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Characterization of the Entomocidal Parasporal Crystal of *Bacillus thuringiensis*

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The parasporal crystalline protoxin of *Bacillus thuringiensis* contains a single glycoprotein subunit that has a molecular weight of approximately 1.2×10^5. The carbohydrate consists of glucose (3.8%) and mannose (1.8%). At alkaline pH, the proendotoxin is apparently solubilized and activated by an autolytic mechanism involving an inherent sulfhydryl protease that renders the protoxin insecticidal. Activation generates protons, degraded polypeptides, sulfhydryl group reactivity, proteolytic activity, and insect toxicity. Chemical modification of the sulfhydryl groups inhibits the proteolytic and insecticidal activities, suggesting that cysteine residues may be present in the active site of the protein.

Bacillus thuringiensis produces an intracellular proteinaceous crystal that has insecticidal (3, 21, 23), antitumor (21), and immune response-enhancing properties (20). In our laboratory, we have been concerned with the first of these properties and are trying to determine the biochemical basis of insect toxicity. Crystal preparations of known composition are required for such a study and, as a first step, we have examined the chemical composition and subunit structure of this inclusion body.

The crystal is formed outside the exosporium within the bacterial cell (Fig. 1) during stages 3 to 5 of sporulation and is readily separated from the endospore and other cellular components by density gradient centrifugation (27). Presumably, subunits are synthesized and progressively assembled to produce a crystalline structure that is refractile to light. This product is a protoxin that is activated after ingestion by an insect susceptible to the toxic component(s) (6).

Most of the evidence reported in the literature indicates that several distinct polypeptide components make up the proendotoxin although their exact number and properties are not well defined. The techniques generally used to solubilize the crystal (6) are rather severe because they involve the use of buffers at alkaline pH or thiol reagents. Such treatments can cause degradation and, consequently, lead to erroneous molecular weight values for the parent protoxin. There is also much uncertainty concerning the molecular weight of the toxin. For example, Herbert et al. (10) electrophoretically separated several polypeptide fractions from the crystal of *B. thuringiensis* that had been solubilized at pH 10.5 (7). Major products generated by this treatment had apparent molecular weights of 5.5×10^4 and 1.2×10^5. These investigators attributed toxicity to the smaller major component but they did not report functionality in any other fractions. By using 1 N NaOH to solubilize the crystal, Prasad and Shethna (21) isolated an insecticidal and antitumor protein with a molecular weight of 1.3×10^4. Sayles et al. (25) employed 8 M urea (pH 6.0), 0.5% dithiothreitol, and gel filtration chromatography to dissociate the crystal into several polypeptides with molecular weights of approximately 10^3. Whether these products were insecticidal was not determined.

It occurred to us that the crystal could very well be composed of a single subunit that is converted to smaller components by the usual conditions of dissolution. To test this hypothesis, we examined the biochemical and biophysical properties of the protoxin and found that it is composed of a single, extremely labile glycoprotein subunit that undergoes autolysis during solubilization and activation. Apparently this degradation is caused by a sulfhydryl protease that is activated when the inclusion body is treated with alkali or thiol reagents.

MATERIALS AND METHODS

Organism and cultural conditions. The strain of *B. thuringiensis* used in this study was HD-1 isolated from a commercial insecticidal formulation called Dipel (Abbott Laboratories, North Chicago, Ill.) and has been identified as *B. thuringiensis* subsp. *kurstaki* by H. deBarjac, Institut Pasteur, Paris, France. This strain is the most toxic for lepidopteran insects. Stock cultures of HD-1 were maintained on modified GYS (18) agar slants. Cells for experimental use were cultured in modified liquid GYS medium at 28°C in 2-liter Erlenmeyer flasks and aerated by rotary agitation at 250 rpm.

Isolation, purification, and solubilization of parasporal crystals. Liquid cultures of strain HD-1 were sporulated in modified GYS medium. The cultures were held 5 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 (27). 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 amending the sporulating cultures with 100 μCi of ^{3}H-labeled amino acid mixture (International Chemical & Nuclear Corp., Waltham, Mass.).

