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NOTES

Separation of Protein Crystals from Spores of *Bacillus thuringiensis* by Ludox Gradient Centrifugation

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A method is described for the purification of *Bacillus thuringiensis* protein crystals by Ludox gradient centrifugation. This method is simple, inexpensive, fast, and efficient compared with other techniques. It has been successfully used to purify and characterize the protein crystals from several *B. thuringiensis* strains.

We are developing transgenic plants containing toxin genes from various strains of *Bacillus thuringiensis*. Each strain synthesizes an intracellular, parasporal protein crystal, which becomes toxic through proteolytic activation after ingestion by insects (9). Strains which produce toxins specific for *Lepidoptera*, *Diptera*, or *Coleoptera* spp. are known. Assays of expression of the transferred genes are based on specific antibody reactions and specific insecticidal activities of the toxin proteins. These assays require pure, biologically active toxin crystals as standards and as antigens. Several methods for purification of toxin crystals or toxin proteins of *B. thuringiensis* have been described. These include germination of spores and dissolution of the crystals (3), extraction by biphasic systems by using an organic solvent or high-molecular-weight polymers (5), isopycnic centrifugation in CsCl (4), and gradient centrifugation in NaBr (1). More recently, a method involving density gradient centrifugation in Renografin (E. R. Squibb & Sons, Princeton, N.J.) has been widely used (11). However, multiple centrifugations through Renografin are needed to achieve acceptable purity. Yields are low, and Renografin is expensive.

We have found that centrifugation through step gradients of Ludox is a very effective method for purifying toxin crystals. Ludox is an aqueous colloidal silica produced by Du Pont Co., Wilmington, Del., in industrial quantities. It has been used to purify organelles (10). The Ludox method is simpler, quicker, more efficient, and less expensive than the Renografin method. Crystals and spores clump less and the band of crystals is sharper in Ludox gradients than in other types of gradients. Consequently, crystals are separated more easily and more completely from spores and debris with fewer centrifugations.

Complete cell lysis to crystals, spores, and cell debris is critical for good separation of the crystals in Ludox gradients. Protein crystals were purified from four subspecies of *B. thuringiensis*: *B. thuringiensis* subsp. *kurstaki* HD-1, *B. thuringiensis* subsp. *kurstaki* HD-73, and *B. thuringiensis* subsp. *aizawai* IC1 (6), which were obtained from the Bacillus Genetic Stock Center, Ohio State University; and *B. thuringiensis* sj, which was isolated at Sungene. For all except sj, the cells were grown in 400 ml of CHES (0.5% Casamino Acids, [Difco Laboratories, Detroit, Mich.], 0.4% yeast extract, 0.2% glucose, 0.5% NaCl, 0.01% $MgSO_4$, 0.05% $CaCl_2$) in a 2-liter flask on a reciprocal shaker (200 rpm) at 28°C overnight. To induce sporulation, the cells were harvested by centrifugation, thoroughly suspended in 400 ml of water, and incubated for another 4 days. For sj, the cells were grown overnight on PYWE (5% peptone, 0.1% yeast extract, 0.5% NaCl [pH 7.5]) and then transferred to NYSM (0.8% nutrient broth, 0.5% yeast extract, 0.5% salt [0.14 M $CaCl_2$, 0.12 M $MgCl_2$, 0.01 M $MnCl_2$]) for 4 days (7). These procedures resulted in virtually complete cell lysis (8).

The lysed cultures were centrifuged at 3,000 rpm (1,600 × *g*) for 10 min. Each pellet was suspended in 200 ml of 1 M NaCl and vigorously shaken to produce foam, which was enriched in spores. After the foam was removed with a spatula, the remaining suspension was centrifuged at 3,000 rpm for 10 min, and the pellet was suspended in 50 ml of H_2O. The crude crystal and spore suspension was disaggregated by sonication at 100 W for 30 s (Braunsonic 2000; Melsungene AG). Aliquots were layered onto Ludox gradients.

