

University of Nebraska - Lincoln

DigitalCommons@University of Nebraska - Lincoln

---

Papers in Microbiology

Papers in the Biological Sciences

---

October 1994

## Comparison of Disulfide Contents and Solubility at Alkaline pH of Insecticidal and Noninsecticidal *Bacillus thuringiensis* Protein Crystals

Cheng Du

*University of Nebraska-Lincoln*

Phyllis A.W. Martin

*Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland*

Kenneth W. Nickerson

*University of Nebraska-Lincoln, knickerson1@unl.edu*

Follow this and additional works at: <https://digitalcommons.unl.edu/bioscimicro>



Part of the [Microbiology Commons](#)

---

Du, Cheng; Martin, Phyllis A.W.; and Nickerson, Kenneth W., "Comparison of Disulfide Contents and Solubility at Alkaline pH of Insecticidal and Noninsecticidal *Bacillus thuringiensis* Protein Crystals" (1994). *Papers in Microbiology*. 31.

<https://digitalcommons.unl.edu/bioscimicro/31>

This Article is brought to you for free and open access by the Papers in the Biological Sciences at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Papers in Microbiology by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

## Comparison of Disulfide Contents and Solubility at Alkaline pH of Insecticidal and Noninsecticidal *Bacillus thuringiensis* Protein Crystals

CHENG DU,<sup>1</sup> PHYLLIS A. W. MARTIN,<sup>2</sup> AND KENNETH W. NICKERSON<sup>1\*</sup>

*School of Biological Sciences, University of Nebraska, Lincoln, Nebraska 68588-0343,<sup>1</sup> and Insect Biocontrol Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705<sup>2</sup>*

Received 24 February 1994/Accepted 1 August 1994

**We compared two insecticidal and eight noninsecticidal soil isolates of *Bacillus thuringiensis* with regard to the solubility of their proteinaceous crystals at alkaline pH values. The protein disulfide contents of the insecticidal and noninsecticidal crystals were equivalent. However, six of the noninsecticidal crystals were soluble only at pH values of  $\geq 12$ . This lack of solubility contributed to their lack of toxicity. One crystal type which was soluble only at pH  $\geq 12$  (strain SHP 1-12) did exhibit significant toxicity to tobacco hornworm larvae when the crystals were presolubilized. In contrast, freshly prepared crystals from the highly insecticidal strain HD-1 were solubilized at pH 9.5 to 10.5, but when these crystals were denatured, by either 8 M urea or autoclave temperatures, they became nontoxic and were soluble only at pH values of  $\geq 12$ . These changes in toxicity and solubility occurred even though the denatured HD-1 crystals were morphologically indistinguishable from native crystals. Our data are consistent with the view that insecticidal crystals contain distorted, destabilized disulfide bonds which allow them to be solubilized at pH values (9.5 to 10.5) characteristic of lepidopteran and dipteran larval midguts.**

*Bacillus thuringiensis* is a gram-positive spore-forming bacterium widely used for the microbial control of insects. The insecticidal activity resides in a proteinaceous parasporal crystal, called the  $\delta$ -endotoxin, which is formed during sporulation. Except for crystal formation, *B. thuringiensis* is indistinguishable from the common soil bacterium *Bacillus cereus* (16). Some taxonomists believe *B. cereus* and *B. thuringiensis* to be a single species (34). Many genes for crystal biosynthesis are plasmid encoded, and these plasmids may be transmissible from *B. thuringiensis* to *B. cereus* by conjugation (15). Initially, strains of *B. thuringiensis* were isolated from insect cadavers, and not surprisingly, those strains were usually toxic to the insect from which they had been isolated (36). These findings led to the view that *B. thuringiensis* makes a crystal to kill insect larvae, thus providing a suitable medium for its subsequent proliferation (2).

More recently, however, many thousands of *B. thuringiensis* variants have been isolated from soil samples (24), animal feed mills (25), and the phylloplane of deciduous and conifer trees (38). Interestingly, many of these crystal-forming isolates (ca. 40% from soil samples [24] and 55% from animal feed mills [25]) have not yet been shown to be toxic to insects. Martin and Travers (24) obtained 8,916 isolates of *B. thuringiensis* from 785 of 1,115 soil samples examined. They concluded that *B. thuringiensis* was a ubiquitous soil organism and that there was no correlation between the presence of *B. thuringiensis* in a particular soil sample and the current presence of insects in that locality. The prevalence of apparently noninsecticidal crystals (24, 25) raises the question of why bacteria would produce noninsecticidal protein crystals. In bacilli, sporulation is triggered by starvation conditions (4), and yet the crystals typically constitute 25 to 30% of the total protein in a sporulated culture of *B. thuringiensis* (26, 36). Furthermore, roughly 80 to 85% of the amino acids used in crystal synthesis

derive from turnover of vegetative-cell proteins (26). On the basis of these considerations, the synthesis of noninsecticidal crystals appears to be extremely wasteful.

Possible reasons that bacteria produce noninsecticidal protein crystals include the following. (i) Insecticidal crystals are only fortuitously insecticidal; they also serve another purpose in nature (23). (ii) The noninsecticidal crystals have not yet been tested against the appropriate host. (iii) The noninsecticidal crystals would exert synergistic toxicity if tested in combination with another strain of *B. thuringiensis* or another insect pathogen. (iv) The noninsecticidal crystals need to be heat activated to become toxic. The existence of an intervening energy barrier in insecticidal crystal proteins would provide a *raison d'être* for the crystal-associated chaperonins recently discovered in *B. thuringiensis* (9, 40). (v) The crystals formed in nature are insecticidal, but those formed by the same bacteria under laboratory conditions are not. That is, crystals formed under different conditions exhibit different properties. Distinctive natural environments might include anaerobic conditions, high nitrate conditions, or extremes of temperature or pH. For instance, *B. thuringiensis* subsp. *aizawai* proteins expressed in *Escherichia coli* formed bipyramidal crystals at 30°C and amorphous inclusions at 37°C (31). (vi) The noninsecticidal crystals are permanently inactive, but they constitute a convenient pool from which insecticidal variants can arise, either by mutation or by having multiple toxin-encoding plasmids resident in the same bacterium. One mechanism by which the noninsecticidal crystals would be permanently inactive would be their insolubility during passage through the lepidopteran and dipteran midguts (pH 9.5 to 10.5).