Solubilization was accomplished by incubating the crystals in: sodium dodecyl sulfate (SDS), 1% (wt/vol); beta-mercaptoethanol (ME), 2% (vol/vol); urea, 6 M; NaH_2PO_4, 0.01 N; at pH 7.0 and 28°C for 15 min. Solubilization at pH 8.4 was done with: SDS, 1% (wt/vol); ME, 2% (vol/vol); tris(hydroxymethyl)aminomethane (Tris), 4.96 mM; and glycine, 38.4 mM; at 70°C for 20 min. Solubilization at higher pH was carried out by using solvents containing NaOH. The effects of pH on crystal solubilization were determined by mixing ^{3}H-labeled crystal with 0.1 N NaOH or universal buffer (8) at pH 3, 5.6, 7, 7.6, 9.4, and 11. After 30 min of incubation at 25°C, the samples were centrifuged to remove undissolved crystal, and samples of the supernatant solution were counted in a liquid scintillation spectrometer using a scintillation cocktail composed of 1 liter of toluene, 1 liter of ethylene glycol monomethyl ether (Pierce Chemical, Rockford, Ill.), and 8 g of Omnifluor (New England Nuclear Corp., Boston, Mass.). The inhibitory effect of various compounds on solubilization at pH 11 was tested after ^{3}H-labeled crystal was treated with the respective compounds at pH 8 for 12 h.

Reduction and *S*-alkylation. Parasporal crystals and protein standards were reduced and *S*-carboxymethylated by "method 2" of Weber et al. (32) in 6 M guanidine hydrochloride and 0.1 M Tris-hydrochloride at pH 8.5. The 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. *S*-carboxymethylation of the crystal in the absence of reducing agent and denaturant was done by using 0.2 M [^{3}H]iodoacetate (Amersham-Searle, Des Plaines, Ill.) in 0.1 M Tris buffer (pH 9) for 12 h at 28°C, after which the crystals were washed three times with water to remove unreacted reagent.

Polyacrylamide gel electrophoresis. Electrophoresis in polyacrylamide containing 0.1% SDS was performed by the methods of Weber and Osborn (31) and Ornstein and Davis (19). Gels were stained with Coomassie brilliant blue (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 reagents (1). Before staining, the gels were incubated overnight in a mixture of 25% isopropyl alcohol and 10% acetic acid to fix the proteins and remove SDS. They were then further preconditioned 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 on 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. Protein was extracted from the gels in 2% SDS at 37°C by the method of Weber and Osborn (31).

Protein determination. Protein was determined by the biuret method (14), the Lowry procedure (16), and by absorbance at 280 nm using an extinction coefficient of 1.0 absorbance unit equal to 1 mg of protein per ml.

Amino acid analysis. Samples were analyzed on a Beckman 120C analyzer after hydrolysis for 24, 48, and 72 h in 6 N HCl. The amounts of labile amino acids were determined by extrapolation to zero time. Cystine and cysteine were determined as cysteic acid and methionine as the sulfone after performic acid oxidation (11). Tryptophan was quantitated in samples hydrolyzed in 3 M *p*-toluenesulfonic acid containing a small amount of tryptamine. The hydrolysis was carried out for 24 h at 110°C in vacuo (15).

Carbohydrate analysis. Total carbohydrates were determined by the anthrone reaction (22). Neutral monosaccharides were determined by gas-liquid chromatography (28) after hydrolysis with 2 N H_2SO_4 and passage of the hydrolysate through coupled columns of Dowex-50 and Dowex-1 resins. Sialic acids were measured with thiobarbituric acid after hydrolysis in 0.1 N H_2SO_4 at 80°C for 60 min (30). Amino sugars were measured in HCl hydrolysates (see above) by ion-exchange chromatography on a Beckman 120C amino acid analyzer and by gas-liquid chromatography by the method of Sloneker (28).

Nucleic acid analysis. Trichloroacetic acid (10%) was added to solubilized crystals (1 mg), incubated for 15 min at 28°C, and centrifuged (10,000 rpm for 30 min). The supernatant solution was removed and the precipitate was subjected to trichloroacetic acid at 80°C for 20 min. The mixture was cooled and centrifuged, and the resulting supernatant solution was removed. Supernatant solutions from both cold and hot trichloroacetic acid treatments were analyzed for deoxyribonucleic acid by the diphenylamine reaction (4) and ribonucleic acid by the method of Schneider (26).

Lipid analysis. Both intact crystal and solubilized material precipitated with cold and hot trichloracetic acid as well as the supernatant solutions were evaporated to dryness in vacuo and extracted three times with chloroform-methanol (2:1, vol/vol). The extracts were centrifuged (13,000 rpm) for 15 min at 4°C. The supernatant solutions were then evaporated under vacuum, the residues were extracted three times with petroleum ether, and the extracts were again centrifuged (13,000 rpm) for 15 min at

4°C. The supernatant solution was dried in vacuo, and neutral lipids and phospholipids were assayed by thin-layer chromatography (TLC) in one-dimensional solvent systems: (i) neutral lipids, hexane-diethyl ether-methanol (80:20:1; vol/vol/vol) and (ii) phospholipids, chloroform-methanol-water (65:25:4; vol/vol/vol). Lipids were detected on TLC plates by charring after spraying with 0.6% $K_2Cr_2O_7$ in 55% H_2SO_4. Phospholipids were detected by the molybdenum blue spray of Dittmer and Lester (Applied Science Laboratories, Inc., State College, Pa.).

