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Delta Endotoxin of *Bacillus thuringiensis* subsp. *israelensis*†

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From *Bacillus thuringiensis* subsp. *israelensis*, a proteinase-resistant protein was purified which exhibited toxicity to larval mosquitoes and cultured mosquito cells, lysed erythrocytes, and was lethal to mice. To extract the protein, a sporulating culture of *B. thuringiensis* subsp. *israelensis* was treated with alkali, neutralized, and incubated with trypsin and proteinase K. It was then purified by gel filtration and DEAE column chromatography. Up to 240 μg of toxic protein was purified from 1 g (wet weight) of culture pellet. Two closely related forms of toxic protein were obtained: the 25a and 25b proteins. The two forms comigrated near 25,000 daltons in a sodium dodecyl sulfate-polyacrylamide gel, were serologically related, and showed similar partial protease digestion profiles, but were distinguishable by DEAE chromatography and nondenaturing polyacrylamide gel electrophoresis. Protein sequencing data indicated the 25b protein lacked the two amino acids at the amino terminus of the 25a protein. A Western blot enzyme-linked immunosorbent assay of alkali-solubilized proteins that were not treated with proteases suggested the toxic 25a and 25b proteins were proteolytically derived from a larger molecule of about 28,000 daltons. Alkali-solubilized proteins from an acrystalliferous strain of *B. thuringiensis* subsp. *israelensis* and from *B. thuringiensis* subsp. *kurstaki* failed to cross-react with antibodies to the 25a protein.

Bacillus thuringiensis subsp. *israelensis* was first isolated as a pathogen of mosquito larvae (10). Since then, the toxic activity against both larval (25) and adult (12) mosquitos has been localized in the parasporal crystals of sporulating cells. When crystals are solubilized, they exhibit toxic effects against other biological targets. These toxic effects include cytolysis of cultured insect cell lines, hemolysis of rabbit erythrocytes, and lethality for mice (22). The molecular species toxic to mosquito larvae has been categorized as a "delta endotoxin," because it conforms to the properties of parasporal, crystal-associated endotoxins from other insecticidal strains of *B. thuringiensis* (11, 14).

Although proteins from alkali-solubilized *B. thuringiensis* subsp. *israelensis* crystals have been studied extensively (3, 22, 24), the delta-endotoxin has not yet been purified, and thus its identity remains unknown. It is also unclear whether the multiple toxic characteristics of the organism are due to a single delta-endotoxin or whether additional molecular species exist which contribute to the toxicity. Reported studies of the biochemistry and mode of action of *B. thuringiensis* subsp. *israelensis* toxicity have been done with protein mixtures from purified crystals (22–24), which could obscure understanding of the specific characteristics of the delta-endotoxin itself.

In this paper, we describe a procedure for the purification of a form of delta-endotoxin from *B. thuringiensis* subsp. *israelensis*. This purified toxin exhibits larvicidal, hemolytic, and cytolytic properties and is lethal to mice. We also report its amino acid composition, present amino acid sequence data from the amino-terminal region, and describe the production of anti-toxin monoclonal antibodies.

MATERIALS AND METHODS

Organisms and cultural conditions. *B. thuringiensis* subsp. *israelensis* HD916 (originally from H. de Barjac, Institut Pasteur, Paris) and *B. thuringiensis* subsp. *kurstaki* HD73 were obtained from the Insect Pathology Research Unit, U.S. Department of Agriculture, Brownsville, Tex. *B. thuringiensis* subsp. *israelensis* Abbott (lot no. 44-219-CD) was an industrially produced culture powder obtained from Abbott Laboratories, North Chicago, Ill. *B. thuringiensis* subsp. *israelensis* Abbott 84-12b Cry$^-$ was isolated from the Abbott strain as a spontaneous asporogenous, acrystalliferous clone. *B. thuringiensis* subsp. *israelensis* HD916 was cultured in G-Tris medium (2) at 30°C for 4 days with rotary agitation before toxin purification.

Mosquito cell cytolytic assay. *Aedes albopictus* cells (from Yale Arbovirus Unit, Yale Medical School, New Haven, Conn.) were maintained at 28°C in 25-cm^2 plastic tissue culture flasks containing 5 ml of medium made as follows: 22 ml of salts-organics medium (see formulation below), 4.4 ml of 10× medium 199 (lacking glutamine and $NaHCO_3$; Microbiological Associates, Bethesda, Md.), 1 ml of 0.2 M glutamine, 10 ml of fetal bovine serum, 1 ml of penicillin (25,000 U/ml)–streptomycin (25,000 μg/ml) solution, and 61.6 ml of distilled water (pH 7.0). The salts-organics medium contained 16 g of NaCl, 0.8 g of KCl, 0.28 g of $CaCl_2 \cdot 2H_2O$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of $MgCl_2 \cdot 6H_2O$, 0.1 g of Na_2HPO_4, 0.12 g of K_2HPO_4, 2 g of dextrose, 10 g of lactalbumin hydrolysate (Difco Laboratories, Detroit, Mich.), and distilled water in 1 liter; the solution was adjusted to pH 6.4 and autoclaved for 15 min.

