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Immunological Relationships among Proteins Making Up the *Bacillus thuringiensis* subsp. *israelensis* Crystalline Toxin

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The immunological relationships among the proteins of the mosquito larvicidal toxin produced by *Bacillus thuringiensis* subsp. *israelensis* have been investigated by using polyclonal antisera specific for the 28-, 70-, and 135-kilodalton proteins. Each of these proteins was immunologically distinct. There was no cross-reaction among the three proteins and the two non-homologous antisera. Treatment of toxin proteins with larval gut enzymes for 20 h identified protease-resistant domains at approximately 65, 38, and 22 kilodaltons. Similar domains were generated by treatment with trypsin and chymotrypsin. Our immunological and kinetic data indicate that the 28-kilodalton protein is degraded successively to protein bands at 26, 25, 23, and 22 kilodaltons, the 70-kilodalton protein is degraded to a protein at 38 kilodaltons, and the 135-kilodalton protein is degraded successively to protein bands at 94, 72, and, probably, 65 kilodaltons. Solubilized toxin possesses two biological activities, larvicidal and general cytolytic (hemolytic). We used nondenaturing gel electrophoresis to show that the hemolytic activity resides in the 28-kilodalton protein. However, higher-molecular-weight proteins are required to achieve the level of toxicity observed in intact toxin.

Bacillus thuringiensis subsp. *israelensis* produces a parasporal crystalline toxin that kills mosquito and black fly larvae (15). These insects are the vectors of such devastating diseases as malaria and encephalitis, and, consequently, their control has great medical and economic impact. The delta-endotoxin of *B. thuringiensis* subsp. *israelensis* differs from those produced by other strains of *B. thuringiensis* in several important respects. (i) Target species for *B. thuringiensis* subsp. *israelensis* are Diptera rather than Lepidoptera. (ii) Upon solubilization, the *B. thuringiensis* subsp. *israelensis* toxin lyses erythrocytes and cultured mammalian cells as well as cultured insect cells (31), whereas solubilized lepidoptera-active toxins display no corresponding cytotoxicity. (iii) The disulfide bonds of *B. thuringiensis* subsp. *israelensis* may be reduced and blocked without loss of toxicity (27). Similar modification of the *B. thuringiensis* subsp. HD-1 crystals resulted in a 160-fold reduction in toxicity (29). (iv) Antiserum specific for *B. thuringiensis* subsp. *israelensis* toxin does not recognize the subsp. *kurstaki* HD-1 toxin (2, 20). (v) The *B. thuringiensis* subsp. *israelensis* crystals contain a wide array of proteins (18, 19, 23, 28, 31, 34), whereas crystals from most other *B. thuringiensis* strains consist of 130- to 150-kilodalton proteins exclusively (9).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of native *B. thuringiensis* subsp. *israelensis* crystal proteins revealed a broad band at 28 kilodaltons, a triplet at 38, 39, and 40 kilodaltons, and doublets at 68 and 70 kilodaltons and 135 and 140 kilodaltons (28). Similar patterns have been observed in other laboratories (18, 19, 23, 31, 34), with the minor differences in protein sizes probably resulting from differences in the gel systems used. The heterogeneity of proteins in *B. thuringiensis* subsp. *israelensis* crystals has hindered identification of the protein(s) responsible for larvicidal and hemolytic activities. The general cytolytic or hemolytic activity is thought to reside in the 28-kilodalton protein subunit (2, 14, 32, 37), but there has been disagreement on the identity of the larvicidal protein(s). The 28-kilodalton (2, 14, 19, 32), the 65-kilodalton (17), and the 130-kilodalton (36) proteins have been identified as being responsible for larval mortality. Cheung and Hammock (11) reported that proteins at 31, 34, and 35 kilodaltons are larvicidal, while Wu and Chang (37) concluded that larvicidal activity was due to a synergistic effect of the 26- to 28-kilodalton and the 65- or 135-kilodalton proteins.