Protease assay. Proteolytic activity was determined by digestion of casein (2). Each reaction was done in duplicate and contained 1 ml of 1% (wt/vol) acid-denatured casein prepared in 0.1 M Tris-hydrochloride buffer (pH 8.4) and 0.05 ml of 0.1% alkaline-solubilized crystal preparation. The mixture was incubated for 12 h at 37°C, and the reaction was stopped by addition of 2 ml of 8% trichloroacetic acid. The precipitate was removed by centrifugation. The absorbance of the supernatant was measured at 280 nm with a Cary 118 spectrophotometer. A blank was prepared by adding trichloroacetic acid to the substrate before adding solubilized crystal. One unit of protease activity is defined as the amount of crystal that produced an absorbance increase of 0.01 per h under the assay conditions. Inhibition of protease activity was measured after preincubating the solubilized crystal overnight with 5 mM $HgCl_2$, 100 mM iodoacetate, 1 mM diisopropyl phosphorofluoridate (DFP), 0.01 mM L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK), or 10 mM ethylenediaminetetraacetic acid (EDTA).

Molecular weight determination. The molecular weight of solubilized crystal protein was determined by ultracentrifugation (33), agarose gel filtration in guanidine hydrochloride (17), and polyacrylamide gel electrophoresis in SDS (32).

Sepharose CL-4B gel (100 to 200 mesh; Pharmacia) was equilibrated in 6 M guanidine-hydrochloride, 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 by 90 cm). 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 by using blue dextran 2000 and ^{3}H-labeled DFP. The elution behavior of *S*-carboxymethylated *B. thuringiensis* crystal was compared under identical conditions with that of protein molecular weight standards (2 mg/0.4 ml) including myosin (2.2×10^5), beta-galactosidase (1.3×10^5), phosphorylase A (9.4×10^4), and gamma globulin subunit (heavy chain, 5×10^4).

For ultracentrifugation, reduced and *S*-carboxymethylated crystal protein, purified by guanidine hydrochloride gel filtration, was dissolved in 0.05 M MES and 6 M guanidine hydrochloride (0.3 mg/ml; pH 6.0) and dialyzed to equilibrium against the solubilizing buffer. The dialysate was used as reference solution, and density of the buffer was determined pycnometrically. Centrifugation (20,000 rpm) was done at 20°C in a Spinco model E ultracentrifuge equipped with electronic speed control and Rayleigh interference optics. Meniscus depletion sedimentation equilibrium was used to determine the apparent weight-average molecular weight by the method of Yphantis (33). Partial specific volume, $\bar{v}$, was calculated by the amino acid and carbohydrate compositions (5).

For electrophoresis, the mobility of the following molecular weight standards was compared in 3% polyacrylamide gels containing 0.1% SDS buffered in phosphate (pH 7.0): myosin (2.2×10^5), beta-galactosidase (1.3×10^5), phosphorylase A (9.4×10^4), bovine serum albumin (6.8×10^4), gamma globulin subunit (heavy chain, 5×10^4), ovalbumin (4.5×10^4), and pepsin (3.5×10^4).

RESULTS

Compositional analysis. The parasporal crystal of *B. thuringiensis* is composed of approximately 95% protein and 5% carbohydrate. The amino acid and carbohydrate compositions are given in Table 1. The values, expressed as grams of amino acid per 100 g of crystal, closely resemble amino acid analyses reported previously for crystals of other strains of *B. thuringiensis* (6). Glutamic acid and aspartic acid residues are the most abundant. Quantitative analysis of the neutral hexoses by gas-liquid chromatography revealed that glucose (3.8%) and mannose (1.8%) account for all of the carbohydrate present. No amino sugars, lipids, nucleic acids, or sialic acid derivatives were detected. The smallest molecular weight possible for the parasporal glycoprotein, based on single integer values of methionine, tryptophan, and mannose, is 1.3×10^4 and consists of 111 amino acids and four sugars.

In neutralized alkaline-solubilized crystal preparations, an unusual ultraviolet-absorbing chromophore was detected. The absorption spectrum revealed the presence of tryptophan and tyrosine as expected, in addition to the novel ultraviolet-absorbing chromophore that is dialyzable and apparently of low molecular weight. Whether the absorption observed is due to a natural component of the crystal (amounting to less than 1% of the total dry weight) or is an artifact produced by alkaline solubilization is not known.

