

## Purification and Characterization of the Entomocidal Protoxin of *Bacillus thuringiensis*\*

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A procedure for purifying the insecticidal parasporal protoxin of *Bacillus thuringiensis* and a description of its biochemical and biophysical properties is provided. Mild alkali titration was necessary to generate a functional protoxin in a soluble form, and anion-exchange chromatography was used to remove contaminating cytoplasmic proteases that are nonspecifically bound to whole native parasporal crystals. Polyacrylamide gel electrophoresis, gel filtration chromatography, and meniscus depletion sedimentation equilibrium analysis revealed an apparent molecular weight for the protoxin of  $1.34 \times 10^5$ . The only  $\text{NH}_2$ -terminal residue found was methionine. The soluble protoxin was 2.5 times more toxic to insect larvae than was the parasporal crystal. At alkaline pH the protoxin slowly converted to a low molecular weight toxin (apparent  $M_r = 6.8 \times 10^4$ ). The molar specific toxicities of the protoxin and toxin were identical.

### MATERIALS AND METHODS<sup>1</sup>

#### RESULTS

**Isoelectric Point and Effect of pH and Time on Crystal Solubilization**—Crystals, isolated by buoyant density centrifugation in Renografin gradients (22) and washed thoroughly in distilled  $\text{H}_2\text{O}$ , were titrated with 1 N NaOH until solubilization was observable by microscopic examination and by an increase in absorbance at 280 nm of the aqueous suspension. For a 0.4% suspension (w/v), complete dissolution occurred when the sodium hydroxide concentration reached 13.5 mM and the pH was about 12. Approximately 4  $\mu\text{mol}$  of alkali were required to dissolve 1 mg of crystal, equivalent to 400 mol of hydroxide ion/mol of subunit (apparent  $M_r = 1.34 \times 10^5$ ). This value was in good agreement with the potential number of ionizable side chains in the crystal (1). At pH 10, 11, and 12, the solubility limits were 0.2, 1.4, and 4 mg/ml, respectively. Apparently, only the highly charged anionic subunit of the crystal was completely soluble. These results are consistent with a crystal subunit that has an isoelectric point near physiological pH. A pI of  $7.2 \pm 0.1$  was determined by isoelectric focusing on polyacrylamide.

Treatment of crystals with excess alkali reduced the yield of soluble protoxin. For example, no biologically active protein was recovered from crystals treated with 1 N NaOH for 20 h. Time of incubation also had an effect. Dissolution was complete in 13.5 mM NaOH after 3 h; however, about 40% of the subunit remained in an aggregated state as revealed by chromatography on DEAE-Bio-Gel A (Fig. 1A, arrow denotes dissociated monomer). Only after 4–5 h was the monomer almost completely dissociated (fraction 48, Fig. 1B). When incubated for periods greater than 24 h at pH 12, the subunit yield decreased such that after 168 h, only 30% of the subunit remained (Fig. 1C). Biological activity of the alkali-treated crystals correlated well with the yield results obtained by ion-exchange chromatography.

**Physical Characterization**—The homogeneity of the protoxin, solubilized for 4 h in alkali, was further analyzed by gel filtration on Sepharose CL-4B at pH 8.4. A nearly symmetrical peak was obtained with apparent molecular weight of  $1.34 \pm 0.20 \times 10^5$  (chromatogram not shown). When this preparation

*Bacillus thuringiensis* is a gram-positive, aerobic, spore-forming bacterium that synthesizes an intracellular parasporal glycoprotein crystal during the sporulation cycle (1, 2). The crystal, which represents 20–30% of the cell dry weight, is toxic to lepidopteran insects (3). In addition to its insecticidal properties, this inclusion body has been reported to cause tumor regression (4, 5) and to enhance the overall immune response in rats (6).

The glycoprotein is a protoxin that is activated after ingestion by an insect susceptible to the toxic product (3). Although information is available about the physical and chemical properties of the crystal (1, 7–17), little is known about its *in vivo* function in the bacterium or about the mechanism of toxicological action (18–21). The primary reason for this lack of understanding is that a reproducible method has not been available for purifying the crystal and rendering the protoxic subunit soluble in a relatively stable form. Here we report a procedure for purifying the protoxin as well as toxin and describe some of their chemical and biological properties.