A structure-function understanding of larvicidal activity requires accurate knowledge of the size and chemical composition of the toxin(s). The ultimate crystal toxins should constitute protease-resistant domains because they must remain active in the intensely proteolytic larval guts (21). *B. thuringiensis* subsp. *israelensis* crystals are produced during bacterial sporulation and they are released into a highly proteolytic environment when the sporangium lyses (16, 26). Crystal-associated bacterial proteases have been a consistent problem in the accurate determination of native crystal parameters for all *B. thuringiensis* strains, and their presence may account for the conflicting reports regarding the toxicity of *B. thuringiensis* subsp. *israelensis* proteins (2, 14, 17, 19, 32, 36). To define the structural relationships among the various proteins, we have prepared polyclonal antisera specific for the 28-, 70-, and 135-kilodalton proteins and assessed their reactivity towards crystal proteins separated by SDS-PAGE. This analysis was also conducted on crystal proteins degraded by larval gut enzymes to identify the size and origin of the protease-resistant domains in the ultimate toxin(s).

MATERIALS AND METHODS

Toxin preparation and production of antisera. A single-colony isolate of *B. thuringiensis* subsp. *israelensis* taken from Bactimos powder (Biochem Products; courtesy of Brian Federici, University of California, Riverside) was grown on GGYS medium (27). After sporulation, the protein crystals were purified on NaBr gradients as described previously (28). The crystals were solubilized for 2 h at 37°C in 50 mM NaOH with 10 mM EDTA at pH 11.7 (28) followed

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by centrifugation at 15,000 × *g* for 10 min. Protein concentrations in the supernatants were determined from the A_{280} ($E^{1\%}_{1\,cm}$ = 11.0) (35).

Solubilized toxin was boiled for 5 min in Laemmli SDS sample buffer (22) and the proteins were resolved on 12.5% preparative gels. Protein standards (Pharmacia, Inc.) were phosphorylase *b* (94,000) bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000), and alpha-lactalbumin (14,400). The gels were stained with Coomassie blue, and the protein bands migrating at 135, 70, and 28 kilodaltons were excised and stored frozen. Gel slices were macerated in a minimum volume of phosphate-buffered saline (10 mM sodium phosphate, 150 mM NaCl, pH 7.2), emulsified with Freund adjuvant, and injected subcutaneously into New Zealand White rabbits in a series of five weekly injections. Serum was collected and used at a 1:50 dilution for immunoblots.

Protease digestion of *B. thuringiensis* subsp. *israelensis* toxin. Mosquito gut proteases were prepared by dissecting food columns from three third-instar *Aedes aegypti* larvae. The food columns were placed in 100 μl of deionized water, vortexed, and incubated at 4°C for 15 min. Particulate material was removed by centrifugation at 15,000 × *g* for 10 min, and the supernatant was retained as the mosquito gut protease extract.

Alkali-solubilized protein toxin (145 μg) was adjusted to pH 8.0 with 50 mM Tris and incubated with trypsin or chymotrypsin (30 μg) in the presence of 5 mM $CaCl_2$. To simulate the high pH conditions present in mosquito larval guts (12), another portion of the solubilized toxin (145 μg) was adjusted to pH 10.15 with 50 mM cyclohexylaminopropane sulfonic acid and incubated with 5 μl of gut protease extract. In all cases, proteolysis occurred at 25°C in a final volume of 255 μl. Reactions were terminated by the addition of 2 μl of 100 mM phenylmethylsulfonyl fluoride and 2 μl of 200 mM EDTA. Laemmli SDS sample buffer was added, and the samples were subjected to SDS-PAGE (22) in 7.5 to 15% gradient gels. Each protease digestion experiment was repeated three times. The relative amounts of the respective proteins were determined by scanning the gels with a Hoeffer densitometer and integrating the areas under the peaks.

