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Purification of the Larvicidal Toxin of *Bacillus sphaericus* and Evidence for High-Molecular-Weight Precursors

PAUL BAUMANN,* BRIAN M. UNTERMAN, LINDA BAUMANN, ANDREW H. BROADWELL, SUSAN J. ABBENE, AND RON D. BOWDITCH

Department of Bacteriology, University of California, Davis, California 95616

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Crystals were purified from spore-crystal complexes of *Bacillus sphaericus* 2362 by disruption in a French pressure cell followed by centrifugation through 48% (wt/vol) NaBr. Crystals from such preparations had a 50% lethal concentration of 6 ng of protein per ml for the larvae of the mosquito *Culex pipiens*. When subjected to polyacrylamide gel electrophoresis under denaturing conditions, the proteins in *B. sphaericus* crystals migrated in positions corresponding to 43, 63, 98, 110, and 125 kilodaltons (kDa); solubilization of the crystal at pH 12 with NaOH eliminated all but the bands at 43 and 63 kDa. Since NaOH-solubilized preparations were toxic to mosquito larvae, these proteins were purified to electrophoretic homogeneity and antiserum was obtained to each. Analysis of the two purified proteins indicated that the 43-kDa protein was toxic to mosquito larvae (50% lethal concentration, 35 ng of protein per ml), whereas the 63-kDa protein was not. Further differences between them were their amino acid compositions, their lack of immunological cross-reactivity, their opposite net charges at pH 7.5, and their susceptibility to digestion by larval midgut proteases (the 63-kDa protein was highly susceptible, whereas the 43-kDa protein was not). The sequence of the 40 N-terminal residues of the 43-kDa protein was determined and found to contain a high percentage of hydrophobic amino acids. The sequence of the 63-kDa protein could not be determined, since it had multiple N termini. By electrophoretically separating the crystal proteins and then electroblotting onto nitrocellulose paper and visualizing the bands with antisera to the 43- and 63-kDa proteins in conjunction with an immunoblot assay, it was found that the high-molecular-mass crystal proteins (98 to 125 kDa) contained antigenic determinants of both proteins. These results suggested that the lower-molecular-weight crystal proteins detected in polyacrylamide gels after electrophoresis under denaturing conditions were derivatives of one or more of the higher-molecular-weight crystal proteins. In vivo studies of the products of crystal degradation by larvae of *Culex pipiens* indicated that the high-molecular-weight proteins and the 63-kDa antigenic determinants were rapidly degraded and that a 40-kDa protein related to the 43-kDa toxin persisted for the duration of the experiment (4 h). Some of the studies performed with *B. sphaericus* 2362 were extended to strains 1593, 1691, and 2297 of this species with results which indicated a high degree of similarity between the crystal proteins of all these larvicidal strains.

Bacillus sphaericus is currently regarded as a promising agent for the biological control of mosquito larvae (27). Toxigenic strains of this species contain a polyhedral body, commonly known as a crystal, which forms during sporulation and is located next to the spore within the exosporeum (7). Investigations in the laboratories of H. de Barjac, E. W. Davidson, and A. A. Yousten have established a correlation between crystal production, sporulation, and larvicidal activity (2, 11, 18, 29). Further implication of the crystal in larval intoxication is the evidence from electron microscopy that it is rapidly solubilized when spore-crystal complexes are ingested by mosquito larvae (29). Recently, Payne and Davidson (20) dissociated the spore-crystal complex by passage through a French pressure cell and separated the crystals from the spores by centrifugation through an NaBr gradient. The highest toxicity for mosquito larvae was found in the fractions enriched for crystal.

The scope of past investigations into the nature of the toxin has been rather limited. A major problem was the failure to isolate crystal in sufficient quantity to permit purification of its toxic components. Soluble toxic material was obtained by freezing and thawing the spore-crystal complex or by solubilizing it with 50 mM NaOH (3, 6, 23). Partial purification of such material by column chromatography indicated that the toxic protein might have a molecular mass of about 35, 55, or 100 kilodaltons (kDa) (5, 6, 23). Evidence suggesting that the lower-molecular-mass proteins are derived from the 100-kDa protein has been reported (6).

B. sphaericus is one of several entomocidal *Bacillus* spp. whose toxicity resides in a parasporal crystal. The most extensively studied organism is *Bacillus thuringiensis* var. *kurstaki* (4), which forms a crystal composed of a nontoxic 130-kDa protein. After solubilization in the alkaline gut of lepidopterous insects (caterpillars), this protoxin is converted into a 64-kDa toxin by proteolytic activity. Another variety of this species (*B. thuringiensis* var. *israelensis*) is active against mosquito larvae; its crystal contains a protoxin which is also activated by proteolysis (22, 25).

In the present study we have purified and characterized two proteins, one of which is larvicidal, from the crystal of *B. sphaericus* 2362 and have established their relationship to other crystal proteins. Strain 2362 was chosen for study because of its ability to make toxin at elevated temperatures, an attribute which may be useful in the commercial manufacture of the toxin (27). Other strains investigated were *B. sphaericus* 1593, the oldest and most extensively used isolate (6, 18, 23); *B. sphaericus* 2297, an isolate recently

* Corresponding author.

proposed for industrial development (11); and *B. sphaericus* 1691, another highly toxigenic strain (7). Strain 2297 belongs to Yousten's bacteriophage group 4, whereas the remaining strains are in group 3 (28).

MATERIALS AND METHODS

Bacterial isolates. Strains of *B. sphaericus* (WHO 1593, 1691, 2297, and 2362) were a gift from A. A. Yousten. *B. thuringiensis* var. *israelensis* HD 567, 4Q3 and *B. thuringiensis* var. *kurstaki* HD-1, 4D1 were obtained from the Bacillus Genetic Stock Center, Ohio State University, Columbus).

Media, conditions of cultivation, and harvesting of cells. The medium used was a slight modification of a formulation of Kalfon et al. (12); it contained (per liter) 100 ml of 1 M Tris-hydrochloride (pH 7.5); 3 ml of 7.5% (wt/vol) K_2HPO_4; 10 ml of a solution containing 3% (wt/vol) $MgSO_4 \cdot 7H_2O$, 0.25% (wt/vol) $CaCl_2 \cdot 2H_2O$, 0.2% (wt/vol) $ZnSO_4 \cdot 7H_2O$, and 0.14% (wt/vol) $FeCl_3 \cdot 6H_2O$; 2 g of yeast extract (Difco Laboratories, Detroit, Mich.); and 10 g of tryptone (Difco). Solid medium was obtained by adding 20 g of Bacto-Agar (Difco) per liter.

Cultures were grown in 2.8-liter Fernbach flasks containing 1-liter portions of the medium. After inoculation with cells from a 24-h slant culture, the flasks were placed on a rotary shaker and incubated at 32°C for 44 to 48 h. Cells were harvested by centrifugation at 20,000 × *g* for 10 min. The pellets from 12 liters of medium were washed twice with 1 liter of 1 M NaCl and twice in 1 liter of 10 mM EDTA (pH 7.5). These and all subsequent operations were performed at 3 to 6°C. Pellets were stored at −20°C until needed.

Separation of crystals and spores. A pellet from 24 liters of culture (120 to 140 g [wet weight]) was suspended in 40 ml of distilled water, and the exosporeum was disrupted by three passages through a French pressure cell at 18,000 lb/in^2. Portions (15 ml) of this suspension were layered onto 25 ml of a 48% (wt/vol) NaBr solution in polycarbonate test tubes (2.5 by 10 cm). After centrifugation at 17,000 × *g* for 3 h in an HB-4 swinging bucket rotor (Sorvall-DuPont, Newton, Conn.), the contents of the tubes were removed from the top by means of a metal tube (diameter, 1.5 mm) attached to Tygon tubing with suction provided by a peristaltic pump. Fractions (20 drops) were collected with a model MFH mini-fraction collector (Gilson, Middleton, Wis.), and the samples were examined by phase microscopy for the presence of crystals. Fractions predominantly containing crystals were combined, washed with six 1-liter portions of 10 mM EDTA (pH 7.5), and stored at −20°C. This preparation is referred to as purified crystal.