The assay is similar to the procedure published by Thomas and Ellar (22). When cells reached 60 to 80% confluent growth, the medium was removed, 2.5 ml of fresh medium warmed to 28°C was added, and the cells were detached from the surface by hitting the flask against the table edge. Two drops (about 0.1 ml) of cell suspension was distributed to wells of a 96-well flat-bottom (1- by 0.6-cm) microtiter plate (Linbro, no. 76-003-05; Flow Laboratories, Hamden, Conn.) and incubated at 28°C for 1 h to permit cell attachment. The medium was then removed by suction with a Pasteur pipette, wells were filled with PBS (0.09% Na_2HPO_4, 0.02% KH_2PO_4, 0.8% NaCl, 0.02% KCl, pH 7.0), the liquid was immediately removed, and 0.1 ml of toxin in PBS was distributed to each well. The assay was terminated after 1 h

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by suctioning the liquid from each well, adding PBS, and again suctioning off the liquid. Two drops of 0.25% safranine in 10% ethanol was added for 1 min. The cells were washed again with distilled water, dried at room temperature, and viewed. The toxin-induced cytolytic reaction caused detachment of cells from the well surface and was assessed by the absence of safranine-stained cells. Cytotoxicity was also monitored by periodic microscopic observation of trypan blue uptake and cytolysis (22). NH_4HCO_3 (150 mM) caused cytolysis of *A. albopictus* cells which was indistinguishable from the cytopathic effect of toxin. Therefore, samples containing NH_4HCO_3 were diluted to 50 mM or less before bioassay. Analyses of serial dilutions of toxin samples were used to determine the minimal protein concentration that produced cytotoxicity.

Larvicidal assay. Wells of a 96-well flat-bottom microtiter plate were filled with 0.2 ml of 15 mM NH_4HCO_3 (pH 8.5) containing toxin or bovine serum albumin (BSA). Five first-instar *Culex pipiens* larvae in 0.1 ml of water were added to each of 12 wells used for each sample analyzed. A few grains of finely ground mouse food pellets (Purina) were also added to feed the larvae. The test was performed at 23°C for 22 h.

Hemolytic assay. Rabbit or human erythrocytes were washed and diluted 1:200 by the procedures of Thomas and Ellar (22). A 100-μl sample of erythrocyte suspension was delivered to each well of a round-bottom well microtiter plate, and 5 μl of toxin sample in 50 mM NH_4HCO_3 (pH 8.5) was added. Plates were incubated at room temperature for 2 h.

Solubilization and proteolysis of proteins. Twenty grams of dry culture powder or 70 g of wet cell pellet was added to 400 ml of water and washed three times by centrifugation at 15,000 × *g* at 4°C. The pellet was then suspended in 160 ml of water and divided into 40-ml volumes. To release crystals from intact bacteria, the suspensions were sonicated in an ice bath for 5 min in a tuned Heat-Systems Ultrasonics sonicator set at 70. The samples were combined, and the pH was adjusted to 12 with 1 N NaOH. After 3 h at 37°C, the pH was lowered to 7.5, and the extract was centrifuged for 20 min at 15,000 × *g*. The supernatant liquid was incubated with trypsin (Sigma Chemical Co., St. Louis, Mo.) at 15 μg/ml for 2 h at 37°C followed by a 2-h incubation with proteinase K (Sigma) at 15 μg/ml. Phenylmethylsulfonyl fluoride (PMSF; 100 mM in absolute ethanol; Sigma) was added to a final concentration of 0.1 mM. Ammonium sulfate (ultra-pure grade; Schwarz/Mann Inc., Spring Valley, N.Y.) was added to 15% saturation, and the solution was stirred for 30 min at 0°C. This was followed by a 15-min centrifugation at 12,000 × *g*. The supernatant liquid was kept on ice, brought to 35% saturation with $(NH_4)_2SO_4$, and centrifuged as before. The pellet was dissolved in 20 ml of 5 mM NaH_2PO_4 (pH 7.5), clarified by another centrifugation, and finally filtered through a 0.45-μm nitrocellulose filter. Filtrates contained up to 15 mg of protein per ml as measured by absorbance at 280 nm (1 absorbance unit was equivalent to 1.1 mg of protein per ml). Filtrates were stored at −20°C until further purification by column chromatography.

Column chromatography. A 4-ml sample of alkali-solubilized, protease-treated filtrate was mixed with 4 μl of 100 mM PMSF and applied to a column containing P-60 Bio-Gel (fine grade; Bio-Rad Laboratories, Richmond, Calif.). Protein was eluted with 50 mM NH_4HCO_3, pH 8.5 (pH adjusted with NH_4OH), in 1.3-ml fractions at 15 to 20 ml/h at 4°C. Toxin-containing fractions were identified with the mosquito cell bioassay and pooled. PMSF was then added at a final concentration of 0.1 mM, and the extract was applied to a 1.4- by 58-cm DEAE Bio-Gel A column (100-200 mesh; Bio-Rad) equilibrated with 50 mM NH_4HCO_3 (pH 8.5). The column was washed with 100 ml of 50 mM NH_4HCO_3 (pH 8.5), and protein was eluted with a linear gradient with 250 ml of 50 mM NH_4HCO_3 and 250 ml of 200 mM NH_4HCO_3 (pH 8.5). The flow rate was 20 ml/h, and 1.3-ml fractions were collected. Toxin eluted from the DEAE in two peaks at about 100 mM NH_4HCO_3. Fractions from each peak were pooled separately, dialyzed overnight against 50 mM NH_4HCO_3 (pH 8.5), and fractionated through the same column with the same gradient. After each gradient run, the column was washed with 200 ml of 500 mM NH_4HCO_3 (pH 8.5) to remove residual bound protein.

PAGE. Polyacrylamide gel electrophoresis (PAGE) analyses were performed with 0.1% sodium dodecyl sulfate (SDS) by the method of Maizel (15) with a 3% acrylamide (acrylamide/bisacrylamide ratio, 30:0.8) upper gel and a 12% lower gel. Samples were diluted in 4% SDS–20% glycerol–10% β-mercaptoethanol–0.002% bromophenol blue–0.125 M Tris-hydrochloride (pH 6.8), heated at 100°C for 90 s, electrophoresed, and stained with silver (18) or Coomassie blue. In PAGE analyses of nondenatured proteins, SDS was omitted from all solutions, the sample buffer lacked β-mercaptoethanol, the samples were not boiled before electrophoresis, and the gel was 7% acrylamide.

