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Stability of the Larvicidal Activity of *Bacillus thuringiensis* subsp. *israelensis*: Amino Acid Modification and Denaturants

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The *Bacillus thuringiensis* subsp. *israelensis* mosquito larvicidal toxin is not a sulfhydryl-activated toxin. The protein disulfide bonds were cleaved and blocked without loss of toxicity. In contrast, modification of the lysine side chains eliminated toxicity. Additionally, the toxin 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). However, it was inactivated by both positively and negatively charged detergents and by guanidine hydrochloride.

The bacterium *Bacillus thuringiensis* subsp. *israelensis* produces a protein crystal that is toxic to the larval stage of many mosquito and blackfly species. This purified crystalline toxin is unlike other *B. thuringiensis* crystal toxins in three respects: (i) it is toxic to dipteran larvae rather than lepidopteran larvae; (ii) it is composed of multiple protein subunits ranging in size from 28 to 135 kilodaltons (9, 21); and (iii) when solubilized, it lyses erythrocytes and all tissue culture cell lines tested (16). This general cytolytic activity resides in the 28-kilodalton subunit of the crystal (1, 3, 4, 17), but there has been disagreement on the identity of the larvicidal protein(s). Both the 28-kilodalton subunit (3) and the 65- to 70-kilodalton subunit (4) have been identified as being responsible for larval mortality.

Because of its potential importance in mosquito abatement programs, *B. thuringiensis* subsp. *israelensis* is being studied extensively in both the field and the laboratory. In both cases the maintenance of toxicity in changing environments is critical. Purification of the larvicidal subunit and identification of its mode of action frequently require chemical manipulations which must be performed without loss of biological activity. Similarly, crystal toxins are exposed to potential denaturants during both commercial formulation and application to polluted waters. For these reasons we studied the stability of larval toxicity after chemical treatments that could potentially inactivate the toxin.

MATERIALS AND METHODS

Toxin preparation. A single-colony isolate of *B. thuringiensis* subsp. *israelensis* taken from a Bactimos powder (courtesy of Brian Federici, University of California, Riverside) was grown on GGYs medium (pH 7.2) containing (per liter of distilled water): glucose, 4 g; glutamic acid, 10 g; yeast extract, 2 g; K₂HPO₄ · 3H₂O, 2.5 g; NaCl, 2 g; MgSO₄, 0.2 g; CaCl₂ · 2H₂O, 0.1 g; and MnSO₄ · 4H₂O, 0.04 g. After sporulation, the protein crystals were purified on NaBr gradients as described previously (9). The crystals (2 to 4 mg/ml of water) were solubilized for 2 h at 37°C in 50 mM NaOH with 10 mM EDTA at pH 11.7 (9) followed by centrifugation at 15,000 × g for 10 min. Protein concentra-

tions in the supernatants were determined from the A₂₈₀ ($E_{1\text{cm}}^{1\%} = 11.0$ [18]).

Bioassay. After solubilization, the toxin was diluted with 0.1 M Tris buffer (pH 7.4) to a volume of 1 ml (resulting in a protein concentration of 25 times the intended final concentration) and mixed with 10 μl of latex beads (12). The beads were pelleted (15,000 × g for 10 min), resuspended in 1 ml of Tris buffer (pH 7.4), and added to 24 ml of deionized water containing 10 to 20 third-instar *Aedes aegypti* larvae. Fifty percent lethal concentrations (LC₅₀s) were determined after 24 h. Control larvae incubated in the absence of protein toxin had no mortality in this time period. Similarly, none of the reagent controls caused larval mortality in the absence of protein toxin.

Protein binding. The bioassay for solubilized toxin is dependent on attachment of the protein to the latex beads (12). Because of the diversity of toxin modifications included in this study, it was necessary to determine whether any of these treatments interfered with protein attachment. Such interference could lead to erroneous increases in the observed LC₅₀s. This possibility was tested for each toxin treatment by measuring the protein remaining in the supernatant after centrifugation of the bead-toxin mixtures. Because of the low protein concentrations to be measured, unbound toxin was quantified on nitrocellulose filters based on the procedure described by the manufacturer (Schleicher & Schuell). The accuracy of this procedure is not affected by reagents commonly used in protein chemistry. The supernatants (1 ml) were mixed with 136 μl of Tris-sodium dodecyl sulfate (SDS) (1 M Tris, 2% SDS; pH 7.5) and 227 μl of trichloroacetic acid (90%) for 2 min. The protein precipitates were collected on nitrocellulose filters in a dot blot apparatus, washed with 6% trichloroacetic acid, stained for 10 min with 0.25% naphthol blue black in 50% methanol-10% acetic acid, and destained in 90% methanol-2% acetic acid. The spots were eluted with 1 ml of 25 mM NaOH-50 μM EDTA in 50% ethanol, and the protein was quantified by the A₆₃₀. Solubilized *B. thuringiensis* subsp. *israelensis* toxin was used as the protein standard. Data on the toxicity of solubilized *B. thuringiensis* subsp. *israelensis* crystal protein are only reported for treatments which did not interfere with binding to the latex beads.

