A Possible Efficient Assay: Low-vacuum SEM Freeze Drying and Its Application for Assaying *Bacillus thuringiensis* Formulations Quality

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A new preparation method for examining the microstructures of *Bacillus thuringiensis* formulations, low-vacuum SEM freeze drying, is described through its application to observations of the formulation sample. Scanning electron micrographs revealed that the drying method preserved the microstructures of BT formulation granules intact condition well. Dehydration in a graded ethanol series is not necessary in this new method. Low-vacuum SEM freeze drying is, therefore, a simple, time-saving and reproducible method for scanning electron microscopy that is applicable to check various commercial BT formulations quality.

*Key word: Bacillus thuringiensis; BT formulation; quality check; low-vacuum SEM.*

Introduction

*Bacillus thuringiensis* (BT) formulations are microbial insecticide commonly used worldwide. Their active ingredients are known to be insecticidal crystalline proteins (ICPs) produced during spore formation. The crystalline protein (δ-endotoxin) is believed to be a protoxin because it is only highly toxic when ingested in susceptible insects midguts [1]. The potency of BT formulation indicates the dose-dependent, lethal activity of the product as compared to that of an accepted standard BT [2].

About 50 years ago, that were appearance of the first commercial products based on BT created a need for methods to compare products in various countries. The first protocols were based on spore counts, but because the number of spores did not reflect the number of crystals present in the bacteria, this method was unreliable and resulted in some major product failures in the field [1].

With the development of commercial BT products in France [3, 4], a titration method based on comparison of the LC50s of a product and standard material was developed. This method led to rapid improvements not only in product reliability, but also in increased biological activity of the products. The new technique became an instrument for industrial fermentation and formulation development.

In Japan, the silkworm bioassay has been used to determine lepidopteran BT product potency [5, 6]. Several bioassay methods have been examined for determination of the potency of BT formulations. For example, two methods are generally used in Japan to examine the oral toxicity of BT formulation; a) mixing with the diet (diet feeding method) [5 to 11], and b) injection into the mouth with a syringe (force-feeding method) [8].

Several insect species have been used for in bioassays for lepidopteran BT formulations for

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Fig. 1. Low-vacuum SEM (LV-SEM) freeze drying method (Suzuki et al., 1995). Black and thick arrows show the general procedure for freeze drying with low vacuum SEM and white arrows show the additional procedure for examining specimens with other SEMs or high vacuum mode of the LV-SEM. *FE SEM: Field Emission SEM.
example; the silkworm, *Bombyx mori* [6, 9 to 13], diamondback moth, *Plutella xylostella* [11], small white butterfly, *Pieris rapae* [12] and common cutworm, *Spodoptera litura* [14]. Heimpel [15] has discussed the merits of using *Bombyx mori* as an internationally standardized test insect species for the assay of insecticidal activity in a lepidopteran BT formulation, where *Pieris brassicae* is not available because of quarantine regulations.

In recent years, a Low-vacuum scanning electron microscope (LV-SEM) has been developed, which allows specimen observation without the preparations needed for conventional SEM, such as chemical fixation, dehydration, drying, and coating. In the present study, we attempted, through LV-SEM observation, to compare and discriminate and control the qualities of several commercial BT formulations. Thus the degradation sample of insecticidal activity of BT formulations after long-term preservation was examined by the LV-SEM for this purpose. The results show that BT-insecticidal activity and several indicators of physical degradation can be detected with this new method. Freeze-drying method in a LV-SEM has been found to be a method suited for observing several commercial BT products such as different manufacturer samples and registration products. We introduce here this new simple method for assaying BT formulations quality.

**Materials and Methods**

**Principle of LV-SEM and SEM/EDS**

LV-SEM neutralizes the charge of nonconductive specimens by utilizing ions generated by the interaction between residual gas molecules in the specimen chamber and the electrons used for observation. For this purpose, the pressure of the specimen chamber can be raised to 270 Pa by using differential pumping. The use of backscattered electrons for imaging allows not only sample surfaces of BT granule structures to be observed.

The schematic diagram of the LV-SEM freeze drying method is shown in Fig. 1. The LV-SEM is a SEM that is equipped with an electron gun that generates an electron beam and a different evacuation system that allows pressure adjustment of the specimen chamber to a low vacuum range (6 to 270 Pa), while the lens system to condense the electron beam is kept at a high vacuum [16].