These ideas are not mutually exclusive. The solubility-insolubility idea in particular is compatible with each of the others. The present paper examines eight noninsecticidal soil isolates of *B. thuringiensis* (24). For six of these isolates, the principal reason that they are noninsecticidal is that their crystals are solubilized only at pH values of  $\geq 12$ .

\* Corresponding author. Phone: (402) 472-2253. Fax: (402) 472-8722.

## MATERIALS AND METHODS

**Organisms and culture conditions.** Crystals of strain HD-1 were purified from a concentrated spore-crystal slurry obtained from a production fermentor (Abbott Laboratories, North Chicago, Ill.). The eight noninsecticidal strains of *B. thuringiensis* were among the 8,916 soil isolates obtained previously (24) by acetate selection (39). They were selected because they produced large, bipyramidal, parasporal crystals containing 130- to 140-kDa protein subunits and had been found (24) to be nontoxic to larvae of *Bombyx mori* (silkworm), *Trichoplusia ni* (cabbage looper), and *Culex pipiens* (mosquito). These insect bioassays (24) used cells grown at 30°C on the T3 medium described by Travers et al. (39). The following strains were isolated from the sources indicated: SHP, from a path in a park in Silver Spring, Md.; HMN, from a cicada exit hole in a yard in Hope Mills, N.C.; CHO, from soil adjacent to the Cho La glacier in Nepal, India; and GST, from a grassy patch over a septic tank in Silver Spring, Md. Each of the eight strains was identified as *B. cereus* with the API (Montalieu-Vercieu, France) 50 CHB Bacillus identification system. These strains are available from Phyllis A. W. Martin (U.S. Department of Agriculture, Beltsville, Md.).

*B. thuringiensis* cultures were grown on a glucose-yeast extract-salts medium (30) at 25 to 27°C on a New Brunswick Scientific G-52 shaker with gyratory agitation at 200 rpm. After sporulation, the cultures were harvested and washed twice in distilled water. The crystals were purified on sodium bromide density gradients (1) modified to contain 7.5% (vol/vol) ethanol. The crystals were harvested at densities between 1.320 and 1.340 g/cm<sup>3</sup>, washed extensively to remove the NaBr, and resuspended in water. Protein contents were determined by the method of Lowry et al. (22). HMN 1-36 crystals did not form a discrete band on NaBr gradients. They were purified by the chloroform extraction method of Murray and Spencer (27). The crystals were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% acrylamide) as described by Pfannenstiel et al. (32) and by electron microscopy as described by Calabrese et al. (7).

**Insect bioassays.** Bioassays for *B. mori*, *C. pipiens*, and *T. ni* were conducted as described by Martin and Travers (24). For *Manduca sexta* (tobacco hornworm), the bioassay procedures followed those recommended by Schesser et al. (37). Eggs were obtained from the USDA Agricultural Research Service (Fargo, N.Dak.), hatched on an artificial diet (41), and grown at 26°C on a 16-h-light-8-h-dark cycle. After 5 days, second-instar larvae were transferred to individual S-100 1 oz. (ca. 28 g) plastic portion cups with LS-1 lids (Prairie Packaging, Inc., Bedford Park, Ill.) containing ca. 10 ml of the same diet but with the formalin omitted. Toxin samples (100 µl) were spread as uniformly as possible over the surface of the agar medium. Larval mortality was recorded daily for 7 days, and larval weight was determined after 7 days. Ten larvae were used for each toxin concentration. Bioassay procedures for the mosquito *Aedes aegypti* followed those recommended by Pfannenstiel et al. (32).

**Protein disulfide.** Free sulfhydryls were measured by the Ellman method as described by Riddles et al. (35). In addition, two methods were used to measure the disulfide content of the crystals. In the first, the total disulfide and sulfhydryl contents were estimated by the disodium 2-nitro-5-thiosulfobenzate (NTSB) procedure (10). Protein disulfide levels were determined indirectly by subtracting the free sulfhydryls from the total disulfide and sulfhydryl content (10). In the second method, protein disulfide levels were also determined directly by the vacuum hydrolysis-high-performance liquid chromatog-

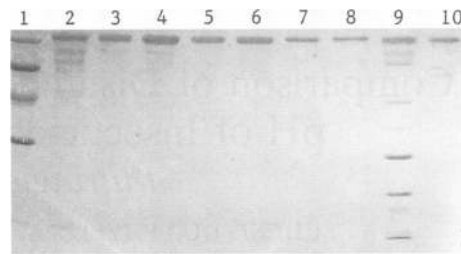


FIG. 1. Protein composition of noninsecticidal *B. thuringiensis* crystals determined by SDS-PAGE. Lanes: 1, molecular mass standards (29, 45, 67, and 116 kDa); 2, SHP 1-4; 3, SHP 1-12; 4, SHP 2-14; 5, SHP 2-17; 6, SHP 2-19; 7, CHO 1-14; 8, GST 2-36; 9, HMN 1-36; 10, HD-1.