Covalent modifications. (i) Disulfide cleavage. The *B. thuringiensis* subsp. *israelensis* crystals were incubated in the normal solubilization system (pH 11.7), after which

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either 2-mercaptoethanol (2%) or dithioerythritol (0.5 M) was added. The reduction was allowed to proceed for 30 min at room temperature.

(ii) **Sulfhydryl modifications.** The *B. thuringiensis* subsp. *israelensis* crystals were incubated for 2 h at 37°C in the solubilization system, pH 11.7, supplemented with dithioerythritol (25 mM) and chilled on ice, and methanol was added to 40% (vol/vol). The function of the methanol was to disaggregate the protein subunits, making all of the disulfide bonds available for reduction (manuscript in preparation). The proteins were then precipitated by titration to pH 5.0 with 0.1 M Na₂HPO₄-citric acid buffer (pH 3.75), washed three times with 2.5 mM Na₂HPO₄-citric acid buffer (pH 5.0), and redissolved in 50 mM Tris, pH 8.0. Methanol was added to 30% (vol/vol), and aliquots containing 300 µg of protein were incubated for 1 h at 4°C with 2,2-dithiopyridine (2 mM), 5,5'-dithiobis(2-nitrobenzoic acid) (0.4 mM), 1,3-propane sultone (0.002% vol/vol), iodoacetamide (2 mM), HgCl₂ (2 mM), or 2-hydroxyethyl-disulfide (2 mM). All solutions were deoxygenated with bubbled N₂ before use.

(iii) **Lysine modifications.** The *B. thuringiensis* subsp. *israelensis* crystals were alkali solubilized in the absence of dithioerythritol or methanol. Aliquots containing 500 µg of protein were then reacted with either dansyl chloride (6 mg/ml in acetone), *O*-methylisourea (guanidination) (as described by Kimmel [5]), potassium cyanate (carbamylation) (as described by Stark [15]) or succinic anhydride (succinylation) (as described by Klotz [6]). Dansylation was terminated by addition of 0.1 M Tris, pH 7.4. The other modified *B. thuringiensis* subsp. *israelensis* proteins were precipitated at pH 4 to 5, washed, and then suspended in 50 mM Tris (pH 8.0) in preparation for the bioassays.

Other treatments. (i) Organic solvents. Equal volumes of organic solvent and solubilized *B. thuringiensis* subsp. *israelensis* protein (pH 11.7) were mixed to give final solvent concentrations of 10, 20, 30, 40, and 50% (vol/vol). The mixtures were incubated for 2 h at 4°C. This low temperature was chosen to minimize denaturation and to prevent solvent evaporation.

(ii) **Potential denaturants.** Solubilized *B. thuringiensis* subsp. *israelensis* protein (pH 11.7) was incubated with 4, 6, and 8 M NaBr, urea, and guanidine hydrochloride for 30 min at room temperature.

(iii) **Detergents.** Intact *B. thuringiensis* subsp. *israelensis* crystals were incubated with 0.1, 0.5, 1.0, 5.0, and 10.0%

TABLE 2. Effect of lysine modification on larval toxicity of solubilized *B. thuringiensis* subsp. *israelensis* crystal protein

Reagent	µg of Reagent/µg of protein	Modification	% Toxicity ^a
<i>O</i> -Methylisourea	0.55	Guanidination	45
Potassium cyanate	16.0	Carbamylation	30
Succinic anhydride	0.20	Succinylation	64
	1.00		36
	5.00		<12
Dansyl chloride	0.04	Dansylation	100
	0.40		50
	1.20		<6

^a See Table 1, footnote a.

Triton X-100, SDS, and tetradecyltrimethylammonium bromide (TTAB) for 3 h at room temperature.

RESULTS AND DISCUSSION

Amino acid modifications. Intact *B. thuringiensis* subsp. *israelensis* crystals exhibit LC₅₀s for *A. aegypti* larvae of 1 to 5 ng/ml (11, 19). Toxicity at such low concentrations implies a high degree of specificity in the host-toxin interaction. One approach to characterizing this specificity is to identify those amino acid side chains which are essential for toxicity. Previously we showed (12) that the tyrosine residues of both intact and solubilized *B. thuringiensis* subsp. *israelensis* proteins can be iodinated without loss of larvicidal activity. We have now examined modifications which block the cysteine-cystine side chains (Table 1) and lysine side chains (Table 2). Solubilized toxin preparations were used to allow unrestricted access to the protein interiors by the modifying reagents. LC₅₀s were determined by using the latex bead assay developed (12) to allow solubilized components to enter the guts of filter-feeding mosquito larvae.