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<sup>1</sup> Portions of this paper (including "Materials and Methods," Figs. 1–5, Tables I and II, and additional references) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 80 M-2085, cite authors, and include a check for \$5.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

was examined by ultracentrifugation under non-denaturing conditions, a time-dependent fringe displacement occurred that indicated that solute was changing irreversibly during the run. Therefore, a sample of the protoxin was reduced and S-carboxymethylated in 6 M guanidinium chloride. At sedimentation equilibrium under denaturing conditions, the modified protoxin gave a molecular weight of  $1.34 \times 10^5$  (Fig. 2). The data plot was essentially linear over the entire solution column. The suggestion of downward curvature at the highest concentrations was to be expected for a homogeneous solute in concentrated guanidinium chloride because, in this solvent, protein solutions show considerable thermodynamic non-ideality (23).

The molecular weight of the alkylated protoxin also was determined by guanidinium chloride gel filtration (24). Eighty-five % of the protein applied to the column eluted at an elution volume which corresponded to that of a protein whose molecular weight is  $1.3 \pm 0.2 \times 10^5$ . Appropriate fractions of the material were combined, dialyzed, lyophilized, and electrophoresed on a sodium dodecyl sulfate polyacrylamide gel. A single band was observed at a molecular weight of  $1.34 \pm 0.20 \times 10^5$  (Fig. 3A, band 1).

This electrophoretic result was in contrast to that obtained when the parasporal crystal was not alkali-solubilized, alkylated, and subjected to guanidinium chloride gel filtration. When the crystal was dissolved in denaturing and reducing agents as described previously (1) and directly electrophoresed on polyacrylamide (Fig. 3B), a major band (band 1) at an apparent molecular weight of  $1.34 \times 10^5$  was observed, together with several minor bands (bands 2, 3, and 4). All but one of the proteins (band 4) were larger in molecular size than the major protein (band 1). The heavier components (bands 2 and 3) apparently arose from molecular association of the glycoprotein because their electrophoretic mobilities corresponded to those expected of oligomeric forms of the  $1.35 \times 10^5$ - $M_r$  subunit.

**Proteolytic and Insecticidal Activities**—Previously, we reported that casein hydrolytic activity was associated with alkali-solubilized crystal (1). It seemed reasonable that because extracts of sporulating bacilli have particularly high proteolytic activity (25), the proteolysis associated with the protoxin might be due to contamination from either extracellular or intracellular proteases. This probably was the case since all of the casein hydrolytic activity was removed from the alkali-solubilized monomer by chromatography on DEAE-Bio-Gel A (Fig. 4). The crude extracts had 15–30 times greater protease-specific activity than the DEAE-Bio-Gel A-purified subunit. The purified subunit still was fully active biologically. The estimated 50 per cent lethal concentration values (based on measurable protein) for the DEAE-Bio-Gel A-purified material, tested against the tobacco hornworm, was approximately 2.5 times more toxic than the native crystal (Table I). This result indicated that casein proteolytic activity was not essential for toxicity as had been suggested previously (1).

After casein hydrolytic activity had been removed, the subunit was still somewhat unstable. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of the purified subunit that had been held at 28°C and pH 8.4 for 72 h (Fig. 5A) showed not only the  $M_r = 1.34 \times 10^5$  subunit (band 1), but it also revealed a lower  $M_r$  component (apparent  $M_r = 6-7 \times 10^4$ ; band 2) which co-migrated with the tracking dye. The latter protein, referred to as toxin, was isolated by anion-exchange chromatography and possessed virtually the same toxicity on a molar basis ( $1.6 \times 10^{-14}$  mol/cm<sup>2</sup>) as the protoxin ( $2.0 \times 10^{-14}$  mol/cm<sup>2</sup>) toward the tobacco hornworm (Table I). Fig. 5B shows a sodium dodecyl sulfate polyacrylamide gradient gel of the toxin purified by DEAE-chromatography.

A single band was observed whose mobility corresponded to that of a protein with an apparent molecular weight of  $6.8 \times 10^4$ .

