

## Characterization of the pH-Mediated Solubility of *Bacillus thuringiensis* var. *san diego* Native $\delta$ -Endotoxin Crystals

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Native crystals of *Bacillus thuringiensis* var. *san diego*, a coleopteran-specific  $\delta$ -endotoxin, were metabolically labelled with [<sup>35</sup>S]methionine. Specific activity was 82,000 CPM/ $\mu$ g (2.44 Ci/mmol). Using a universal buffer formulated with the same ionic strength at every pH, we determined that native crystals dissolve above pH 10 and below pH 4. At the acidic pH, the rate of solubilization was substantially slower than at the alkaline pH. Recrystallization rates for the toxin were similar regardless of solubilization conditions. The banding patterns in denatured polyacrylamide gel electrophoresis were unaffected by solubilization conditions. Toxicity was higher for soluble toxin compared to crystal toxin, but virtually identical for the acidic and alkaline produced solutions. Acid solubilization is significant because of the acidic midgut of susceptible Coleoptera. © 1992 Academic Press, Inc.

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Interest in the  $\delta$ -endotoxin protein of *Bacillus thuringiensis* (*B.t.*) arises out of its insecticidal properties. The protein is packaged in a parasporal crystal that is released with the spore during cell lysis. It was over 40 years after the discovery of the insecticidal properties of *B.t.* that Angus (1) demonstrated that toxicity was attributable to this crystal inclusion. Numerous natural variations in the primary structure of the  $\delta$ -endotoxin exist and are responsible for differences in susceptible host range of each toxin (2). The earliest investigations of the chemical properties of the crystal inclusion were directed toward the lepidopteran-active CryI gene  $\delta$ -endotoxins. They share many physical properties (reviewed by 3) including shape of the crystal (bipyramidal), size of the protoxin (ca. 130 kDa), size of the enzymatically activated toxin (ca. 66 kDa), reliance on S-S bonding to maintain crystal integrity, and the pH required for solubilization of the crystal (alkaline).

The earliest success in solubilizing the crystals, which would not dissolve in standard buffers, was with 0.1 N NaOH (pH 13). The addition of reducing agents or gut juice allowed the pH to be lowered to the 7-10 range while preserving the toxicity of the protein (3, 4). These conditions are compatible, generally, with the midgut environment of susceptible insects. Differential, gut-mediated solubility, however, does contribute to *B.t.* toxicity (5).

More recently, crystal inclusions of the *B.t.* varieties *tenebrionis*, *san diego*, and EG2158 were discovered, which have coleopteran toxicity (6,7,8). These crystals, products of CryIII genes (2), are rhomboid and flat in shape (7), synthesized as a 73 kDa protein, cleaved in the crystal to ca. 66 kDa (9), and lack the S-S bonds (10), which obviate the requirement for reducing agents in solubilization. Crystals were, however, soluble using the same alkaline buffers that were used for CryI toxin crystals (11). The relevance of this response comes into question, given the acidic midgut environment of susceptible Coleoptera (12). Solubility, while generally not thought to be a barrier to toxicity in susceptible insects, is an essential step in its expression, and is potentially a factor in the development of resistance. This led us to make a careful investigation of the solubility properties of native crystals of the *B.t.* var. *san diego*.

## MATERIALS AND METHODS

**Reagents.** The [<sup>35</sup>S]methionine (1112 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA) and [<sup>14</sup>C]methemoglobin (20 μCi/mg) from New England Nuclear (Boston, MA). Components of bacterial growth media were from Difco (Detroit, MI). Safety-Solve scintillation cocktail was purchased from Research Products International (Prospect, IL). Other chemicals were analytical grade and obtained from Sigma or Baker.

**Organisms.** All experiments were performed with toxin produced by *B.t.* var. *san diego* obtained from Mycogen Corp. (San Diego, CA). This strain was reported to be identical to *B.t.* var. *tenebrionis* (13). Cultures are maintained in our lab on NYS/CAA agar slants and stored at 4°C. A laboratory colony of cottonwood leaf beetle, *Chrysomela scripta* (Coleoptera:Chrysomelidae), maintained on poplar foliage, was the source of insect material.