Purified *B. thuringiensis* subsp. *israelensis* crystals are reported to contain 2.1% cysteine-cystine (18). The effect on larval toxicity of modifying these residues was examined (Table 1). The disulfide bonds could be cleaved with full retention of toxicity, and once cleaved, the resulting sulfhydryl molecules could be blocked with a very slight reduction in toxicity. Two of the sulfhydryl blocking procedures substantially increased toxicity. Both Hg²⁺ and 2-mercaptoethanol (the cleavage product of 2-hydroxyethyl-disulfide) show intrinsic toxicity to *A. aegypti* larvae (7, 13). These compounds may have entered the larval gut by attaching to the toxin. The nonessential nature of the toxin sulfhydryl molecules in *B. thuringiensis* subsp. *israelensis* is in marked contrast to that of the lepidoptera-active *B. thuringiensis* toxins which resemble sulfhydryl-activated toxins (8). For example, blocking of the sulfhydryl molecules generated by disulfide cleavage of strain HD-1 (isolated from a commercial insecticidal formulation called Dipel [Abbott Laboratories, North Chicago, Ill.]) toxin resulted in a greater than 160-fold decrease in toxicity (10).

Purified *B. thuringiensis* subsp. *israelensis* crystals contain 4.2% lysine (18). Unlike sulfhydryl modification, modification of the exposed lysines led to a loss of toxicity (Table 2). The extent of that loss was dependent on the extent of the modification. The chemical or ionic nature of the modified residue did not appear to influence the loss of larval toxicity: guanidination was positive, carbamylation was neutral, suc-

TABLE 1. Effect of cysteine and cystine modification on larval toxicity of solubilized *B. thuringiensis* subsp. *israelensis* crystal protein

Reagent	Modification	% Toxicity ^a
2-Mercaptoethanol	Disulfide cleavage	100
Dithioerythritol	Disulfide cleavage	100
5,5'-dithiobis(2-nitro benzoic acid) (Ellman's reagent) ^b	Blocks sulfhydryls	95
1,3-Propane sultone ^b	Blocks sulfhydryls	95
Iodoacetamide ^b	Blocks sulfhydryls	90
HgCl ₂ ^b	Blocks sulfhydryls	350
2,2-Dithiopyridine ^b	Disulfide interchange	95
2-Hydroxyethyl-disulfide ^b	Disulfide interchange	120

^a Percent toxicity = LC₅₀ (untreated toxin)/LC₅₀ (modified toxin). Values reported are the averages of two or three determinations. Percent toxicity is reported because the control LC₅₀s for independent experiments varied (100 to 220 ng/ml) depending on minor variations in the latex bead assay (12).

^b Disulfide bonds were cleaved with dithioerythritol before treatment.

cinylation was negative, and dansylation was hydrophobic. It appeared that free lysine residues were necessary for larvicidal activity of *B. thuringiensis* subsp. *israelensis* protein toxin, but firm conclusions must await quantification of the lysine residues modified by each procedure.

Organic solvents. Separation of the multiple *B. thuringiensis* subsp. *israelensis* subunits (9, 21) is essential to identification of the larvicidal toxin(s). This separation is not trivial and techniques such as high-pressure liquid chromatography in organic solvents may be necessary (20). We tested the effect of 10 organic solvents commonly used in high-pressure liquid chromatography on the larval toxicity of solubilized crystal protein. Sulfolane was chosen because of its recent use in the separation of hydrophobic proteins (20). Also chosen were four of the eight solvent types recognized by Snyder (14). The sensitivity of the toxin to solvent depended on the solvent type (Table 3). The toxin exhibited a high tolerance to methanol and ethylene glycol but was inactivated by >10% acetone, sulfolane, or acetonitrile. This inactivation was probably due to protein denaturation since the extent of protein adsorption to the latex beads remained unchanged. No larval mortality was observed in the controls with organic solvents but no protein toxin. The organic amines were not included in the present study of organic solvents because they have LC₅₀s of ≤0.01% (vol/vol) for *A. aegypti* larvae (7).