**Chemical Composition**—The amino acid compositions of the parasporal crystal and protoxin are presented in Table II. For comparison the compositions were normalized to a molecular weight of  $6.8 \times 10^4$ . The parasporal crystal and protoxin had similar amino acid compositions. The minor differences probably were due to contaminating proteases associated with the crystal. Both compositions were characterized by large amounts of aspartic acid or asparagine, glutamic acid or glutamine, and arginine. Analysis of the NH<sub>2</sub>-terminal residue by Edman degradation using thin layer and high performance liquid chromatography revealed methionine as the only detectable amino acid in both the solubilized subunit and native crystal. No hexose analysis was conducted on the solubilized subunit, but we assume on the basis of identical physical behavior, that the carbohydrate composition is the same for both (3.8% glucose and 1.8% mannose; Ref. 1). Solubilized protoxin produced a positive anthrone reaction. Furthermore when 1 mCi of D-[2-<sup>3</sup>H]mannose was added to a sporulating culture of *B. thuringiensis* and the crystals were subsequently isolated and solubilized by alkali titration (see "Materials and Methods"), the solubilized subunit was radioactive, indicating glycosylation had occurred, probably via mannose-1-PO<sub>4</sub>. No sialic acid derivatives or amino sugars were detected using thiobarbituric acid and ion-exchange chromatography, respectively.

The toxin had a very different amino acid composition when compared to the protoxin and, therefore, is truly unique (Table II). Major differences were found in the amounts of tryptophan, aspartic acid, serine, alanine, valine, and methionine. Periodic acid-Schiff staining of the toxin purified by polyacrylamide gel electrophoresis revealed that it also is glycosylated.

#### DISCUSSION

We have found that the solubility and toxicity of the parasporal crystal of *B. thuringiensis* varies with both pH and time. Nishiitsutsuji-Uwo *et al.* (26) obtained similar results with regard to pH using another strain (subspecies *aizawai*) of the bacterium. Maximum solubility of the *kurstaki* crystal occurred about 5 h after the crystal was titrated with 400 eq of base. The subunit was stable for several hours thereafter, but then began to degrade into smaller fragments with a concomitant loss in insecticidal activity. Reaggregation also occurred, especially after the pH was lowered to near neutrality. These findings are all consistent with the experimentally determined pI of the protoxin, pH 7.2.

Previously, we reported a range of molecular weights for the native subunit from  $0.9-1.3 \times 10^5$ , depending on the method used for solubilization and for size determination. When we examined material solubilized by the mild titration procedure detailed in this report, all molecular weight determinations gave essentially the same result of  $1.34 \times 10^5$ . Also, a single NH<sub>2</sub>-terminal residue was detected in quantitative yield. The crystal protein may be an intact product of translation since methionine was the NH<sub>2</sub>-terminal residue. Preparations that we used previously apparently were contaminated with small amounts of lower molecular weight components. This probably was the result, in part, of a contaminant protease that was not fractionated away from the crystal by the density centrifugation method. Now, by ion-exchange chromatography of the alkali-solubilized crystal, this protease has been excluded. The protease was probably an intracellular protease, generated during sporulation, that bound nonspecifically to the crystal. Previously, Chestukina *et al.* (8) detected

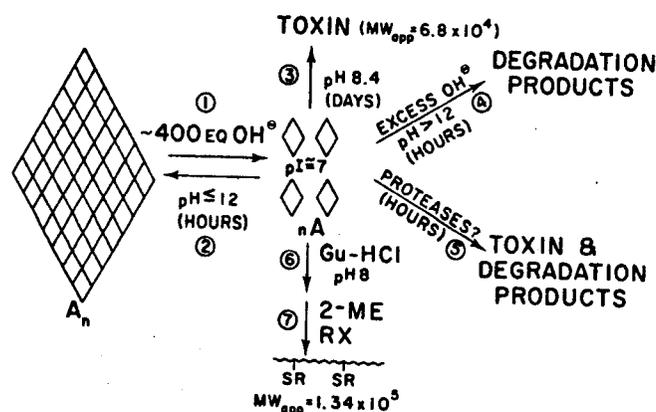


FIG. 6. Schematic diagram of the behavior of parasporal crystal subunit in solution.

several proteases associated with parasporal crystals derived from *B. thuringiensis* subsp. *galleriae* and *infectum*.