raphy (HPLC) method of Chang and Knecht (8). Dried crystals (20 to 25 µg) were placed in a vacuum hydrolysis tube with 240 µl of 6 N HCl (sequanal grade; Pierce Chemical Co., Rockford, Ill.), flushed with argon, and evacuated to 0.03 mm Hg (ca. 3.9 Pa). After hydrolysis at 110°C for 24 h the samples were dried, mixed with 30 µl of 50 mM sodium bicarbonate (pH 8.1) and 50 µl of 4 mM dabsyl chloride in acetonitrile, and heated at 70°C for 10 min. The derivatized samples were then diluted to 500 µl with 50 mM potassium phosphate (pH 8.1)-ethanol (1:1) and applied (50 µl) to a Vydac C<sub>18</sub> reverse-phase column (5-µm bead diameter, 4 by 250 mm; The Separations Group, Hesperia, Calif.). Solvent A was 40 mM sodium acetate (pH 6.60) containing 3% dimethylformamide. Solvent B was acetonitrile. With the use of an ISCO (Lincoln, Nebr.) model 2350 pump and model 2360 gradient programmer, solvent B was increased from 16 to 40% over the first 18 min, held at 40% from 18 to 22 min, increased from 40 to 90% over the next 6 min, and then held at 90% for a further 5 min. The column was run at 44°C, and peaks were detected at 436 nm. For both methods, lysozyme (EC 3.2.1.17; Sigma, St. Louis, Mo.) and amino acid standard H (Pierce Chemical Co.) were used as the disulfide standards. Values are expressed as 10<sup>-9</sup> mol of disulfide per mg of protein.

**Crystal solubilization and presolubilization.** In the solubilization assay, 75 µl of an intact crystal suspension (2 mg/ml in distilled water) was mixed with 75 µl of a buffer containing 10 mM EDTA and 50 mM CAPS (3-cyclohexylaminopropanesulfonic acid) adjusted to pH values from 8.8 to 12.9 with NaOH. The pH values were measured with a Beckman Altex 41 pH meter; values were checked before and after crystal addition. The mixtures were incubated at 37°C for 2 h and then centrifuged for 10 min in an Eppendorf 5441 centrifuge. The supernatant (50 µl) was removed, and its protein content was determined by the method of Lowry et al. (22). Bovine serum albumin was used as the protein standard. All readings were performed in duplicate and averaged. For those solubilization assays which included β-mercaptoethanol (4.6 mM), 100 mM Tris was used instead of CAPS because of the lower pK<sub>a</sub> value of Tris. For presolubilization prior to bioassay, the crystals were solubilized in 50 mM Na<sub>2</sub>CO<sub>3</sub> (pH 10) with 4.6 mM β-mercaptoethanol.

## RESULTS

**Protein subunit composition.** The eight noninsecticidal strains of *B. thuringiensis* used in this study were chosen because they produced large bipyramidal crystals ca. 1 to 2 µm in length. All of the crystals contained 130- to 135-kDa protein subunits (Fig. 1); this feature may be necessary for the assembly of bipyramidal crystals. However, a given crystal

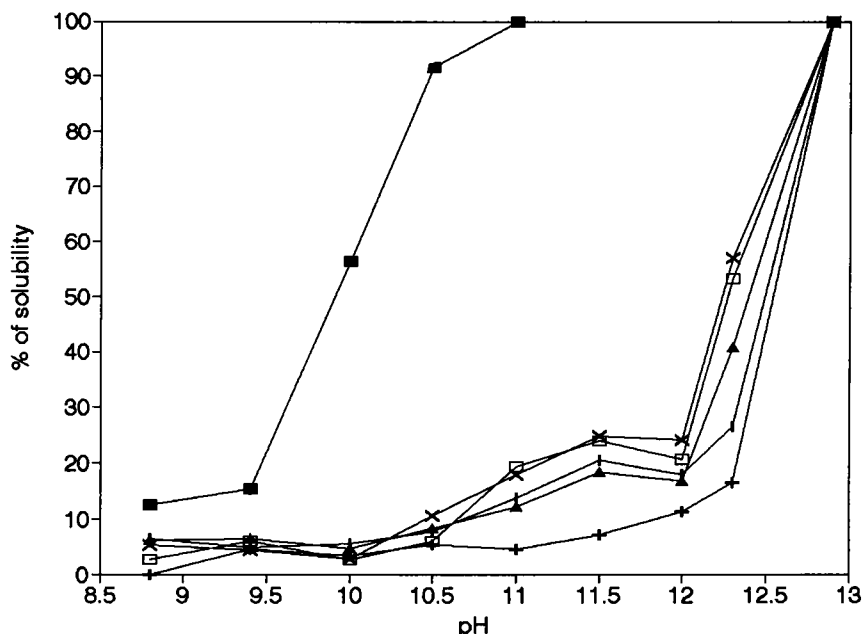


FIG. 2. Comparison of alkaline solubilities of crystals from insecticidal HD-1 and noninsecticidal SHP strains of *B. thuringiensis*. No reducing agents were included in the solubility assays. ■, HD-1; ▲, SHP 1-4; ▨, SHP 1-12; □, SHP 2-14; ×, SHP 2-17; +, SHP 2-19.

shape does not presuppose a given protein composition (7). For the eight noninsecticidal crystals examined in Fig. 1, CHO 1-14, GST 2-36, and the five SHP strains contained only 130- to 135-kDa protein subunits while HMN 1-36 (lane 9) contained proteins that were ca. 130 to 135, 75, 47, 27, and 18 kDa in size. The pattern for HMN 1-36 resembles that seen for mosquitoicidal *B. thuringiensis* subsp. *israelensis* crystals (32). As expected, the well-studied *B. thuringiensis* subsp. *kurstaki* HD-1 crystals (lane 10) contained both P1 proteins at 130 to 135 kDa and P2 proteins at 70 kDa. Some preparations of CHO 1-14 crystals also exhibited presumptive P2 proteins at 70 kDa (data not shown).