Western blots and immunoblots. The reactivity of the three antisera with native *B. thuringiensis* subsp. *israelensis* toxin was determined by using a dot blot apparatus (Schleicher & Schuell). Solubilized *B. thuringiensis* subsp. *israelensis* toxin (200 ng) was applied to nitrocellulose membranes by vacuum filtration. Immobilized proteins were detected with the antisera described above and protein A-horseradish peroxidase conjugate (Bio-Rad Laboratories) diluted 1:3,000. Incubation times, buffers, and development conditions were performed as recommended by Bio-Rad.

The specificity of the antisera to both denatured and protease-digested *B. thuringiensis* subsp. *israelensis* proteins was determined with Western blots. After SDS-PAGE, the proteins were transferred to nitrocellulose with a Hoeffer transblot apparatus operating at maximum voltage for 45 min. Transfer buffer consisted of 192 mM glycine, 25 mM Tris, and 20% methanol. Immunoglobulin binding was again detected by the protein A-horseradish peroxidase method (Bio-Rad).

RESULTS

Specificity of the antisera. The three antisera produced in response to denatured *B. thuringiensis* subsp. *israelensis* toxin reacted strongly with alkali-solubilized toxin (Fig. 1). No reactions were observed with the corresponding preimmune sera. The specificity of the three antisera was tested by their reactivity towards *B. thuringiensis* subsp. *israelensis* crystal proteins separated on reducing SDS-polyacrylamide gels. Antiserum produced in response to the 28-kilodalton protein recognized only the 28-kilodalton protein (Fig. 2, lane a), antiserum produced in response to the 70-kilodalton protein recognized both the 70-kilodalton protein and the 38-kilodalton protein (Fig. 2, lane c), and antiserum produced in response to the 135-kilodalton protein recognized both the expected 135-kilodalton protein and a protein of approximately 94 kilodaltons (Fig. 2, lane e). The 94-kilodalton protein may or may not be visible on Coomassie blue-stained gels depending on the crystal purification procedure used. These results suggest that the 38- and 94-kilodalton proteins are proteolysis products of the 70- and 135-kilodalton proteins, respectively.

Protease-resistant domains. The in vitro susceptibility of solubilized *B. thuringiensis* subsp. israelensis crystal proteins to exogenous mosquito gut enzymes was determined throughout a 20-h digestion period (Fig. 3, lanes b to f). The 135- and 94-kilodalton proteins were degraded within the first 0.5 h of incubation (Fig. 3, lanes b and c), but discrete protease-resistant domains were still present at 65, 31, and 22 kilodaltons after 20 h of incubation (Fig. 3, lane f).

It is well known that the same protein can exhibit different apparent molecular weights in gradient and nongradient gels (3, 33). Our protease digestion experiments were run in 7.5 to 15% gradient gels to obtain better separation of the protein fragments. The apparent sizes in these gradient gels were similar to those reported previously for 12.5% gels (28) with the exception of the triplet at 38, 39, and 40 kilodaltons. Other laboratories have confirmed that these proteins migrate in the 35- to 42-kilodalton range in nongradient gels (19, 23, 32). However, in gradient gels, the 38-, 39-, and 40-kilodalton proteins exhibited apparent molecular weights of 31,000, 34,000, and 36,000, respectively (Fig. 3, lane b). For purposes of consistency, these native crystal proteins are referred to as 38 to 40 kilodaltons in this manuscript.

Mosquito guts are intensely proteolytic (21). They contain both trypsin-like and chymotrypsin-like enzymes (38), and we were able to simulate gut enzyme digestion of the crystal

