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Biphasic System for Separation of Spores and Crystals of *Bacillus thuringiensis*

N. S. GOODMAN, R. J. GOTTFRIED, AND M. H. ROGOFF
International Minerals & Chemical Corporation, Chemical Division, Wasco, California 93280

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Bacillus thuringiensis and related strains of sporeforming insect pathogens form a true microcrystal of protein within the cell at the time of sporulation. These crystals are toxic to certain lepidopterous larvae (T. A. Angus, Can. J. Microbiol. **2**:122, 1956; A. M. Heimpel and T. A. Angus, J. Insect Pathol. **1**:152, 1959). Methods for isolating the crystals free from contaminating spores have not been efficient enough to base precise biological and biochemical analyses on the resulting preparations. To obtain highly purified preparations of crystals free from spores in sufficient quantity for precise quantitative study, we have investigated separation in two-phase systems, as generally described by P. A. Albertsson (*Partition of Cell Particles and Macromolecules*, John Wiley & Sons, Inc., New York, 1960) and as applied to spore separation by L. E. Sacks and G. Alderton (J. Bacteriol. **82**:331, 1961). The latter investigators used a two-phase aqueous system consisting of polyethylene glycol 4,000 and potassium phosphate, in which free spores entered the polyethylene glycol-enriched phase. The spore separation they obtained indicated the feasibility of a two-phase system for isolating the crystals from a sporulated culture of *B. thuringiensis* by selective spore removal. The method developed is as follows.

A two-phase system is prepared to contain 4.68% polyethylene glycol 6,000 (Union Carbide Corp., New York, N. Y.) and 6.68% sodium dextran sulfate 500 (Pharmacia Fine Chemicals, Inc., New York, N. Y.), and contains the following components: sodium dextran sulfate 500, 334 ml of a 20% (w/v) solution; polyethylene glycol 6,000, 234 ml of a 20% (w/v) solution; sodium chloride, 100 ml of a 3 M solution; spore-crystal suspension, 10 to 20 g, wet weight (previously frozen, thawed, distilled water-washed, and resuspended in distilled water to a convenient volume). Final volume is brought to 1 liter with distilled water.

Fresh polyethylene glycol-rich upper phase is prepared by adding the following components (grams) to sufficient distilled water to make 6 liters: sodium dextran sulfate 500, 1.8; polyethylene glycol 6,000, 421.8; sodium chloride, 105.0.

One liter of the complete system is placed in a separatory funnel. Approximately 1 liter of fresh upper phase is added, the funnel is shaken vigorously, and the liquid phases are allowed to separate. At this point, the upper phase contains large quantities of spores and some cell debris; the lower phase and interface contain crystals, many residual spores, and debris. The upper phase is carefully aspirated off without disturbing the interface. Fresh upper phase (1 liter) is added, and the process ("pass") is repeated until few or no spores are observed microscopically in the upper phase. The number of passes required depends on the quantity of starting material and the degree of purity desired in the final crystal preparation; usually four are required.

The viscous crystal-rich lower phase is diluted with an equal volume of distilled water and centrifuged (12,000 to 27,000 $\times$ *g*, 30 min, 5 C). Supernatant fluids are discarded. The sediment is washed by resuspension in distilled water and centrifugation in 12-ml glass centrifuge tubes for 20 min (2,000 $\times$ *g* at 5 C). Remaining spores and debris layer onto the pellet or remain suspended. They can then be carefully removed with a pasteur pipette. The crystals are washed in this manner until less than one spore per densely packed microscopic field of crystals remains. A difficult preparation can be reintroduced into the complete system, and passes can be made until a relatively spore-free, clear upper phase is obtained.

Yields of pure crystal prepared by the above method may vary with the starting preparation. Typically, about 250 mg of crystals is isolated from 10 g of spore-crystal paste, with 99.94% crystals and 0.06% spores in the final product. Crystals, purified and isolated in this manner, have been demonstrated to retain their toxicity, by ingestion, for larvae of the wax moth, cotton bollworm, cabbage looper, and lucerne moth.