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Improved Technique for Refining the Crystal of *Bacillus thuringiensis* by NaBr Gradient Centrifugation

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An improved method is described for the purification of *Bacillus thuringiensis* Cry protein crystals by sodium bromide (NaBr) gradient centrifugation. This method is simple, low cost, and efficient for separating cry gene products from spores and cell debris. The purified crystal proteins retained biological activities.

Bacillus thuringiensis synthesizes parasporal crystals, made of proteins (Cry), which may be activated into insecticidal toxins through proteolytic digestion following ingestion by susceptible insects. Assays of Cry proteins require pure, biologically active crystals as standards. Several methods for the purification of crystals of *B. thuringiensis* through germination of spores and dissolution of the crystals, and extraction by biphasic systems have been reported (3, 6). However, multiple extractions are needed to achieve acceptable purity, and final yields are low. Crystal purification methods using isopycnic centrifugation in CsCl (4), density gradient centrifugation in Renografin (7), step gradient centrifugation in Ludox (9), and in sugar (8), were also described. These methods give crystal preparations of high purity, but the reagents required in these methods are often expensive. Ang and Nickerson (2) described a method employing zonal gradient centrifugation in sodium bromide (NaBr). Although NaBr is an inexpensive reagent, zonal centrifugation is a special process that is labor intensive. We describe here an improved method using NaBr gradient centrifugation simplified at a small scale.

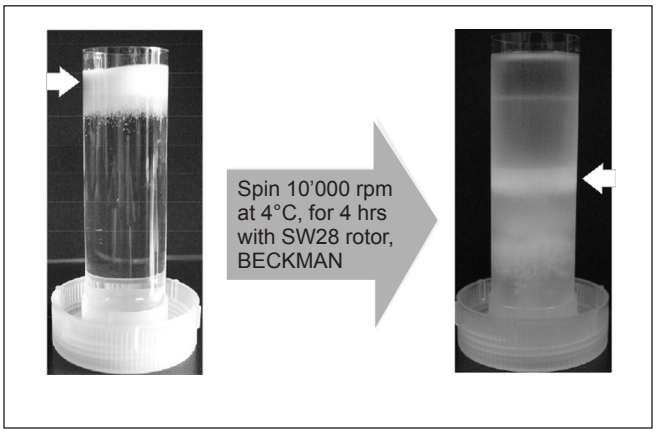

FIG. 1. Separation of crystals, spores, and debris in continuous 30 - 40% NaBr gradients. Cell extracts, top of the tube (left), were separated into three bands after the centrifugation (right). The crystal band was indicated by an arrow (right).

Bacillus thuringiensis serovar *israelensis* HD522 was obtained from the U.S. Department of Agriculture Research Service. *B. thuringiensis* serovar *israelensis* 4Q7 was obtained from the *Bacillus* Genetic Stock Center and used as the host cell of the recombinant plasmid pIS431. pIS431 carries a 5.03-kbp fragment with an intact *cry4A* gene. It was inserted into the *Bacillus – E. coli* shuttle vector pHY300PLK (TAKARA BIO Inc.) (5). Crystals and spores were harvested from *B. thuringiensis* serovar *israelensis* HD522 strain grown in Shaeffer's sporulation medium (SSM) until autolysis. Cry4A protein and spores were obtained from *B. thuringiensis* serovar *israelensis* 4Q7 bearing pIS431. Culture medium was pelleted by centrifugation, and cell debris was removed from the pellet by washing with 2% Triton X-100-0.5 M NaCl, with 0.5 M NaCl, and with deionized water (1). Final pellet was resuspended in water and was layered on top of a 30 ml linear NaBr gradient, comprising 30%-40% NaBr solutions. Centrifugation was carried out with a Beckman ultracentrifuge in a SW-28 rotor operating at 10,000 rpm, 4°C for 4 hrs. Crystals formed a single band at about 33% NaBr solution (Fig. 1). Scanning-transmission electron microscopy indicated that crystals purified by NaBr gradient centrifugation were free of contaminating spores and were morphologically pure (Fig. 2). SDS-polyacrylamide gel electrophoresis indicated that the proteins composing the crystals were stable and that negligible proteolysis occurred during the purification