**Determination of midgut pH and assay of protease activity.** Midgut and hindgut pH was determined by feeding third-instar *C. scripta* larvae pH indicator dyes in 12% sucrose. After 15 minutes, the digestive tracts were excised and viewed under a stereo microscope. A quantitative measure of proteolytic activity was made with [<sup>14</sup>C]methemoglobin as a substrate using the procedure described by Murdock et al. (12). The [<sup>14</sup>C]methemoglobin was diluted with unlabelled methemoglobin to yield 10 μg and about 1,000 cpm/μL. Midguts, with contents, were excised from third instar larvae and stored at -85°C until needed. Extracts were collected in ddH<sub>2</sub>O by centrifugation. For each assay, 87 μL of universal buffer (Table 1), 3 μL of midgut extract, and 10 μL of [<sup>14</sup>C]methemoglobin were combined in a 1.5 mL microcentrifuge tube at 30°C for 15 minutes. The reaction was terminated by the addition of 100 μL of ice-cold 10% TCA. After standing on ice for 20 minutes, the tubes were centrifuged at 16,000 x g for 10 minutes. A 20 μL sample of each supernatant was pipetted into 3 mL of scintillation cocktail and counted.

**Synthesis of native crystals.** For the production of native [<sup>35</sup>S]crystals, 50 mL of vegetative liquid growth media (PWYE): peptone (50 g/L), yeast extract (1 g/L), and NaCl

(5 g/L), adjusted to pH 7.5, was inoculated from a *B.t.* slant. All incubations were in a water-bath shaker, at 30°C, 200 OPM, and in Ehrlemeyer flasks with a five-fold capacity over the incubated volume. After 6 h, a 10% transfer to a second flask of PWYE (50 mL) was then grown overnight (16 h). These cells were pelleted at 3000 RPM for 20 min. The pellet was resuspended in an equal volume of protein-free glucose-salt medium (GSM), which supports sporulation. This medium, adjusted to pH 7.2, contained: glucose (1 g/L);  $\text{KH}_2\text{PO}_4$  (25 mM);  $\text{K}_2\text{HPO}_4$  (25 mM);  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  (1.25 mM);  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (500  $\mu\text{M}$ );  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  (10  $\mu\text{M}$ );  $\text{ZnCl}_2$  (50  $\mu\text{M}$ );  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  (50  $\mu\text{M}$ );  $\text{CuCl}_3$  (20  $\mu\text{M}$ ). After a brief vortexing the cells were repelleted and then suspended in GSM (20 mL) to which [ $^{35}\text{S}$ ]methionine was added at the rate of 70  $\mu\text{Ci}/\text{mL}$ . After four days, spore and crystal formation was complete and the cells were lysed. To make unlabelled toxin the [ $^{35}\text{S}$ ]methionine was omitted in favor of 7.5 g/l peptone (PGSM). This media received a 10% inoculation directly from the overnight culture.

**Purification of Native Crystals.** The contents of the flasks, containing spores, crystals, and lysed cells, were collected and centrifuged (20,000 x g, 20 min). The supernatant with unincorporated [ $^{35}\text{S}$ ]methionine was discarded. The pellet was washed twice with cold  $\text{ddH}_2\text{O}$ , once with 2 M NaCl, and finally twice more with cold  $\text{ddH}_2\text{O}$ . The final pellet was resuspended in 6 mL of 0.02% Triton X-100 and dispersed with sonication for 1 min. This material was layered onto a discontinuous sucrose gradient (79%, 72%, 67% w/v) and ultracentrifuged at 80,000 x g and 4°C for 3 h, in a swinging-bucket rotor (Sorvall AH 627). The CryIII A  $\delta$ -endotoxin crystals formed a distinct band at the interface of the 72% and 79% sucrose. This material was collected and washed free of sucrose with several centrifugations and resuspensions in cold  $\text{ddH}_2\text{O}$  and stored in  $\text{ddH}_2\text{O}$  at 4°C. Purity was then analyzed with SDS-PAGE (14) on 4% to 15% gradient gels visualized on x-ray film after fluorographic enhancement.

**Protein determination and specific activity.** A sample of the crystal suspension was solubilized for 2 h at 37°C in 100 mM pH 11.5  $\text{NaCO}_3$  buffer. Following a centrifugation at 16,000 x g for 10 minutes, protein in an aliquot of supernatant was determined using the BioRad Protein Reagent in the microassay protocol. A standard curve was constructed using BSA. Another aliquot of supernatant was used for scintillation counting. Specific activity was reported as CPM/ $\mu\text{g}$ .