**Crystal solubility.** Insecticidal and noninsecticidal crystals were characterized with regard to the percent protein solubilized at progressively more alkaline pH values (Fig. 2). As expected, crystals from the highly insecticidal *B. thuringiensis* HD-1 started to solubilize at pH 9.5 and were fully solubilized by pH 11.0 (Fig. 1). Insecticidal crystals from strain HD-73 were also readily solubilized (Table 1). In contrast, all five of the noninsecticidal SHP crystals were insoluble at pH values of  $\leq 12$  (Fig. 1), as were the crystals from strain HMN 1-36 (Table 1). Crystals from strain CHO 1-14 exhibited an intermediate solubility while those from strain GST 2-36 were equally as soluble as those from strain HD-1 (Table 1). For each crystal type, solubilization at pH 10 could be achieved by including 5 mM  $\beta$ -mercaptoethanol in the solubilization buffer. Crystals from strain SHP 2-19 were selected for three variations on these solubility studies. The absence of solubilization at pH  $\leq 12$  was still observed (i) in an atmosphere of  $N_2$  gas instead of air, (ii) by using freshly prepared nonlyophilized crystals instead of lyophilized crystals, and (iii) after all heat treatments tested (10 min), ranging from 60 up to 121°C.

**Disulfide content.** Protein interchain disulfide bonds are thought to be responsible for the insolubility of *B. thuringiensis* crystals (5, 20, 29). Lecadet (20) observed that bipyramidal crystals were converted into large, swollen spheres when suspended in 8 M urea and then reverted to their original

bipyramidal shapes when the urea was removed by dialysis. This sequence of shape changes was observed for all eight noninsecticidal protein crystals. In 8 M urea, they formed spheres that were ca. 6 to 10  $\mu$ m in diameter. Along with the enhanced solubility in the presence of  $\beta$ -mercaptoethanol, these data indicate that the noninsecticidal crystals are held together by interchain disulfide bonds.

Purified crystals from the eight noninsecticidal strains were also examined with regard to their free sulfhydryl and protein disulfide contents (Table 2). No free sulfhydryls were found in any of the crystals. This absence of free sulfhydryls is in agreement with previous findings on insecticidal *B. thuringien-*

TABLE 1. Alkaline solubilities and toxicities of *B. thuringiensis* protein crystals to *M. sexta*

Strain	Alkaline solubility (pH)	Toxicity <sup>a</sup> of:	
		Intact crystals	Solubilized crystals
SHP 1-4	$\geq 12$	Nontoxic	Nontoxic
SHP 1-12	$\geq 12$	Partially toxic	1.3 $\mu$ g/cm <sup>2</sup>
SHP 2-14	$\geq 12$	Nontoxic	Nontoxic
SHP 2-17	$\geq 12$	Nontoxic	Nontoxic
SHP 2-19	$\geq 12$	Nontoxic	Nontoxic
GST 2-36	9.5–10.5	2 $\mu$ g/cm <sup>2</sup>	ND
CHO 1-14	10.5–11.5	2 $\mu$ g/cm <sup>2</sup>	ND
HMN 1-36	11.5–12.5	Nontoxic	Nontoxic
HD-1			
Native	9.5–10.5	10 ng/cm <sup>2</sup>	ND
Urea dialyzed	$\geq 12$	Nontoxic	ND
Autoclaved	$\geq 12$	Nontoxic	—
HD-73 (native)	10–11.5	40 ng/cm <sup>2</sup>	ND

<sup>a</sup> Nontoxic, no toxicity or larval weight loss at a protein concentration of 5  $\mu$ g/cm<sup>2</sup>. At 1 to 2  $\mu$ g/cm<sup>2</sup>, SHP 1-4, 2-14, and 2-17 actually enhanced larval weight gain. Partially toxic, at 5  $\mu$ g/cm<sup>2</sup>, the average weight gain was 70% of that of the control group. Values given are the 50% lethal concentrations. ND, not determined. —, crystals were insoluble.

TABLE 2. Disulfide contents of insecticidal and noninsecticidal *B. thuringiensis* protein crystals

Strain	No. of assays	Disulfide content (nmol/mg of protein) <sup>a</sup>	% Free sulfhydryl	No. of disulfide bonds/130-kDa protein
SHP 1-4	3	71.5 ± 0.8	0	9.2 ± 0.1
SHP 1-12	3	72.3 ± 2.00	0	9.4 ± 0.3
SHP 2-14	3	57.0 ± 0.4	0	7.4 ± 0.1
SHP 2-17	3	69.0 ± 4.2	0	9.0 ± 0.5
SHP 2-19	3	73.1 ± 1.4	0	9.5 ± 0.2
GST 2-36	3	65.0 ± 3.3	0	8.4 ± 0.4
CHO 1-14	3	64.5 ± 2.7	0	8.4 ± 0.4
HMN 1-36	3	58.3 ± 2.7	0	7.6 ± 0.4
HD-1	3	73.2 ± 7.4	0	9.5 ± 1.0
HD-73	4	58.7 ± 3.8	ND <sup>b</sup>	7.6 ± 0.5

<sup>a</sup> By the HPLC method of Chang and Knecht (8).

<sup>b</sup> ND, not done.

sis crystals (5, 13). Also, the protein disulfide contents of noninsecticidal crystals ranged from 57 to 73 nmol/mg of protein (Table 2), equivalent to 7.4 to 9.5 disulfide bonds per 130-kDa protein. The data in Table 2, obtained by the method of Chang and Knecht (8), were confirmed for HD-1 and the five SHP crystals by the NTSB method of Damodaran (10). The disulfide contents of the insecticidal and noninsecticidal crystals agreed within ±20% (data not shown). Thus, the altered solubility properties of insecticidal and noninsecticidal crystals cannot be attributed to quantitative differences in the numbers of disulfide bonds present.