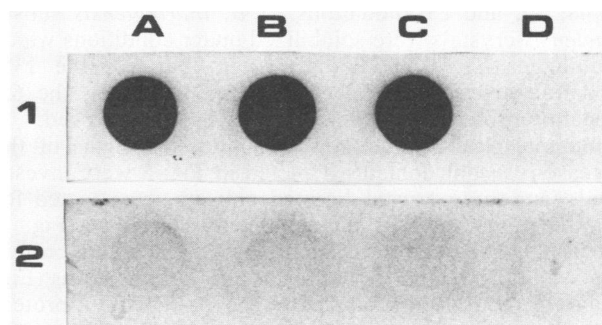

FIG. 1. Reaction of antisera with nondenatured *B. thuringiensis* subsp. *israelensis* proteins. A 200-ng portion of alkali-solubilized *B. thuringiensis* subsp. *israelensis* proteins (A–C) or Tris-saline buffer (D) was applied to nitrocellulose membranes. The nitrocellulose was incubated with preimmune sera (2) or antisera (1) produced to the 135-kilodalton (A), 70-kilodalton (B), or 28-kilodalton (C and D) protein. Antigen-antibody reactions were detected by the protein A-horseradish peroxidase method described in Materials and Methods. Nonspecific attachment of serum immunoglobulins to nitrocellulose did not occur (D).

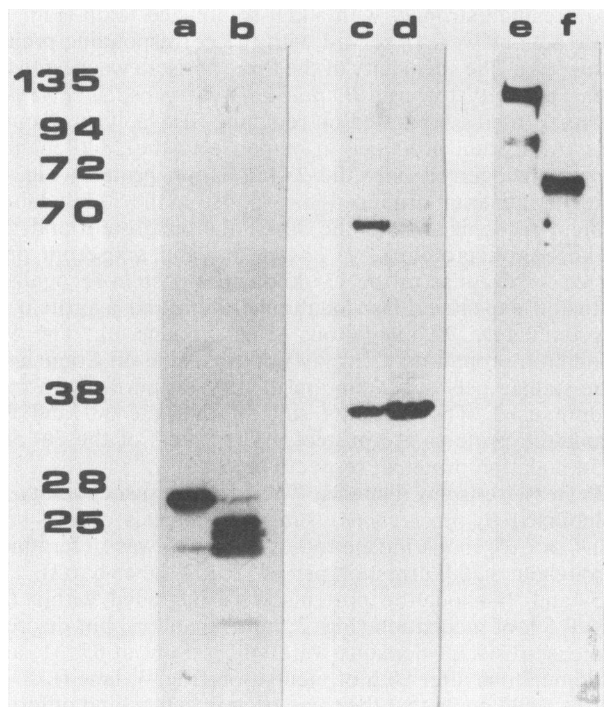

FIG. 2. Reaction of antisera with *B. thuringiensis* subsp. *israelensis* crystal proteins separated by gel electrophoresis. Following gradient SDS-PAGE, the proteins were electrophoretically transferred to nitrocellulose and incubated with antisera produced to the 28-kilodalton (lanes a and b), 70-kilodalton (lanes c and d), and 135-kilodalton (lanes e and f) protein. Lanes a, c, and e contained solubilized toxin, while lanes b, d, and f contained toxin subjected to 1 h of proteolysis with mosquito gut enzymes. Antigen-antibody reactions were detected by the protein A-horseradish peroxidase method described in Materials and Methods. The numbers on the left represent the size (in kilodaltons) of the visible bands.