Table 1. Formulation of a universal buffer having constant ionic strength at all pH values. Stock contained 2.7 mL  $\text{H}_3\text{PO}_4$ , 2.29 mL  $\text{CH}_3\text{COOH}$ , and 2.48 g  $\text{H}_3\text{BO}_3$  brought to 100 mL with  $\text{ddH}_2\text{O}$ .

pH	Stock	KCl	2M NaOH	$\text{H}_2\text{O}$
30°C	( $\mu\text{L}$ )	(mg)	( $\mu\text{L}$ )	(mL)
2.3	1,111	71.0	67	10.6
2.8	1,000	61.8	180	10.62
4.01	1,000	55.6	245	11.20
5.03	833	42.2	292	10.12
6.01	833	35.3	350	10.65
7.04	833	25.8	437	11.43
7.98	714	8.6	429	10.29
9.05	714	1.8	486	10.80
9.98	714	0	557	11.44
11.3	625	0	530	10.31

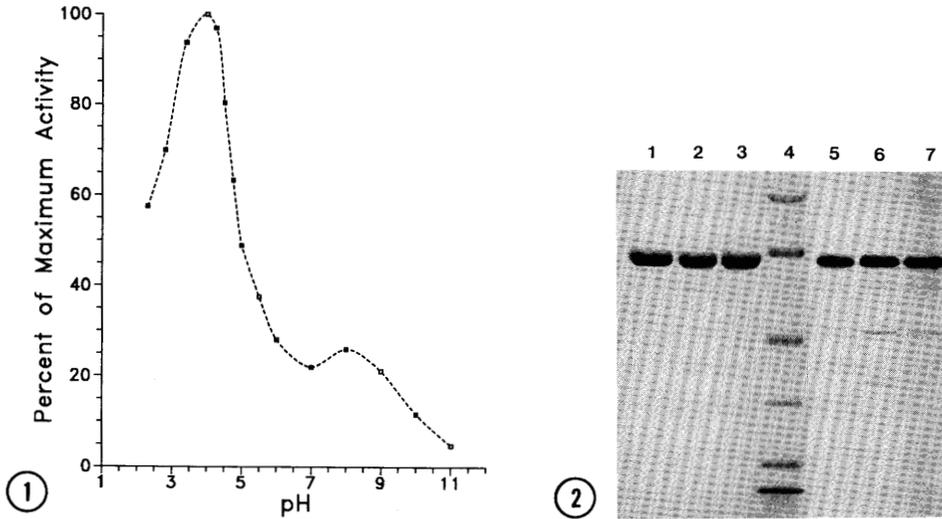
**Assay of pH-mediated solubility of native  $\delta$ -endotoxin crystals and their recrystallization.** For solubilization assays, 2  $\mu$ L of labelled crystals, (ca. 400,000 CPM) suspended in ddH<sub>2</sub>O, were added to 48  $\mu$ l of buffer (15), formulated according to Table 1. Intermediate pH values were achieved by proportional mixing of buffers. The pH of the buffers was determined using a Beckman PHI 40 meter. Incubations were done in 1.5 mL microcentrifuge tubes, at 30°C, with rapid shaking (1200 rpm) on an Eppendorf Thermomixer. The incubation period was 24 h for pH effects and for appropriate intervals for a determination of solubilization rate. After incubation, tubes were centrifuged at 16,000 x g for 10 min and 20  $\mu$ L of each supernatant was added to scintillation cocktail and counted. For recrystallization assays, soluble toxin was utilized in a similar protocol. The percent solubilization and recrystallization was based on the amount in the supernatant relative to the total radioactivity added to each tube. We assessed the integrity of each solubilized and recrystallized toxin with SDS-PAGE (14) and fluorography.

**Bioassay protocol.** The toxicity of solubilized crystal toxins was determined in insect bioassays. Toxicity was assayed in third-instar *C. scripta* larvae. Each larva was given, individually, a 2  $\mu$ L droplet of 22% sucrose with 3.75  $\mu$ g of unlabelled toxin on a poplar leaf disk. After ingestion of the dose, larvae were left on the disk overnight and transferred onto fresh foliage as needed.

## RESULTS AND DISCUSSION

In the *C. scripta* midgut, bromthymol blue and bromcresol purple were yellow, indicating a pH <6 and pH <5, respectively. Bromcresol green was blue-green, which estimated midgut pH between 4.5 and 5.0. The hindgut appeared slightly less acidic. These measurements were consistent with other Coleoptera (12). The peak of protease activity, measured by methemoglobin proteolysis, was consistent with this pH (Figure 1). It was clear from these results that the var. *san diego*  $\delta$ -endotoxin must express its toxicity under acidic conditions. There was almost none of the alkaline protease activity, typical of Lepidoptera, detected in the midgut. Acidic optima are characteristic of the cysteine proteases that predominate in guts of leaf-feeding beetles (17,18). Previously, the effect of midgut homogenate on processing of the CryIIIA  $\delta$ -endotoxin was determined at alkaline pH (9). Degradation was limited. The effect of coleopteran and lepidopteran homogenates was indistinguishable, perhaps due to the minimal alkaline protease activity in Coleoptera. It is expected that an acidic assay, which we show to be compatible with this toxin, would provide a different result.

