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Purification of the Mosquitocidal and Cytolytic Proteins of *Bacillus thuringiensis* subsp. *israelensis*†

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Two proteins from parasporal crystals of *Bacillus thuringiensis* subsp. *israelensis* were purified to electrophoretic homogeneity by gel filtration and anion-exchange chromatography. The larger of the two proteins (molecular weight, 68,000) was not cytolytic, whereas the smaller protein (molecular weight, 28,000) was highly cytolytic when assayed against rat erythrocytes. When these proteins were assayed against larvae of the yellow fever mosquito, *Aedes aegypti*, the larger protein was at least 100-fold more toxic than the smaller protein. Although proteolytic activity was not detected in solubilized crystals nor in purified protein preparations, the toxin (molecular weight, 68,000) was readily degraded to smaller, nontoxic molecules, even when maintained at 4°C. Mixtures of the two purified proteins were significantly more toxic to mosquito larvae than was either protein alone. Thus, it is likely that both the mosquitocidal and the cytolytic protein play roles in the overall insecticidal action of the parasporal crystal produced by this bacterium.

Bacillus thuringiensis subsp. *israelensis* is a gram-positive, spore-forming bacterium that is best known for the potent mosquitocidal parasporal crystal that it produces. Intact parasporal crystals produced by *B. thuringiensis* subsp. *israelensis* are toxic to larvae of several dipteran insects, including mosquitoes, black flies, and horn flies (19, 24, 27, 28), and solubilized preparations of the crystal also have been shown to kill adult dipteran insects, such as mosquitoes (14, 16), black flies (15), and stable flies (29). Furthermore, solubilized crystal proteins are lytic to several types of mammalian erythrocytes and other cultured cells of insect and mammalian origin (25). Separation of the mosquitocidal and cytolytic activities has been achieved by gel filtration chromatography; the molecular weights of the two corresponding proteins were estimated to be 68,000 and 28,000, respectively (11).

This paper describes the purification of the *B. thuringiensis* subsp. *israelensis* toxin and reports an instability of the purified mosquitocidal protein. The techniques described herein use conditions that approximate those found in nature, and therefore they preserve the toxic activity of the 68,000-molecular-weight protein.

MATERIALS AND METHODS

Organism and culture conditions. The *B. thuringiensis* subsp. *israelensis* strain used in this study, LB2, was previously described by Tyrell et al. (26, 27). Cultures were maintained on GYS (32) agar slants. Cells were grown in liquid GYS medium, and completely sporulated cells were harvested as described elsewhere (22, 27).

Purification and solubilization of crystals. Parasporal crystals were isolated by isopycnic centrifugation at 25,000 × *g* in a Sorvall (Du Pont Co., Wilmington, Del.) SS34 rotor for 3 h in 50% (wt/vol) NaBr (3). With this method, crystals are buoyant at the top of the centrifuge tube, whereas spores and spore-crystal aggregates are formed into a pellet at the bottom of the tube. Alternatively, crystals were purified on velocity gradients of 15 to 40% (wt/vol) NaBr by centrifugation at 10,000 × *g* in a Sorvall HB-4 swinging-bucket rotor for 20 min. Washed and lyophilized crystals were solubilized in alkali (pH 12) for 5 h at room temperature (5). The pH of the resulting mixture, soluble crystal protein plus insoluble crystal residue, was lowered to pH 8.0 by using 0.5 M Tris hydrochloride (pH 8.0), and the mixture was clarified by centrifugation (2).

Protease determinations on solubilized crystal preparations and on fractions containing purified protein were done by using azocasein and azoalbumin by the method of Andrews et al. (1) and by using protease substrate gel tablets (Bio-Rad Laboratories, Richmond, Calif.). Although protease activity was readily measured in the culture supernatant, in vegetative cells, and in sporulating cells, activity was not detected in purified crystal preparations, nor was it detected during any further purification steps. These results are consistent with those of Ang and Nickerson (3), who reported removal of contaminating proteases from *B. thuringiensis* parasporal crystals by using NaBr density gradient centrifugation.