Protease digestion. Proteolytic cleavage fragments were compared by the method of Cleveland et al. (8), except toxin at 56 μg/ml was incubated for 1 h at 37°C in 0.125 M Tris–0.1% SDS–0.001 M EDTA with or without 0.5 μg of *Staphylococcus aureus* V8 protease (Miles Laboratories, Inc., Elkhart, Ind.) per ml before analysis by SDS-PAGE.

Amino acid composition. Amino acid composition was determined as described by Moore and Stein (16). The protein hydrolysate was chromatographed in a Beckman Spinco model 120B amino acid analyzer.

Amino acid sequence. Amino acid sequence analysis was done as reported by Rohrmann et al. (20).

Production of monoclonal antibodies. A 20-μg sample of DEAE-purified toxin (dose not lethal to mice) was emulsified in complete Freund adjuvant and injected intraperitoneally into 6-week-old BALB/c mice. After 21 days, mice were boosted intraperitoneally with 30 μg of toxin in incomplete Freund adjuvant, and 6 days later they were boosted with another 25 μg of toxin. Spleen cells were harvested 3 days after the last boost and were fused (19) to BALB/c myeloma cell line SP2/0 in polyethylene glycol 1500 (M. A. Bioproducts, Walkersville, Md.). Two weeks after the fusion, antibody-producing cells were identified with an indirect enzyme-linked immunosorbent assay (ELISA; see below and reference 19), minicloned, and then cloned (17). Antibody-containing ascites fluids were produced as explained by Oi and Herzenberg (19).

ELISA. The method of Voller et al. (26) was followed for the ELISA, except Immulon 2 plates (Dynatech Laboratories, Inc., Alexandria, Va.) were used. All incubations were done at 37°C, and wells were treated for 30 min with 1% BSA in buffer and washed before the addition of toxin antibodies.

Western blot ELISA. Samples were electrophoresed in SDS-polyacrylamide gels and transferred at 4°C (Trans-Blot Cell; Bio-Rad Laboratories) to nitrocellulose paper for 3 h at 185 mA. The paper was incubated overnight at room temperature in BSA buffer (3% BSA, 150 mM NaCl, 10 mM $Na_2HPO_4 \cdot H_2O$, pH 7.4), washed extensively in the buffer, and incubated for 4 h in 50 ml of BSA buffer containing ascites fluid (1:500 dilution). After extensive washing in

buffer, the paper was incubated in 50 ml of BSA buffer containing a 1:1,000 dilution of goat anti-mouse immunoglobulin G-conjugated horseradish peroxidase (Sigma). After another wash, 50 ml of 50 mM Tris (pH 7.4) containing 15 mg of 3,3'-diaminobenzidine and 30 μl of H_2O_2 was added to develop the color.

RESULTS

Toxin purification. Our initial attempts to purify the delta endotoxin were hindered by the tendency of alkali-solubilized proteins from *B. thuringiensis* subsp. *israelensis* to form aggregates. Since we observed that alkali-solubilized extracts retained cytotoxicity after digestion with various proteases, we considered use of these enzymes to eliminate contaminating proteins. *S. aureus* protease, proteinase K, trypsin, chymotrypsin, pepsin, papain, thermolysin, and grade B pronase were tested for the most effective cleavage of *B. thuringiensis* subsp. *israelensis* proteins, but with maximum retention of biotoxicity. Alkali-solubilized proteins were dialyzed against 20 mM NaH_2PO_4 (pH 7.5), diluted to 1 to 3 mg/ml, mixed with each protease at 0.1 mg/ml, and incubated at 37°C for 1 h. SDS-PAGE was used to verify proteolytic activity. All digests retained toxicity to mosquito cells, whereas none of the proteases alone caused cytolysis. Proteinase K at 100 μg/ml appeared to give maximum digestion of the proteins without a decrease in toxicity. Trypsin and proteinase K were selected for use in tandem, since trypsin was sensitive to proteinase K and both could be inhibited by PMSF.

To increase yield, toxin was prepared from alkali-solubilized *B. thuringiensis* subsp. *israelensis* culture material containing cells, crystals, and spores in lieu of gradient-purified crystals typically used by others (1, 22–24). Since toxin was sensitive to extended exposure to proteases, PMSF was added immediately after proteinase K digestion. Ammonium sulfate fractionation permitted recovery of toxin with a minimum of protease contamination, since both trypsin and proteinase K were soluble in 35% $(NH_4)_2SO_4$. Elution of crude protein extract from *B. thuringiensis* subsp. *israelensis* HD916 through a P-60 Bio-Gel column is shown in Fig. 1a, whereas that from *B. thuringiensis* subsp. *israelensis* Abbott is shown in Fig. 1b. A 5-μl sample of each column fraction was mixed with 45 μl of 20 mM NaH_2PO_4 (pH 7.5) and 50 μl of 2× PBS and bioassayed on *A. albopictus* cells to locate toxin in peak III. For *B. thuringiensis* subsp. *israelensis* HD916, this peak contained about 25% of total absorbance applied to the column. With strain Abbott, peak III represented about 15% of the applied sample.