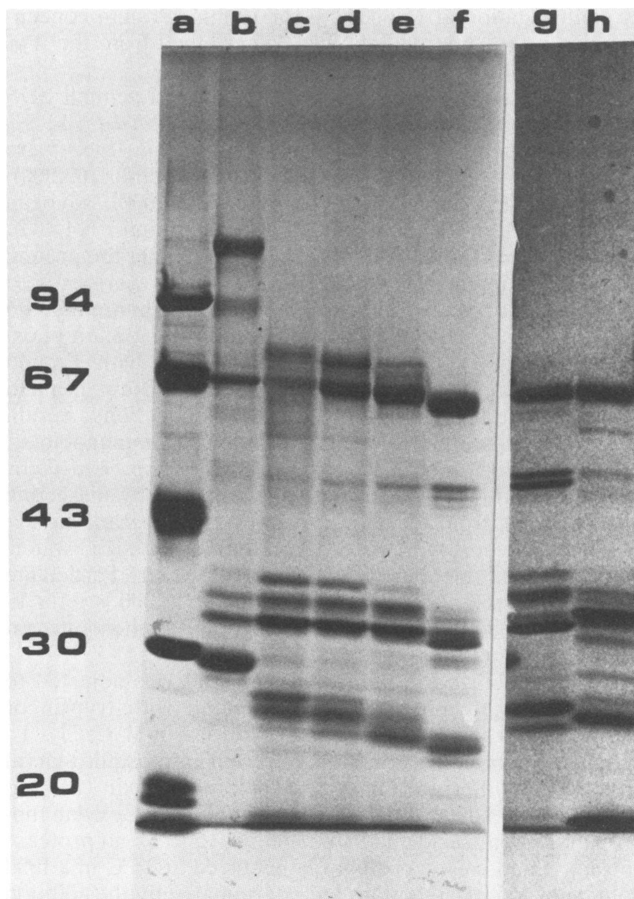

FIG. 3. Proteolytic digestion of *B. thuringiensis* subsp. *israelensis* toxin proteins. Solubilized crystal toxin (20 μg per lane) was subjected to gradient SDS-PAGE followed by Coomassie blue staining. Lane a contains molecular weight standards whose sizes (in kilodaltons) are indicated at the left. Solubilized *B. thuringiensis* subsp. *israelensis* toxin (b) was compared with toxin digested with the following proteases: mosquito gut enzymes for 0.5 h (c), 1 h (d), 2 h (e), and 20 h (f); trypsin (g) and chymotrypsin (h), both at 118 μg/ml, for 20 h. Lanes g and h are aligned so that the molecular weight standards (lane a) are applicable.

proteins with purified trypsin and chymotrypsin (Fig. 3, lanes g and h). Chymotrypsin digestion duplicated the mosquito gut enzymes almost exactly, giving major protease-resistant domains at 65, 31, and 22 kilodaltons, while trypsin digestion resulted in major protease-resistant domains of 65, 50, 48, 30, and 23 kilodaltons. If *B. thuringiensis* subsp. *israelensis* crystals were solubilized under conditions which solubilized the 70-kilodalton protein but not the 135-kilodalton protein (pH 10 or 2% SDS at pH 7), the 65-kilodalton protease-resistant domain was not observed.

Immunological relationships. The protein origins of the protease-resistant domains depicted in Fig. 3 were investigated. Our three crystal-directed antisera were tested for their reactivity towards the 1-h-digested proteins (Fig. 3, lane d). Antiserum produced to the 28-kilodalton protein recognized protein bands at 26, 25, and 23 kilodaltons (Fig. 2, lane b), antiserum produced to the 70-kilodalton protein recognized a single protein band at 38 kilodaltons (31 kilodaltons on a gradient gel; Fig. 2, lane d), and antiserum to the 135-kilodalton protein recognized a protein band at 72 kilodaltons (Fig. 2, lane f). This 72-kilodalton protein was present only after 0.5 to 2 h of proteolysis (Fig. 3, lanes c to e) and had disappeared after 20 h of proteolysis (Fig. 3, lane f). The protease-resistant domain migrating at 65 kilodaltons (Fig. 3, lane f) did not react with any of the antisera (Fig. 2, lanes b, d, and f). The 65-kilodalton protein was successfully transferred to nitrocellulose during Western blotting (as determined by naphthol blue-black staining), but it was not recognized immunologically. Consequently, the blots were stripped of the antibody-protein A-horseradish peroxidase complex (24) and retested with fresh antisera and iodinated protein A to increase sensitivity. However, even after extended exposure to X-ray film, no reaction with the 65-kilodalton protein could be detected.