Column chromatography. Bio-Gel P-150 (Bio-Rad) gel filtration chromatography of alkali-solubilized *B. thuringiensis* crystals was modified from conditions described previously (11). The packed column (2.5 × 100 cm) was equilibrated with column buffer (50 mM triethanolamine hydrochloride buffer [pH 8.0] containing 10 mM KCl and 0.04% sodium azide) and was maintained at 4°C. The column flow rate was approximately 15 ml/h. Commercially purchased molecular weight standards for gel filtration (Bio-Rad) were used to calibrate the column.

Certain samples of insecticidal protein were separated on hydroxyapatite. These samples were dialyzed into phosphate buffer (10 mM NaH_2PO_4 adjusted to pH 7.5 with 1 M NaOH) and applied to a column (1 × 20 cm) of hydroxyapatite (Hypatite-C; Clarkson Chemical Co., Williamsport, Pa.) equilibrated with phosphate buffer. After sample loading and subsequent elution of flowthrough material, a gradient of 50 to 500 mM sodium phosphate buffer (pH 7.5) was used to release any components bound to the column. The column flow rate was approximately 50 ml/h.

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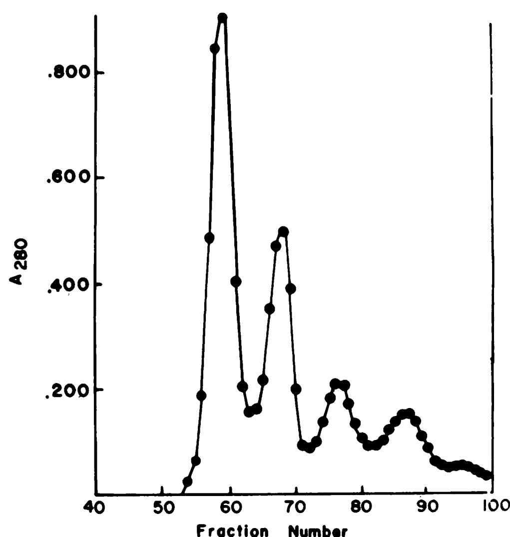

FIG. 1. Gel filtration chromatography of alkali-solubilized crystal toxin proteins. The *B. thuringiensis* crystals were solubilized by titration to pH 12.0 with NaOH and then loaded on the Bio-Gel P-150 column.

DEAE anion-exchange chromatography was on a column (1 × 25 cm) of DEAE Bio-Gel A (Bio-Rad) equilibrated with the same triethanolamine buffer used for gel filtration chromatography. Bound protein was eluted from the anion-exchange resin by using a gradient of 10 to 500 mM KCl in column buffer. The column flow rate was approximately 15 ml/h. Column effluent protein content was determined by measurement of the A_{280}.

Bioassays. Toxicity of soluble material was determined by using early-fourth-instar larvae of the yellow fever mosquito, *Aedes aegypti*. Several investigators have reported that soluble proteins from *B. thuringiensis* subsp. *israelensis*, although not as toxic as particulate proteins, are active under these conditions (4, 11, 16, 26). At each dilution, 10 larvae were suspended in 10 ml of water (tap/distilled ratio, 1:1, vol/vol) containing 2.5 mg of larval diet (16) at 23°C. Each assay was done in triplicate. Mortality was recorded after 16 h. Bioassay controls included solubilized crystal as a positive control and column buffer as a negative control. The concentrations which killed 50% of the larvae (LC_{50}s), slope, and 95% confidence limits were determined by probit analysis (8) by using commercial programs from the SAS Institute, Cary, N.C.

Determination of cytolytic activity. Aliquots of column fractions (10 μl) were assayed for lytic activity against freshly obtained rat erythrocytes (150 μl) that had been washed three to four times and diluted 10-fold in 100 mM phosphate-buffered saline, pH 7.2 (11). Cell lysis was measured by release of material at A_{420}.

Reconstitution experiments with purified proteins. To test the effect of purified cytolysin on the activity of purified mosquitocidal protein, a constant, sublethal concentration of cytolytic protein, 1,000 ng/ml, was mixed with various concentrations of the mosquitocidal protein (5 to 1,250 ng), and the LC_{50} was determined. Ten *A. aegypti* fourth-instar larvae were treated with mixtures of the two proteins in 10 ml of water as described above. Mortality was recorded after 16 h. The bioassay was performed on three separate occasions. Control experiments used mixtures of the two proteins at the same levels, except that the cytolysin was heat denatured by boiling for 5 min.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli and Favre (17), and proteins were visualized by staining with Coomassie brilliant blue.