We also have isolated a toxic protein (apparent  $M_r = 6.8 \times 10^4$ ) that is generated from protoxin upon prolonged incubation (4–6 days) at slightly alkaline pH. This glycoprotein remained toxic at room temperature for several months at neutral pH. It was the smallest toxic component that we found and any further breakdown was detrimental to toxic activity. When compared to the protoxin, the 68,000- $M_r$  polypeptide had very similar toxicity (50 per cent lethal concentration  $\approx 2 \times 10^{-14}$  mol/cm<sup>2</sup>) and, like the protoxin, was 2.5 times more insecticidal than the native crystal. Apparently, alkali titration of crystal and subsequent incubation of protoxin is an efficient *in vitro* method for toxin production.

Fig. 6 is a schematic diagram that summarizes the behavior of the protoxin and toxin in solution. As depicted, the scheme points out certain precautions that are necessary to preserve both subunit structure and biological activity. The crystal ( $A_n$ ) is made up of many subunits that are dissociated in native conformation ( $nA$ ) by mild alkali titration (reaction 1). Reaggregation occurs slowly (reaction 2), especially at pH  $\leq 12$ ; also, the subunit may break down to smaller fragments (reaction 4). Degradation may be further stimulated by contaminating proteases that co-fractionate with the crystals during isolation. To properly characterize the physical and chemical properties of the subunit, it was necessary to use the material within several hours of preparation. Otherwise, the subunit must be stabilized by placing it in a denaturing solvent and alkylating the disulfide linkages (reactions 6 and 7). The toxin can be generated by prolonged incubation at slightly alkaline pH (reaction 3). Whether this reaction is similar to the *in vivo* mechanism of activation (reaction 5) is not known. We now are investigating further the chemical and physical properties of the toxin and its mechanism(s) of activation.

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Additional references are found on p. 3003.

Supplemental Material  
toPurification and Characterization of the Entomocidal Protoxin of  
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## MATERIALS AND METHODS

**Organism and cultural conditions:** *B. thuringiensis* subsp. *kurstaki* was isolated from Dipel<sup>®</sup>, a commercial bacterial insecticide produced by Abbott Laboratories, North Chicago, IL, and maintained on modified GYS (27) agar slants. Cells for experimental use were cultured in modified liquid GYS medium at 28°C in 2-liter Fernbach flasks and aerated by rotary agitation at 250 rpm.

**Isolation of parasporal crystals:** Liquid cultures of *B. thuringiensis* subsp. *kurstaki* were sporulated in the modified liquid GYS medium. Cultures were held for 3 h after sporulation to allow individual cells to lyse and release spores and parasporal crystals. Spores and crystals were removed from the culture medium by centrifugation (10,000 rpm for 20 min). The crystals subsequently were separated from spores and cellular debris by buoyant density centrifugation in Renografin gradients (22). Crystals isolated in this manner were washed at least three times in water and lyophilized to constant weight. Radiolabeled crystals were obtained in the same manner by growing a sporulating culture (contained in one liter of culture medium) with one mCi of D-[2-<sup>14</sup>C] mannose (2 Ci/mmol; Amersham Corp.).

**Solubilization:** Solubilization was accomplished by titrating a suspension of wet crystals (0.4% wt/vol) with aliquots of 1 N NaOH to pH 12 at 28°C. After various times of incubation, samples were subjected to either bioassay, dialysis, or chromatography as described below.

**Reduction and S-alkylation:** Alkali solubilized parasporal crystals and protein standards were reduced and S-carboxymethylated by method 2<sup>a</sup> of Weber et al. (28) in 6 M guanidinium chloride and 0.1 M Tris-hydrochloride at pH 8.5. 300 mg material was subsequently dialyzed against 0.1 M Tris buffer containing 9 M urea (pH 8.0) for 48 h and against water for 120 h before it was lyophilized.