Further purification of toxin was accomplished by pooling fractions on the right side of peak III and applying them to a DEAE–Bio-Gel A column equilibrated with 50 mM NH_4HCO_3 (pH 8.5). Some nontoxic proteins were eluted when the column was washed. Toxin was eluted with a 50 to 200 mM gradient of NH_4HCO_3. Figure 2a and b depict fractionation of protein from *B. thuringiensis* subsp. *israelensis* strains HD916 and Abbott, respectively, into peak I containing the 25a protein and peak II containing the 25b protein. About half the protein from *B. thuringiensis* subsp. *israelensis* HD916 applied to the column eluted in peaks I and II, whereas 30% of the sample from the Abbott strain eluted in these peaks. The 25a protein fractions in peak I were pooled, dialyzed, and again passed through the DEAE Bio-Gel A column. The second elution of 25a protein from *B. thuringiensis* subsp. *israelensis* Abbott is depicted in the inset of Fig. 2b.

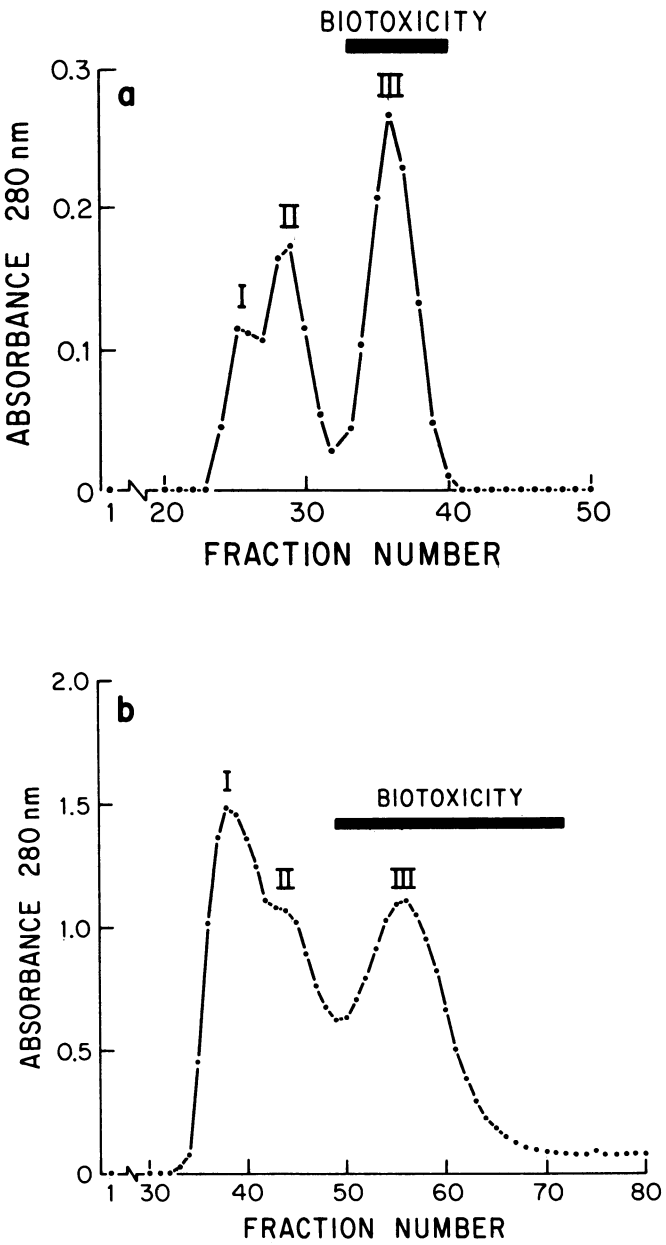

FIG. 1. P-60 Bio-Gel chromatography of alkali-solubilized, protease-treated *B. thuringiensis* subsp. *israelensis* proteins. (a) Protein from strain HD916 (4.8 mg) was applied to a 1.3- by 81-cm column; (b) protein from strain Abbott (19.2 mg) was applied to a 2- by 70-cm column.

PAGE analyses of proteins. Electrophoresis of proteins from *B. thuringiensis* subsp. *israelensis* Abbott during different stages in the purification procedure is shown in Fig. 3 and 4. Figure 3, lane a, illustrates SDS-PAGE analysis of more than 20 proteins, ranging in size from 20,000 to 130,000 daltons, present in the crude, alkali-solubilized *B. thuringiensis* subsp. *israelensis* Abbott extract used for toxin purification. The most abundant protein is about 28,000 daltons. This pattern of protein bands is similar to that reported by others (22, 24) who solubilized proteins from purified crystals. Figure 4, lane a, shows electrophoresis of the same extract in a nondenaturing polyacrylamide gel. Nondenaturing gels were useful in distinguishing forms of the toxin,