#### Native, lyophilized, urea-dialyzed, and autoclaved crystals.

The solubility differences observed (Fig. 2) between HD-1 crystals and the five noninsecticidal SHP crystals do not indicate which of them is typical and which is atypical. Evidence that the insecticidal crystals exhibit atypical solubility characteristics is presented in Fig. 3. When native HD-1 crystals were analyzed immediately after purification on sodium bromide gradients, they were completely solubilized at pH values of ≤10 (Fig. 3). However, chemical or physical perturbation of the native crystals greatly altered their solubility properties. Crystals which had been frozen (−80°C) and lyophilized were only 50% solubilized at pH 10 (Fig. 3), while

crystals which had been subjected to high temperatures (Fig. 3) or 8 M urea (Table 2) were even less soluble. The temperatures tested ranged from 75 to 121°C (Fig. 3). The autoclaved HD-1 crystals were biologically inactive (Table 1) and insoluble at pH values of ≤12 (Fig. 3). Thus, in both regards the autoclaved crystals and crystals dialyzed with 8 M urea were indistinguishable from SHP crystals. At the time the urea dialysis procedure was introduced (20), the toxicity of the resulting crystals was not examined (21). However, we found that the urea-dialyzed crystals also were nontoxic (Table 1).

Interestingly, the HD-1 and SHP crystals which had been autoclaved (121°C for 20 min) were morphologically indistinguishable from native crystals when examined by light microscopy; the denatured crystals were still distinctly bipyramidal in shape. This point was studied in greater detail by electron microscopy (Fig. 4). Crystals which had been denatured by dialysis with 8 M urea (Fig. 4B) were indistinguishable from native crystals (Fig. 4A), but those which had been autoclaved while still bipyramidal were somewhat damaged in appearance (Fig. 4C).

**Toxicity to *M. sexta*.** None of the eight noninsecticidal *B. thuringiensis* strains had shown toxicity to larvae of the silkworm *B. mori* (24), and, as expected, purified crystals from six of the eight strains (HMN 1-36 and all of the SHPs) were nontoxic to larvae of the tobacco hornworm *M. sexta* (Table 1). However, the more easily solubilized GST 2-36 and CHO 1-14 crystals exhibited limited toxicity towards *M. sexta* larvae. They were 200-fold less toxic than HD-1 crystals and 50-fold less toxic than HD-73 crystals. Because of the similarity of their protein patterns to those of crystals from *B. thuringiensis* subsp. *israelensis*, HMN 1-36 crystals were also bioassayed versus the mosquito *A. aegypti*. However, no mosquito larval mortality was seen, even at crystal doses as high as 4 µg/ml.

Considering the remarkable alkaline stability of the SHP crystals (Fig. 2), we wondered whether in vitro, presolubilized crystal protein would be toxic to *M. sexta* larvae. For SHP 1-12, the answer was clearly yes. Presolubilized SHP 1-12 crystals gave a 50% lethal concentration of 1.3 µg/cm<sup>2</sup> (Table 1). Finally, in no case did heat treatments increase the toxicity of the five SHP crystals. Intact and presolubilized crystals were heated for 10 min at 60, 70, 80, 90, and 100°C. No toxicity was

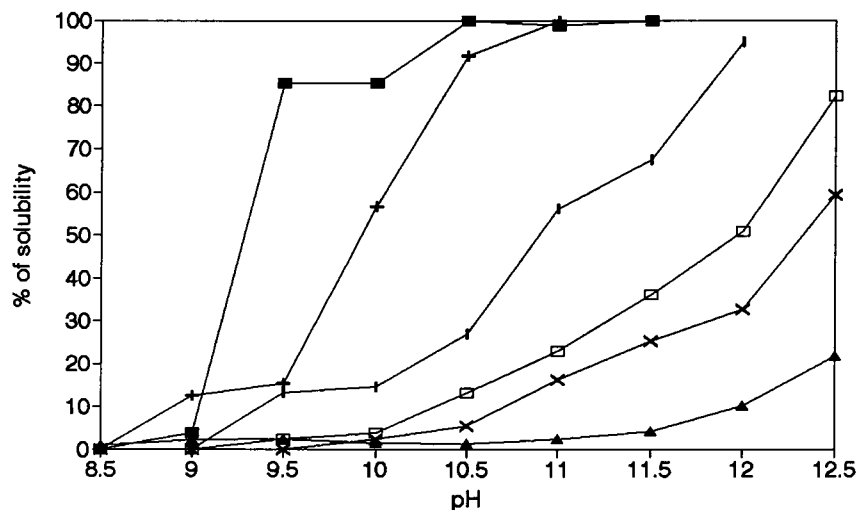


FIG. 3. Effect of temperature on alkaline solubilities of HD-1 protein crystals. Heat treatments were 10-min exposures to 75°C (○), 85°C (□), 90°C (×), and 121°C (▲) (autoclave conditions, 121°C for 20 min). ■, native crystals; +, lyophilized crystals.