RESULTS

When alkali-solubilized parasporal crystalline proteins were separated by gel filtration chromatography (Bio-Gel P-150), four peaks of UV light-absorbing material were observed (Fig. 1). Peaks 1, 2, and 3 were toxic when bioassayed against early-fourth-instar *A. aegypti* larvae. Peaks 2 and 4, on the other hand, contained cytolytic activity, whereas peaks 1 and 3 did not (Table 1).

To determine what crystal protein components were present in fractions from the gel filtration column, selected fractions were further analyzed by SDS-PAGE. Peak 1 (Fig. 2, lanes A and B) contained mainly a polypeptide doublet with a molecular weight of 130,000 to 137,000 plus several smaller polypeptides. Peak 2, (lanes C to F) and peak 4 (lanes M to O) contained the 28,000-molecular-weight protein, whereas peak 3 (lanes F to L) contained primarily the 68,000-molecular-weight protein. Peak 2 (lanes C to E) evidently contained aggregates that consisted almost entirely of cytolysin. Peak 4 also contained cytolytic activity but eluted in fractions corresponding to a molecular weight of approximately 28,000 (Fig. 2).

Previous work showed that, after successive separations of the two major proteins found in parasporal crystals by gel filtration chromatography, mosquitocidal protein (molecular weight, 68,000) could be prepared that was essentially devoid of cytolytic protein (molecular weight, 28,000) (11). Unfortunately, these fractions again contained proteins with molecular weights greater than that of cytolytic protein but smaller than that of the mosquitocidal protein (molecular weight, 34,000). To further purify the mosquitocidal material, protein from peak 3 was separated by hydroxyapatite column chromatography. A single, symmetrical peak eluted from the column (data not shown). This material was highly toxic (LC_{50} = 180 ng/ml) but not cytolytic. SDS-PAGE of this material (which had been stored for 24 h at 4°C) showed that it consisted mainly of a 68,000-molecular-weight polypeptide and minor polypeptides of intermediate size (Fig. 3).

Because hydroxyapatite chromatography did not appear to improve the purity of the mosquitocidal protein, anion-exchange chromatography of pooled peak 3 fractions was used to further purify this protein. Peak 3 material from gel filtration chromatography resolved into two peaks of UV light-absorbing material on DEAE Bio-Gel A (Fig. 4). Peak

TABLE 1. Cytolytic and mosquitocidal activity of major peaks after separation by gel filtration chromatography on Bio-Gel P-150 (Fig. 1)

Peak no.	Relative toxicity[a]	Sp cytolytic act[b]
1	100	111
2	31.8	12,980
3	76.7	1,190
4	0	48,333

[a] Percent mortaility when 10 μl of material from the peak fraction was added to the 10-ml bioassay containing mosquito larvae.

[b] Specific cytolytic activity calculated as release of A_{420}-absorbing material from rat erythrocytes per microgram of protein per 5 min.

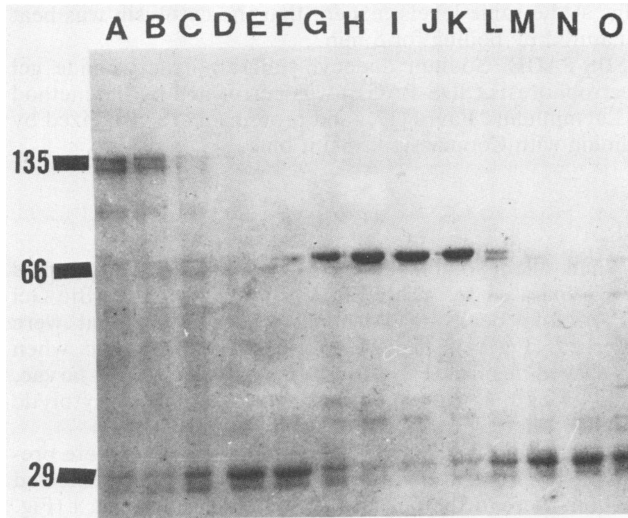

FIG. 2. SDS-PAGE of fractions from Bio-Gel P-150 gel filtration. The gel (12.5% acrylamide) shows selected fractions from the various peaks from gel filtration. Lanes A to C, peak 1, fractions 58 to 60; lanes D to F, peak 2, fractions 64 to 66; lanes G to L, peak 3, fractions 71 to 75; lanes M to O, peak 4, fractions 82 to 88. The markers show the migration of molecular weight standards (weight in thousands).