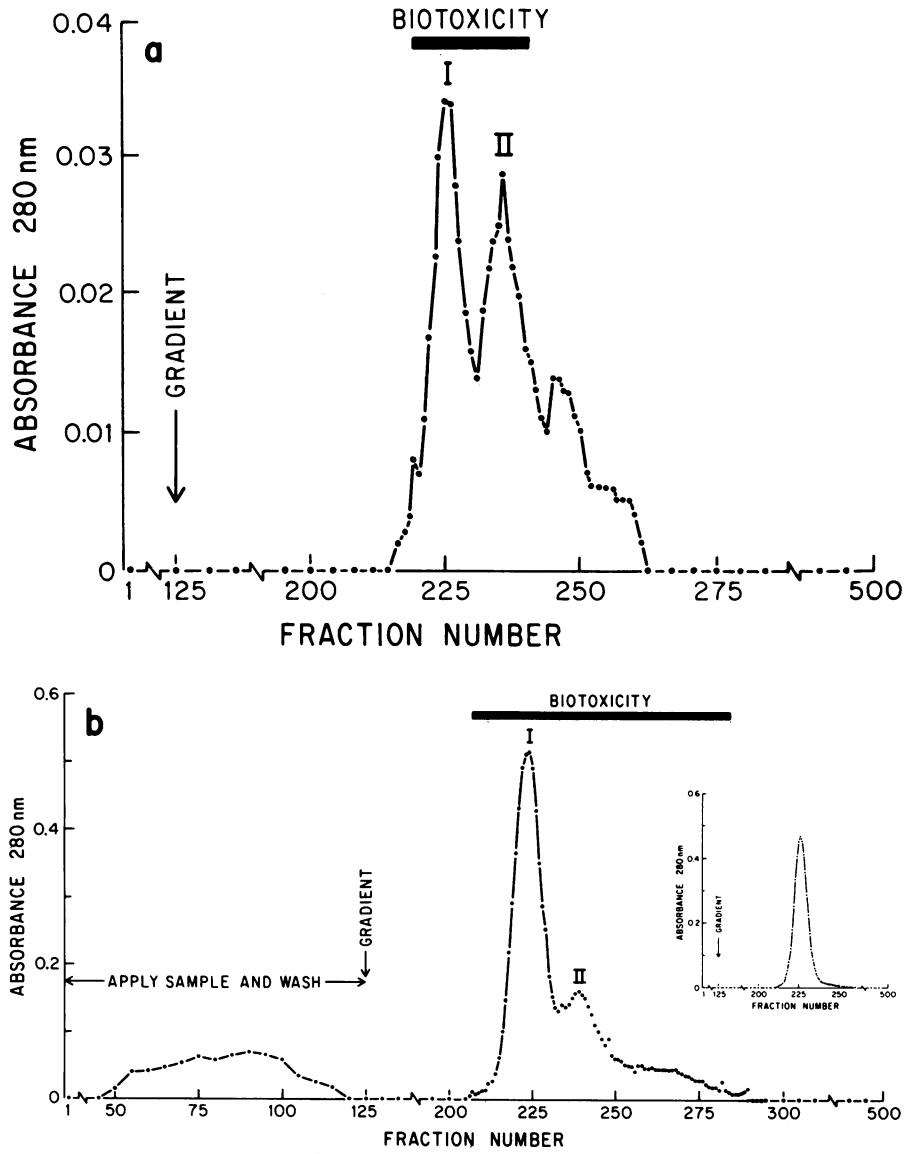

FIG. 2. DEAE Bio-Gel A chromatography of toxic fractions from P-60 Bio-Gel column. (a) Sample (1.1 mg) of strain HD916 protein from the elution shown in Fig. 1a. No protein was observed in the wash, fractions 1 through 124. (b) Sample (19.2 mg) of strain Abbott protein from the elution shown in Fig. 1b. The inset shows the second elution of peak I through DEAE Bio-Gel A column.

which appeared identical in denaturing gels. Similarly, analyses of proteins from peak III of the P-60 Bio-Gel elutions (Fig. 1b) are shown in Fig. 3, lane b, and Fig. 4, lane b. The predominant component in peak III of the P-60 Bio-Gel column was a 25,000-dalton molecule. It is of interest that a protein of this size was not visible in the SDS-PAGE analyses of alkali-solubilized protein extract before digestion with the proteases (Fig. 3, lane a), suggesting that it is a product of proteolysis. Figure 3, lane c, and Fig. 4, lane c, illustrate SDS-PAGE and nondenaturing PAGE analyses, respectively, of the 25b protein from peak II of the DEAE Bio-Gel A column elution. Similarly, Fig. 3, lane e, and Fig. 4, lane e, depict the 25a protein from peak I, whereas Fig. 3, lane d, and Fig. 4, lane d, show electrophoresis of a mixture of the proteins from each peak. The 25a protein eluted twice through DEAE Bio-Gel A is shown in Fig. 3, lane f, and Fig. 4, lane f. The 25a and 25b proteins from *B. thuringiensis* subsp. *israelensis* HD916 were also about 25,000 daltons and indistinguishable in SDS-PAGE, but were separable by nondenaturing PAGE (data not shown).

Biotoxicity studies. *B. thuringiensis* subsp. *israelensis* Abbott 25a protein from the left side of peak I of the DEAE Bio-Gel A elution and 25b protein from the right side of peak II were both toxic to *A. albopictus* cells at a concentration of 0.1 μg/ml. Also, 22 μg of toxin per ml hemolyzed human or rabbit erythrocytes within 5 min.

The 25a toxin from *B. thuringiensis* subsp. *israelensis* Abbott that was purified through DEAE Bio-Gel A was also larvicidal against first-instar *C. pipiens* larvae. After exposure to 50 μg of toxin per ml for 22 h, 6 of 60 larvae lived, whereas control tests with BSA at 50 μg/ml exhibited 57 of 60 survivors. Assays with toxin at 5 μg/ml gave 57 of 60 survivors and were thus indistinguishable from BSA controls.

In an initial attempt to prepare toxin antibody, 100 μg of DEAE-purified toxin in complete Freund adjuvant was in-

