abbott, however, showed a significant level of this toxin. It is possible that this contamination resulted from sequential use of a fermenter to produce *B. thuringiensis* subsp. *kurstaki* and *israelensis* since it is difficult and possibly not necessary to sterilize equipment used to produce spore-forming bacteria. However, researchers should be very cautious when using commercial formulations to study species specificity. Several WHO unknown samples which were reported to contain *B. thuringiensis* subsp. *israelensis* toxin were also included in Table 2 and they were found to contain high levels of the Bacillus *B. thuringiensis* *israelensis* except unknown D which had mainly the subspecies *kurstaki* determinants rather than subspecies *israelensis* determinants. Possibly *B. thuringiensis* subsp. *kurstaki* was used deliberately to dilute the *B. thuringiensis* subsp. *israelensis* when the standard unknowns were prepared. LC₅₀ values obtained on *Aedes aegypti* larvae according to WHO methods were compared with the toxin levels detected by direct ELISA. For instance, the WHO IPS-78 standard with an LC₅₀ value of 0.24 mg/mL had 115 μg/mL of subspecies *israelensis* toxin and undetectable levels of subspecies *kurstaki* toxin. The unknowns were all derived from diluted sample A and the results indicate that LC₅₀ values correlated with the experimentally determined *B. thuringiensis* *israelensis* toxin levels. Thus, WHO unknown D which had an LC₅₀ of > 100 mg/mL had negligible subspecies *israelensis* toxin (0.1 μg/mL) and large amounts of subspecies *kurstaki* toxin (240 mg/mL). Samples B, D and E all had subspecies *israelensis* toxin in addition to *kurstaki* toxins (Table 2).

Similarly, using direct ELISA, the amount of subspecies *kurstaki* toxin in commercial tank mixes prepared from Dipel 14/706 CF was determined and found to be proportional to the amount of *B. thuringiensis* subsp. *kurstaki* formulation added to the sample in a blind experiment. When identical formulation ingredients without *B. thuringiensis* were assayed, false positives were not found and the ingredients did not interfere with the positive ELISAs.

The present study demonstrates that a simple