**BT formulation samples**

Commercial BT formulations, servers and main δ-endotoxin proteins were as follows: No. 1. Selectzin® (Kyowa Hakko Kogyo Co. Ltd., Tokyo, serovar *aizawai*, Cry1C) : A.

No. 2. Bacilex® (Shionogi & Co. Ltd., Osaka: two-strain formulation of serovar *kurstaki* and *aizawai*, Cry1Aa and Cry1C) : B.

No. 3. Dipole® (Sumitomo Chemical, Tokyo, serovar *kurstaki*, Cry1Aa) : C.

No. 4. Thricide® (SDS Bio-tech K. K., Tokyo, serovar *kurstaki*, Cry1Aa) : D.

No. 5. Xentary® (Tomen Co., Tokyo, serovar *aizawai*, Cry1C) : E.

**Silkworm bioassay**

Insecticidal activities shown in the present study were from the original data [17]. The method of bioassay was the same as Matsumoto [10, 18].

**Observation in the solution of BT formulation by light microscopy**

Prepared BT solution (ten times) samples were observed by a light microscopy. The light micrographs was taken by an Olympus bright-field microscope.

**Observation in the solution of BT formulation by electron microscope (Suzuki Method, Suzuki et al., 1995)**

A scanning electron microscope (JSM-5300LV, JEOL) was used to observe the BT formulations. The colony specimen of *B. thuringiensis* on culture medium and BT formulation samples were prepared by the method of Suzuki et al. [16]. Namely, each BT formulation was prepared by dissolving it in ten times of solute water (DW).

The BT solutions were dropped onto a specimen stub, which a piece of double-side adhesive carbon tape had been attached. These specimens on the stub were then rapidly frozen using liquid nitrogen. The formulation samples were left until they are completely frozen and frost covered them (Fig. 4a). The stub was then attached to a specimen holder for use in the LV-SEM, and freeze-dried in the specimen chamber. As needed, samples were sputter-coated with Pt-Pd alloy with an ion sputter before SEM-observation (Fig. 1).

**Observation on the surface of BT formulation granules by electron microscope (Improved Suzuki method, Matsumoto et al., 2002b, in press)**

A scanning electron microscope (JSM-5300LV, JEOL) was used to observe the sur-
face of the BT formulation granules. The specimens were prepared by the Improved Suzuki method [17]. Namely, each BT formulations were prepared as it is, but the dissolving step was omitted (see Fig. 5a).

Analysis of BT formulation sample with LV-SEM/EDS

The studies on the electrolyte composition of BT granule surface have been attempted using SEM and energy dispersive X-ray analysis (EDS) based on Gould et al. [19]. An atomic element analyzer JED-2140 (JEOL) was used for this analysis. As the electron beam of SEM is exposed on samples and penetrated, it generates characteristic X-ray from the atoms in its path (Fig. 7; upper left). The EDS microanalysis system collects the X-ray, spots them by energy, and automatically identifies and labels the elements responsible for the peaks in this energy distribution [20]. Scanning electron micrograph show the point X-ray analysis of BT formulation granules. EDS analyses of points A and B are provided in this figure (Fig. 7 upper right).

Result

Suzuki Method was used at first to observe BT formulation samples simply and rapidly (Fig. 4a). One minute after the start of vacuum evaporation, it was possible to observe ice sublimation [16, 21]. BT spores and crystals, in the BT solution with cultivated specimen (with B. thuringiensis colony on medium) exposed during ice sublimation, appeared to be similar to observations by light microscope (Fig. 2 and Fig. 3). However, BT formulation samples and toxin proteins underwent obvious gelatins so we were unable to observe the spores and crystals in the solution of BT formulation (Fig. 4b). This is like of gelation in protein solution (e.g. soybean 7S globulin) [22].

SEM observation of intact Bacillex® formulation granule revealed wall-like envelope on the surface of a normal active BT formulation (Improved Suzuki Method [17]) (Fig. 5a).

Five kinds of BT formulations by the scanning electron micrographs are shown in Fig. 5b. In this method spores and crystalline protoxins were not observed as same as a light microscope, but the surface of BT formulation granules were observed without any collapses of surface on the granules (Fig. 5b, Fig. 6). This suggests the shapes of the granules are characteristic to BT formulations.