Native crystals produced by *B.t.* var. *san diego*, with (Lane 7) or without (Lane 3) [<sup>35</sup>S]methionine had identical banding patterns in SDS-PAGE, confirming the quality of our probe (Figure 2). Specific activity was calculated at 82,000 CPM/ $\mu$ g, which permitted detection of ng amounts of toxin in assay supernatants. Metabolic incorporation of a radioactive amino acid is the ideal method of labelling the crystals to study their properties, because the characteristics of the native crystals are maintained. In contrast to the initiation of  $\delta$ -endotoxin expression during the midsporulation stage in other *B.t.* strains, initial expression of *B.t.* var. *tenebrionis*  $\delta$ -endotoxin occurs during vegetative growth (16). Since the CryIIIA gene has been completely sequenced and contains only 9 methionine residues

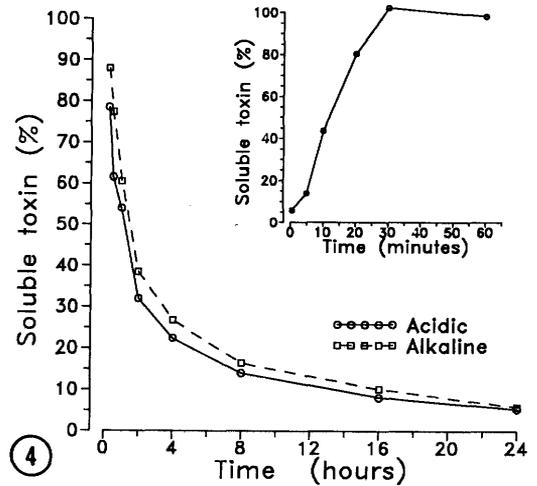
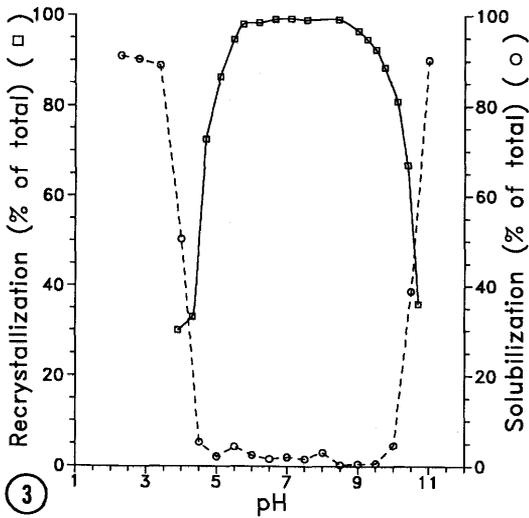


**Figure 1.** Effect of pH on proteolytic activity in *Chrysomela scripta* larval midgut extracts. Midgut extracts were assayed using [ $^{14}\text{C}$ ]methemoglobin dissolved in universal buffer adjusted to the pH indicated. The values are a mean of 3 determinations.

**Figure 2.** Solubilized and native crystal  $\delta$ -endotoxin of *B.t.* var. *san diego*. Unlabelled toxin (Lanes 1-3) was stained with Coomassie blue, and [ $^{35}\text{S}$ ]methionine incorporated toxin (Lanes 5-7) was visualized by X-ray fluorography following SDS-PAGE. Lanes 1 and 5 pH 11.3 solubilized; Lanes 2 and 6 pH 3.7 solubilized; lanes 3 and 7 unsolubilized crystals; Lane 4 contains molecular weight standards (Biorad): 94,400 phosphorylase B, 66,200 BSA, 42,700 ovalbumin, 31,000 carbonic anhydrase, 21,500 soybean trypsin inhibitor, and 14,400 lysozyme.

(8,19,20), we were concerned that our labelling would be insufficient with so few methionine residues. Fortunately, there was enough *de novo* synthesis to yield ample activity.