Molecular weight determination. The molecular weight of the crystalline inclusion was determined by guanidine hydrochloride gel filtration chromatography (17), sedimentation equilibrium in guanidine hydrochloride (33), and SDS-polyacrylamide gel electrophoresis (32). Because of the extreme insolubility of the protoxin, a solvent mixture containing denaturants such as SDS, urea, or guanidine hydrochloride and a reducing agent such as 2-mercaptoethanol was required to render it soluble as described by Bulla, Kramer, Bechtel, and

TABLE 1. *Amino acid and carbohydrate compositions of B. thuringiensis proendotoxin and SDS-polyacrylamide gel electrophoresis components*

Amino acid or sugar	Proendotoxin[a]			Residues (g) of SDS-PAGE components 1, 2, or 3[c] (Fig. 4)
	Residues (g)/100 g of crystal[b]	Mol/minimal mol wt	Mol/subunit	
Aspartic acid	12.4	14.0 (14)	129	11.9
Threonine	5.5	7.1 (7)	65	4.9
Serine	5.9	8.5 (9)	83	6.2
Glutamic acid	13.1	13.2 (13)	120	15.2
Proline	2.8	3.8 (4)	37	3.4
Glycine	3.6	8.2 (8)	74	4.8
Alanine	3.3	6.0 (6)	55	3.3
Half-cystine[d]	1.4	1.8 (2)	18	
Valine	5.8	7.6 (8)	74	5.8
Methionine[d]	0.9	0.9 (1)	9	
Isoleucine	5.4	6.2 (6)	55	5.0
Leucine	7.7	8.6 (9)	83	8.8
Tyrosine	5.7	4.5 (5)	46	
Phenylalanine	5.0	4.4 (4)	37	4.9
Lysine	2.8	2.8 (3)	28	3.6
Histidine	1.9	1.8 (2)	9	1.8
Arginine	10.3	8.6 (9)	83	6.6
Tryptophan[e]	1.7	1.2 (1)	9	
Polypeptide molecular weight		12,400	114,400	
Glucose[f]	3.8	3.0 (3)	28	
Mannose[f]	1.8	1.4 (1)	13	
Mol wt[g]		13,100	121,100	

[a] Mean values determined from six analyses performed in 24-, 48-, and 72-h hydrolysates; nearest nanomole integer values are expressed in parentheses. Values from individual analyses fall within 10% of the mean.

[b] Mean values determined from three analyses each performed in 24-h hydrolysates. Half-cystine, methionine, tyrosine, and tryptophan were not stable to hydrolysis.

[c] Normalized to 3.3 g of alanine for comparison to gram residues of proendotoxin (column 1). Polyacrylamide gel electrophoresis.

[d] Determined as cysteic acid and methionine sulfone after performic acid oxidation (11).

[e] Determined by hydrolysis in *p*-toluenesulfonic acid (15).

[f] Determined by gas-liquid chromatography of acid hydrolysate (23).

[g] Minimal molecular weight value based on nearest integer composition. Subunit molecular weight determined by physical analyses described in Materials and Methods.

Davidson (*Microbiology–1976*, p. 534–539, American Society for Microbiology, Washington, D.C.). When appropriate, the reduced protein was carboxymethylated with iodoacetate to prevent disulfide bond reformation during analysis. Gel filtration of reduced and *S*-carboxymethylated crystal in 4% Sepharose and 6 M guanidine-hydrochloride at pH 6.0 yielded a single peak, suggesting the presence of only one subunit in the protoxin. The elution volume was nearly identical to that of beta-galactosidase whose molecular weight is 1.3×10^5 (Fig. 2).

Meniscus depletion sedimentation equilibrium analysis of reduced and *S*-carboxymethylated protoxin purified by guanidine hydrochloride filtration produced a linear plot of the natural logarithm of the fringe displacement versus the square of the radius of rotation for the entire length of the cell (Fig. 3), indicating homogeneity and the absence of molecular association in 6 M guanidine hydrochloride. The molecular weight of the subunit calculated from the linear plot is 1.01×10^5.

Figure 4 displays results of SDS-polyacrylamide gel electrophoretic analyses of the parasporal crystal. Tubes A and B are 5% gels buffered in Tris-glycine at pH 8.4 (19); tube C is a 3% gel buffered in phosphate at pH 7.0 (32). Gels in tubes A and C are stained with Coomassie brilliant blue; gel B is stained with periodic acid-Schiff reagents. Electrophoresis of the solubilized protoxin produces a major band of glycoprotein in SDS gels (band 1) with an apparent molecular weight of 1.35×10^5, as well as several minor bands, all but one (band 4) of which are larger in molecular size. The migration pattern of the heavier components (bands 2 and

