

PURIFICATION OF THE INSECTICIDAL TOXIN FROM THE
PARASPORAL CRYSTAL OF BACILLUS THURINGIENSIS SUBSP. KURSTAKI

Lee A. Bulla, Jr., Loren I. Davidson, Karl J. Kramer, and Berne L. Jones

U.S. Grain Marketing Research Laboratory, Agricultural Research, Science
and Education Administration, U.S. Department of Agriculture, Manhattan,
Kansas 66502

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Summary: The insecticidal toxin of Bacillus thuringiensis subsp. kurstaki was isolated from parasporal crystals. The toxin, which is stable for several months, is a glycoprotein with an apparent molecular weight of 68,000 that is generated upon solubilization and activation of a higher molecular weight protoxin ($MW_{app} = 1.3 \times 10^5$) at alkaline pH. The toxin was purified by gel filtration and anion exchange chromatography and its molecular weight was established by gel filtration chromatography and SDS polyacrylamide gel electrophoresis.

Bacillus thuringiensis forms a parasporal crystalline inclusion adjacent to the endospore during post-exponential cellular development (1, 2). The crystal, which comprises 20-30 percent of the cell dry weight, is toxic to lepidopteran insects (3) and is the basis for commercial development of B. thuringiensis as a microbial insecticide (4).

The parasporal crystal is a glycoprotein composed of a repeating subunit whose molecular weight is approximately 1.3×10^5 (5, 6). The glycoprotein is a protoxin that is activated to the toxic product after ingestion by a susceptible insect (7). Although information is available about the physical and chemical properties of the crystal (7), little is known about the biochemical basis of its toxicological action. The main reason for this lack of understanding is that no method has been developed to purify the toxin from the crystal in a soluble and stable form. In this paper, we report for the first time a procedure for purifying the soluble toxin and describe some of its biochemical properties.

MATERIALS AND METHODS

B. thuringiensis subsp. *kurstaki* was isolated from Dipel[®], a commercial bacterial insecticide produced by Abbott Laboratories, North Chicago, IL, and maintained on modified GYS (8) agar slants. Sporulated cells containing parasporal crystals were cultured in modified liquid GYS medium at 28 C in 2.8-liter Fernback flasks and were aerated by rotation at 200 rpm. Cultures were held for 1-3 days 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 10 min) and washed three times in water. The crystals subsequently were separated from spores and cellular debris by buoyant density centrifugation in Renografin gradients (9). Crystals isolated in this manner were washed at least three times in water and lyophilized to constant weight.

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

Electrophoresis in polyacrylamide gels containing 0.1% SDS was performed by the method of Weber et al. (10). Gels were stained with Coomassie brilliant blue (0.25%, wt/vol) and destained by washing in methanol-acetic acid-water (25:7.5:67.5, vol/vol/vol) for 16 to 20 hr. Glycoprotein (vicinal hydroxyl groups) was visualized directly on the gels by staining with periodate-Schiff (PAS) reagent (11). 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 further preconditioned with 0.5% sodium arsenite and 5% acetic acid. Destaining was accomplished by soaking the gels for 16 hr in a solution of 0.1% sodium metabisulfite and 0.01 N HCl. Densitometer tracings of the gels were made 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. Protein was determined by the procedure of Lowry et al. (12) or by absorbance at 280 nm assuming an extinction coefficient of 1.1 absorbance units equal to 1.0 mg of protein per ml.

Amino acid analyses were conducted on samples excised from SDS-polyacrylamide gels. Samples were hydrolyzed *in vacuo* in 6N HCl for 24 hr and were analyzed on a Beckman 120C analyzer.

Toxicity of protein fractions purified by chromatography was determined by the method of Schesser et al. (13), utilizing neonate larvae of the tobacco hornworm, *Manduca sexta*.

The molecular weight of the purified soluble toxin was determined by Sephadex G-100 (Pharmacia) gel filtration and polyacrylamide gel electrophoresis in the presence of SDS (10). The elution of the protein from Sephadex G-100 and the mobility on polyacrylamide gels containing 0.1% SDS buffered in phosphate were compared to those of the following molecular weight standards: beta-galactosidase (1.3×10^5), phosphorylase A (9.4×10^4), bovine serum albumin (6.8×10^4), and gamma globulin subunit (heavy chain, 5×10^4).