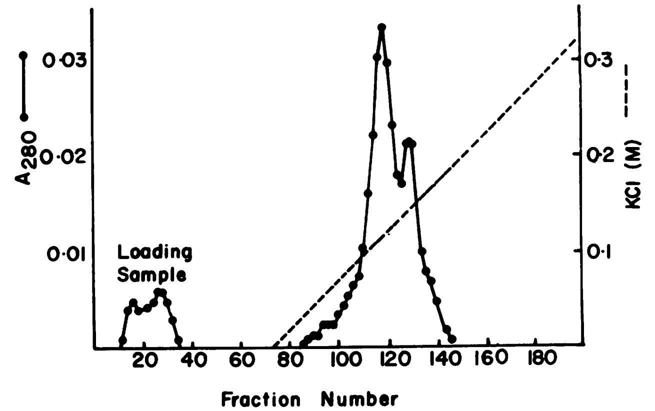

FIG. 4. Anion-exchange chromatography of a mosquitocidal peak from gel filtration. Peak 3 (fractions 71 to 75) from a Bio-Gel P-150 column was chromatographed on DEAE Bio-Gel A. Peak A contains fractions 117 to 119, and peak B contains fractions 122 to 125. Elution was with a 10 to 500 mM KCl gradient

A and peak B fractions contained single polypeptides the molecular weights of which, as determined by SDS-PAGE, were 68,000 and 28,000 respectively (Fig. 5).

Whole, solubilized crystal toxin proteins were toxic to mosquito larva (LC_{50} = 1,280 ng/ml). Purified 68,000-molecular-weight protein (peak A) was very active against early-fourth-instar *A. aegypti* larvae (LC_{50} = 180 ng/ml), whereas the smaller polypeptide displayed no lethality after 16 h at concentrations as high as 25,000 ng/ml. At these extremely high levels of purified cytolysin, however, some larval mortality was observed after 32 h. Conversely, peak B from anion-exchange chromatographya lysed to rat erythrocytes, but peak A was not. Each of the two proteins demonstrated increased specific activity when compared

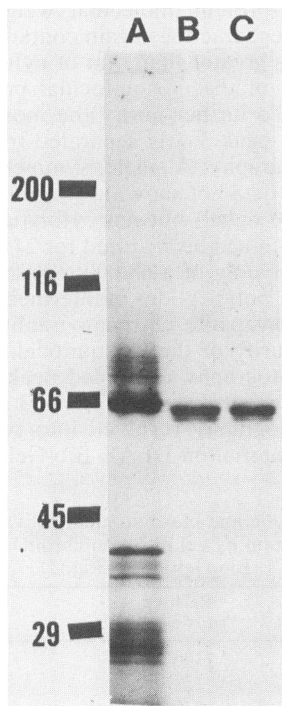

FIG. 3. SDS-PAGE of fractions from hydroxyapatite. The gel is a 7.5 to 12.5% acrylamide gradient which shows the single, toxic peak that eluted upon hydroxyapatite column chromatography. Lane A shows alkaline-solubilized crystal toxin, and lanes A and B are consecutive toxic fractions that eluted from this column.

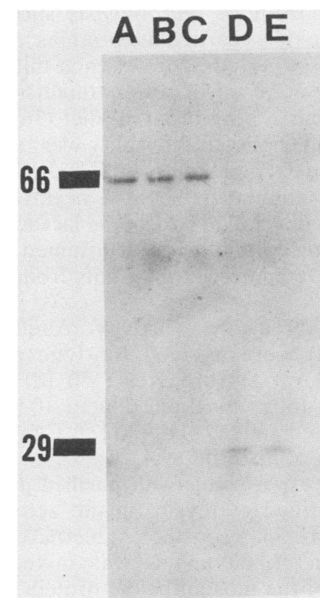

FIG. 5. SDS-PAGE of fractions from anion-exchange chromatography. The gel (12.5% acrylamide) shows toxin in peak A from DEAE Bio-Gel A (lanes A to C, fractions 117 to 119) and cytolysin in peak B (lanes D to E, fractions 123 and 124). The molecular weight of each protein (in thousands) is shown by the markers.