When sporulation is complete, crystals of *B. thuringiensis* var. *israelensis* and *B. thuringiensis* var. *kurstaki* are not enclosed within an exosporeum. With these strains, crystals were separated from spores by centrifugation through Renografin (E. R. Squibb & Sons, Princeton, N.J.) gradients as previously described (21).

Solubilization of the crystal. Purified crystals derived from 24 liters of culture were suspended in 20 ml of water and incubated at 25°C with stirring. The pH of the suspension was brought to 12 with 1 N NaOH. Incubation was continued for an additional 3 h, and the pH was monitored periodically and adjusted with 1 N NaOH as needed. The pH was lowered by adding 2 ml of 1 M Tris-hydrochloride (pH 7.5), and the suspension was centrifuged at 40,000 × *g* for 30 min. The supernatant was frozen at −20°C overnight and thawed, and the precipitate was removed by centrifugation. This procedure is similar to that developed by Bulla et al. (4) for the solubilization of crystal from *B. thuringiensis* var. *kurstaki*.

Column chromatography. Subsequent purification of solubilized crystal proteins involved chromatography through Sephadex G-200 (Pharmacia, Uppsala, Sweden) in 2.5- by 100-cm columns with 20 mM K_2HPO_4–KH_2PO_4 (pH 7.5) (KPB) as the buffer; 3.2-ml fractions were collected at a flow rate of 14 to 16 ml per h. DEAE-agarose (Bio-Rad Laboratories, Richmond, Calif.) chromatography, which followed, was performed in 2.5- by 50-cm columns. The sample was applied in KPB and washed with 500 to 700 ml of the buffer. Protein was eluted with KPB containing 100 mM NaCl. The flow rate was 16 to 18 ml per h, and the fraction size was 6.2 ml. NaCl concentration was monitored by means of a model 10419 refractometer (American Optical Corp., Buffalo, N.Y.). Fractions selected for further purification on Bio-Rad P-100 polyacrylamide columns were combined and concentrated by filtration through PM-10 filters (Amicon Corp., Lexington, Mass.). The dimensions of the P-100 columns were 2.5 × 100 cm, and the buffer was KPB containing 80 mM NaCl. Fractions of 1.6 ml were collected at a flow rate of 11 to 13 ml per h.

SDS-PAGE. Slab gel electrophoresis under denaturing conditions was performed as described by Laemmli (13), with gels 1.5 mm thick. The molecular weight standards were purchased as a mixture from Sigma Chemical Co., St. Louis, Mo. Bovine albumin, which has a molecular mass of 66 kDa, migrated at a distance corresponding to 74 kDa and was given this value in all of the figures where these standards were used. Unless otherwise stated, the concentration of acrylamide was 8.5% (wt/vol), and the gels were stained for protein by the methods of Fairbanks et al. (8). In the purification of the proteins, purity was judged by electrophoresis of the fractions in SDS-PAGE.

Protein and dry-weight determinations. In the course of purifying the crystal proteins, approximate protein concentrations were obtained by measuring the optical density at 280 nm (OD_{280}) (see exception below). Protein was also determined by the method of Lowry et al. (15), with bovine serum albumin as the standard; the alkaline conditions used in this method caused solubilization of any crystals present. Dry weight was determined after samples of washed crystals or crystal-spore complex had been heated at 105°C for 3 days. Unless otherwise stated, all the amounts of protein given in the text are based on determinations by the Lowry method.

Toxin assay with mosquito larvae. The concentration of toxin resulting in 50% mortality (LC_{50}) was determined by using second- to third-instar larvae of *Culex pipiens* (18). Each concentration of toxin was tested in triplicate in a plastic cup containing 10 larvae in 30 ml of water. About 10 mg of 1:1 mixture of TetraMin fish food (Melle, Federal Republic of Germany) and Guinea Pig Chow 5025 (Purina, St. Louis, Mo.) was added to each cup before incubation at 25°C for 2 days with a photoperiod of 14 h of light and 10 h of dark. After correction for mortality of the controls (always less than 7%), the line of best fit was determined for the relation between probit mortality (16) and the logarithm of toxin concentration by means of linear regression analysis. In each determination of LC_{50} for *B. sphaericus*, a parallel determination was made of the toxicity of Renografin-purified crystals from *B. thuringiensis* var. *israelensis*. From a total of 12 such determinations, it was found that the mean LC_{50} for this crystal was 3.5 ng (dry weight) or 2.6 ng of Lowry protein per ml. All the determinations of LC_{50} for *B. sphaericus* were normalized to this value. The 95% confi-

dence limits were calculated as described by Matsumura (16). In cases for which only a rough indication of toxicity was required, 10 larvae were used in a cup containing 10 ml of water.

Immunization of rabbits and purification of immunoglobulins. Four male New Zealand White rabbits, each weighing between 5 and 6 lb (2.27 and 2.72 kg), were injected intradermally with 1 mg of the 43-kDa protein mixed with an equal volume of complete Freund adjuvant (Difco). Similarly, two rabbits were injected with the 63-kDa protein. In both cases, booster doses of 1 mg of the appropriate antigen in an equal volume of incomplete Freund adjuvant (Difco) were injected intradermally at 5 and 7 weeks. After a 3-week interval, the rabbits were given intravenous injections on three alternate days, each injection containing 0.75 mg of antigen. One week after the last injection, the rabbits were bled by cardiac puncture, and the serum was stored overnight at 4°C. Serum was separated from clotted blood by filtering through Whatman no. 1 filter paper by gravity. The immunoglobulins in the filtered serum were purified by passage through a Bio-Rad DEAE Affi-Gel Blue column as described by the manufacturer. The presence of immunoglobulins reacting with either the 43- or 63-kDa protein was determined by Ouchterlony immunodiffusion. Fractions which gave precipitin bands were combined, and the protein was precipitated at 45% $(NH_4)_2SO_4$ saturation. The pellet was dissolved in 10 mM Tris-hydrochloride (pH 7.5)–150 mM NaCl, dialyzed against two 2-liter portions of the same buffer, and stored at −20°C.

Since the solubilized toxin of *B. thuringiensis* var. *israelensis* is highly toxic to mammals (22), an autopsy of the four rabbits injected with the 43-kDa protein was performed by a veterinarian from the Animal Resources Services of the University of California, Davis. The results indicated no detectable pathological abnormalities.

Immunoelectrophoresis and Ouchterlony immunodiffusion. Immunoelectrophoresis was performed in gels containing 25 mM K_2HPO_4–KH_2PO_4 (pH 7.8), 0.02% (wt/vol) thimerosal, and 1% (wt/vol) Ionager (Wilson Diagnostics, Glenwood, Ill.) as described by Williams (26). Antigens (15 μg; 10 μl) were electrophoresed for 3 h at 3 V/cm before addition of 150 μl of antiserum to the central trough (by using either a 1/2 dilution of the anti-43-kDa crystal protein antiserum [A-43] or a 1/4 dilution of the anti-63-kDa protein antiserum [A-63]). The gels were incubated at 4°C and observed for a period of 4 days.

Ouchterlony immunodiffusion, which was performed as described by Munoz (17), involved the use of gels containing 10 mM K_2HPO_4–KH_2PO_4 (pH 7.5), 0.58% (wt/vol) NaCl, 1% (wt/vol) Ionagar, and 0.02% (wt/vol) thimerosal. The wells were 4 mm apart and contained 20 μl of the antigen or antiserum. The dilution of the latter was the same as for immunoelectrophoresis; however, the concentration of the NaOH-solubilized crystal was adjusted so that the precipitin band was approximately equidistant from the antiserum and antibody wells (20 to 30 μg of protein). The plates were incubated at 4°C and observed for 2 days.