Du Pont kindly provided a sample of Ludox HS-40 which was sufficient for this study. Ludox HS-40 is 40% (wt/wt) sodium silica with a density of 1.295 g/ml and pH 9.7. This sol irreversibly precipitates below pH 7, at salt concentrations above 0.1 N, or upon freezing. The pH of 100 ml of Ludox HS-40 was adjusted at room temperature to 8.0 with about 10 ml of 1 M Tris hydrochloride (pH 2.5), with rapid stirring. Desired Ludox concentrations were then made by diluting the pH-adjusted Ludox HS-40 (defined as 100% solution) with distilled water. These Ludox solutions were stable for weeks at 4°C. Typical Ludox gradients were made by layering 5 ml of 40% (vol/vol) Ludox on 5 ml of 50% (vol/vol) Ludox in 30-ml Corex tubes (Corning Glass Works, Corning, N.Y.), all at room temperature. Usually, 2 to 5 ml of the crude suspension of crystals and spores described above was layered onto the Ludox gradient. The gradient was centrifuged in a swinging bucket rotor (no. JS-13; Beckman Instruments, Inc., Fullerton, Calif.) in a centrifuge (no. J2–21; Beckman) at 8,000 rpm (10,000 × *g*) at 4°C for 1 h. Large-scale Ludox gradients with similar geometry should

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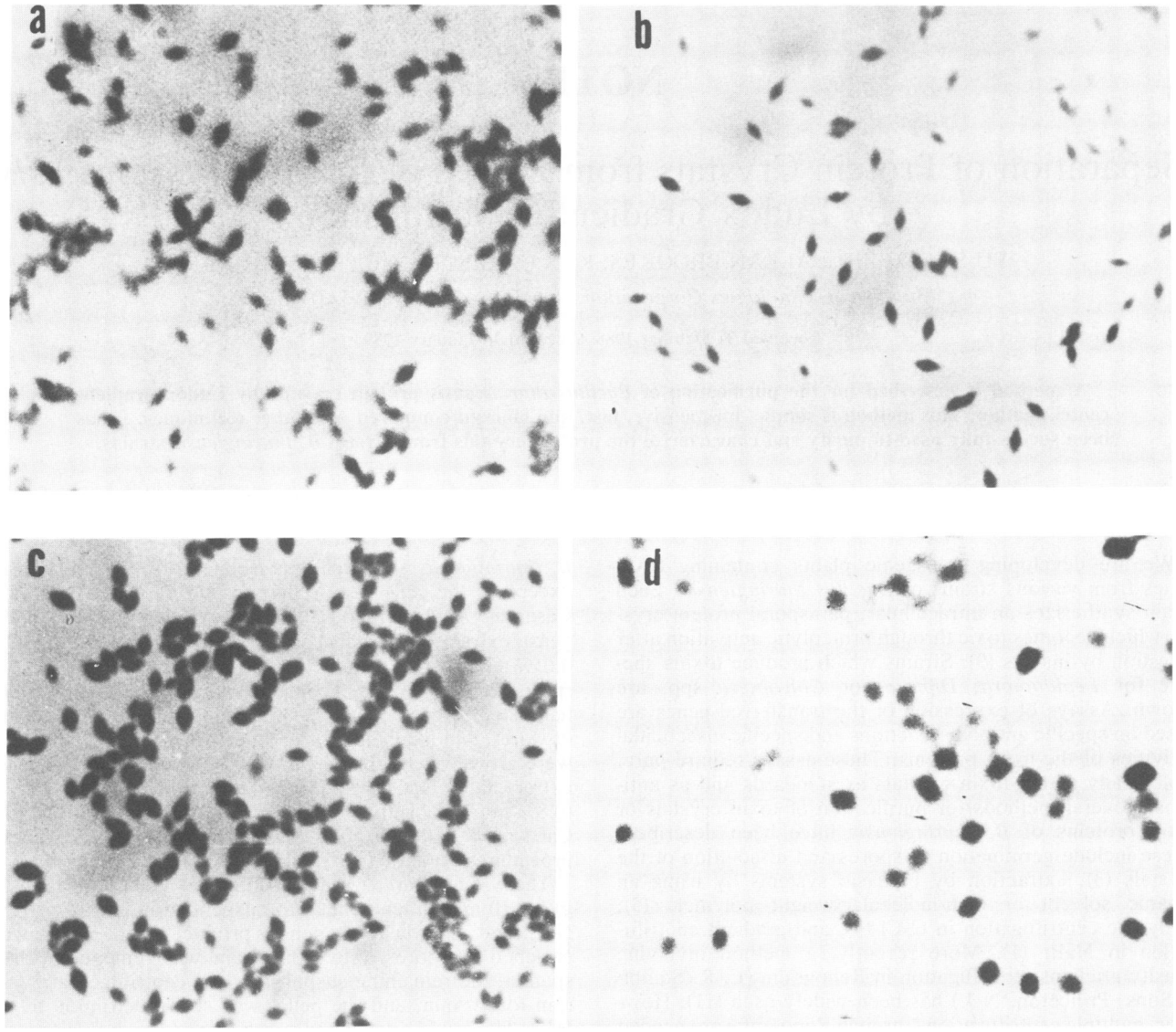