TABLE 2. Effects of purified mosquitocidal and cytolytic proteins on early-fourth-instar *A. aegypti* mosquito larvae and rat erythrocytes

Protein type	LC_{50}[a]	95% confidence limits	Sp cytolytic act[b]
Alkaline-solubilized, unfractionated	1,280	1,040–1,610	8,000
Mosquitocidal	180	115–252	0
Cytolytic	>25,000		111,400

[a] Calculated by probit analysis.
[b] Specific cytolytic activity calculated as release of A_{420}-absorbing material from rat erythrocytes per microgram of protein per 5 min.

with the toxic or cytolytic action of unfractionated solubilized crystal protein preparations (Table 2).

Both activities occur naturally within intact *B. thuringiensis* subsp. *israelensis* parasporal crystals, and, because the activities have been assigned to different proteins, it is logical to propose that both proteins have a function. To determine whether these two proteins act in a coordinate manner, reconstitution experiments involving both proteins were designed. When the mosquitocidal protein (molecular weight, 68,000) was bioassayed in buffer that contained a sublethal concentration (1,000 μg/ml) of cytolytic protein (molecular weight, 28,000), the LC_{50} of the mosquitocidal protein was 138 ng/ml (average from three bioassays), whereas the LC_{50} of the mosquitocidal protein plus the same concentration of heat-denatured cytolysin was 317 ng/ml. The LC_{50} of the mosquitocidal protein plus denatured cytolytic protein was higher (i.e., less toxic) than the LC_{50} reported for purified mosquitocidal protein alone (180 ng/ml), probably because some activity was lost during storage at −20°C. Further, the toxicity of the preparations decreased through the three separate assays. The paired Student's *t* test, however, showed that these results are significant at the 95% confidence level.

As indicated in the previous paragraph, an instability of

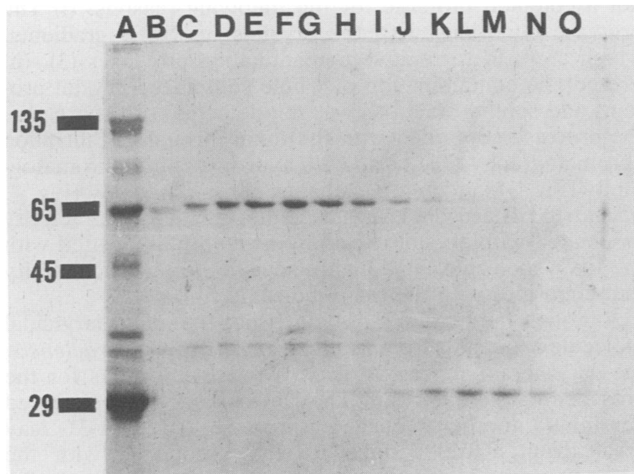

FIG. 6. SDS-PAGE of DEAE Bio-Gel A fractions after 24 h at 4°C. Fractions from DEAE Bio-Gel were separated by SDS-PAGE (12.5% acrylamide). These same samples, separated by SDS gel electrophoresis, are shown in Fig. 5, except that the fractions shown in Fig. 6 are now 24 h old. Peak A, lanes B to I, DEAE Bio-Gel A fractions 114 to 121; Peak B, lanes J to O, DEAE Bio-Gel A fractions 122 to 127.

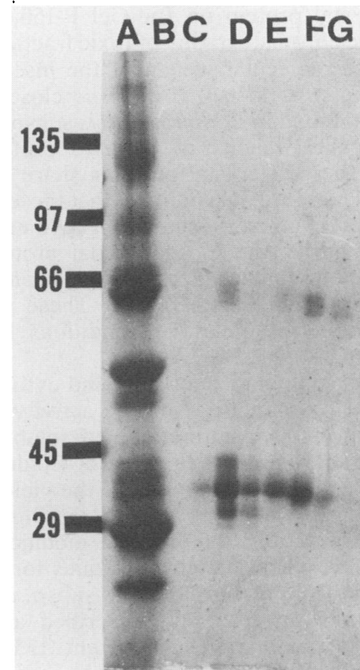

FIG. 7. SDS-PAGE of highly degraded insecticidal toxin. The gel (12.5% acrylamide) shows purified mosquitocidal protein (fractions 115 to 117 from DEAE Bio-Gel A) that has been concentrated and then chromatographed on Bio-Gel P-150. Two peaks (lanes B to D and C to G) eluted from this column. Lane A shows alkaline-solubilized toxin, and the markers indicate the migration of molecular weight standards (weight in thousands).

