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NOTE

A Method for *in Vivo* Radiolabeling *Bacillus thuringiensis* Native δ-Endotoxin Crystals[1]

The entomocidal CryIIIA δ-endotoxin protein of *Bacillus thuringiensis* var. *tenebrionis* is distinctive in chemistry and host range. In contrast to other δ-endotoxins, the CryIIIA parasporal crystals are toxic within the acidic midgut environment of several coleopteran species, particularly those in the family Chrysomelidae. Resistance to this toxin was successfully developed by laboratory selection of *Leptinotarsa decemlineata* (Whalon *et al.*, 1993) and *Chrysomela scripta* (L. S. Bauer, D. L. Miller, and C. N. Koller, unpublished). Our investigations of the *B. thuringiensis* var. *tenebrionis* CryIIIA crystal toxin targets both the mode of action and the mechanisms of resistance in these insect species.

To study steps in the intoxication pathway, we required a sensitive probe for the CryIIIA δ-endotoxin protein in the form of its native crystal. This probe is used for assessing crystal solubilization, digestive processing, and other toxin–insect interactions, e.g., gut retention time. Radiolabeling techniques that require substantial manipulations of the protein, including solubilization, recrystallization and, in the case of iodination, the incorporation of a nonnative molecule could result in artifacts. The recrystallization of *B. thuringiensis* var. *tenebrionis* δ-endotoxin after solubilization produces crystals with nonnative appearance (Bernhard, 1986) and solubility properties.

The synthesis of δ-endotoxin during sporulation suggested that the basic method of incorporation of a radiolabeled amino acid during protein synthesis could be effectively used. We developed an *in vivo* labeling method based on the incorporation of [^{35}S]methionine/cysteine into the δ-endotoxin during sporulation of *B. thuringiensis* var. *tenebrionis* cultures. By separating cell growth from crystal formation, we were able to produce milligram quantities of radioactively labeled native *B. thuringiensis* var. *tenebrionis* δ-endotoxin protein containing most recently 200,000 cpm/μg. The procedure may be applied with equivalent success to other *B. thuringiensis* isolates (D. Bradley, personal communication). The level of activity we achieved with ^{35}S-labeled amino acid incorporation will detect nanogram quantities of toxin or its decomposition products in SDS–PAGE, or extremely low levels of crystal solubility, *in vivo*.

Crystal yield and label incorporation into *B. thuringiensis* var. *tenebrionis* crystal protein were dependent on the *B. thuringiensis* var. *tenebrionis* vegetative growth medium used. Vegetative growth in PWYE (Herrnstadt *et al.*, 1986.) yielded less toxin, but with higher activity than 2× LB medium (Davis *et al.*, 1986) at pH 7.4. The latter medium produced a better toxin yield with significantly less activity in the protein. Medium is inoculated from a *B. thuringiensis* var. *tenebrionis* slant and grown through two additional 10% transfers at 4-hr intervals before a final 10% transfer for overnight growth to mid stationary phase (12 to 16 hr). Growth conditions included incubation at 30°C, 200 orbits per minute agitation, and a minimum flask size to medium volume ratio of 5:1. A baffled flask also enhanced crystal yield. All manipulations were done at room temperature. The culture was then centrifuged at 1800*g* (Beckman GPR Centrifuge, GH-3.7 rotor) for 15 min. The supernatant was decanted, and the pellet was washed by resuspention in an equal volume of protein-free glucose–salt medium (GSM: 0.1% glucose, 1.25 mM $CaCl_2 \cdot 6H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 0.05 mM $FeCl_3 \cdot 6H_2O$, 0.05 mM $ZnCl_2$, 0.02 mM $CuCl_2$, 0.01 mM $MnCl_2 \cdot 4H_2O$, filter sterilized and added to autoclaved pH 7.2 buffer of 25 mM K_2HPO_4 and KH_2HPO_4). The cells were recentrifuged and resuspended in half the original volume of GSM in a culture flask. The [^{35}S]methionine/cysteine labeling mixture (ICN or NEN) was added at the rate of 100 μCi methionine/ml. The optimal time for addition of the labeled amino acids after transfer of cells to GSM was determined (Table 1). The trace elements in GSM are supplied by chloride salts; the absence of sulfates insures maximum incorporation of ^{35}S. Culture in this medium induced sporulation and crystal formation, which was carried to completion. Unlabeled toxin can be made by omitting the ^{35}S-labeled amino acids and adding tryptone (7.5 g/liter).