FIG. 1. Light micrographs of stained crystals, purified by Ludox gradient centrifugation, from various *B. thuringinesis* strains. (a) HD-1; (b) HD-73; (c) IC1; (d) sj. A few cuboidal P2 crystals are evident in the preparation from HD-1. Magnification, ×2,805.

work as well, but we did not attempt this because we lacked a suitable rotor. To optimize separations, we sometimes varied the Ludox concentrations between 30 and 70%, depending on the *B. thuringiensis* strain.

Excellent separation of HD-73 or sj crystals was obtained in the 30-ml tubes under the conditions described, with the spores at the bottom of the tube, the crystals at the interface between the Ludox layers, and the debris mostly at the interface between the crude suspension layer and the upper layer of Ludox. For HD-1 and IC1, the positions of crystals and spores were reversed. The sharp band of crystals could easily be collected with a pipette. To estimate purity, we stained the crystals red with safranin and the spores green with malachite green (2). A single Ludox gradient centrifugation sometimes produced apparently pure crystals by this criterion (about 5 to 10 mg, containing less than 0.1% spores, from 100 ml of culture) (Fig. 1).

Both light microscopy (Fig. 1) and scanning-transmission electron microscopy (Fig. 2) indicated that the preparations of the bipyramidal crystals from HD-73 and IC1 and the flat, rhomboid crystals from sj were free of contaminating particles and were morphologically pure. In addition to the bipyramidal P1 crystals, the HD-1 cultures produced small amounts of cuboidal P2 crystals (Fig. 1a). All of these crystal types were stable in Ludox.

The crystal preparations were also biochemically pure, judging from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3) and their immunochemical properties. The predominance of the 130-kilodalton (kDa) polypeptide in the HD-73 and IC1 crystals and the 130-kDa (P1) and 60-kDa (P2) polypeptides in HD-1 crystals indicates that negligible proteolysis occurred during purification. The crystals from sj had a major polypeptide of 64 kDa and a minor one of 58 kDa. Antibodies against each of the crystal preparations have been raised in rabbits. Ouchterlony tests, enzyme-linked immunosorbent assays, and Western immu-