RESULTS

The procedure for solubilizing parasporal crystals of *B. thuringiensis* and purifying the insecticidal toxin is outlined in Fig. 1. Specifically,

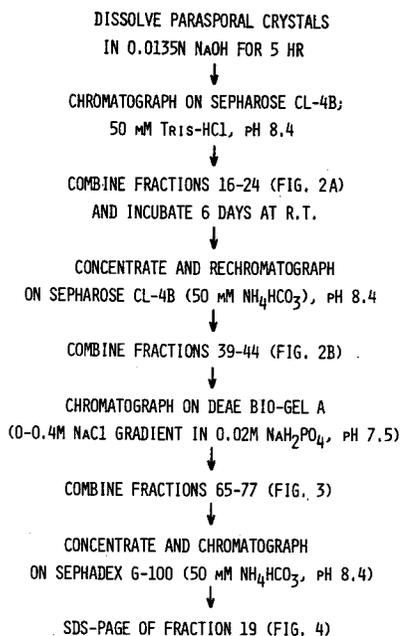


Fig. 1. An outline of the procedure for solubilizing parasporal crystals and purifying the resulting insecticidal toxin.

solubilization was accomplished by titrating a suspension of wet crystals (0.4%, wt/vol) with 400 molar equivalents of 1N NaOH (to pH 12) at 28 C. After 5 hr of incubation in alkali, one ml of the solubilized crystal solution was subjected to gel filtration on Sepharose CL-4B. A single major component was obtained (Fig. 2A, peak fraction 21) whose elution volume was similar to that of beta-galactosidase ($MW = 1.3 \times 10^5$). Fractions 16-24 were combined and incubated 6 days at 28 C. Chromatography of the incubated material on a larger Sepharose CL-4B column yielded three major peaks (Fig. 2B): the 130,000-dalton component (peak fraction 32), some material that eluted in the inclusion volume (peak fraction 47), and a third component that eluted at an intermediate position (peak fraction 40). Fractions 32 and 40, but not 47, were toxic to neonate larvae of the tobacco hornworm.

Fractions 39-44 (Fig. 2B) were combined and applied to a column of DEAE-Bio-Gel A equilibrated with 20 mM Na_2HPO_4 , pH 7.5. A gradient of 0 to 0.4 M NaCl in the same buffer was applied to elute the insecticidal material.

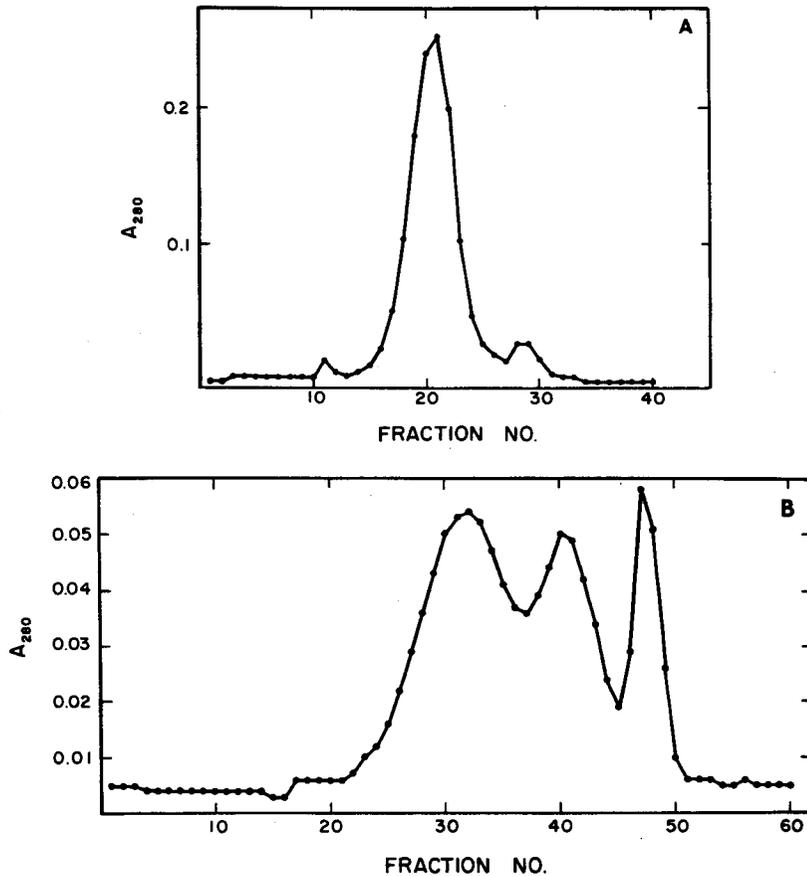


Fig. 2. Sepharose CL-4B gel filtration of (A) 5-hr, alkali solubilized parasporal crystals and (B) combined fractions 16-24 from A after a further 6-day incubation.