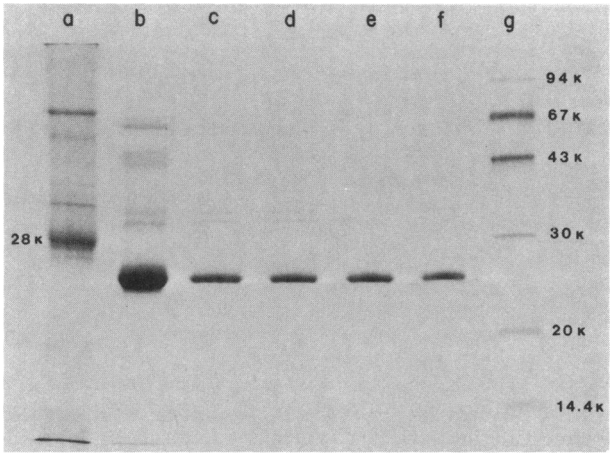

FIG. 3. SDS-PAGE analysis of *B. thuringiensis* subsp. *israelensis* Abbott toxin at various stages in purification. Lanes: (a) 15 μg of crude, alkali-solubilized protein extract (28K marks the position of the 28,000-dalton protein); (b) 2.5 μg from peak III of P-60 Bio-Gel column (fraction 58, Fig. 1b); (c) 0.5 μg of 25b protein from DEAE Bio-Gel A column (fraction 245, Fig. 2b); (d) mixture of 0.25 μg of 25b protein and 0.25 μg of 25a protein from DEAE Bio-Gel A column; (e) 0.5 μg of 25a protein from DEAE Bio-Gel A column (fraction 216, Fig. 2b); (f) 0.5 μg of 25a protein twice purified through DEAE Bio-Gel A; (g) protein standards (in decreasing order of molecular weight: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and alpha-lactalbumin; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). The negative electrode is at the top.

jected intraperitoneally into five 6-week-old BALB/c mice. All mice died within 6 h. However, a dose of 20 μg of toxin per mouse was not lethal and was used for production of monoclonal antibodies.

Partial proteolytic cleavage of 25a and 25b proteins. The 25a and 25b proteins from *B. thuringiensis* subsp. *israelensis* Abbott were subjected to partial proteolysis using *S. aureus* protease to determine their structural relationship (8). Electrophoresis of the protease cleavage fragments is shown in Fig. 5, lanes c and d. Similarities in migratory rates of fragments indicated both proteins were very similar in primary structure.

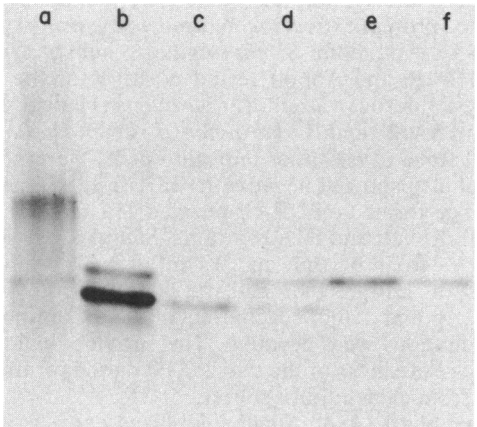

FIG. 4. Nondenaturing PAGE analysis of *B. thuringiensis* subsp. *israelensis* Abbott toxin at various stages in purification. Lanes: (a) 15 μg of crude, alkali-solubilized protein extract; (b) 2.5 μg from peak III of P-60 Bio-Gel column (fraction 58, Fig. 1b); (c) 0.5 μg of 25b protein from DEAE Bio-Gel A column (fraction 245, Fig. 2b); (d) mixture of 0.25 μg of 25b protein and 0.25 μg of 25a protein from DEAE Bio-Gel A column; (e) 0.5 μg of 25a protein from DEAE Bio-Gel A column (fraction 216, Fig. 2b); (f) 0.5 μg of 25a protein twice purified through DEAE Bio-Gel A. The negative electrode is at the top.

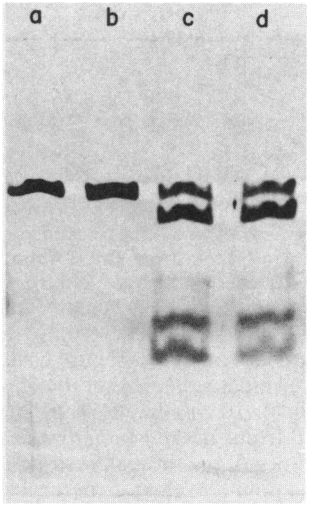

FIG. 5. Partial protease digestion and SDS-PAGE analysis of *B. thuringiensis* subsp. *israelensis* Abbott 25a or 25b proteins in the absence or presence of *S. aureus* protease. Lanes: (a) undigested 25a protein; (b) undigested 25b protein; (c) digested 25a protein; (d) digested 25b protein.

Amino acid composition. The amino acid composition of the 25a protein purified from *B. thuringiensis* subsp. *israelensis* Abbott is presented in Table 1. The data are similar to the composition of a crude, alkali-solubilized extract of *B. thuringiensis* subsp. *israelensis* crystal proteins as determined by Tyrell et al. (24).

TABLE 1. Amino acid composition of 25a protein from *B. thuringiensis* subsp. *israelensis* Abbott

Amino acid	mol/100 mol of amino acids	No. of residues[a] per 25a toxin molecule
Aspartic acid[b]	15.0	38
Threonine	8.2	20
Serine	6.5	16
Glutamic acid[b]	11.5	29
Proline	3.7	9
Glycine	4.8	12
Alanine	8.4	21
1/2-Cysteine[c]	0.1	0
Valine	10.7	27
Methionine	2.5	6
Isoleucine	6.4	16
Leucine	8.4	21
Tyrosine	3.2	8
Phenylalanine	5.3	13
Histidine	0	0
Lysine	3.4	10
Arginine	1.5	4

[a] Based on molecular weight of 25,000.
[b] Aspartic and glutamic acid values include asparagine and glutamine, respectively.
[c] Determined as cysteic acid.

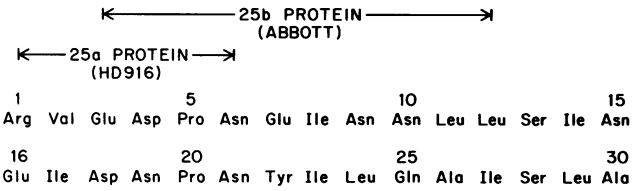

FIG. 6. Partial amino acid sequences of toxic proteins from *B. thuringiensis* subsp. *israelensis*. The sequence of 30 residues was obtained from the 25a protein from strain Abbott. Arrows indicate lengths of partial sequences of 25b protein from strain Abbott and 25a protein from strain HD916. The amino termini are to the left.