mosquitocidal activity was observed in purified preparations. To determine the extent and nature of such instability, older preparations were analyzed by SDS-PAGE. An SDS-PAGE electrophoretagram of DEAE Bio-Gel A peaks A and B after storage of this material at 4°C for 24 h is shown in Fig. 6. After 24 h, polypeptides migrating with a molecular weight similar to that of the intermediate bands from hydroxyapatite chromatography (Fig. 3) and gel filtration chromatography (Fig. 2) were observed in what had previously been a pure preparation (Fig. 5).

To confirm that the mosquitocidal protein was breaking down into polypeptides of lower molecular weight, fractions from DEAE Bio-Gel A peak A (Fig. 4) were combined, concentrated 100-fold by evaporation at room temperature (a technique that was used successfully with the *B. thuringiensis* subsp. *kurstaki* toxin [5]), and then separated by gel filtration chromatography (Bio-Gel P-150). Column fractions (from two symmetrical peaks of UV light-absorbing material that no longer corresponded to a molecular weight of 68,000; data not shown) were analyzed by SDS-PAGE. The mosquitocidal protein (molecular weight, 68,000) apparently was converted to lower-molecular-weight proteins (molecular weight, approximately 34,000) (Fig. 7). Significantly, this material was not toxic to mosquito larvae.

DISCUSSION

This report describes the purification of the mosquitocidal protein (molecular weight, 68,000) and of the cytolytic protein (molecular weight, 28,000) from *B. thuringiensis* subsp. *israelensis* parasporal crystals. This purification was accomplished by gel filtration chromatography of alkali-

solubilized crystal protein on Bio-Gel P-150, followed by anion-exchange chromatography of toxic fractions on DEAE Bio-Gel A. The molecular weight of the insecticidal toxin from *B. thuringiensis* subsp. *israelensis* closely resembles that of the toxin found in *B. thuringiensis* subsp. *kurstaki* (5), but the molecular weights of the two proteins are not identical, and the two crystal proteins share few common epitopes (26). Therefore, it is unlikely that these two proteins are identical or even very similar. The method used to solubilize and purify the mosquitocidal protein does not require the use of denaturing or reducing agents and therefore results in a highly active toxin. These solubilization conditions closely resemble the conditions found in the insect midgut (6, 9, 20, 23).

The identities of the mosquitocidal and cytolytic proteins were confirmed by increased specific activity observed as they were purified from unfractionated solubilized crystal preparations (Table 1). As in the case of lepidopteran toxin of *B. thuringiensis* subsp. *kurstaki* (5), the yields of purified toxin were poor; however, as with *B. thuringiensis* subsp. *kurstaki* (1), it is likely that further modification of the procedures will result in better techniques for the purification of the toxin from *B. thuringiensis* subsp. *israelensis*.

Recently, Andrews et al. (1) described a method for converting the protoxin (molecular weight, 135,000) from *B. thuringiensis* subsp. *kurstaki* crystals to insecticidal toxin (molecular weight, 68,000) by using commercially prepared trypsin. Such a procedure resulted in stoichiometric conversion of protoxin to toxin. Attempts to use this same procedure to prepare mosquitocidal protein from alkaline-solubilized *B. thuringiensis* subsp. *israelensis* crystal protein resulted in complete destruction of the 68,000-molecular weight protein and a corresponding loss of toxicity to mosquito larvae (R. E. Andrews, Jr., unpublished data). This result is consistent with those obtained by Armstrong et al. (4), who purified a 25,000-molecular-weight protein from *B. thuringiensis* subsp. *israelensis* by treatment of alkali-solubilized crystals with trypsin and proteinase K. They reported an LC_{50} of between 5 and 50 μg/ml against first-instar *Culex pipiens* mosquito larvae. Furthermore, Davidson and Yamamoto (7) have reported a similar LC_{50}, 12.5 μg/ml, for the 25,000-molecular-weight crystal protein against first-instar *Culex pipiens* var. *quinquefasciatus* larvae. The LC_{50} reported herein are approximately 100-fold lower (more toxic) than are those reported in previous reports. The estimated 100-fold difference in toxicities in probably conservative, because first-instar larvae are more sensitive to the *B. thuringiensis* subsp. *israelensis* toxin than are fourth-instar larvae and because *A. aegypti* mosquitoes are probably slightly more sensitive to the crystal toxin than are *C. pipiens* mosquitoes (27).