Electroblots and immunoblot assay. Proteins which had been electrophoresed in SDS-PAGE gels were transferred to nitrocellulose paper by using the Bio-Rad Trans-Blot Cell; sponges were used in the transfer cassette assembly. Optimal protein concentrations ranged from 15 to 20 μg for crystals and 0.2 to 0.5 mg (dry weight) for crystal-spore samples. The buffer, which contained 25 mM Tris-hydrochloride (pH 8.3), 192 mM glycine, and 20% (vol/vol) methanol, was precooled to 4 to 6°C, the temperature at which the cell was kept during the 3-h transfer at 70 V. Total protein was detected by means of the Amido Black stain (24). Bands containing antigenic determinants of the 43- or 63-kDa proteins were visualized by exposing the nitrocellulose paper to the appropriate antiserum and subsequently detecting bound antiserum with the Bio-Rad Immun-Blot Assay Kit, which contains an anti-rabbit immunoglobulin G conjugated to horseradish peroxidase as well as a color development solution. The A-43 and A-63 antisera were used at dilutions of 1/200 and 1/400, respectively, and the anti-rabbit immunoglobulin G was used at a dilution of 1/3,000. In the immunoblot assay, the buffer contained 20 mM Tris-hydrochloride (pH 7.5), 500 mM NaCl, and 0.05% (vol/vol) Tween-20, except during the final color developing stage when the Tween-20 was omitted. The stained nitrocellulose paper was immediately photographed with Kodak Technical pan film 2415 (Eastman-Kodak Co., Rochester, N.Y.), which was developed and printed for maximal contrast.

Amino acid composition and N-terminal sequence determination. Amino acid composition and N-terminal sequence were determined in the Protein Structure Laboratory of the University of California, Davis, School of Medicine. To find the total amino acid composition, three samples with 25 μg of protein were hydrolyzed in 5.7 N HCl at 110°C for 24, 48, and 72 h. The concentrations of serine and threonine were determined from these three time points by extrapolating to zero time. Separate samples were used to assay cysteine and methionine by performic acid oxidation. To establish amino acid sequences, samples of 2 mg of protein were subjected to automated Edman degradation. Phenylthiohydantoin-derivatized amino acids were identified and quantitated by both gas-liquid chromatography and by high-pressure liquid chromatography. Qualitative identifications were also made by two-dimensional thin-layer chromatography.

Larval gut protease digestion of crystal protein solubilized by freezing and thawing. A crystal preparation was thawed and centrifuged at 27,000 × *g* for 15 min. A total of 3 mg/ml of the initial 17-mg/ml (dry weight) crystal protein was recovered in soluble form in the supernatant. This protein was digested in a reaction mixture containing 150 μl of the solubilized crystal, 25 μl of a mixture of 1 M *N*-[2-(cyclohexylamino)]ethanesulfonic acid (CHES), 1 M [3-(cyclohexylamino)]-1-propanesulfonic acid (CAPS) (pH 9.5), and 25 μl of the appropriate amount of the larval midgut extract. After an incubation of 30 min at 30°C, the reaction mixture was centrifuged at 12,800 × *g* for 15 min at 4°C, and 6-μl samples were subjected to SDS-PAGE at an acrylamide concentration of 10% (wt/vol). The electrophoretically separated proteins were transferred to nitrocellulose paper as described in the procedure on electroblotting and subsequently analyzed with A-43 and A-63 by the immunoblot assay.

Extracts containing larval midgut proteases were obtained by dissecting out the midgut of third- and fourth-instar larvae of *Culex pipiens*. The midguts from 25 larvae were placed in 0.5 ml of *Aedes aegypti* saline (9) and homogenized in a 0.1- to 1-ml Radnoti tissue homogenizer (Radnoti Glass Technology, Monrovia, Calif.). The homogenate was centrifuged at 12,800 × *g* for 15 min, and the supernatant was assayed for protease activity by the Azocoll method (19). The activity of the proteases in the extract resulted in an increase of 0.26 OD_{580} per 30 min per 10 μl of the extract.

Products of in vivo digestion of crystals by mosquito larvae. Eight identical toxin assays were set up with each cup containing 40 second- and third-instar larvae of *Culex pipiens* in 30 ml of water and 50 μg of purified crystal from

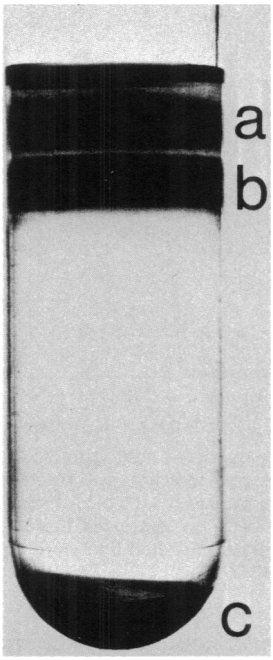

FIG. 1. Centrifugation of dissociated spore-crystal complexes through 48% NaBr. Layers: a, predominantly membrane fragments, some crystals present; b, predominantly crystals, some membrane fragments present; c, spores and some crystals.

strain 2362 per ml. At specific times, the contents of a single cup were poured into a funnel lined with cheesecloth and the larvae were washed with 50 to 60 ml of ice-cold water. The larvae were then homogenized in 0.25 ml of 50 mM CAPS and 50 mM CHES (pH 9.5). After centrifugation at 12,800 × *g* for 15 min, the supernatant (about 0.15 ml) was removed and the pellet was resuspended in 0.2 ml of additional buffer. To determine the crystal proteins present in these two fractions, 80-μl portions were electrophoresed by SDS-PAGE (12% [wt/vol] acrylamide), and then analyzed by the electroblot and immunoblot procedures described above.

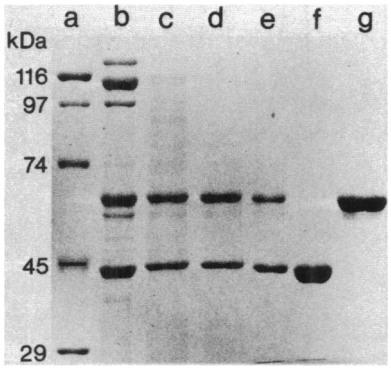

FIG. 2. SDS-PAGE of preparations obtained in the course of purifying the 43- and 63-kDa proteins from the crystals of *B. sphaericus* 2362. Lanes: a, molecular weight standards (solid line indicates the position of the faint band of the 45-kDa standard); b, crystal, 60 μg; c, NaOH-solubilized crystal, 30 μg; d, Sephadex G-200, combined fractions 80 through 120 (Fig. 3), 30 μg; e, DEAE-agarose, combined fractions 114 through 140 (Fig. 4), 30 μg; f, 43-kDa protein, Bio-Rad P-100, combined fractions 112 through 121 (Fig. 5), 50 μg; g, 63-kDa protein, 50 μg.

RESULTS

Purification of the 43- and 63-kDa proteins from *B. sphaericus* 2362. Spore-crystal complexes that were disrupted by passage through a French pressure cell separated into three distinct layers when centrifuged through 48% (wt/vol) NaBr (Fig. 1). Macroscopically and microscopically, the two upper layers (a and b) appeared to be identical to fractions C1 and C2 obtained by Payne and Davidson with NaBr gradients (20). Layer a was composed primarily of membrane fragments, and layer b contained most of the crystal; each was contaminated with small amounts of material from the adjacent band. The majority of the spores were found in layer c (the pellet), usually free of the exosporeum, although many were still loosely associated with crystal.

Layer b provided the starting material for all subsequent protein purifications. An estimate of the number of spores present was obtained from a viable spore count determined after heating a sample of the purified crystal for 10 min at 80°C. Only 4.3×10^5 CFU were detected per mg of sample compared with 1.6×10^9 CFU/mg (dry weight) in the original spore-crystal complex and 1.5×10^9 CFU/mg (dry weight) of the pellet (c).