Column buffer for A was 50 mM Tris-HCl, pH 8.4, bed volume = 97 ml; column buffer for B was 50 mM NH_4HCO_3 , pH 8.4, bed volume = 150 ml.

As seen in Fig. 3, a single major UV-absorbing peak was obtained (fractions 65-77). Each of the fractions comprising this peak was toxic to hornworm larvae. Fractions 65-77 were combined, concentrated and chromatographed on Sephadex G-100, yielding a peak (chromatogram not shown) whose elution volume was nearly identical to that of bovine serum albumin ($\text{MW} = 6.8 \times 10^4$). Material in the peak fraction was lethal to the insect larvae. The peak fraction was dialyzed against water overnight and concentrated. The resulting preparation was dissolved in 1% SDS, 2% β -ME, and 6 M urea at pH 7.0 as

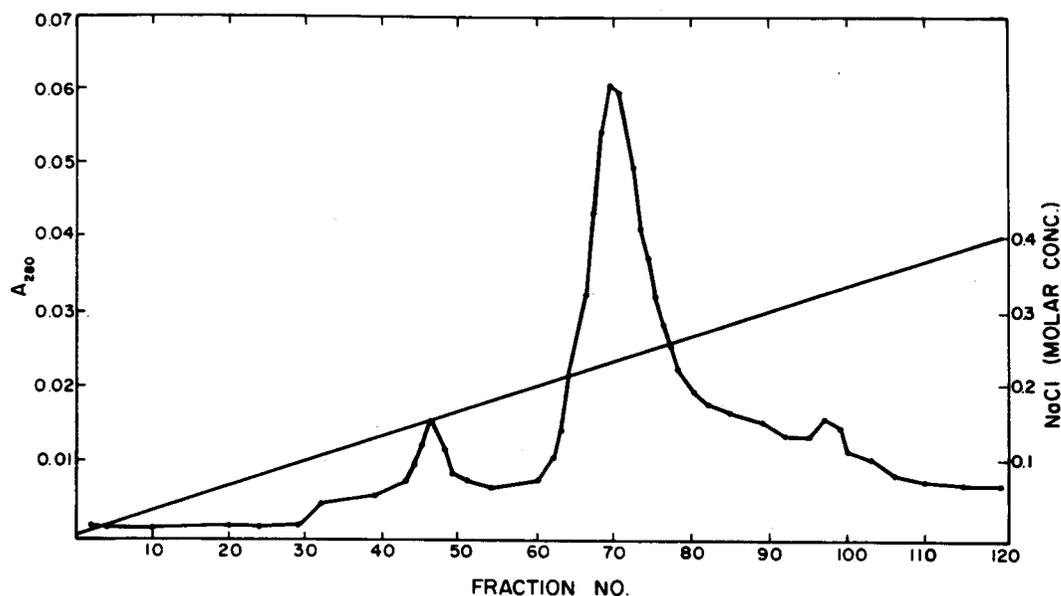


Fig. 3. DEAE Bio-Gel A chromatography of fractions 39-44 from Sepharose gel filtration (Fig. 2B).

Column buffer was 20 mM Na_2HPO_4 , pH 7.5, bed volume = 32 ml. A gradient of 0 to 0.4 M NaCl in the phosphate buffer was applied to elute the toxin.

previously described (14) and electrophoresed in polyacrylamide containing 0.1% SDS (10). Fig. 4 shows electrophoretograms of the insecticidal toxin (Fig. 4B) and of native crystal (Fig. 4A). Our toxin preparation yielded a single band ($R_m = 0.78$, Fig. 4B) with an apparent molecular weight of 68,000. PAS staining of the gel revealed that the toxin was glycosylated. The amino acid compositions of the *B. thuringiensis* toxin and protoxin are presented in Table 1. Due to the small amount of material available for analysis and the instability of certain residues during acid hydrolysis, cysteine, cystine, methionine, tryptophan, and carbohydrate were not determined for the toxin. For comparison with protoxin, the toxin composition was normalized to 3.3 g of alanine. It is obvious from Table 1 that the compositions of the protoxin and toxin are very different and that the two proteins are unique species.