**Polycrylamide gel electrophoresis:** Electrophoresis in polyacrylamide containing 0.1% SDS was performed by the methods of Weber et al. (28) and Ornstein and Davis (29). Gels were stained with Coomassie Brilliant Blue G-250, 0.25% (wt/vol) and destained by washing in methanol-acetic acid-water (25:7.5:62.5, vol/vol/vol) for 16 to 20 h. Glycoprotein (vicinal hydroxyl groups) was visualized directly on gels by staining with periodate-Schiff reagent (PAS, ref. 30). Before staining, the gels were incubated overnight in a mixture of 25% isopropyl alcohol and 5% acetic acid to fix the proteins and remove SDS. They were further preincubated with 0.5% sodium arsenite and 5% acetic acid. Destaining was accomplished by soaking the gels for 16 h in a solution of 0.1% sodium metabisulfite and 0.01 N HCl. Densitometer tracings of the gels were obtained with a gel scanning attachment of a Gilford 250 spectrophotometer (Gilford Instruments Lab, Inc., Oberlin, Ohio). Gels stained with Coomassie Brilliant Blue were scanned at 550 nm and gels stained with periodate-Schiff reagents were scanned at 560 nm.

**Isoelectric focusing (31)** was accomplished by high resolution gradient gel slab electrophoresis (Pharmacia Gel Electrophoresis Apparatus GE-4) using pH 5 to 9 Ampholine carrier ampholytes (2%, w/v, LKB). Electrofocusing occurred in 10-cm gels at 15°C and 1000 V for 2 1/2 h. Approximately 200 µg of solubilized protein yielded bands that gave optimal staining.

**Protein determination:** Protein concentration was estimated by the Lowry procedure (32) and by absorbance at 280 nm using an extinction coefficient of 1.0 absorbance unit equal to 1.1 mg of protein per ml in a 1 cm cell.

**Amino acid analysis:** Samples were analyzed on a Beckman 120C analyzer after hydrolysis for 24 h in 6 N HCl containing 0.1% phenol. The amounts of labile amino acids were calculated by extrapolation of analyses for 24, 48, and 72 hours to zero time. Cystine and cysteine were determined as cysteic acid and methionine as the sulfone derivative after performic acid oxidation (33). Tryptophan was quantitated in samples hydrolyzed in 3 M p-toluene sulfonic acid containing a small amount of tryptamine. The hydrolysis was carried out for 24 h at 110°C in vacuo (34).

Protein hydrolysates were also analyzed as their D-phthalaldehyde derivatives (35) using a high performance liquid chromatography system consisting of a Varian 5020 pump, a Rheodyne 7120 injector valve, a Waters 8 Bondapak C<sub>18</sub> column (30 cm x 3.9 mm, i.d.), a Turner fluorometer, and a Hewlett-Packard 3385A printer-plotter automation system.

**Carbohydrate analysis:** Total carbohydrates were determined by the anthrone reaction (36). Sialic acid derivatives were measured with thiobarbituric acid after hydrolysis in 0.1 N H<sub>2</sub>SO<sub>4</sub> at 80°C for 60 min (37). Amino sugars were measured in HCl hydrolysates (see amino acid analysis above) by ion-exchange chromatography on a Beckman 120C amino acid analyzer.

**Amino terminal analysis:** The amino terminal residue was determined by automated sequencer analysis (Beckman Model 890C) using the method of Edman and Berg (38) with dimethylbenzylamine (DMBA) buffer and the "general" program published by Hermodson et al. (39). Ten mg of protein (approximately 80 nmoles) was suspended in 500 µl of water, dissolved by adding three drops of concentrated NH<sub>4</sub>OH, placed into the spinning cup, and dried with the Beckman sample application subroutin program. The sample was treated with heptafluorobutyric acid (HFBA) and washed with chloroform prior to sequencing by starting the first sequencer cycle (39) with the HFBA addition step. The protein fragment was somewhat insoluble in DMBA coupling buffer and was therefore subjected to two cycles of coupling prior to cleavage.

Sequencer fractions were converted as previously reported (40) except that no ethanethiol was added to the HCl and the chloroform that was used to extract the 3-phenyl-2-thiohydantoin (PTH)-amino acid from the spinning cup contained dithiothreitol (15 mg/l). The resulting PTH sample was dissolved in 20 µl of methanol, and 0.5 µl was examined on high performance thin-layer chromatographic plates (E. Merck) coated with silica gel using the solvent systems of Bucher (41). The remainder of the sample was diluted to 50 µl with methanol and a 5 µl aliquot was analyzed by gas-liquid chromatography using an SP-400 column as reported previously (40). The remaining PTH material was diluted to 150 µl with methanol and analyzed by high performance liquid chromatography (HPLC).