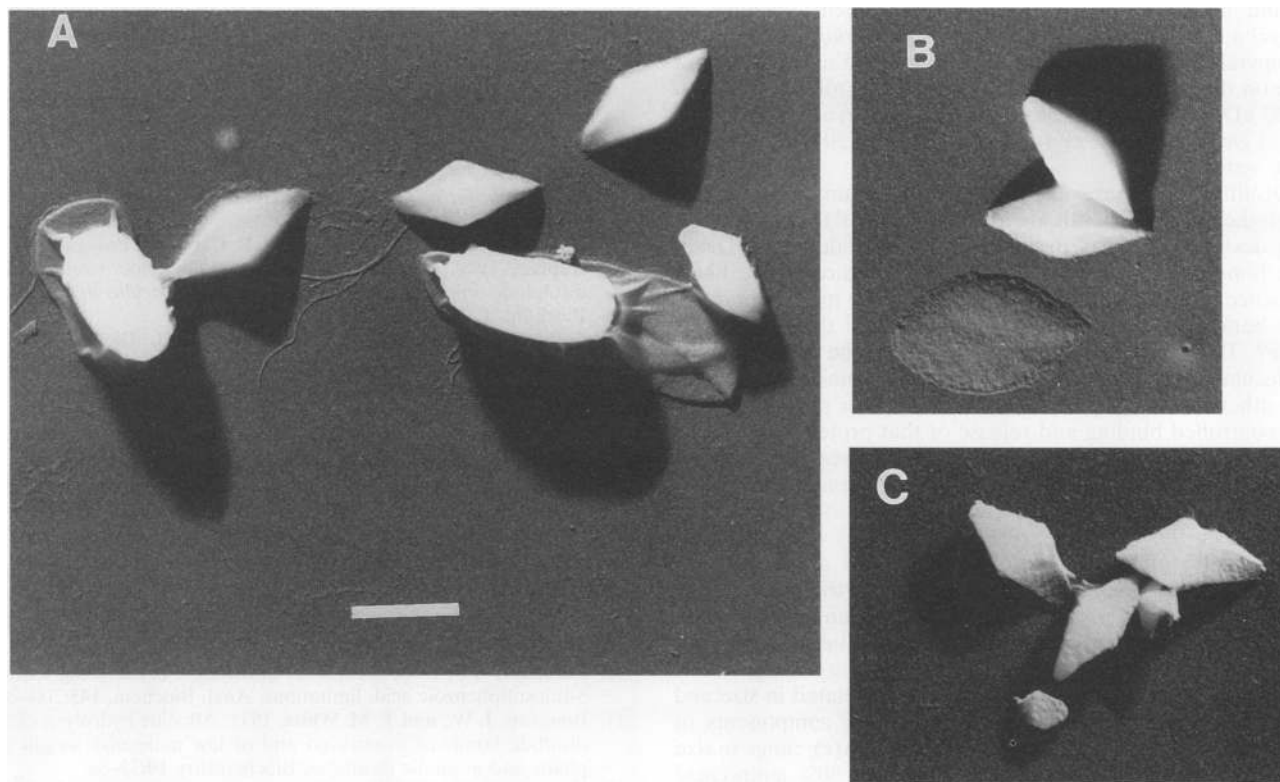


FIG. 4. Reversed-image shadow-casting transmission electron micrographs of crystals from *B. thuringiensis* SHP 2-14. (A) Native crystals; (B) crystals dialyzed with 8 M urea; (C) autoclaved crystals. Bar, 1  $\mu\text{m}$ . Magnification,  $\times 14,000$ .

observed. Thus, we have no evidence for the heat activation of noninsecticidal crystals.

#### DISCUSSION

There is a dichotomy in the solubility properties of the crystals produced by different strains of *B. thuringiensis*. Even though they had similar sizes and shapes and were composed of proteins with similar sizes and disulfide contents, the insecticidal and noninsecticidal crystals differed dramatically in the pH at which they were solubilized. Highly insecticidal crystals were solubilized at pH 9 to 10.5 (6, 14, 36), corresponding to the highly alkaline midguts of mosquito and lepidopteran larvae, whereas six of eight randomly selected noninsecticidal crystals were not solubilized until pH  $\geq 12$ . Similarly, Pietrantonio and Gill (33) reported that protein crystals from *B. thuringiensis* subsp. *shandongensis* were not solubilized until the pH reached  $\geq 12$  and that these crystals were not toxic to any insect larvae tested (*T. ni*, *Spodoptera exigua*, *Helicoverpa virescens* and the mosquitoes *A. aegypti* and *Culex quinquefasciatus*). It is likely that nonsolubility during passage through the larval gut is a frequent feature of noninsecticidal *B. thuringiensis* crystals. This idea is a variation of the earlier observation that insects with only slightly alkaline midguts (pH 8 to 8.5), such as the Mediterranean flour moth (*Anagasta kuehniella*), were not susceptible to intact crystals but were susceptible to presolubilized crystals (43).

Our crystal solubility data are consistent with the existence of two types of interchain disulfide bonds, one cleaved at pH 9 to 10.5 and the other cleaved at pH  $\geq 12$ . However, these solubility differences by themselves do not indicate which bond type is typical and which is atypical. Evidence that it is the pH

9 to 10.5 labile disulfides which are atypical is provided by the conversion of native HD-1 crystals to the form labile at pH  $\geq 12$  when they were denatured by either high temperatures or 8 M urea. This conclusion is in agreement with previous studies on disulfide bond stability in a wide range of protein model systems which found that alkaline disulfide bond cleavage occurred only at pH  $\geq 12$ . The model proteins studied include ovomucoid (11); chymotrypsin, chymotrypsinogen, trypsinogen, and RNases A and S (12); and lysozyme,  $\alpha$ -lactalbumin, and  $\alpha_{s1}$ - and  $\beta$ -casein (17). Denatured HD-1 crystals can now be added to this list. Note that our work concerns the 130- to 135-kDa CryI proteins, which are also known as the P1 proteins (42). The 70-kDa CryII (P2) proteins also produced by HD-1 are solubilized only at pH 11.5 to 12 (28, 42), even in their native form. Indeed, this difference in crystal solubilities is used as a convenient means to separate the P1 and P2 proteins (28).

The values reported in Table 2 are for total disulfide bonds; they do not distinguish between interchain and intrachain bonds. However, Bietlot et al. (5) have shown that for two crystal types, *entomocidus* and HD-1, all the disulfide bonds are interchain bonds. We assume that this generalization also holds true for the crystals that we have studied. Furthermore, the interchain disulfides are thought to remain intact during protein denaturation; they should not break and reshuffle. This view is supported by the restoration of bipyramidal morphology in denatured crystals (Fig. 4). We conclude that the alignment of disulfide bridges in native bipyramidal crystals is necessary and sufficient for the maintenance of that shape. That is, if the disulfide bridges remain intact, the tertiary structure of the protein subunits is irrelevant. This conclusion

should be valid for any protein crystal held together by interchain disulfide bonds, not just the *B. thuringiensis* crystals. A bipyramidal crystal that is 1.6  $\mu\text{m}$  in length and 0.8  $\mu\text{m}$  per side on the pyramid base should contain one million copies of a 130-kDa protein. This calculation is based on a crystal density of 1.3  $\text{g}/\text{cm}^3$  (1) and a crystal composition of 50% protein and 50% water.