The protein pattern resulting from SDS-PAGE of intact crystals at 8.5% acrylamide is presented in Fig. 2, lane b, with bands at about 125, 110, 98, 63, 57, and 43 kDa. Although this is a typical representation of the crystal proteins of strain 2362, the relative amounts of the high-molecular-weight proteins varied considerably from preparation to preparation, and an additional band at 37 kDa was observed on occasion in different batches. Similar results were obtained when the crystal preparation was solubilized in 10 mM 2-mercaptoethanol or 10 mM dithiothreitol, with NaOH used to adjust the pH to 9.0, 10.0, and 11.0. When the crystals were solubilized with NaOH at pH 12, all the higher-molecular-weight bands were eliminated and only the 63- and 43-kDa proteins could be detected in substantial quantity (Fig. 2, lane c). Similar results were obtained at pH 12 in the presence of 10 mM 2-mercaptoethanol or 10 mM dithiothreitol.

An outline of the purification scheme for the 43-kDa protein is presented in Table 1. The solubilized preparation was divided and layered onto two Sephadex G-200 columns; the results from one column are shown in Fig. 3. The yellow-orange material in peak A absorbed strongly at 280 nm but did not stain with Coomassie blue or react with Lowry protein reagents. The size of this peak was considerably reduced by freezing the preparation overnight at −20°C and then centrifuging it before loading the supernatant onto Sephadex G-200. Peak B contained primarily the 63- and 43-kDa proteins as well as some additional minor bands (Fig. 2, lane d). The shoulder on the right side of this peak (Fig. 3)

TABLE 1. Purification of the 43-kDa toxic protein from the crystal of *B. sphaericus* 2362

Preparation	Fractions used	Total vol (ml)	Total Lowry protein (mg)
Crystal			403
NaOH-solubilized crystal		23	223
G-200	2(80–120)	254	133
DEAE-agarose	114–140	84	49
P-100 (1st)	114–124	17	8.3
P-100 (2nd)	112–121	16	5.8

appeared to be due to somewhat larger amounts of the 43-kDa protein, as determined by SDS-PAGE. When the toxicity of these crystal components was determined, as much as 1 ml of the peak fraction of A failed to kill 10 mosquito larvae in 10 ml of water within 2 days, whereas 10 μl of the combined fractions from peak B were larvicidal under the same conditions.

The results of DEAE-agarose chromatography of the pooled fractions indicated by the dashed line in Fig. 3 are presented in Fig. 4. Peak A consisted primarily of the 63-kDa protein, whereas peak B contained both the 63- and 43-kDa proteins (Fig. 2, lane e). Selected fractions were combined, concentrated, and placed on a P-100 column; the results are presented in Fig. 5. Both proteins were present in peak A but eluted separately in the two peaks that followed: the fractions in the region of peaks B and C contained mainly the 63-kDa protein and the 43-kDa protein, respectively. The dashed line over peak C indicates the fractions that were pooled, concentrated, and rechromatographed on a P-100 column; the results are shown under peak C (Fig. 5). The 43-kDa protein in the final combined fractions was considered pure, since it was electrophoretically homogeneous in 7, 8.5, 10, and 12% (wt/vol) polyacrylamide gels (shown for 8.5% gels in Fig. 2, lane f).

To obtain the 63-kDa protein, fractions 78 through 90 of peak A resulting from DEAE-agarose chromatography (Fig. 4) were placed onto a P-100 column. A single, major peak in a position identical to that of peak B (Fig. 5) resulted (data not shown). Five fractions from the left side of the peak, including the fraction with the highest OD_{280}, were combined. SDS-PAGE (Fig. 2, lane g) revealed one major band at 63 kDa as well as a minor band with a slightly lower molecular mass. Repeated attempts to separate these two proteins were unsuccessful. The total amount of 63-kDa protein recovered from 403 mg of crystal protein was 4.5 mg.

Both purification protocols were repeated with batches of cells harvested from 24 liters of medium until the necessary amounts of 63- and 43-kDa proteins had been purified. It should be noted that one of the primary goals in the purification of the 43- and 63-kDa proteins was to obtain preparations to be used for immunization. After each procedure, only fractions judged to be the most pure by SDS-PAGE were combined and purified further; this greatly reduced the final yields.

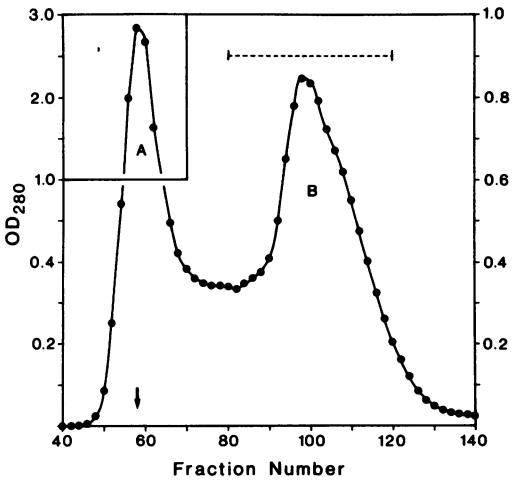

FIG. 3. Sephadex G-200 chromatography of NaOH-solubilized crystals. Fraction volume = 3.2 ml; arrow indicates void volume. Peak A, non-protein OD_{280} absorbing material; peak B, 63- and 43-kDa proteins. Fractions indicated by the dashed line were combined and purified further.

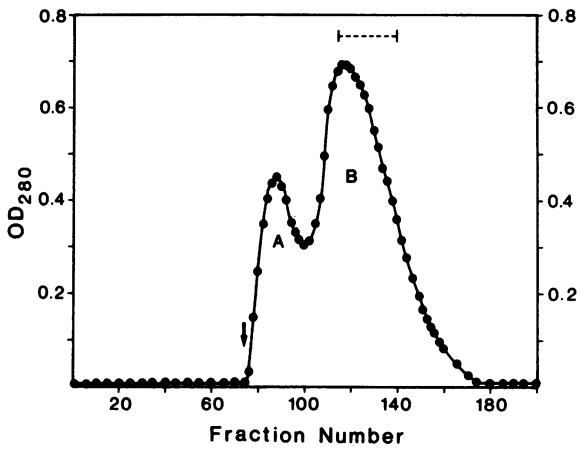

FIG. 4. DEAE-agarose chromatography of the combined fractions from Fig. 3. Arrow indicates the fraction at which the NaCl concentration was increased by 100 mM. Fraction volume = 6.2 ml. Peak A, primarily 63-kDa protein; peak B, 63- and 43-kDa proteins. Fractions indicated by the dashed line were combined and purified further.

Toxicity of the *B. sphaericus* 2362 preparations. The toxic activity of the spore-crystal complex, the purified crystals, and various fractions obtained during the protein purifications are presented in Fig. 6. The data derived from these assays are provided in Table 2. It should be noted that mosquito larvae ingest particulate matter at a higher rate than dissolved material, and it is therefore not possible to compare the LC_{50} values of the crystal and crystal-spore complex with the LC_{50} values for the solubilized material. The high LC_{50} for the NaOH-solubilized crystals may be due

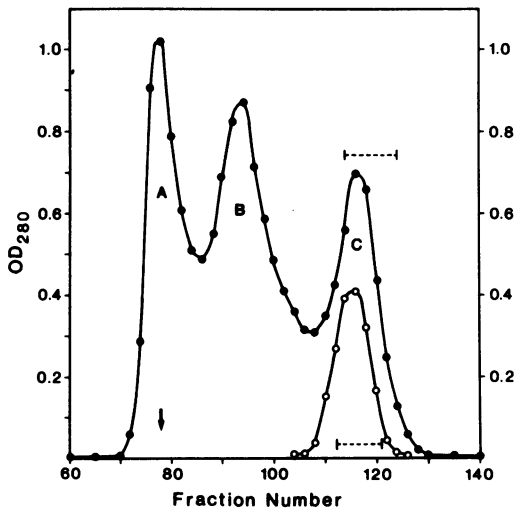

FIG. 5. Bio-Rad P-100 polyacrylamide gel chromatography of the combined fractions from Fig. 4. Fraction volume = 1.6 ml; arrow indicates void volume. Peak A, 63- and 43-kDa proteins; peak B, primarily 63-kDa protein; peak C, primarily 43-kDa protein. The fractions indicated by the dashed line over peak C (closed circles) were combined and rechromatographed on P-100. The results are shown under peak C (open circles). The fractions indicated by the lower dashed line had only the 43-kDa protein.