Scanning electron micrographs show the point X-ray analysis of BT formulation granules. EDS analyses of point A and B, respectively in Fig. 7 upper right. Recognition of individual components in the BT sample (e.g. Organic components (with C atoms; originated from BT 6-endotoxins) and other additive inorganic components (with Si atoms; diatomaceous earth) patterns was simple (Fig. 4b) in visible and EDS permitted rapid identification of these component of BT samples observed (Fig. 7 lower).

Discussion

Freeze-drying in a low-vacuum SEM has been found to be a method suited for observing the characteristic structures of commercial BT formulations. LV-SEM provided satisfactory results by the conventional methods in both the total images and microstructures of BT formulations (Fig. 5b). The most important point of this method is high preservation on BT granule surface of SEM specimens. Namely, quality of specimen was not influenced by the preparation procedure of each BT sample. Furthermore, the preparation procedure has the advantage that it drastically reduces labor and time required, because no dehydration process with a graded ethanol series is needed. All normal BT formulation has a steady characteristic surface on their granules in intact observation by LV-SEM (Fig. 5b). The surface of such BT granules is easily damaged by contraction or dissolution, especially during fixation with solutions or dehydration. Special care is needed; to avoid such damage, such specimens should be examined without troublesome processing as in the present study.

When checking BT formulations, it is essential to discriminate the target formulation and other formulations. Discrimination of BT formulations is needed for BT formulation quality control. With the advantage of this new method, it becomes possible to observe the microstructure and surface of BT formulation granules and small-sized granules which has so far been hard to observe without a LV-SEM. Namely, it was shown that by using this LV-SEM observation method, the possibility of discriminating the respective BT formulations was opened through obtaining the differences in structures on surfaces of BT formulation granules, which have fine and unique morphology. In order to achieve much more detailed observation of BT granule surface structure, it is highly advisable to sputter-coat the specimen coated by the improved method, and then observe it at the high vacuum mode of a LV-SEM and by
an atomic element analyzer (e.g., JSM-5410) (Fig. 7). BT δ-endotoxin is a crystalline glycoprotein. Thereby, we tried to study more by the atomic element analyzer the components on surface of the formulation granules (Fig. 7 upper right). The result showed that the ratios of Carbon atoms (C) to Silicon atoms (Si) in different parts of BT granule are dramatic change (Fig. 7 lower). Namely, the large ratio of C to Si means that the BT granule includes much glycoprotein serving as active ingredients. And molecular component C amounts of the degraded BT sample have been rapidly decreased (Matsumoto, Data not shown). This method may applicable not only to discrimination of commercial formulations, but also to use for quality check of them (e.g. active degradation of BT after long-term storages) [17]. The new method can be useful not only for observing BT to discriminate but also detection of degradation in BT formulations. The mechanisms of deterioration of BT are still not completely elucidated. Further study on the mechanisms of inhibition and deterioration of the BT insecticidal activity are needed.

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![Fig. 5a.](image1) Fig. 5a. We show the procedure for specimen preparation when the LV-SEM observation of the five BT formulation granules shown in Fig. 5b was performed. Improved Suzuki method (2002b, Matsumoto et al, in press), which was reviewed by the method of Suzuki et al., (1995), (Fig. 1).

![Fig. 5b.](image2) Fig. 5b. LV-SEM views of five formulation granules of Bacillus thuringiensis by the new method by the Improved Suzuki method (Matsumoto et al, 2002b, in press) (×5000); 5 µm

![Fig. 6.](image3) Fig. 6. Scanning electron micrographs of outer surface of envelope at a higher magnification (×5000), showing the wall-like envelope. Scale bars = 5 µm. Comparison of scanning electron micrographic observations of the granule surfaces of BT powders. B_new means new sample. And B_old means the old sample with degradation (Bacilex®; Fig. 5b-D) after 21 years preservation at 5°C under dark conditions.


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Fig. 7. The micrograph at the upper right of Fig. 7 shows a granule formed after a BT formulation was dissolved. Points A and B in the micrograph are analysis points where element analysis was performed using the EDS. We presume that the reason why the EDS signal from carbon was strong at point A is due to the granule at point A being large and ICP (insecticidal crystal proteins), an insecticidal component of the BT formulation, remaining on the surface in abundance. In contrast, we consider that the reason why the signal of silicon (from a diatom), which is used as an additive to BT formulations, was stronger at point B than the carbon signal is probably due to point B being in an area consisting of residual components where ICP was completely dissolved out.