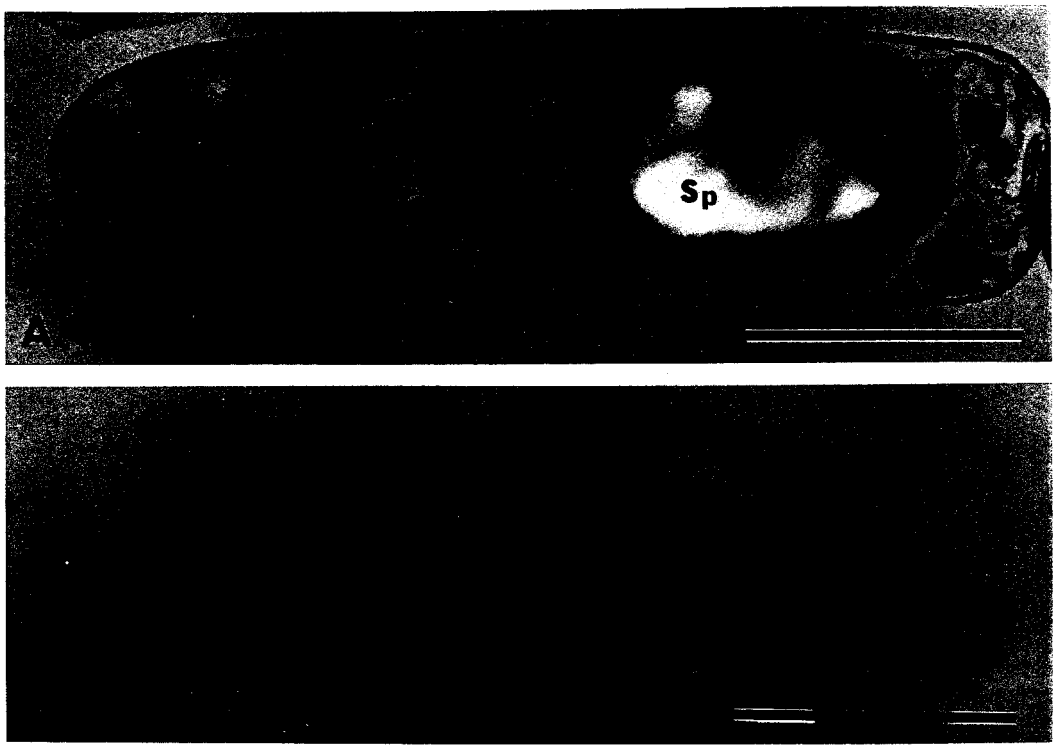

FIG. 1. *Spore and entomocidal parasporal crystal of B. thuringiensis. (A) Fully sporulated cell containing dormant spore (Sp) and crystal (Cr); (B) parasporal crystal showing crystal lattice fringes from end to end. Bar = 1.0 and 0.5 μm for A and B, respectively.*

3) indicates molecular association of the major glycoprotein because their relative mobilities correspond to that of oligomeric forms of the 1.35×10^5-dalton subunit. The estimated molecular weight of material in band 4 varies from 1×10^4 to 7×10^4, depending upon the pH of the solubilizing and running buffers. All of the individual components (bands 1 to 3) separated by SDS-gel electrophoresis displayed an amino acid profile nearly identical to that of the whole crystal (Table 1, columns 1 and 4). This evidence supports the hypothesis that bands 2 and 3 are aggregates of the glycoprotein subunit. The periodic acid-Schiff staining procedure showed that polypeptides 1 and 4 in gel B (Fig. 4) contain vicinal hydroxyl groups characteristic of the neutral hexoses that we determined by gas-liquid chromatography as glucose and mannose. The carbohydrate staining technique was not sensitive enough to determine whether the components in bands 2 and 3 were also glycosylated. Similar results were obtained with the phosphate-buffered gel (not shown).

The average molecular weight of the crystal subunit derived from the three independent analyses used here is 1.2×10^5. The amino acid

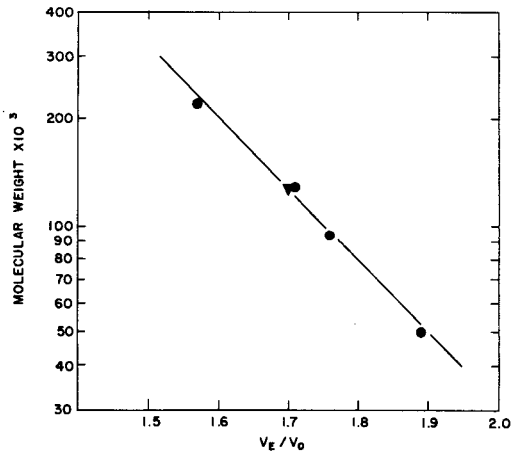

FIG. 2. *Molecular weight of the glycoprotein subunit of the parasporal crystal from B. thuringiensis as determined by 4% Sepharose-guanidine hydrochloride gel filtration. Plot of $\log_{10}$ molecular weight versus elution weight of polypeptide V_e, divided by elution weight of blue dextran, V_0. The standard proteins (from left to right) used and their molecular weights were myosin (2.2×10^5), beta-galactosidase (1.3×10^5), phosphorylase A (9.4×10^4), and gamma-globulin subunit (heavy chain, 5×10^4).*