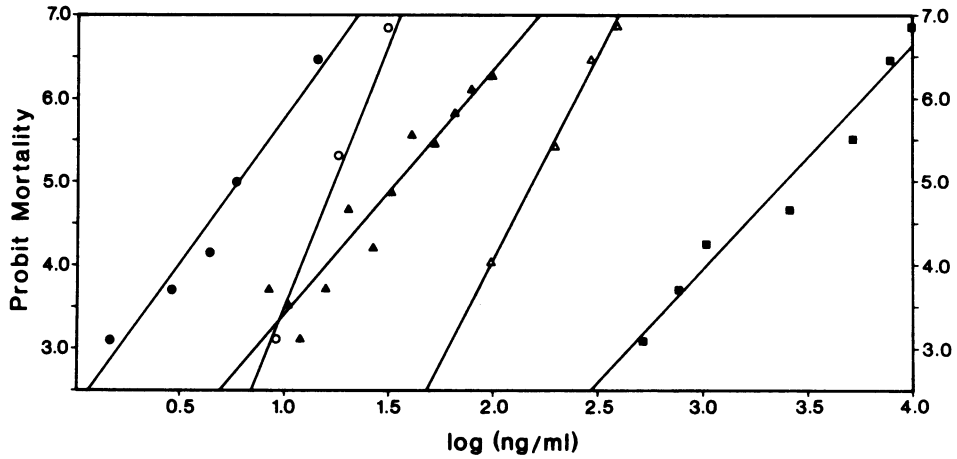

FIG. 6. Effect of different preparations of *B. sphaericus* 2362 on the mortality of second- and third-instar larvae of *Culex pipiens*. Symbols: ●, purified crystal (dry weight); ○, spore-crystal complex (dry weight); ▲, purified 43-kDa toxin from fractions 112 through 120, Fig. 5 (Lowry protein); △, DEAE-agarose, fractions 112 through 140, Fig. 4 (Lowry protein); ■, NaOH-solubilized crystal (Lowry protein).

to interference caused by the material which eluted as peak A of Fig. 3, since a rough estimation from the combined fractions from peak B indicated an approximately 12-fold reduction in the LC_{50}. The 63-kDa protein, tested up to a concentration of 100 μg/ml, had no larvicidal activity.

Amino acid composition and molecular mass determination. The amino acid contents of the purified 43- and 63-kDa proteins are presented in Table 3. The molecular masses of the two proteins, based on SDS-PAGE determinations, were 43.2 ± 1.2 kDa (26 determinations) and 63.0 ± 0.9 kDa (18 determinations).

N-terminal amino acid sequence. The N-terminal sequence of the 43-kDa protein is presented in Fig. 7. A similar sequence determination for the 63-kDa protein could not be performed, since the molecules in this preparation had different N termini. Nevertheless, a comparison of the sequence of the first five residues of the 43-kDa protein with the pattern of release of amino acids during the first five sequencing cycles of the 63-kDa protein suggested that none of the N-terminal sequences of this protein corresponded to that of the 43-kDa protein.

Antiserum specificity. The antigenic specificity of the two antisera was determined by immunoelectrophoresis. A-43 was tested against the purified 63- and 43-kDa proteins and found to produce a single precipitin line with the latter antigen (Fig. 8a and b). A similar experiment with A-63 (Fig. 8c and d) indicated that this antiserum was specific for its homologous antigen. When NaOH-solubilized extract at 30, 60, and 90 μg of protein was tested against A-43 and A-63, single bands were found, indicating that within the limits of detection, these antisera were directed against single proteins (results not shown). It should be noted that under these test conditions the 43-kDa protein migrated toward the anode and the 63-kDa protein migrated toward the cathode.

Another test of the specificity of the antisera was based on their ability to neutralize the larvicidal activity of an NaOH-solubilized crystal preparation. The solubilized crystal (100 μl) was reacted overnight at 4°C with 200 μl of either a 1/2 dilution of A-43 or a 1/4 dilution of A-63. After centrifugation for 10 min at 17,000 × *g*, a series of 10-fold dilutions of the supernatant was prepared. Toxicity remaining in these samples was determined by testing each concentration in single cups containing 10 larvae. The A-43 antiserum completely eliminated the toxicity of the solubilized crystal preparation, whereas A-63 had no significant effect. Identical results were obtained when purified 43-kDa protein was substituted for the solubilized crystal preparation.

Antigenic relationships of crystal proteins. An indication that proteins antigenically related to the 43- and 63-kDa crystal proteins of strain 2362 were present in other *B. sphaericus* strains (1593, 1691, and 2297) was obtained by Ouchterlony immunodiffusion. Sodium hydroxide-solubilized crystals from these strains not only cross-reacted with the A-43 and A-63 antisera, but also produced reactions of

TABLE 2. Larvicidal activities of preparations of *B. sphaericus* 2362[a]

Preparation	Dry wt (ng/ml) of preparation for:		Wt in Lowry protein (ng/ml) for:		LC_{50} 95% confidence limits	
	LC_{50}	LC_{90}[b]	LC_{50}	LC_{90}	Lowest	Highest
Crystal plus spore	17.5	27.9			13.7	22.3
Crystal	8.1	19.1	6.1	14.2	5.2	7.0
NaOH-solubilized crystal			2,500	7,520	2,330	2,670
DEAE-agarose (fractions 114–140)			155	285	138	174
43-kDa toxic protein			35.0	95.3	33.5	36.5
B. thuringiensis var. israelensis crystal	3.5±1.6	20.8±11.6	2.6±1.2	15.5±8.7		

[a] All values for *B. sphaericus* normalized to the mean LC_{50} of *B. thuringiensis* var. *israelensis*.
[b] LC_{90}, Concentration at which 90% of larvae were killed.

TABLE 3. Amino acid composition of the purified 43-kDa and 63-KDa proteins[a]

Amino acid	Mol of acid per 1,000 mol in:	
	43-kDa protein	63-kDa protein
Alanine	45	87
Valine	49	85
Leucine	62	85
Isoleucine	86	63
Proline	62	36
Methionine	4	3
Phenylalanine	54	39
Glycine	68	57
Serine	80	62
Threonine	111	96
Cysteine	7	10
Tyrosine	54	38
Aspartic	114	127
Glutamic	89	84
Lysine	48	86
Arginine	46	32
Histidine	21	10

[a] Aspartic acid and asparagine, glutamic acid and glutamine, cysteine and 1/2 cystine are not differentiated; the tryptophan content was not determined.

identity among themselves (Fig. 9; all possible combinations tested, only one set shown for each antiserum). Solubilized crystals from two other insecticidal bacilli, *B. thuringiensis* var. *israelensis* and *B. thuringiensis* var. *kurstaki*, did not cross-react with either antiserum.

Further similarities, as well as some differences, among the crystal proteins were revealed by separating the proteins by SDS-PAGE and then electroblotting onto nitrocellulose paper and visualizing the proteins with the amido black stain or the immunoblot assay with A-43 or A-63 (see Fig. 10 and 11). Since both antisera were specific, it was possible to establish which protein bands had antigenic determinants of the 43-kDa protein and which were related to the 63-kDa protein. Amido black, which stains all proteins, is not as sensitive as the immunoblot assay and occasionally did not stain bands detected by the antisera.