Amino acid sequence analyses. Figure 6 shows 6 residues of the amino acid terminal sequence of the 25a protein from *B. thuringiensis* subsp. *israelensis* HD916 and 30 residues of the 25a protein from *B. thuringiensis* subsp. *israelensis* Abbott. The identity of the first six residues in toxic proteins from both strains points to their structural relatedness. Also shown in Fig. 6 are the 10 residues at the amino terminus of the 25b protein from *B. thuringiensis* subsp. *israelensis* Abbott, which are identical to residues 3 through 12 of the 25a protein of the Abbott strain. This is further evidence that both forms of toxin in this strain are close in primary structure and may be proteolytic cleavage products of the same protein. Of interest are two groups of five amino acids, residues 6 through 10 and 15 through 19 in the 25a protein, which are identical except for substitution of amino acids asparagine (residue 9) and aspartic acid (residue 18). The frequent occurrence of asparagine in the 25a protein sequence is similar to that noted in the partial sequence of *B. thuringiensis* subsp. *kurstaki* delta-endotoxin reported by Schnepf and Whiteley (21). A comparison of our sequence data with those of Schnepf and Whiteley did not reveal any regions of homology in either structure.

TABLE 2. Comparison of antigenic cross-reactivity and biotoxicity among toxins of *B. thuringiensis* strains

B. thuringiensis subsp. (serovar)	Toxin purity	Bioassays			ELISA[a]
		Mosquito larvae[b]	Mosquito cells[c]	Erythrocytes[d]	
israelensis Abbott (14)	Impure[e]	+	+	+	+
	Pure[f]	+	+	+	+
israelensis HD916 (14)	Impure	+	+	+	+
	Pure	n.d.	+	+	+
israelensis Abbott 84-12b (14)[g]	Impure	−	−	−	−
kurstaki HD73 (3a, b)	Impure	n.d.	−	−	−

[a] For impure toxin, 5 μl of alkali-solubilized protein (footnote *e*) per well was analyzed. For pure toxin, 0.1 ng of toxin per well was tested; +, cross-reaction; −, no cross-reaction.

[b] For impure toxin, a loopful of colony material (footnote *e*) was suspended in 0.25 ml of water containing 5 *C. pipiens* larvae and incubated at 25°C; +, all larvae dead in 1.5 h; −, all larvae alive after 19 h. Pure toxin at 50 μg/ml was tested as described in the text; +, toxicity (6 of 60 survivors); −, no toxicity (57 of 60 survivors).

[c] For impure toxin, alkali-solubilized proteins (footnote *e*) were mixed with an equal volume of 2× PBS and incubated with cultured *A. albopictus* cells for 1 h at 28°C. Pure toxin at 0.1 μg/ml was tested as described in the text; +, cytolysis; −, no cytolysis.

[d] For impure toxin, alkali-solubilized proteins (footnote *e*) were mixed with 25 μl of 2× PBS containing a 1:200 dilution of human erythrocytes and incubated for 30 min at room temperature; +, hemolysis; −, no hemolysis. Pure toxin at 1 μg/ml was tested as described in the text; +, hemolysis; −, no hemolysis.

[e] Bacteria were streaked on G-Tris medium (2) containing 1.5% agar and incubated at 30°C for 48 h. Alkali-solubilized proteins were prepared by suspending a loopful of colony material in 50 μl of NaOH and incubating at 37°C for 2 h. A 25-μl volume of PBS (see the text) was added, the suspension was centrifuged for 5 min in a Beckman microfuge, and the pellet was discarded.

[f] The 25a protein was purified through DEAE Bio-Gel A chromatographic step (see the text).

[g] An asporogenous, acrystalliferous strain.

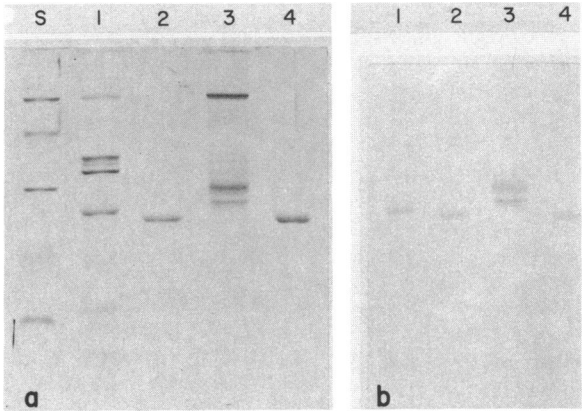

FIG. 7. Western blot ELISA of *B. thuringiensis* subsp. *israelensis* proteins, using monoclonal antibodies to toxin. (a) Gel stained with Coomassie blue; (b) Western blot of (a). Lanes: S, protein standards as listed in Fig. 3 (phosphorylase *b* not visible); 1, strain HD916 alkali-solubilized proteins; 2, strain HD916 25a protein; 3, strain Abbott alkali-solubilized proteins; 4, strain Abbott 25a protein. The negative electrode is at the top.