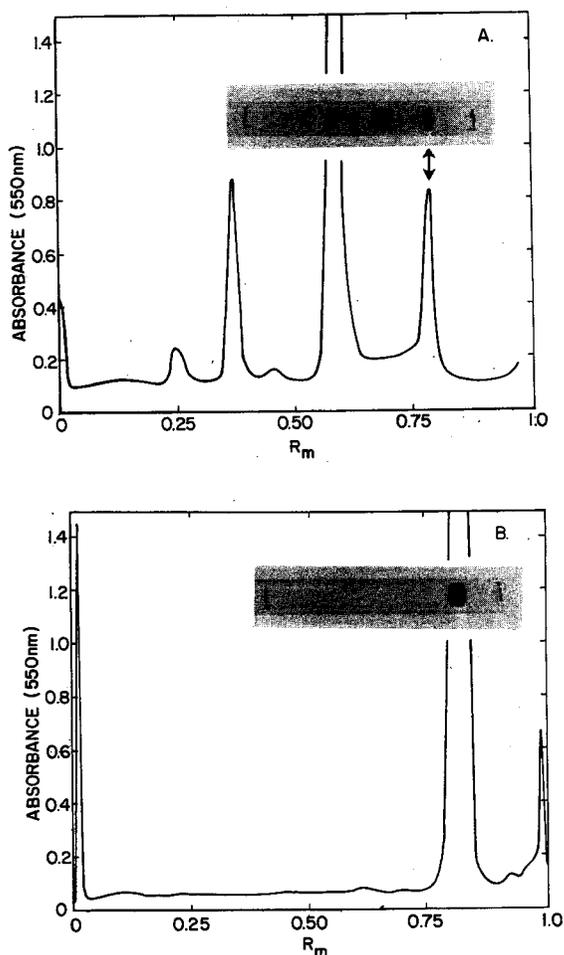


Fig. 4. Stained gels and densitometer scans of solubilized parasporal crystal (A) and purified toxin (B) electrophoresed in 3% SDS polyacrylamide gels.

DISCUSSION

We have isolated a purified insecticidal toxin from *B. thuringiensis* subsp. *kurstaki* whose molecular weight is approximately 68,000 as determined by gel filtration chromatography and SDS polyacrylamide gel electrophoresis. Earlier, we hypothesized (5) that the parasporal crystal of *B. thuringiensis* is composed of a repeating subunit (protoxin) that is converted to a smaller toxic component by dissolution with reducing and denaturing agents. In the present study, we obtained maximum solubility of the parasporal crystal

Table 1. Amino acid composition of *B. thuringiensis* protoxin and toxin.

Amino acid	Toxin ^a	Protoxin ^b
Aspartic acid	9.1	12.4
Threonine	4.2	5.5
Serine	11.4	5.9
Glutamic acid	18.4	13.1
Proline	3.0	2.8
Glycine	8.9	3.6
Alanine	3.3	3.3
Cysteine	--	1.4
Valine	3.7	5.8
Methionine	--	0.9
Isoleucine	2.8	5.4
Leucine	5.4	7.7
Tyrosine	--	5.7
Phenylalanine	2.8	5.0
Lysine	6.5	2.8
Histidine	2.1	1.9
Arginine	3.7	10.3
Tryptophan	--	1.7
Glucose	--	1.8
Mannose	--	0.9

^aNormalized to 3.3 g alanine for comparison to protoxin.

^bGram residues per 100 g crystals. From Bulla et al. (1977).

subunit in about five hours after it had been titrated with 400 molar equivalents of base. Upon prolonged incubation (6 days), the protoxin was converted to toxin (Fig. 2B). The activation mechanism is unknown.

The 68,000-dalton toxin which is lethal to tobacco hornworm larvae upon ingestion, remains toxic at room temperature for several months at neutral pH. Although lower molecular weight components were also generated by our procedure, none of these were entomocidal. Apparently, the 68,000-dalton glycoprotein is the smallest toxic component and any further breakdown probably is detrimental to toxic activity. A more extensive investigation of the biochemical and biophysical properties of the toxin is now being conducted in our laboratory.

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