The results of the electroblots developed with A-43 are presented in Fig. 10. Every one of the strains, as well as each

Asp	Phe	Ile	Asp	Ser	Phe	Ile	Pro	Thr	Glu
1	2	3	4	5	6	7	8	9	10
Gly	Lys	Tyr	Ile	Arg	Val	Met	Asp	Phe	Tyr
11	12	13	14	15	16	17	18	19	20
Asn	Ser	Glu	Tyr	Pro	Phe	Ser	Ile	X	Ala
21	22	23	24	25	26	27	28	29	30
Pro	Ser	Ala	Pro	Asn	Gly	Asp	Ile	Met	Thr
31	32	33	34	35	36	37	38	39	40

FIG. 7. N-terminal amino acid sequence of the 43-kDa protein. X designates a residue which was not identified.

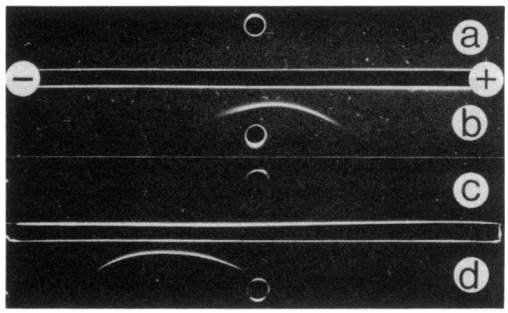

FIG. 8. Immunoelectrophoresis of the 43- and 63-kDa proteins. Lanes: a, 63-kDa protein; b, 43-kDa protein, tested against A-43; c, 43-kDa protein; d, 63-kDa protein, tested against A-63. +, −, Anode and cathode, respectively.

preparation, contained a number of high-molecular-mass proteins, ranging from 98 to 125 kDa, with antigenic determinants related to those of the 43-kDa protein from strain 2362. Another finding was that the crystal preparations of all the strains contained the 43-kDa protein, which has been shown to be toxic for mosquito larvae in strain 2362. The main difference among the strains was the presence or absence of individual bands. As mentioned above, strain 2362 contained a band at 37 kDa which appeared in some crystal preparations but not in others. This band was present in three of the four *B. sphaericus* strains (1593, 1691, and 2362). Other bands detected in only some of the strains were a 72-kDa protein in spore-crystal preparations of strains 1593 and 1691, a 130-kDa protein in occasional preparations of strain 1593, and a few minor bands below 32 kDa in strains 2362 and 2297. Two spore-crystal preparations (strains 1593 and 1691) did not contain a detectable 43-kDa protein. From the amido black stain it appears that the 63-kDa protein was not present either.

An electroblot of crystal proteins of *B. sphaericus* strains developed with A-63 or stained with amido black is presented in Fig. 11. Each crystal contained high-molecular-mass proteins at 98 to 125 kDa, with antigenic determinants of the 63-kDa protein; all of these appeared to be the same molecules as those which reacted with A-43. Strain 1593 occasionally had a band at 130 kDa. In strains 1593 and 2362, the antibody interacted with a protein at about 37 kDa, which might be the same as the 37-kDa band detected with A-43 (Fig. 10).

No reaction was observed when the crystal proteins from *B. thuringiensis* var. *israelensis* and *B. thuringiensis* var. *kurstaki* were developed with A-43 and A-63 (results not

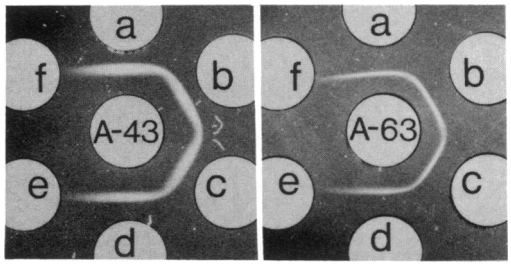

FIG. 9. Ouchterlony immunodiffusion experiments involving NaOH-solubilized crystals from different strains tested against A-43 and A-63. a, *B. sphaericus* 2362; b, *B. sphaericus* 1593; c, *B. sphaericus* 1691; d, *B. sphaericus* 2297; e, *B. thuringiensis* var. *israelensis*; f, *B. thuringiensis* var. *kurstaki*.

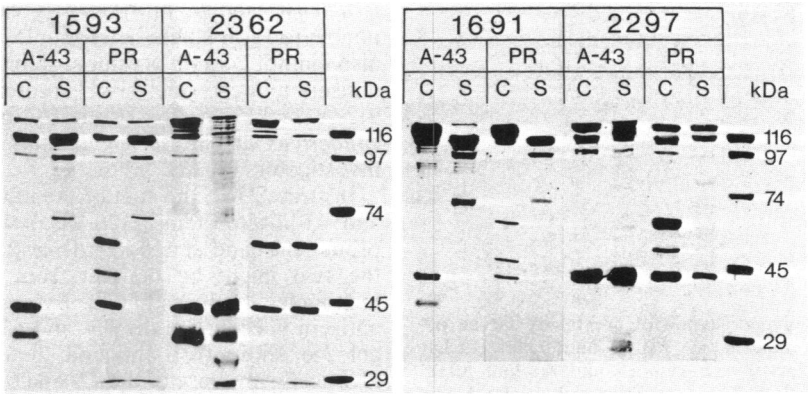

FIG. 10. Proteins detected in an electroblot of gels from SDS-PAGE. A-43, Bands detected by antiserum to the 43-kDa protein; PR, protein stained by amido black; C, crystal; S, spore-crystal complex.

shown). This is consistent with the results of the Ouchterlony immunodiffusion experiments.

In vitro digestion of solubilized crystal by larval midgut proteases. Aliquots of crystal protein solubilized by freezing and thawing (a procedure which leaves intact the high-molecular-weight proteins) were digested with different amounts of an extract containing larval midgut proteases, and the degradation products having antigenic determinants of the 43- and 63-kDa proteins were established (Fig. 12). The bands present before digestion or after incubation for 30 min without protease are shown in lanes A-43a and A-63a. With increasing protease, a gradual decrease in the large band corresponding to the 110- to 125-kDa proteins was accompanied by the appearance of several new bands, between 68 and 88 kDa, with antigenic determinants of both the 43- and 63-kDa proteins. The electroblot developed with A-43 revealed other intermediates, ranging from about 48 to 62 kDa, which were derived from the higher-molecular-mass proteins. Only two additional bands appeared below 43 kDa: one at 40 kDa and a small band at 24 kDa. From this experiment it was not possible to establish whether these degradation products were derived from the 43-kDa protein or some larger molecule. Similarly, for the bands related to the 63-kDa protein, it was not possible to distinguish its derivatives from the digestion products of the higher-molecular-mass proteins. At the highest enzyme concentration (Fig. 12, lane A-63e), no trace of the 63-kDa protein remained; however, there were numerous low-molecular-mass bands present which were probably derived from it.