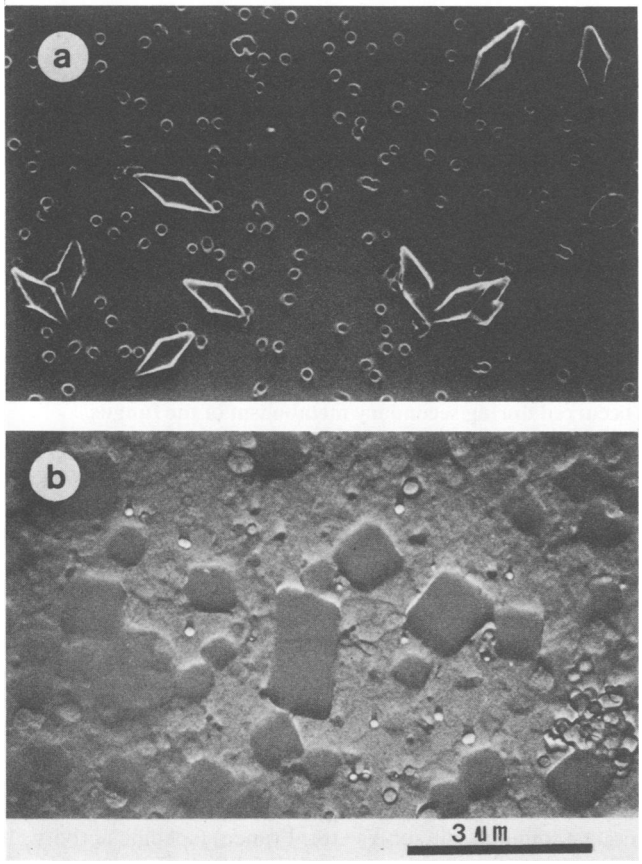

FIG. 2. Electron micrographs of *B. thuringinesis* crystals. (a) HD-1 (scanning electron micrograph); (b) sj scanning-transmission electron micrograph. Magnification, ×8,300. Bar, 3 µm. Scanning-transmission electron microscopy makes images from both the elastically and inelastically forward-scattered electrons. The result is a combination image of the surface and the interior of the sample. The small circular objects are pores in the supporting film.

noblots showed that the protein in each crystal preparation specifically immunoreacted with the corresponding antibodies.

The purified crystals retained their activities as substrates for insect gut proteases and as toxins to insects. The crystal proteins from HD-73 and sj were processed to polypeptides of the sizes expected for the active toxins, when purified crystals were mixed with gut juice from tobacco hornworms (*Lepidoptera*) or gut extracts from *Tenebrio molitor* mealworms (*Coleoptera*), respectively. Crystals purified from HD-1 and HD-73 were highly toxic to tobacco hornworm larvae (50% lethal dose, 0.01 µg per third-instar larva), and crystals purified from sj were toxic to mealworm larvae (50% lethal dose, 4 µg per second-instar larva). At 4 µg per larva, HD-1 cyrstals were not toxic to mealworms and sj crystals were not toxic to hornworms.

We thank B. Clark for his electron microscopy work.

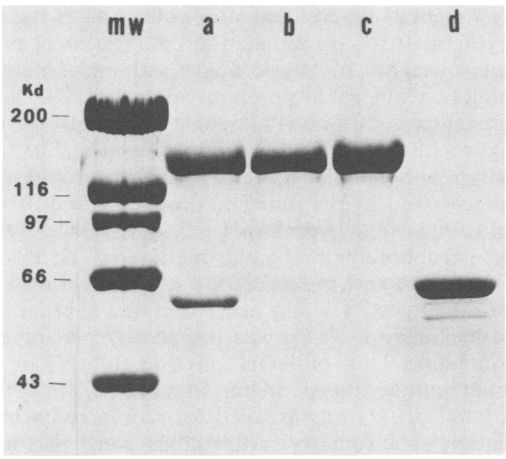

FIG. 3. Coomassie blue-stained sodium dodecyl sulfate–10% polyacrylamide gel after electrophoresis of crytal proteins from various *B. thuringinesis* strains. (a) HD-1; (b) HD-73; (c) IC1; (d) sj. Lane mw, Molecular weight standards. To ensure solubilization of the P2 component (60 kDa) of HD-1, we dissolved the crystals by boiling them for 5 min in 10 mM Tris hydrochloride–10 mM dithiothreitol–8 M urea (pH 8.5).

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