Previous investigations by Tyrell et al. (26), on the biochemistry of the parasporal crystals of different *B. thuringiensis* subspecies, including *B. thuringiensis* subsp. *israelensis*, indicated that crystal proteins could be efficiently solubilized in SDS-containing buffers to render reproducible SDS-PAGE polypeptide profiles. This material was not toxic. Huber et al. (10) have also reported lack of toxic activity of SDS-denatured crystal toxin proteins. Insell and Fitz-James (13) solubilized different *B. thuringiensis* subsp. *israelensis* crystal proteins in buffers containing 1% SDS and 50 mM dithiothreitol at different alkaline pHs and assessed their relative toxicities against mosquito larvae after acid precipitation. Acid precipitation and SDS denaturation of the mosquitocidal protein probably damage its biological activity, whereas the cytolytic activity is not affected. Therefore, it is not surprising that, in these experiments, the 70,000-molecular-weight crystal protein was not toxic to mosquito larvae (13).

Ibarra and Federici (12) recently reported separation of small crystals that they termed polyhedral inclusions from *B. thuringiensis* subsp. *israelensis* sporulating cells. These polyhedral inclusions were not toxic to mosquito larvae (12). The results of Ibarra and Federici conflict with those previously published by Lee et al. (18) and those from this study. The observations in this study are somewhat consistent with those of Wu and Chang (30), who observed toxicity in column fractions containing the 68,000-molecular-weight protein, but not the 28,000-molecular-weight protein, of *B. thuringiensis* subsp. *israelensis*. Wu and Chang (3) did not observe high toxicity associated with purified preparations of the 68,000-molecular-weight protein but observed that both proteins were required. In this study, we have shown high toxicity associated with the 68,000-molecular-weight protein and found more-modest synergistic activity resulting from the cytolytic protein.

The degradation of the mosquitocidal protein at room temperature (Fig. 7) and at 4°C (Fig. 6) suggests a possible explanation for the conflicting reports that identify other molecules as the toxin. The cytolysin, which may be somewhat toxic in its own right (Table 2), is relatively stable, whereas the toxin is relatively unstable in soluble form. The toxic molecule probably degrades easily during routine biochemical manipulations, and those investigators who did not obtain insecticidal activity from preparations containing this molecule may have degraded the toxin during preparation and bioassay.

Recently, Pfannenstiel et al. (21) used Western blotting to show that the 68,000-molecular-weight protein from *B. thuringiensis* subsp. *israelensis* can be degraded by trypsin and mosquito gut enzymes to form a 34,000-molecular-weight degradation product. We have shown that this same conversion occurs spontaneously in purified preparations of toxin. Pfannenstiel et al. (21) concluded that toxin instability may be due to contamination by proteases from *B. thuringiensis* subsp. *israelensis*. The results of our present study indicate that, if this is the case, the contaminating protease is not a common protease for the following reasons. (i) The protease copurifies with the crystals on NaBr gradients, which are said to remove contaminating proteases (3). (ii) Proteolytic contamination of whole solubilized crystal proteins and column fractions was not detected. (iii) If present, the protease copurified with the toxin through gel filtration chromatography (Fig. 1 and 2), hydroxyapatite chromatography (Fig. 3), and anion exchange chromatography (Fig. 4, 5, and 6). These data suggest either an autolytic activity associated with crystal toxin, an instability associated with the toxin molecule, or a highly specific proteolytic activity that copurifies with the toxin molecule.

Although the cytolytic protein is not the major larvicidal molecule in crystals from *B. thuringiensis* subsp. *israelensis*, the data herein suggest at least two possible roles for the presence of the cytolysin. The larvicidal activity observed herein and in other published reports (4, 7, 12, 13, 31) may result from a well-defined toxicity associated with the cytolytic protein or contamination of these preparations by toxin at levels below the limits of detection. On the basis of current information, it is not possible to distinguish between these two possibilities.

We are continuing detailed investigations of the functional, biochemical, and genetic properties of both the mosquitocidal and cytolytic proteins.

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