Products of in vivo digestion of crystals by mosquito larvae. Larvae of *Culex pipiens* were incubated with crystal from strain 2362 for zero time, 15 min, 30 min, 1, 2, 3, or 4 h before they were homogenized and the supernatant and pellet fractions of the larval extracts were analyzed for the presence of antigenic determinants of the 43- and 63-kDa proteins. The results at 15 and 60 min are presented in Fig. 13. The crystal preparation that had been fed to the larvae contained the high-molecular-mass bands and the 43- and 63-kDa proteins, as well as the 37-kDa band occasionally seen in crystals of this strain. As in previous experiments, the latter protein reacted with A-43 and A-63, suggesting a single molecule with some antigenic determinants of each. At time zero (within 20 s of adding the crystal), no bands were detected with either antiserum (data not shown). The only protein occurring in the supernatant at 15 min was a small band corresponding to 40 kDa, which was related to the 43-kDa protein. All but one of the proteins in the pellet had antigenic determinants of both the 43- and 63-kDa proteins. Of these, two corresponded to higher-molecular-mass bands at 110 and 98 kDa, present in the undigested crystal, and two others were lower-molecular-mass intermediates. A band at about 54 kDa only had antigenic determi-

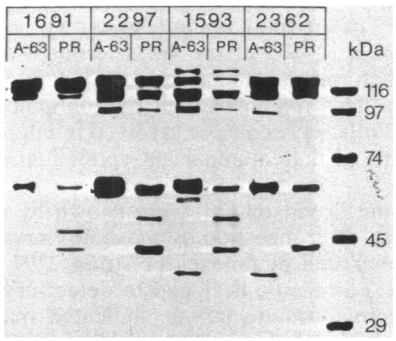

FIG. 11. Crystal proteins detected in an electroblot of gels from SDS-PAGE. A-63, Bands detected by antiserum to the 63-kDa protein; PR, protein stained by amido black. The crystal preparations are the same as used for Fig. 10.

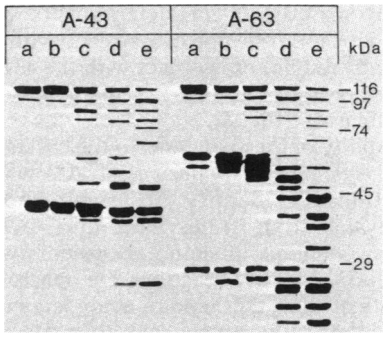

FIG. 12. Electroblot of crystal proteins digested with different amounts of midgut proteases from larvae of *Culex pipiens* and separated by SDS-PAGE. A-43, Proteins detected by antiserum to the 43-kDa protein; A-63, proteins detected by antiserum to the 63-kDa protein; *a*, incubation for 30 min without protease; b through e, incubation for 30 min with protease. Final dilution of the midgut protease preparation; b, 1/1,024; c, 1/256; d, 1/32; e, 1/4.

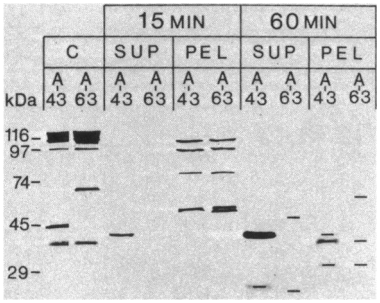

FIG. 13. Products of in vivo digestion of crystal by larvae of *Culex pipiens*. Proteins in supernatant (SUP) and pellet (PEL) fractions of homogenized larval extracts, 15 and 60 min after feeding, were separated by SDS-PAGE and electroblotted. A-43, Bands detected by antiserum to the 43-kDa protein; A-63, bands detected by antiserum to the 63-kDa protein; C, undigested crystal. Bars represent faint bands.

nants of the 63-kDa protein. At 60 min, A-43 revealed a prominent 40-kDa band in the supernatant, along with a small band of 24 kDa. The relative size and intensity of the two were the same at 30 min, 60 min, and 2, 3, and 4 h. At 60 min, only two faint bands related to the 63-kDa protein were observed in the supernatant. In the pellet, three light bands reacted with each antiserum. Of these, two shared antigenic determinants of the 43- and 63-kDa proteins and one corresponded to the 37-kDa protein observed in the crystal preparation.

DISCUSSION

Our selection of the 43- and 63-kDa proteins of *B. sphaericus* 2362 as the focus of this study was based on the observation that these were the only proteins remaining in substantial quantities in larvicidal, NaOH-solubilized crystal preparations. Analysis of the proteins after they had been purified to electrophoretic homogeneity revealed major differences between them. Most importantly, the 43-kDa protein was toxic for larvae of *Culex pipiens*, whereas the 63-kDa protein was not, a finding consistent with the fact that the latter protein was highly susceptible to digestion by larval midgut protease, whereas the former was not (Fig. 12). In addition, their amino acid compositions were very different (Table 3). Especially significant were the lower content of alanine, valine, leucine, and lysine and the higher content of isoleucine and proline in the 43-kDa protein. The absence of any shared antigenic determinants was indicated by the fact that A-43 did not cross-react with the 63-kDa protein and A-63 did not cross-react with the 43-kDa protein (Fig. 8). Furthermore, at pH 7.5 the two proteins had opposite net charges (Fig. 8).

The applicability of these findings to the crystal proteins of other highly larvicidal *B. sphaericus* strains was confirmed by the reactions of identity observed among NaOH-solubilized crystals from strains 2362, 1593, 1691, and 2297 in Ouchterlony immunodiffusion experiments with A-43 as well as with A-63. Identity reactions are generally regarded as an indication that the amino acid sequences of the antigens compared differed by less than 5% (1). This is consistent with the results of Louis et al. (14), who used cloned DNA containing the gene (or part of the gene) for crystal protein of strain 1593 to show that heteroduplexes of high thermal stability were formed between the probe DNA and the DNA from strains 1691, 2297, and 2362, indicating extensive sequence complementarity. *B. thuringiensis* var. *israelensis* and *B. thuringiensis* var. *kurstaki*, which were unable to form stable heteroduplexes with the DNA probe, also did not contain crystal proteins able to cross-react with antisera to the 43- and 63-kDa proteins of *B. sphaericus* (Fig. 9). A lack of cross-reactivity between crystals of *B. sphaericus* and these species has been reported by other investigators (6, 27).

In strain 2362, the sum of the molecular masses of the 63- and 43-kDa proteins (106 kDa) was similar to that of a prominent band at 110 kDa (Fig. 2, lane b), suggesting that the two might be derived from this or another of the high-molecular-mass bands. By analyzing electroblots with antisera specific for the 43- and 63-kDa proteins, we were able to show that antigenic determinants related to or identical with those of the 43- and 63-kDa proteins resided in an array of high-molecular-mass proteins ranging from 98 to 125 kDa. The same was true for strains 1593, 1691, and 2297, except that in strain 1593 the largest molecule in some preparations was 130 kDa. This observation suggests that all of the bands detected in Fig. 10 through 12 were degradation products of the higher-molecular-mass proteins. This is reinforced by the results of the in vitro digestion experiment in which the disappearance of the higher-molecular-mass proteins was paralleled by the appearance of new bands of lower molecular mass (Fig. 12). Further evidence comes from the protein patterns of the spore-crystal complexes of strains 1593 an 1691, which contained the high-molecular-mass proteins but no detectable 43- and 63-kDa bands; both of the lower-molecular-mass proteins were present in purified crystal preparations (Fig. 10). Degradation during purification is the most likely explanation for this observation, since spore-crystal complexes were subjected to far less manipulation than were the purified crystals. We have not yet systematically examined the effect of various protease inhibitors on crystal from sporulating cells. In the case of strain 2362, the omission of EDTA from the washes had no effect on the pattern of protein bands in SDS-PAGE.

Since all the evidence suggests that the 43- and 63-kDa proteins are degradation products of a larger molecule, it is of interest to know which of the two is at the N terminus. Two facts pertinent to this question were established during the amino acid sequence determinations: the 43-kDa protein had a single N terminus, and the 63-kDa protein had multiple N termini. These findings suggest that the 43-kDa protein is at the N terminus and that peptide bonds between it and the C-terminal 63-kDa protein were cleaved by alkali.

A striking attribute of the N-terminal portion of the 43-kDa protein that was sequenced (Fig. 7) is the presence of a large number of hydrophobic amino acids. Alanine, valine, isoleucine, proline, methionine, and phenylalanine, which make up 30% of the total amino acid content of the 43-kDa protein, represent 45% of the 40 N-terminal amino acids, indicating that this peptide is enriched with hydrophobic amino acids. This region of high hydrophobicity may, by analogy with other systems, be involved in interactions with the membrane of the columnar gut epithelium of the larval midgut (10).