The CryIIIA crystals were collected by centrifugation of the sporulated medium at 20,000*g* (Sorvall SS-34) for 15 min and washed five times to remove unincorporated ^{35}S-labeled amino acids—twice with ice-cold water, once with 2 M NaCl, and twice more with water. The pellet is resuspended in a small volume of

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TABLE 1
Optimal Timing of Addition of [^{35}S]Methionine/Cysteine to *B. thuringiensis* var. *tenebrionis* Sporulation Culture in GSM

Time[a] (hr)	Protein[b] (μg/μl)	Specific activity[c] (cpm/μg protein)
0	21.9	4080
2	18.9	4823
4	19.4	7247
6	18.2	4248
8	16.5	2790
10	20.8	2084
12	17.3	905
24	19.0	417

[a] The 100-ml vegetative culture was grown as indicated in the text. The cell pellet was washed and resuspended in 50 ml GSM and distributed into eight equal aliquots of 6 ml each in 50-ml flasks. At the times indicated following resuspension, 180 μCi [^{35}S]methionine was added and cultured until sporulation and cell lysis were complete.

[b,c] The protein content and specific activity in purified crystals from each culture were determined according to methods cited in the text.

distilled water with 0.02% Triton X-100 and briefly sonicated to disperse the spores. Crystals were separated from spores via a sucrose gradient (65, 72, and 79% w/v). The white crystals formed a band at the interface between the 72 and 79% sucrose. Specific activity was determined as cpm/μg protein by scintillation counting and Pierce BCA microassay for protein using BSA as a standard. When labeled crystal toxin was solubilized and then recrystallized, all of the radioactivity was recovered in the crystal, indicating that the label was incorporated in the protein and not merely associated with it (Koller *et al.*, 1992). Purity was analyzed with SDS–PAGE (Laemmli, 1970) and fluorography (Fig. 1). Toxicity was verified in bioassays of cottonwood leaf beetle, *C. scripta*. Toxicity and SDS–PAGE were similar for labeled and unlabeled CryIIIA crystal δ-endotoxin.

The use of metabolically labeled toxin enhances aspects of our research by ensuring that observations based on our probe are identical to those exhibited by the native toxin. Given the apparent complexity and specificity of the interaction, this is crucial.

KEY WORDS: *Bacillus thuringiensis; Leptinotarsa decemlineata; Chrysomela scripta;* [^{35}S] incorporation; insecticidal crystal protein; δ-endotoxin crystals; radiolabeling.

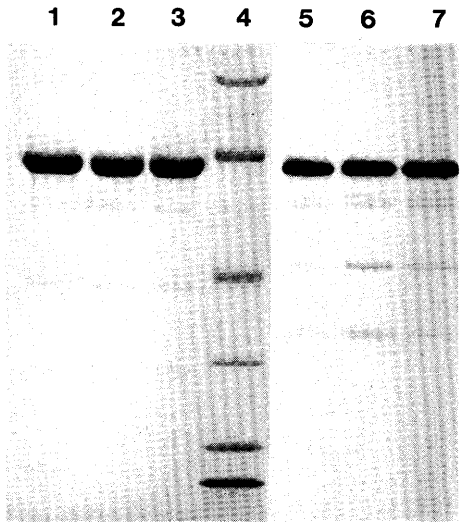

FIG. 1. Labeled and unlabeled crystal. *B. thuringiensis* var. *tenebrionis* δ-endotoxin. Purified native toxin in lanes 1–3 was stained with Coomassie blue. Labeled toxin in lanes 5–7 was visualized by X-ray flourography following SDS–PAGE. The lanes differ only in their handling following crystal purification. Lane 4 contains molecular weight standards (Bio-Rad): 94,400 phosphorylase B; 66,200 BSA; 42,700 ovalbumin; 31,000 carbonic anhydrase; 21,500 soybean trypsin inhibitor; and 14,400 lysozyme.

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