A solubility profile was produced over a range of pH from 2.3 to 11.3. It showed that over 90% of the administered CPM was in the supernatant after 24 h, at both ends of the range (Figure 3). However, between pH 5.0 and pH 9.5 over 95% of the  $\delta$ -endotoxin remained in crystal form. This pH-mediated change in solubility was very abrupt. Between pH 3.9 and 4.2 solubility fell from 85% to 11%. Alkaline solubility showed a similar steep response above pH 10.0. Fluorography of the SDS-PAGE gel of the supernatants collected at acidic and alkaline pH indicated that the toxin peptide remained intact regardless of the pH used in the solubilization (Figure 2). The solubility below pH 4, has not been reported for any other *B.t.*  $\delta$ -endotoxin. It still fails, however, to explain dissolution of the crystals at midgut pH values approaching 5. Other factors in the midgut, e.g. surfactants, could modify this curve sufficiently to improve solubilization. It is notable that pH peaks of enzymatic activity and toxin solubility are very close. A significant difference in solubility rate was observed between acid and alkaline conditions. At pH 11.3 solubility was virtually instantaneous. A period of 30 minutes of gradual crystal dissolution was required to achieve



**Figure 3.** Effect of pH on the solubilization and recrystallization of *B.t.* crystal toxin. For solubilization, [ $^{35}\text{S}$ ]toxin crystals (400,000 CPM) were added to universal buffer at the pH indicated. For recrystallization, acid soluble toxin was added to the buffer to yield the pH indicated. Radioactivity in supernatants was determined after 24 h at 30°C. Values are a mean of 3 determinations.

**Figure 4.** Rate of recrystallization of both acid and alkaline solubilized toxin. Identical aliquots of solubilized toxin were adjusted to pH 9 by the addition of the appropriate universal buffer. At intervals, samples were pelleted by centrifugation and radioactivity in the supernatant was determined. **Inset:** Rate of solubilization of native crystals at pH 3.7. Crystals added to pH 11.3 buffer dissolved instantly. Values are means of 3 determinations.

maximum solubility at pH 3.7 (Figure 4, inset). It must be noted that the alkaline soluble toxin did, on several occasions, unpredictably and spontaneously recrystallize within hours of exposure to the high pH. When this happened, these crystals resisted subsequent alkaline resolubilization. The acid-solubilized toxin, in contrast, stayed in solution indefinitely. A slow *in vivo* rate of solubilization at acidic pH may help explain the delay in the visible expression of intoxication at the ultrastructural level (21). The reduction in mortality with a lowering of rearing temperature also is (22) consistent with a diminished solubility rate.

Recrystallization of both acid and alkaline toxin occurred as pH was adjusted toward neutrality from both directions. This behavior was shown already with alkaline soluble Cry IIIA toxin (11). The pH-induced change was slightly more gradual than the solubilization process (Figure 3). On the acidic side, a pH change from 4.3 to 5.5 resulted in a change from 30% to 95% recrystallization. On the alkaline end, a corresponding change from pH 10.7 to pH 9.0 was required for a similar result. The rate of recrystallization at pH 9 was virtually identical for the both acid and alkaline toxin solutions (Figure 4). It took 85 minutes to reach 50% recrystallization, and 15 h to attain the 90% level.

The toxicity of both the acid and alkaline preparation of var. *san diego*  $\delta$ -endotoxin was bioassayed at an approximate  $\text{LD}_{50}$  dose for crystals (3.75  $\mu\text{g}$ ), using third-instar *C. scripta*.

**Table 2.** Cottonwood leaf beetle mortality, after 96 h and 144 h, caused by a single 3.75  $\mu\text{g}$  dose of *B.t.*  $\delta$ -endotoxin fed in a 22% sucrose solution on a 3 mm leaf disk. Control doses contained buffer without toxin.

Type of dose (n=36)	Mortality (%)		No. of assay disks consumed within 24 hours (%)
	96 h	144 h	
Crystals	48.6	51.4	89
pH 11.3 soluble	51.4	67.6	38
pH 3.8 soluble	50.0	77.3	3
Controls (all)	0	0	100

Solubilization of the toxin, regardless of pH, enhanced total mortality (Table 2). The acid-soluble  $\delta$ -endotoxin was slightly more toxic to larvae than the alkaline-solubilized toxin. Offering dose droplets on leaf disks allowed some indication of the onset of paralysis, an early symptom of intoxication. The paralysis of larvae treated with crystals was sufficiently delayed to enable them to eat the foliage disk (Table 2). When given the pH 11.3 dose, only 39% consumed the whole leaf disk after the toxin (44% of disks were partially consumed). Yet, only a single insect on the acid soluble dose was able to consume any foliage at all.

This study provides pertinent information on the characteristics of CryIII $\delta$ -endotoxins. Knowledge of acid solubility and gut pH will permit investigation of the mode of action of this  $\delta$ -endotoxin under physiologically relevant conditions. The rate of solubilization suggests that crystal dissolution could be a critical factor governing the potency of *B.t.* toxins affecting Coleoptera.

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