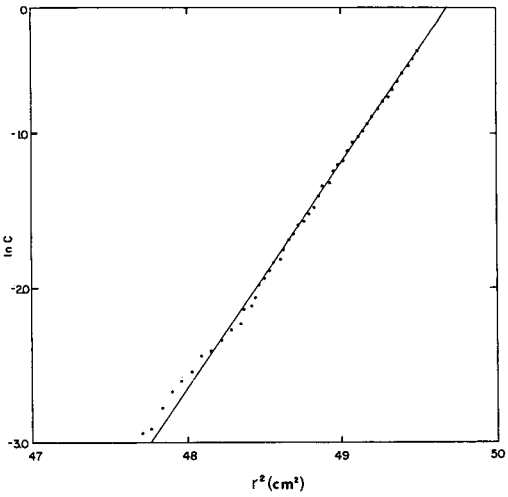

FIG. 3. *Sedimentation equilibrium of S-carboxymethylated parasporal crystal. The natural logarithm of the fringe displacement (InC) is plotted against the square of the radial distance (r^2). Protein concentration was 0.3 mg/ml in 0.1 mM MES, 6 M guanidine hydrochloride (pH 6.0). Centrifugation was at 20°C and 20,000 rpm using a double-sector interference cell.*

and carbohydrate compositions of the subunit are listed in Table 1.

Solubilization. Crystal solubilization is dependent upon hydroxide ion concentration and is accompanied by a release of protons, necessitating the use of a solvent of relatively high buffering capacity or the addition of hydroxide ions to maintain constant pH. Soluble components are generated from the glycoprotein by increasing the pH of the solvent (Fig. 5). Radiolabeled peptides (molecular weight $\leq 7 \times 10^4$) appear in solution after the protoxin is incubated for 30 min at pH values greater than 9, whereas none appears at pH values near neutrality or lower. At pH 11.5, only a trace amount of the major glycoprotein subunit survives after 30 min. Electrophoresis of this preparation shows a rather broad smear at the bottom of a gel (not shown) revealing that substantial degradation occurs. The glycoprotein is also unstable at neutral or slightly alkaline pH during prolonged incubation in the presence of denaturing and reducing agents. Polyacrylamide gel electrophoresis of radioactive protoxin that was incubated at 28°C for several days disclosed the build-up of low-molecular-weight polypeptide material, possibly the result of autolytic activity. As shown in Fig. 6, there is a significant decrease in the relative amount of subunit polypeptides 1 and 2 (corresponding to bands 1 and 2 of gel A, Fig. 4), with a simultaneous increase in the lower-molecular-weight component in band 4.

The mechanism of solubilization was examined by testing the inhibitory potential of a number of compounds. Crystals were treated with the respective compounds in aqueous buffer at pH 8 to 9 for 12 h, washed with water, and then titrated to alkaline pH. Interestingly, the most effective inhibitors (Table 2) were either those that form a complex with thiol groups, e.g., Hg^{2+}, or those that chemically modify them, e.g., iodoacetate. Reagents specific for serine (DFP), histidine (TPCK), or metal ions (EDTA) had no effect. The presence of 50 mM Hg^{2+} prevented solubilization at pH 9.5 and 11.0 and to a lesser degree at pH 13 (Fig. 5). Agents such as iodoacetate and phenylmercuric acetate as well as other heavy metal salts which modify sulfhydryl groups were also quite effective in maintaining the inclusion body insoluble at pH 11. Mercuric ion inhibition can be reversed by washing the treated crystals with thiol compounds such as 2-mercaptoethanol or dithiothreitol prior to treatment with alkali. To determine whether thiol groups are generated when the pH is increased, the extent