To identify the origin of the 65-kilodalton domain, the kinetics of proteolysis of the high-molecular-weight proteins was determined by scanning densitometry of the gel depicted in Fig. 3. After 1 h of protease digestion, no 70-kilodalton protein was detected by immunoblots, and after 2 h of digestion all of the original 70-kilodalton protein could be accounted for by the increased amounts of 38-kilodalton protein. Similarly, the amount of 135- and 94-kilodalton proteins in undigested toxin was quantitatively converted to a 65-kilodalton domain. Taken together, these observations demonstrate that the 65-kilodalton protein is a proteolysis product of the 135-kilodalton protein rather than the 70-kilodalton protein.

Biological activity. Alkali-solubilized *B. thuringiensis*

TABLE 1. Biological activity of *B. thuringiensis* subsp. *israelensis* crystal proteins

Protein fraction[a]	Protein composition (%)[b]				Hemolytic activity (LC_{50}, μg/ml)[c]	Larvicidal activity (LC_{50}, ng/ml)[d]
	135 kilodaltons	65–70 kilodaltons	38–40 kilodaltons	28 kilodaltons		
Solubilized crystal protein	15	32	15	38	8	112
Band 1 (135 kilodalton enriched)	64	22	5	9	>31	168
Band 2 (65–70 kilodalton enriched)	13	64	19	4	>64	120
Band 3 (28 kilodalton enriched)	0	5	0	95	8	>400

[a] The *B. thuringiensis* subsp. *israelensis* crystal proteins were alkali solubilized (28) and analyzed by 10% nondenaturing gel electrophoresis. The resolved bands were cut from the gel and electroeluted.

[b] After electroelution, the proteins were subjected to SDS-PAGE. The silver-stained gels were scanned, and the area under the peak was integrated to give the percentage of protein at each molecular mass.

[c] The assay for erythrocyte lysis was conducted as described previously (28).

[d] Solubilized proteins were attached to latex beads (30) to determine their toxicity to *A. aegypti* larvae. Bioassays were performed as described previously (30).

subsp. *israelensis* toxin possesses both larvicidal and hemolytic activity. To determine which proteins are responsible for the biological activities, the proteins must be separated under nondenaturing conditions. However, the *B. thuringiensis* subsp. *israelensis* toxin proteins have a high affinity for one another, and when proteins from apparently separated chromatography peaks or gel bands are examined by silver-stained SDS-PAGE, they are found still to consist of protein mixtures (37; our unpublished data). Consequently, only "enriched" protein fractions were available for comparison of biological activities. Total solubilized *B. thuringiensis* subsp. *israelensis* toxin lysed erythrocytes (50% lethal concentration [LC_{50}] = 7.5 μg/ml) and was toxic to mosquito larvae (LC_{50} = 112 ng/ml). After separation of the proteins on nondenaturing gels, the 28-kilodalton enriched fraction lysed erythrocytes but was unable to kill mosquito larvae at the highest concentration tested (400 ng/ml). In contrast, the 65- to 70- and 135-kilodalton enriched fractions possessed near-normal larvicidal activity but no detectable hemolytic activity (Table 1). Similar results were obtained following chromatography on a Bio-Gel P-100 column. The "resolved" peaks consisted of protein mixtures, with the 28-kilodalton enriched peaks exhibiting hemolytic activity and the 65- to 70-kilodalton enriched peaks exhibiting larvicidal activity (E. J. Ross, M.S. thesis, University of Nebraska, Lincoln, 1984).

DISCUSSION

We have used three specific antisera to demonstrate the immunological relationships among the multiple protein subunits present in native *B. thuringiensis* subsp. *israelensis* crystals. It has been suggested that the multiple protein sizes observed might not represent unique protein species but instead result from proteolysis or the dimerization, trimerization, etc., of smaller proteins (19, 28). Immunological relationships were observed between the 70- and 38-kilodalton proteins and the 135- and 94-kilodalton proteins (Fig. 2), and these relationships likely result from proteolysis (Fig. 3). Once the disulfide bonds have been cleaved, discrete protein multimers do not appear to exist.