Disulfide bonds are at an energy minimum (most stable) when their two C-S bonds are at an angle of 90° to one another. Any deviation from 90° destabilizes the disulfide bonds. Disulfide bond cleavage at pH 9 to 10.5 (6) indicates the likely presence of distorted, comparatively unstable interchain disulfide bonds (29). How might those distorted disulfide bonds arise? Two mechanisms seem feasible. The first involves molecular chaperones (18). These proteins bind to and stabilize otherwise-unstable conformers of another protein, and by the controlled binding and release of that protein, they facilitate its fate in vivo, be it folding, oligomeric assembly, transport to a particular subcellular compartment, or controlled switching between active and inactive conformations (18). They could steer the *B. thuringiensis* Cry proteins to metastable, more easily solubilized conformations. Two genes for presumptive chaperones closely associated with the genes for *B. thuringiensis* crystal proteins have recently been found (9, 40). The second mechanism involves the number of proteins present in a given crystal type. Many crystals are composed of multiple protein subunits which are closely related in size and sequence homology. For instance, the three components of HD-1 crystals, CryIA(a), CryIA(b), and CryIA(c), range in size from 131.0 to 133.3 kDa and show more than 80% amino acid identity (19). Significantly, the interactions of structurally related protoxins within an inclusion are probably important in determining its ease of solubility under alkaline conditions. As an example, *B. thuringiensis* subsp. *aizawai* HD133 produces inclusions containing three protoxins, CryIA(b), CryIC, and CryID. Aronson et al. (3) observed that a plasmid-cured strain of HD133 produced inclusions comprising only the CryIC and CryID protoxins and that these inclusions were less toxic because they were less soluble under alkaline conditions (3). The protein composition of multisubunit crystals would determine their solubility if the homologous proteins were sufficiently alike to cocrystallize into a single inclusion yet sufficiently different to cause mismatches or torsional stress in the resulting interchain disulfide bonds.

We do not yet know whether the nonsoluble-therfore-nontoxic model should be considered a paradigm for all noninsecticidal isolates or only for those isolates which produce bipyramidal crystals. Also, for the strains studied in this paper, GST 2-36 clearly does not fit the nonsoluble-therfore-nontoxic model (Table 1), and another explanation must be sought. Our search will now focus on those noninsecticidal isolates which produce amorphous, rhomboid, and irregular parasporal bodies rather than bipyramidal parasporal bodies.

#### ACKNOWLEDGMENTS

This research was supported by grants to K.W.N. from Entotech, Inc. (Davis, Calif.), the National Science Foundation (DCB-8919166), and the Consortium for Plant Biotechnology Research (593-0009-02).

We thank James Buckner (USDA, Fargo, N.Dak.) for providing the *M. sexta* eggs used in these experiments and Kit Lee for performing the electron microscopy.

#### REFERENCES

- Ang, B. J., and K. W. Nickerson. 1978. Purification of the protein crystal from *Bacillus thuringiensis* by zonal gradient centrifugation. *Appl. Environ. Microbiol.* **36**:625-626.
- Aronson, A. I. 1993. The two faces of *Bacillus thuringiensis*: insecticidal proteins and post-exponential survival. *Mol. Microbiol.* **7**:489-496.
- Aronson, A. I., E.-S. Han, W. McGaughey, and D. Johnson. 1991. The solubility of inclusion proteins from *Bacillus thuringiensis* is dependent on protoxin composition and is a factor in toxicity to insects. *Appl. Environ. Microbiol.* **57**:981-986.
- Bernlohr, R. W., and C. Leitzman. 1969. Control of sporulation, p. 183-213. In G. W. Gould and A. Hurst (ed.), *The bacterial spore*. Academic Press, London.
- Bietlot, H. P. L., I. Vishnubhatla, P. R. Carey, M. Pozsgay, and H. Kaplan. 1990. Characterization of the cysteine residues and disulphide linkages in the protein crystal of *Bacillus thuringiensis*. *Biochem. J.* **267**:309-315.
- Bulla, L. A., Jr., K. J. Kramer, and L. I. Davidson. 1977. Characterization of the entomocidal parasporal crystal of *Bacillus thuringiensis*. *J. Bacteriol.* **130**:375-383.
- Calabrese, D. M., K. W. Nickerson, and L. C. Lane. 1980. A comparison of protein crystal subunit sizes in *Bacillus thuringiensis*. *Can. J. Microbiol.* **26**:1006-1010.
- Chang, J.-Y., and R. Knecht. 1991. Direct analysis of the disulfide content of proteins: methods for monitoring the stability and refolding process of cysteine-containing proteins. *Anal. Biochem.* **197**:52-58.
- Crickmore, N., and D. J. Ellar. 1992. Involvement of a possible chaperonin in the efficient expression of a cloned Cry IIA  $\delta$ -endotoxin gene in *Bacillus thuringiensis*. *Mol. Microbiol.* **6**:1533-1537.
- Damodaran, S. 1985. Estimation of disulfide bonds using 2-nitro-5-thiosulphobenzoic acid: limitations. *Anal. Biochem.* **145**:200-204.
- Donovan, J. W., and T. M. White. 1971. Alkaline hydrolysis of the disulfide bonds of ovomucoid and of low molecular weight aliphatic and aromatic disulfides. *Biochemistry* **10**:32-38.
- Florence, T. M. 1980. Degradation of protein disulphide bonds in dilute alkali. *Biochem. J.* **189**:507-520.
- Ghosh-Dastidar, P., and K. W. Nickerson. 1979. Interchain crosslinks in the entomocidal *Bacillus thuringiensis* protein crystal. *FEBS Lett.* **108**:411-414.
- Gill, S. S., E. A. Cowles, and P. V. Pietrantonio. 1992. The mode of action of *Bacillus thuringiensis* endotoxins. *Annu. Rev. Entomol.* **37**:615-636.
- Gonzalez, J. M., Jr., B. J. Brown, and B. C. Carlton. 1982. Transfer of *Bacillus thuringiensis* plasmids coding for  $\delta$ -endotoxin among strains of *B. thuringiensis* and *B. cereus*. *Proc. Natl. Acad. Sci. USA* **79**:6951-6955.
- Gordon, R. E., W. C. Haynes, and C. H.-N. Pang. 1973. The genus *Bacillus*. *Agricultural handbook no. 427*. United States Department of Agriculture, Washington, D.C.
- Hasegawa, K., N. Okomoto, H. Ozawa, S. Kitajima, and Y. Takado. 1981. Limits and sites of lysinoalanine formation in lysozyme,  $\alpha$ -lactalbumin, and  $\alpha_1$ - and  $\beta$ -caseins by alkali treatment. *Agric. Biol. Chem.* **45**:1645-1651.
- Hendrick, J. P., and F.-U. Hartl. 1993. Molecular chaperone functions of heat-shock proteins. *Annu. Rev. Biochem.* **62**:349-384.
- Höfte, H., and H. R. Whiteley. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* **53**:242-255.
- Lecadet, M. M. 1967. Action comparée de l'urée et du thioglycolate sur la toxine figurée de *Bacillus thuringiensis*. *C. R. Acad. Sci. Ser. D* **264**:2847-2850.
- Lecadet, M. M. Personal communication.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
- Martin, P. A. W. 1994. An iconoclastic view of *Bacillus thuringiensis* ecology. *Am. Entomol.* **40**:85-90.
- Martin, P. A. W., and R. S. Travers. 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. *Appl. Environ. Microbiol.* **55**:2437-2442.
- Meadows, M. P., D. J. Ellis, J. Butt, P. Jarrett, and H. D. Burges. 1992. Distribution, frequency, and diversity of *Bacillus thuringiensis* in an animal feed mill. *Appl. Environ. Microbiol.* **58**:1344-1350.
- Monro, R. E. 1961. Protein turnover and the formation of protein