Yousten and Davidson (29) have shown by electron microscopy that, after ingestion by mosquito larvae, the crystals in spore-crystal complexes of strain 2297 rapidly dissolve. Our experiments, designed to detect crystal-derived proteins within mosquito larvae, indicated that the major protein which accumulated and persisted had a molecular mass of 40 kDa (Fig. 13); a minor band at 24 kDa was also evident. In contrast, the high-molecular-mass proteins and antigenic determinants of the 63-kDa protein rapidly disap-

peared. In vitro, where larval midgut proteases were used to digest solubilized crystal proteins of strain 2362, the fate of the crystal proteins was similar. Accumulation of a 40-kDa protein as well as a 24-kDa band appeared to accompany the gradual reduction of the 43-kDa toxin. Since digestion of the crystal proteins was far slower in vitro, we could follow the disappearance of the 63-kDa and high-molecular-mass proteins and the appearance of multiple, lower-molecular-mass bands. It was apparent that the 63-kDa protein was more rapidly digested than were the high-molecular-mass bands.

The persistence in vivo of a 40-kDa molecule related to the toxic 43-kDa protein and the evidence that the 43- and 63-kDa proteins are derived from one or more larger molecule(s) suggest the existence of a high-molecular-mass protoxin which is proteolytically cleaved in the larval gut to an active 40-kDa toxin. Precedents for this are the toxins from *B. thuringiensis* var. *israelensis* and *B. thuringiensis* var. *kurstaki*, which have been shown to be derived from protoxins (4, 22). We are currently investigating this possibility in *B. sphaericus*.

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LITERATURE CITED

1. **Baumann, P., L. Baumann, M. J. Woolkalis, and S. S. Bang.** 1983. Evolutionary relationships in *Vibrio* and *Photobacterium*: a basis for a natural classification. Annu. Rev. Microbiol. **37**:369–398.
2. **Bourgouin, C., J.-F. Charles, A. R. Kalfon, and H. de Barjac.** 1984. *Bacillus sphaericus* 2297. Purification and biogenesis of parasporal inclusions, toxic for mosquito larvae, p. 389–390. *In* J. E. Alouf, F. J. Fehrenbach, J. F. Freer, and J. Jeljaszewicz (ed.), Bacterial protein toxins. Academic Press, Inc. (London), Ltd., London.
3. **Bourgouin, C., R. Tinelli, J.-P. Bouvet, and R. Pires.** 1984. *Bacillus sphaericus* 1593-4. Purification of fractions toxic for mosquito larvae, p. 387–388. *In* J. E. Alouf, F. J. Fehrenbach, J. F. Freer, and J. Jeljaszewicz (ed.), Bacterial protein toxins. Academic Press, Inc. (London), Ltd., London.
4. **Bulla, L. A., K. J. Kramer, D. J. Cox, B. L. Jones, L. I. Davidson, and G. L. Lookhart.** 1981. Purification and characterization of the entomocidal protoxin of *Bacillus thuringiensis*. J. Biol. Chem. **256**:3000–3004.
5. **Davidson, E. W.** 1982. Purification and properties of soluble cytoplasmic toxin from the mosquito pathogen *Bacillus sphaericus* strain 1593. J. Invertebr. Pathol. **39**:6–9.
6. **Davidson, E. W.** 1983. Alkaline extraction of toxin from spores of the mosquito pathogen, *Bacillus sphaericus* strain 1593. Can. J. Microbiol. **20**:271–275.
7. **Davidson, E. W., and P. Myers.** 1981. Parasporal inclusions in *Bacillus sphaericus*. FEMS Microbiol. Lett. **10**:261–265.
8. **Fairbanks, G., T. L. Steck, and D. F. H. Wallach.** 1971. Electrophoretic analysis of the major peptides of the human erythrocyte membrane. Biochemistry **10**:2606–2617.
9. **Hagedorn, H. H., S. Turner, E. A. Hagedorn, D. Pontecorvo, P. Greenbaum, D. Pfeiffer, G. Wheelock, and J. R. Flannagan.** 1977. Postemergence growth of the ovarian follicles of *Aedes aegypti*. Insect. Physiol. **23**:203–206.
10. **Houslay, M. D., and K. K. Stanley.** 1982. Dynamics of biological membranes, p. 92–151. John Wiley and Sons, Chichester, United Kingdom.
11. **Kalfon, A., J.-F. Charles, C. Bourgouin, and H. de Barjac.** 1984. Sporulation of *Bacillus sphaericus* 2297: an electron microscope study of crystal-like inclusion biogenesis and toxicity to mosquito larvae. J. Gen. Microbiol. **130**:893–900.
12. **Kalfon, A., I. Larget-Thiéry, J.-F. Charles, and H. de Barjac.** 1983. Growth, sporulation and larvicidal activity of *Bacillus sphaericus*. Eur. J. Appl. Microbiol. Biotechnol. **18**:168–173.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Louis, J., K. Jayaraman, and J. Szulmajster.** 1984. Biocide gene(s) and biocidal activity in different strains of *Bacillus sphaericus*. Expression of the gene(s) in *E. coli* maxicells. Mol. Gen. Genet. **195**:23–28.
15. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
16. **Matsumura, F.** 1975. Toxicology of insecticides, p. 20–22. Plenum Publishing Corp., New York.
17. **Munoz, J.** 1971. Double diffusion in plates. Methods Immunol. Immunochem. **3**:146–160.
18. **Myers, P., A. A. Yousten, and E. W. Davidson.** 1979. Comparative studies of the mosquito-larval toxin of *Bacillus sphaericus* SSII-1 and 1593. Can. J. Microbiol. **25**:1227–1231.
19. **Oakley, C. L., G. H. Warrack, and W. E. van Reymingen.** 1946. The collagenase (K-toxin) of *Cl. welchii* type A. J. Pathol. Bacteriol. **58**:229–235.
20. **Payne, J. M., and E. W. Davidson.** 1984. Insecticidal activity of the crystalline parasporal inclusions and other components of *Bacillus sphaericus* 1593 spore complex. J. Invertebr. Pathol. **43**:383–388.
21. **Sharpe, E. S., K. W. Nickerson, L. A. Bulla, Jr., and J. N. Aronson.** 1975. Separation of spores and parasporal crystals of *Bacillus thuringiensis* in gradients of certain X-ray contrasting agents. Appl. Microbiol. **30**:1052–1053.
22. **Thomas, W. E., and D. J. Ellar.** 1983. *Bacillus thuringiensis* var. *israelensis* crystal δ-endotoxin: effects on insect and mammalian cells *in vitro* and *in vivo*. J. Cell Sci. **60**:181–197.
23. **Tinelli, R., and C. Bourgouin.** 1982. Larvicidal toxin from *Bacillus sphaericus* spores. FEBS Lett. **142**:155–158.
24. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**:4350–4354.
25. **Ward, E. S., D. J. Ellar, and J. A. Todd.** 1984. Cloning and expression in *Escherichia coli* of the insecticidal δ-endotoxin gene of *Bacillus thuringiensis* var. *israelensis*. FEBS Lett. **175**:377–382.
26. **Williams, C. A.** 1971. Immunoelectrophoretic analysis. Methods Immunol. Immunochem. **3**:234–294.
27. **Yousten, A. A.** 1984. *Bacillus sphaericus*: microbiological factors related to its potential as a mosquito larvicide. Adv. Biotechnol. Processes **3**:315–343.
28. **Yousten, A. A.** 1984. Bacteriophage typing of mosquito pathogenic strains of *Bacillus sphaericus*. J. Invertebr. Pathol. **43**:124–125.
29. **Yousten, A. A., and E. W. Davidson.** 1982. Ultrastructural analysis of spores and parasporal crystals formed by *Bacillus sphaericus* 2297. Appl. Environ. Microbiol. **44**:1449–1455.