Protease-resistant domains are common in extracellular proteins produced by bacilli. They have been found in cereolysin (5), and the delta-endotoxins produced by *B. thuringiensis* subsp. *kurstaki* (10) and *B. sphaericus* (13). We have now found that *B. thuringiensis* subsp. *israelensis* crystal proteins also contain protease-resistant domains. Domains at approximately 65, 31, and 22 kilodaltons were found following 20 h of incubation with trypsin, chymotrypsin, or mosquito larval gut enzymes (Fig. 3). This indication of the stability of the *B. thuringiensis* subsp. *israelensis* proteins complements our previous observation (27) that the larval toxicity of solubilized *B. thuringiensis* subsp. *israelensis* proteins was resistant to high concentrations of salt (8 M NaBr), organic solvents (40% methanol), denaturants (4 M urea), and neutral detergents (10% Triton X-100).

The number of proteins present in native *B. thuringiensis* subsp. *israelensis* crystals coupled with the diversity of their protease-resistant domains and proteolytic intermediates has led to substantial confusion in the literature regarding the identity and significance of the crystal proteins detected by gel electrophoresis. In an attempt to clarify these relationships, we have purified toxin crystals which are essentially protease-free (26). The crystals were harvested from 30% NaBr and solubilized in the presence of 10 mM EDTA. Our immunological and kinetic data indicate the following. (i) The 28-kilodalton protein is degraded successively to protein bands at 26, 25, 23, and 22 kilodaltons. Using monoclonal antibodies, Armstrong et al. (2) similarly concluded that the 28-kilodalton protein was degraded to multiple bands in the vicinity of 25 kilodaltons. (ii) The 70-kilodalton protein is degraded to a protein band at 38 kilodaltons; a substantial amount of the 38-kilodalton protein is normally present in solubilized *B. thuringiensis* subsp. *israelensis* crystals. (iii) The 135-kilodalton protein is degraded successively to protein bands at 94 and 72 kilodaltons and, probably, 65 kilodaltons. Extensive proteolysis appears to remove the surface epitopes recognized by the immunoglogulins of the antisera (4) and, consequently, this conclusion is based on the kinetics of appearance. The conclusion that the 65-kilodalton protease-resistant domain is derived from the 135-kilodalton protein is consistent with three additional considerations: (i) it is unlikely that antiserum produced to the 70-kilodalton protein would recognize a 38-kilodalton proteolysis product but not a 65-kilodalton product; (ii) the 65-kilodalton protease-resistant domain is not detected if the 135-kilodalton protein is not solubilized; and (iii) a 135- to 65-kilodalton proteolytic conversion would be directly analogous to that in which the 135- to 145-kilodalton protoxin from Lepidoptera-active *B. thuringiensis* crystals is converted to a 68-kilodalton toxin in susceptible larval guts (1, 6–8, 25).

With regard to biological activity, our data indicate that the general cytolytic activity resides in the 28-kilodalton protein of the crystal. This conclusion is in agreement with several previous reports (2, 14, 32, 37). However, the 28-kilodalton protein alone is not larvicidal at low concentrations. The 28-kilodalton protein may be toxic when consumed at abnormally high concentrations, but only when it is mixed with high-molecular-weight components does the LC_{50} approach that found for the unfractionated solubilized toxin.

This study demonstrates the importance of inhibiting proteases during purification and solubilization of *Bacillus*

toxins. If proteolysis has occurred during isolation of the *B. thuringiensis* subsp. *israelensis* toxin, discrete protein bands migrating at 135, 94, 72, and 65 kilodaltons may be found. Without immunological data such as we have generated, it would be difficult to determine that these four proteins are related, while the proteins at 135, 70, and 28 kilodaltons are unique. Our results suggest that the *B. thuringiensis* subsp. *israelensis* crystal toxin consists of at least three distinct proteins and that the ultimate larvicidal activity resides in multiple protease-resistant domains obtained from these proteins.

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