HPLC analyses were performed with a Varian Model 5020 pump equipped with a 20-µl sample loop on a Rheodyne 7120 injector valve and a Tracor Model 870 UV-VIS variable wavelength detector (wavelength = 257 nm). A DuPont 5-microparticle Zorbax-ODS column (25 cm x 4.6 mm) was used in the reverse phase mode at 60°C. The detector output was plotted and peaks were integrated by a Hewlett-Packard Model 3385A printer-plotter. Sequencer samples were analyzed with two different HPLC solvent systems, one a gradient and one an isocratic method. The gradient method was a modification of that of Brown et al. (42) which involved substituting acetonitrile for methanol to reduce back pressure and to increase mass transfer effects. The column was developed by starting with a 9 min gradient of 95% acetonitrile (A)-5% sodium acetate, 0.1 M, pH 4.5 (B) to 80% A-20% B (flow rate = 2.5 ml/min), operating isocratically (70% A-30% B) for 9 min at 1 ml/min and immediately returning to the initial gradient conditions. This procedure allowed a complete chromatographic analysis every 20 min. The isocratic method used that described by Zimmerman (43).

**Protease assay:** Proteolytic activity was determined by digestion of casein (44). Each reaction was conducted in duplicate and contained 1 ml of 1% (w/v) acid denatured casein prepared in 0.1 M Tris-hydrochloride buffer (pH 8.4) and 0.05 ml of 0.1% alkali-solubilized crystal preparation. The mixture was incubated for 12 h at 37°C and the reaction was terminated by addition of 0.1 ml of 6% trichloroacetic acid. The precipitate was removed by centrifugation. The absorbance of the supernatant was measured at 280 nm with a Cary 118C spectrophotometer. A blank was prepared by adding trichloroacetic acid to the substrate before adding solubilized crystal. One unit of protease activity was defined as the amount of crystal that produced an absorbance increase on 0.01 per h under the assay conditions.

**Insect bioassay:** Toxicity of purified material was determined by the method of Schesser et al. (45), which utilizes neonate larvae of the tobacco hornworm, *Manduca sexta* L. Freshly prepared artificial agar-based diet (46) was poured into disposable tri-pour beakers to the 30-ml level and, after it had congealed, one ml of appropriately diluted material was applied uniformly to the diet surface (16 cm<sup>2</sup>) and allowed to air-dry. The application resulted in surface concentrations ranging from 2.5 ng/cm<sup>2</sup> (0.04 µg/ml) to 62.8 ng/cm<sup>2</sup> (1 µg/ml).

One neonate larva of *M. sexta* was placed on the treated surface of each container. The cups containing the larvae were kept at constant temperature (27°C) and humidity (60%) until the observation period ended. Mortality count was made after 7 days of exposure to the treated surface and the larvae were characterized according to stadium development. Control larvae were incubated in cups containing diet without purified material. All data were subjected to probit analysis (47).

**Molecular weight determination:** The molecular weight of solubilized crystal protein was determined by agarose gel filtration in guanidinium hydrochloride (24), ultracentrifugation (48), and polyacrylamide gel electrophoresis in SDS (28).

Sephacrose CL-4B gel (100 to 200 mesh; Pharmacia) was equilibrated with 6 M guanidinium chloride in 0.05 M 2-(N-morpholino)ethanesulfonic acid (MES) (pH 6.0). The gel slurry was poured to a column bed height of 85 cm in a glass column (Pharmacia, 1.5-cm inner diameter). The agarose bed was further equilibrated by passing two column volumes of the above buffer through the column at a flow rate of 4 ml/h. The void and inclusion volumes were determined using blue dextran 2000 and [<sup>3</sup>H]-labeled diisopropyl phosphorofluoridate, respectively. The elution behavior of S-carboxymethylated *B. thuringiensis* crystal protein was compared under identical conditions with that of protein molecular weight standards (2 mg/0.4 ml) including myosin (Mw=2.2 x 10<sup>5</sup>), beta-galactosidase (1.3 x 10<sup>5</sup>), phosphorylase A (9.4 x 10<sup>4</sup>), and gamma globulin subunit (heavy chain, 5 x 10<sup>4</sup>).