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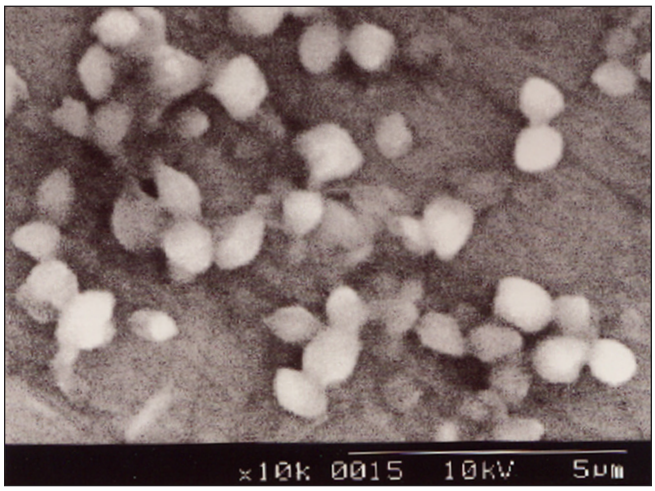
A *Bti.HD522*



B *pIS431/Bti.4Q7*

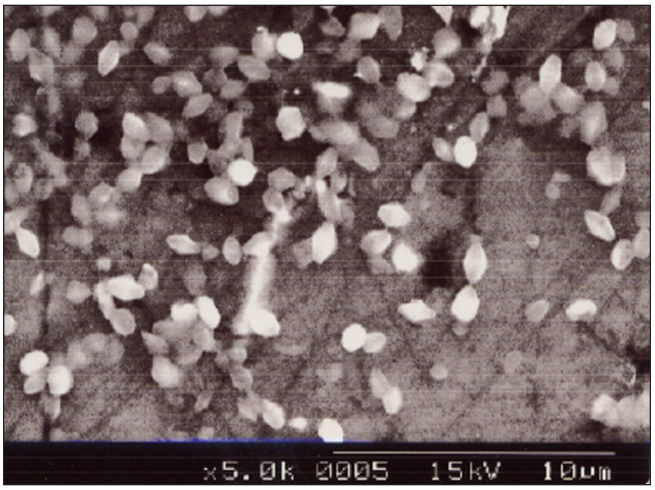


FIG. 2. Electron micrographs of *Bacillus thuringiensis* crystals. Crystals from *B. thuringiensis* serovar *israelensis* HD522 (Panel A) or pIS431/*B. thuringiensis* serovar *israelensis* 4Q7 (Panel B) prepared by NaBr gradient centrifugation (indicated by arrow in Fig. 1) were observed by scanning electron microscopy.

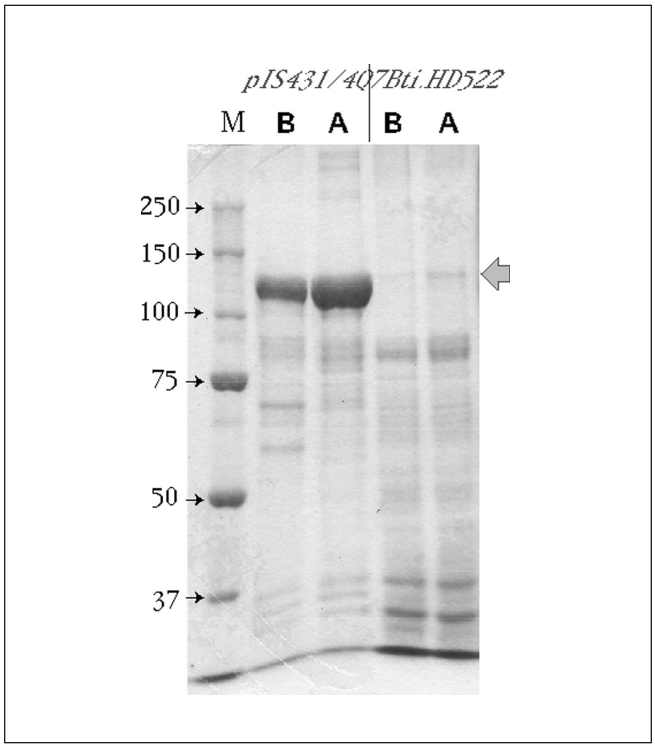


FIG. 3. SDS-polyacrylamide gel electrophoresis of crystals before (B) or after (A) NaBr gradient centrifugation. Samples were solubilized with 50 mM carbonate buffer (pH10) contained 10mM DTT at 37ºC for 2hrs, before loaded on 8% gel (3 µg proteins/lane). Arrow on the right side indicates 135 kDa proteins.

process (Fig. 3). The purified crystals retained their insecticidal activities (data not shown).

The procedure described here does not require ultracentrifugation. Our method is inexpensive compared to methods that require CsCl or Renografin. It is simple and more rapid than zonal gradient centrifugation.

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