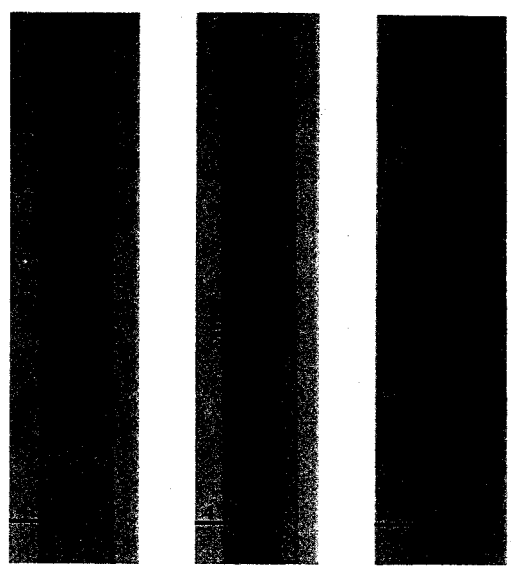

FIG. 4. *Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the B. thuringiensis parasporal crystal. (A) 5% polyacrylamide buffered in Tris-glycine (pH 8.4) and stained with Coomassie brilliant blue, 12 μg of protein applied; (B) same as (A) except stained with periodic acid-Schiff reagents, 100 μg of protein applied; (C) 3% polyacrylamide buffered in phosphate (pH 7.0) and stained with Coomassie brilliant blue, 12 μg of protein applied. Arrows indicate point of migration of tracking dye during electrophoresis.*

of radiolabeling of the crystal with tritiated iodoacetate was determined by liquid scintillation counting. Also, the amount of *S*-carboxymethylated cysteine was measured by ion-exchange chromatography on an amino acid analyzer. Four cysteine residues of the protoxin subunit became reactive to iodoacetate at pH values above 9.

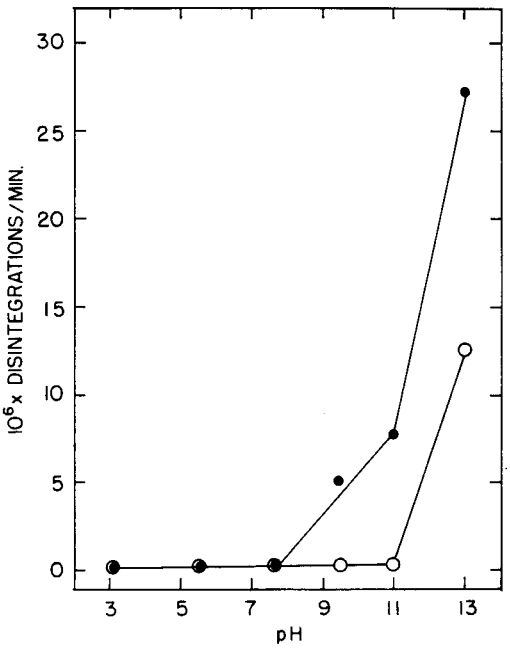

FIG. 5. *Solubility of B. thuringiensis protoxin as a function of pH (●) and effect of 50 mM $HgCl_2$ (○).*

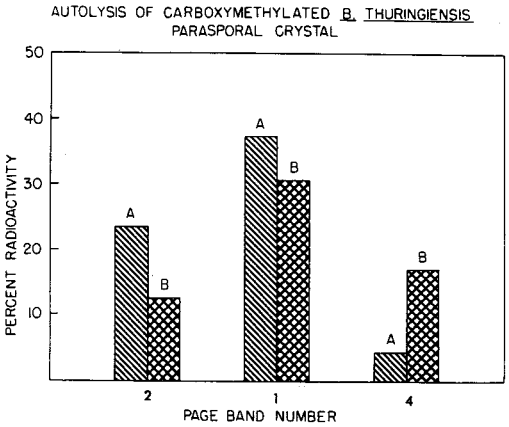

FIG. 6. *Degradation of radioactive B. thuringiensis parasporal crystal in the presence of reducing and denaturing solvent. Crystals were solubilized in 1% SDS and 2% ME at pH 8.4. (A) Amount of radioactivity in polyacrylamide gel electrophoresis (PAGE) bands 1, 2, and 4 (see gel A, Fig. 4) after 20 min of incubation; (B) same as (A) except after 7 days of incubation.*

Proteolytic and insecticidal activity. Because there are thiol groups and degradation products in the solubilized crystal, it occurred to us that the protoxin may be "dissolved" and activated by an autolytic mechanism involving a sulfhydryl protease that renders the protoxin insecticidal. We found that casein is hydrolyzed at pH 9.0 by the solubilized crystal. Five units of protease activity was observed with 1 mg of alkali-treated crystal under our assay conditions. The addition of 5 mM Hg^{2+}, but not 1 mM DFP, 100 μM TPCK, or 10 mM EDTA, completely inhibits casein hydrolysis.