A sample of protoxin dissolved in 6 M guanidinium chloride at a concentration of 0.34 mg/ml was examined by Rayleigh interference optics after sedimentation to equilibrium in a Beckman Model E analytical ultracentrifuge. A double sector cell with sapphire windows was filled so as to give a 3 mm solution column. The rotor temperature was 20°C and the rotor speed was 22,000 rpm; at this speed, the solute was well depleted at the meniscus (48), as indicated by an extensive region of negligible concentration gradient high in the solution column. The fringe pattern did not change detectably between 30 and 45 hours.

The electrophoretic mobilities of the following molecular weight standards were compared with that of protoxin in 5% polyacrylamide gels containing 0.1% SDS buffered in phosphate (pH 7.0): myosin (Mw=2.2 x 10<sup>5</sup>), beta-galactosidase (1.3 x 10<sup>5</sup>), phosphorylase A (9.4 x 10<sup>4</sup>), bovine serum albumin (6.8 x 10<sup>4</sup>), gamma globulin subunit (heavy chain, 5 x 10<sup>4</sup>), ovalbumin (4.5 x 10<sup>4</sup>), and pepsin (3.5 x 10<sup>4</sup>).

**Anion exchange chromatography:** Solubilized crystal was dialyzed against 20 mM NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.5) and subsequently applied to a column of DEAE-Bio-Gel A (1.5 x 40 cm) equilibrated with 20 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.5) and fractions (3 ml) of effluent were collected. After washing the column with 2 column volumes of buffer, a gradient of 0 to 0.4 M NaCl in the phosphate buffer was applied to elute the protoxin subunit.

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[References 1-26 are in the parent paper]

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## RESULTS

Table I. Lethal concentration of *B. thuringiensis* parasporal crystal, alkali-solubilized subunit, and purified toxin for *Manduca sexta*.

Preparation	Lethal concentration	
	ng/cm <sup>2</sup>	10 <sup>-14</sup> mole/cm <sup>2</sup>
Parasporal crystal	7.0 (5.5-8.5) <sup>a</sup>	
Protoxin	2.7 (1.4-3.4)	2.0 (1.0-2.5)
Toxin	1.1 (0.7-1.7)	1.6 (1.0-2.5)

<sup>a</sup>95% confidence limits in parentheses.Table II. Amino acid composition of *B. thuringiensis* native parasporal crystal, alkali solubilized protoxin, and toxin.

Amino acid	Residues per mol wt of 68,000 <sup>a</sup>		
	Parasporal crystal	Protoxin	Toxin
Lysine	16	15	12
Histidine	10	13	8
Arginine	48	46	37
Aspartic acid	77	80	98
Threonine	39	36	38
Serine	47	39	61
Glutamic acid	73	72	69
Proline	21	32	25
Glycine	45	41	49
Alanine	33	32	45
Half-cystine <sup>b</sup>	10	10	8
Valine	42	44	33
Methionine <sup>b</sup>	5	5	8
Isoleucine	34	33	30
Leucine	48	45	48
Tyrosine	25	23	21
Phenylalanine	24	29	31
Tryptophan <sup>c</sup>	7	6	1

<sup>a</sup>For comparison, compositional data is presented based on an apparent molecular weight of 6.8 x 10<sup>4</sup>. Since the molecular weight for the crystal and solubilized subunit is 1.34 x 10<sup>5</sup>, the integer values should be doubled for these proteins. Mean values determined from duplicate or more analyses. Values from individual analyses fall within 10% of the mean.<sup>b</sup>Amino acid composition data for the parasporal crystal were obtained using a Beckman 120C amino acid analyzer. Amino acid compositions of protoxin and toxin were obtained from high performance liquid chromatography after derivatization with o-phthalaldehyde (35) except for threonine, proline, and glycine, which were quantitated by ion exchange chromatography.<sup>c</sup>Determined by hydrolysis in p-toluenesulfonic acid (34).