Toxicity and ELISA testing of various *B. thuringiensis* toxins. Monoclonal antibodies to 25a toxin protein from *B. thuringiensis* subsp. *israelensis* Abbott were used to study the relationship between the occurrence of toxin and biological activity among four strains of *B. thuringiensis*. Table 2 summarizes toxic effects of these strains on *C. pipiens* larvae, *A. albopictus* cells, and human erythrocytes and the results of ELISA tests. Either a cell culture suspension or a sample of pure 25a protein was used for larval assays. Alkali-solubilized protein from culture suspensions or a sample of pure 25a protein was used for assays with mosquito cells and erythrocytes (Table 2). When impure, alkali-solubilized protein extracts were used as the primary antigen in ELISA tests, both *B. thuringiensis* subsp. *israelensis* strains HD916 and Abbott tested positive for the antigen. The acrystalliferous variant *B. thuringiensis* subsp. *israelensis* Abbott 84-12b and *B. thuringiensis* subsp. *kurstaki* HD73 were not toxic to larvae or mosquito cells, were not hemolytic, and also showed negative ELISA results. The ELISA was also performed on DEAE-purified 25a and 25b proteins from both Abbott and HD916 strains. Samples were adjusted to 20 μg of toxin per ml, and 0.1 ml of each was tested at dilutions of 1:10, 1:100, and 1:1,000. All four samples, diluted 1:10 and 1:100, reacted equally with antibody. The 1:1,000 dilutions were negative. This provided evidence for antigenic relatedness of the two 25,000-dalton forms of toxin purified from each culture source.

Western blot ELISA. Alkali-solubilized extracts and purified 25a proteins from *B. thuringiensis* subsp. *israelensis* HD916 and Abbott were analyzed by SDS-PAGE and stained with Coomassie blue (Fig. 7a) or tested further by Western blot ELISA (Fig. 7b). A 26,000-dalton protein in crude, alkali-solubilized *B. thuringiensis* subsp. *israelensis* HD916 (lane 1, Fig. 7b) cross-reacted with antibody against 25a protein from strain Abbott. Lane 2, Fig. 7b, shows reaction with DEAE-purified 25a protein from strain HD916. Lane 3, Fig. 7b, shows 26,000- and 28,000-dalton alkali-solubilized

proteins from strain Abbott that cross-reacted with antibody to 25a protein and can be compared with the purified 25a protein from the Abbott strain in lane 4, Fig. 7b.

DISCUSSION

We describe the purification from *B. thuringiensis* subsp. *israelensis* of a proteinaceous toxin that lyses rabbit and human erythrocytes and cultured mosquito cells, is insecticidal to first-instar mosquito larvae, and is lethal to mice. Monoclonal antibodies to purified toxin used in ELISA tests cross-react only with proteins of toxic *B. thuringiensis* subsp. *israelensis* strains. Therefore it appears the toxicological properties of *B. thuringiensis* subsp. *israelensis* are determined by a single protein and that this protein is the delta endotoxin.

The purification scheme we developed is well suited for obtaining large quantities of each of two forms of the toxin. The method depends on the alkali-soluble and protease-tolerant properties of the toxin, which make it possible to extract toxin directly from sporulated cultures containing a mixture of cells, spores, and crystals. Digestion of alkali-solubilized *B. thuringiensis* subsp. *israelensis* proteins with trypsin followed by proteinase K eliminates most of the proteins associated with the toxin and permits efficient purification of toxin by column chromatography. The two closely related forms of purified toxin coelute from a P-60 Bio-Gel column, migrate similarly in SDS-polyacrylamide gels, exhibit identical cleavage products when partially digested with *S. aureus* protease in the presence of SDS, and contain an identical sequence of 10 amino acids. Furthermore, they are equally toxic and cross-react equivalently in an ELISA with monoclonal antibodies to the 25a protein. These results point to the many similarities between the two forms of toxin. A difference between the 25a and 25b proteins, however, is the presence of arginine and valine residues at the amino terminus of the 25a molecule.

Western blot ELISA studies with monoclonal antibodies to the 25a protein indicate that the 25a and 25b proteins are proteolytic cleavage products derived from a larger protein of about 26,000 daltons in strain HD916 and 28,000 daltons in the Abbott strain. Therefore the 25a and 25b proteins appear to be proteolytic cleavage fragments of a larger protein. Both the use of proteases during purification and the occurrence of proteases endogenous to the *B. thuringiensis* subsp. *israelensis* crystal (7) probably contribute to the proteolysis. Yamamoto et al. (27) reported partial purification of a 25,000-dalton protein, which they also suggest is proteolytically derived from a 28,000-dalton molecule.

Crude alkali-soluble protein extracts from the two *B. thuringiensis* subsp. *israelensis* strains exhibited different patterns in SDS-polyacrylamide gels (Fig. 7a). They also showed qualitative differences in relative peak size and location during gel filtration and elution from DEAE. However, the purified 25a proteins extracted from these strains were similar in toxic properties, size, and antigenicity and contained six identical amino-terminal residues. This emphasizes the specificity of the purification procedure. It also suggests the structure of the 25a protein may be similar among various strains of *B. thuringiensis* subsp. *israelensis*.

The prevalence of protease-tolerant proteins is a remarkably common phenomenon among species of *Bacillus*. For example, the protease-resistance of the delta-endotoxin of *B. thuringiensis* subsp. *kurstaki* has been well characterized (6, 13). Davidson (9) has purified from *B. sphaericus* a toxin that is toxic to mosquito larvae and exhibits tolerance to trypsin and chymotrypsin. Also, the cereolysin of *Bacillus cereus* is inactivated little or not at all by trypsin, chymotrypsin, or papain (4). The protease-tolerant feature may permit the bacilli to successfully invade the protease-rich environment of the insect gut (5). Both from unpublished data (J. L. Armstrong), which indicated the purified 25a protein was tolerant to trypsin, and from results presented here, it is apparent the *B. thuringiensis* subsp. *israelensis* toxin is also protected from proteolysis. This is particularly interesting, since the amino acids associated with proteinase K and trypsin cleavage sites can be demonstrated by amino acid composition and terminal amino acid sequence analyses.

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