Preliminary results of bioassays with neonate larvae of the tobacco hornworm, *Manduca sexta*, indicate that toxicity resides with alkaline-solubilized protoxin and is associated with polypeptide material, the molecular weight of which has not been established. Alkaline-solubilized crystal is extremely toxic, exhibiting kill of the larvae at concentrations as low as 200 μg/kg of artificial insect diet (Schesser, Kramer, and Bulla, Appl. Environ. Microbiol., in press). Carboxymethylation of solubilized crys-

TABLE 2. *Effect of various compounds on B. thuringiensis parasporal crystal solubility at pH 11.0*

Compound[a]	Concn (mM)	Solubility of crystal (%)
NaOH[b]	1	97
$HgCl_2$	5	60
	50	0
$HgCl_2$; ME or DTT[c]	50	95
ICH_2COOH	50	20
$Cd(OAc)_2$	50	35
$MnCl_2$	50	39
$ZnCl_2$	50	61
$FeCl_2$	50	68
$CaCl_2$	50	73
$CuCl_2$	50	92
CuCl	50	93
$MgCl_2$	50	97
$Pb(OAc)_2$	50	95
$NiCl_2$	50	89
EDTA[d]	10	93
TPCK[e]	0.1	82
DFP[f]	1	94

[a] Pretreated with respective compound at pH 8.5 for 12 h before addition of NaOH.

[b] Titrated with 10 mM NaOH to maintain pH 11.0.

[c] ME, Mercaptoethanol; DTT, Dithiothreitol.

[d] EDTA, Ethylenediaminetetraacetic acid.

[e] TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

[f] DFP, Diisopropyl phosphorofluoridate.

tals renders them nontoxic to *M. sexta* and nonproteolytic.

DISCUSSION

The results of these experiments show that the parasporal crystal of *B. thuringiensis* is composed of a single glycoprotein subunit with a molecular weight of about 1.2×10^5 (average value determined by sedimentation, electrophoretic, and gel chromatographic analyses). On the basis of X-ray diffraction analysis, Holmes and Monro (12) concluded that the asymmetric unit of the proteinaceous inclusion has a molecular weight of 2.3×10^5. Their calculation, along with our results, indicates that the basic crystal asymmetric unit is a dimer of the glycoprotein monomer we identified. Holmes and Monro (12) also detected 0.5% carbohydrate by using a β-naphthol-sulfuric acid chromagenic reagent. The highly sensitive and specific gas-liquid chromatographic technique (28) that we used reveals 10 times more carbohydrate in our crystal preparations. We have found that carbohydrate comprises 5.6% of the total crystal weight and consists of 20 glucose and 10 mannose residues per subunit. The structure of the individual carbohydrate side chains or their specific site(s) of attachment to the protein are not known. Glycoproteins lacking hexosamines generally are attached to the hydroxyl side-chain groups of serine or threonine (13). Mycodextranase secreted by *Penicillium melinii* (24) is the only other glycoprotein reported that contains a heteroglycoside composed exclusively of glucose and mannose. Whether the use of different strains of *B. thuringiensis* in the respective laboratories accounts for the discrepancy in carbohydrate content is not known. We believe, as Somerville (29) surmises, that there is little difference in physical structure, chemical composition, and functional properties of crystals from all the closely related strains because their amino acid compositions and insecticidal activities are virtually the same (6).

The large number of polypeptides with widely ranging molecular weight values (1×10^3 to 7×10^5) that have been reported in the literature (9, 10, 12, 21, 25, 29) for the solubilized crystal may be the result of subunit autolysis brought about by the traditional alkali treatment (pH $\geq$10) or aggregation of the highly associating glycoprotein. Our study reveals that the parasporal crystal has the capacity for autolytic activity, apparently involving a sulfhydryl protease that is activated under alkaline conditions. In addition to proteolysis, other phenomena such as insect toxicity, proton release, and sulfhydryl group reactivity are all generated by alkaline treatment. Reagents that complex or modify sulfhydryl groups inhibit all of these activities. The presence of cysteine residues in the activated protoxin apparently is essential for both toxicity and proteolytic activity.

A common feature of all insects susceptible to the proendotoxin of *B. thuringiensis* is an extremely alkaline midgut pH, often exceeding a value of 10 (6). In vivo activation of the protoxin probably is identical to the in vitro activation described above and the proteolytic activity of the toxin could cause the pathological disorders ascribed to the crystal: (i) separation of gut cells and detachment from the basement membrane; (ii) enhancement of secretory activity of gut epithelial cells; (iii) increase in permeability of the gut wall to sodium ions with a slower rate of glucose uptake into the hemolymph; (iv) elevated level of potassium ion concentration in the hemolymph; and (v) gut paralysis and sometimes general paralysis of the body (6). For example, the protease itself could digest the insect gut tissue and bring about a general breakdown of the epithelial cells with consequent changes in the pattern of molecular uptake, release, and transport of various materials throughout the insect. On the other hand, the quantity of protease activity per milligram of crystal seems rather low and may simply be sufficient for crystal autolysis rather than digestion of insect gut tissue. In view of the results presented here, we believe that previously published biochemical properties and postulated mechanisms (6) for toxicity of the parasporal crystal need to be further reevaluated. Certainly the elucidation of the mechanism of insect pathogenicity is dependent upon a more extensive biochemical characterization of the toxin.

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