Denaturants. Three potential denaturants were examined (Table 4). Solubilized *B. thuringiensis* subsp. *israelensis* toxin was exposed to 4, 6, and 8 M concentrations of NaBr, urea, and guanidine hydrochloride, respectively. Complete toxicity was maintained in 8 M NaBr. This result was not surprising since the intact crystals were harvested from 3.8 to 4.2 M NaBr. The remarkable stability of the *B. thuringiensis* subsp. *israelensis* toxin also extended to urea but not to guanidine hydrochloride. After treatment with 6 M guanidine hydrochloride the toxin was inactivated; the LC₅₀ was so high that it could not be precisely determined.

Detergents. *B. thuringiensis* subsp. *israelensis* toxins may encounter detergents in several ways: as antifoam during their production; by application to polluted waters; or through commercial formulations which are oil emulsions for the solubilization of lipophilic sunscreens or antimicrobial agents (procedures designed to increase the persistence of the toxin). Additionally, *B. thuringiensis* subsp. *israelensis* toxin may be encapsulated in lipid (2) so that its increased buoyancy will make it effective against surface-feeding mos-

TABLE 3. Effect of organic solvents on larval toxicity of solubilized *B. thuringiensis* subsp. *israelensis* crystal protein

Organic solvent	% Tolerated ^a
Ethylene glycol.....	50
Methanol.....	40
Dioxane.....	30
Ethanol.....	25
2-Propanol.....	25
Acetone.....	10
Acetonitrile.....	10
Sulfolane.....	10
Dimethylformamide.....	10 ^b
Dimethyl sulfoxide.....	10 ^b

^a Concentrations from 0 to 50% (vol/vol) were tested. The concentration indicated is the maximum which retained 80% or more of the toxicity of the untreated solubilized control. Toxicity was determined by the latex bead assay (12) at a toxin concentration of 400 ng/ml.

^b Maximum concentration tested.

TABLE 4. Effect of denaturants on larval toxicity of solubilized *B. thuringiensis* subsp. *israelensis* crystal protein

Potential denaturant	Concn (M)	% Toxicity ^a
NaBr	4	100
	6	100
	8	100
Urea	4	87
	6	50
	8	16
Guanidine hydrochloride	4	10
	6	<6
	8	<6

^a See Table 1, footnote a.

quito larvae such as *Anopheles* sp. However, the effects of detergents could not be studied with solubilized *B. thuringiensis* subsp. *israelensis* toxin, because even low levels of detergent prevent protein binding to the latex beads (G. A. Couche and K. W. Nickerson, manuscript in preparation). Instead, intact *B. thuringiensis* subsp. *israelensis* crystals have been employed. This choice is also more appropriate for identifying incompatible constituents of commercial formulations or polluted waters.

The sensitivity of the toxin to detergents was strongly dependent on the type of detergent employed (Table 5). The neutral detergent Triton X-100, unlike the charged detergents SDS (-) and TTAB (+), did not affect toxicity at any of the concentrations tested. TTAB completely prevented *B. thuringiensis* subsp. *israelensis* toxicity at all concentrations tested, whereas 0.1% SDS decreased toxicity by 73%. SDS is itself toxic to *A. aegypti* larvae (LC₅₀ = 0.1% [7]), but the residual larval mortality observed at higher SDS concentrations (Table 5) was not due to SDS because the final SDS concentrations in the bioassays were well below the level toxic to larvae (7). Also, controls with detergents but without *B. thuringiensis* subsp. *israelensis* toxin did not kill the larvae.

TABLE 5. Effect of detergents on larval toxicity of intact *B. thuringiensis* subsp. *israelensis* crystal protein

Detergent	Concn (%) ^a	% Larval mortality ^b
Triton X-100	0.1	100
	0.5	100
	1.0	100
	5.0	100
	10.0	100
SDS	0.1	27
	0.5	14
	1.0	8
	5.0	15
TTAB	10.0	6
	0.1	0
	0.5	0
	1.0	0
	5.0	0
	10.0	0

^a Triton X-100 expressed as vol/vol; SDS and TTAB expressed as wt/vol.

^b Larval mortality was determined with intact crystals at 10 ng/ml. In the absence of detergent treatment, this toxin level resulted in 100% larval mortality.

Armstrong et al. (1) reported that the *B. thuringiensis* subsp. *israelensis* toxin contains protein domains which are extremely resistant to proteolysis. Our findings extended their stability observations to include many potential denaturants. The unusual stability of the *B. thuringiensis* subsp. *israelensis* toxin should be an important feature in its use as a biological control agent.

The *B. thuringiensis* subsp. *israelensis* toxin differs from other *B. thuringiensis* toxins in several major characteristics, e.g., in its target organisms, in the multiplicity of the protein subunits in the crystal, and in the general toxicity of solubilized toxin to eucaryotic cells. This list of important distinctions must now include the fact that the *B. thuringiensis* subsp. *israelensis* toxin is not a sulfhydryl-activated toxin.

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