- inclusions during sporulation of *Bacillus thuringiensis*. *Biochem. J.* **81**:225–232.
27. Murray, E. D., and E. Y. Spencer. 1966. A simplified purification technique for parasporal inclusions from certain varieties of *Bacillus thuringiensis*. *J. Invertebr. Pathol.* **8**:418–420.
  28. Nicholls, C. N., W. Ahmad, and D. J. Ellar. 1989. Evidence for two different types of insecticidal P2 toxins with dual specificity in *Bacillus thuringiensis* subspecies. *J. Bacteriol.* **171**:5141–5147.
  29. Nickerson, K. W. 1980. Structure and function of the *Bacillus thuringiensis* protein crystal. *Biotechnol. Bioeng.* **22**:1305–1333.
  30. Nickerson, K. W., G. St. Julian, and L. A. Bulla, Jr. 1974. Physiology of spore forming bacteria associated with insects: radiorespirometric survey of carbohydrate metabolism in the 12 serotypes of *Bacillus thuringiensis*. *Appl. Microbiol.* **28**:129–132.
  31. Oeda, K., K. Inouye, Y. Ibuchi, K. Oshie, M. Shimizu, K. Nakamura, R. Nishioka, Y. Takada, and H. Ohkawa. 1989. Formation of crystals of the insecticidal proteins of *Bacillus thuringiensis* subsp. *aizawai* IPL7 in *Escherichia coli*. *J. Bacteriol.* **171**:3568–3571.
  32. Pfannenstiel, M. A., E. J. Ross, V. C. Kramer, and K. W. Nickerson. 1984. Toxicity and composition of protease-free *Bacillus thuringiensis* var. *israelensis* crystals. *FEMS Microbiol. Lett.* **21**:39–42.
  33. Pietrantonio, P. V., and S. S. Gill. 1992. The parasporal inclusion of *Bacillus thuringiensis* subsp. *shandongensis*: characterization and screening for insecticidal activity. *J. Invertebr. Pathol.* **59**:295–302.
  34. Priest, F. G., M. Goodfellow, and C. Todd. 1988. A numerical classification of the genus *Bacillus*. *J. Gen. Microbiol.* **134**:1847–1882.
  35. Riddles, P. W., R. L. Blakeley, and B. Zerner. 1983. Reassessment of Ellman's reagent. *Methods Enzymol.* **91**:49–60.
  36. Rogoff, M. H., and A. A. Yousten. 1969. *Bacillus thuringiensis*: microbiological considerations. *Annu. Rev. Microbiol.* **23**:357–386.
  37. Schesser, J. H., K. J. Kramer, and L. A. Bulla, Jr. 1977. Bioassay for homogeneous parasporal crystals of *Bacillus thuringiensis* using the tobacco hornworm, *Manduca sexta*. *Appl. Environ. Microbiol.* **33**:878–880.
  38. Smith, R. A., and G. A. Couche. 1991. The phylloplane as a source of *Bacillus thuringiensis* variants. *Appl. Environ. Microbiol.* **57**:311–315.
  39. Travers, R. S., P. A. W. Martin, and C. F. Reichelderfer. 1987. Selective process for efficient isolation of soil *Bacillus* spp. *Appl. Environ. Microbiol.* **53**:1263–1266.
  40. Wu, D., and B. A. Federici. 1993. A 20-kilodalton protein preserves cell viability and promotes CytA crystal formation during sporulation in *Bacillus thuringiensis*. *J. Bacteriol.* **175**:5276–5280.
  41. Yamamoto, R. T. 1969. Mass rearing of the tobacco hornworm. II. Larval rearing and pupation. *J. Econ. Entomol.* **62**:1427–1432.
  42. Yamamoto, T., and T. Iizuka. 1983. Two types of entomocidal toxins in the parasporal crystals of *Bacillus thuringiensis kurstaki*. *Arch. Biochem. Biophys.* **227**:233–241.
  43. Yamvriasis, C. 1962. Contribution à l'étude du mode d'action de *Bacillus thuringiensis* berliner vis-a-vis se la teigne de la farine. *Entomophaga* **7**:101–159.