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〈論　文〉

Scanning Electron Microscopy of the Tissues of *Helicoverpa assulta* Larvae intoxicated with *Bacillus thuringiensis* Protein Crystals.

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Abstract

Surface changes of tissues caused by *B. thuringiensis* var. *kurstaki* δ-endotoxin intoxication of *Helicoverpa assulta* were observed by scanning electron microscopy. *Bt*-endotoxin crystals induced the erosion and disruption on the surface of all tissues tested. The results revealed that the toxin binds to all exposed plasma membranes without apparent specificity for particular membrane domains.

Key words : *Bacillus thuringiensis*, *Helicoverpa assulta*, δ-endotoxin, scanning electron microscopy

INTRODUCTION

Bacillus thuringiensis is one of the crystalliferous bacteria. The crystal-protein produced during sporulation acts as a toxin on a number of insects. *B. thuringiensis* has been used in the control of defoliating lepidopterous larvae as a biocide (Percy and Fast, 1983). The accumulated evidence is quite convincing that action of the crystal toxin is restricted to the gut epithelium which swells and eventually disintegrates.

For nearly three decades investigators have studied the mode of action of *B. thuringiensis* and an extensive literature is available on the various aspects of the pathogenesis of *B. thuringiensis*. Such pathogenetic changes have been analysed in *Bombyx. mori* by scanning electron microscopy (Spies & Spence, 1985; Chiang *et al.*, 1986) and by transmission electron microscopy after 1 to 2 day interval following sublethal doses (de Lello *et al.*, 1984). The most prominent modification observed was swelling of the goblet cell cavities. However, other studies on thin-sectioned material have been made 15～90 min after oral administration of the toxin to *B. mori* (Endo & Nishiitsutsuji-Uwo, 1980) or 1～30 min after feeding either *B. mori* (Percy & Fast, 1983) or *P. brassicae* larvae with it (Lüthy & Ebersold, 1981), using highest toxin doses. The toxin is thought to bind to plasma membrane-associated receptors and form lytic pores(Knowles and Ellar, 1987), although the precise mechanism of binding and the cytological distribution of binding sites remain obscure.

B. thuringiensis var. *kurstaki* HD-73 δ-endotoxin inhibits fluid transport and disrupts cell structure in insect Malpighian tubules (Reisner *et al.*, 1989). The extensive histolysis and accompanying cessation of urine secretion in two physiologically distinct Malpighian tubule regions following serosal and mucosal exposure is consistent with the idea that the toxin acts as a general pore-former rather than by poisoning histospecific membrane proteins.

We attempted to clarify the mode of action of the δ-endotoxin on the tissues of *Helicoverpa assulta*. The present report deals with the toxic response of various tissues including midgut and

nonmidgut.

Materials and Methods

1. Insects

The larvae of *H. assulta* used for the experiment were obtained from a colony maintained in the laboratory of Insect Pathology, Seoul National University and reared on artificial diet at 27℃.

2. Bacterial strain and toxin preparation

B. thuringiensis var. *kurstaki* HD-1 was obtained from Dr. Faust (USDA, Maryland). The growth and crystal purification were performed as described by Thomas and Ellar (1983). The δ-endotoxin inclusions from *Bt* were separated from spores and cell debris by ultracentrifugation on discontinuous sucrose density gradients. The fraction of crystal protein was concentrated and the protein content was estimated by the method of Bradford (1976).

3. Administration

After starvation of 12h, the last instar larvae of *H. assulta* were transferred to individual vials containing toxin-mixed diet (100 μg/g, w/w). Then larvae were examined for ingestion during the first 2h after treatment. Vials containing toxin-ingested larvae were selected and kept at 27℃ for 12h. After overnight, living larvae were classified as the sequence of overt pathological symptoms (Endo and Nishitsutsuji-Uwo, 1980) : Stage 0, appearance and locomotion normal but no feeding behavior; Stage 1, locomotion slightly sluggish; Stage 2, extremely sluggish; Stage 3, completely paralyzed.

3. Scanning electron microscopy

Excised organs were fixed with 2.5% buffered glutaraldehyde for 2h, using 0.1 M phosphate buffer pH 7.5. The tissues were then washed several times with the same buffer. Postfixation was done with 1% buffered OsO_4 for 1h at 4℃. Thereafter the samples were dehydrated in the ethanol series, treated with acetone, critical point dried from CO_2, mounted, and sputter gold coated (Brandt *et al.*, 1978; Cohen, 1974). Samples were observed using a JSM 6400 operated at 15KV.

Results and Discussion

Since the manifestation of toxic effects is va riable among individuals, we followed an arbitrary classification depending on the pathological symptoms (Endo and Nishiitsutsuji-Uwo, 1980). In the present experiment with administration, all the larvae survived after 12h exposure to the toxin. However they showed slightly sluggish locomotion and no feeding behavior, which were classified as between Stage 0 and Stage 1.

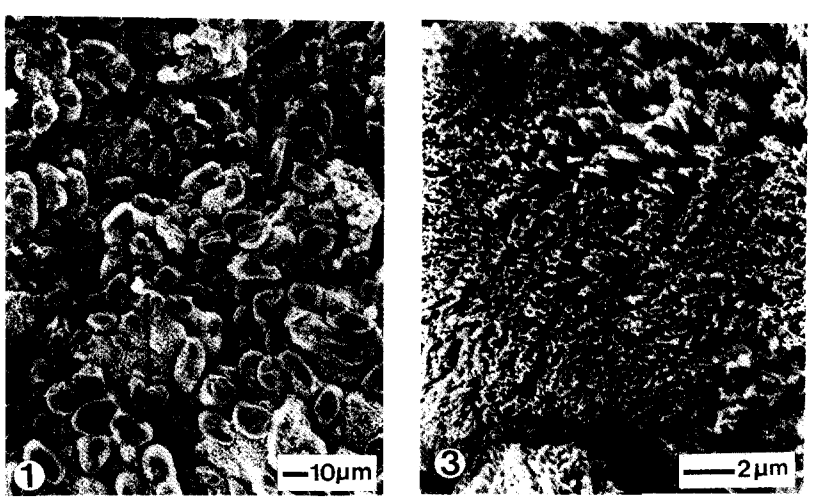

Fig. 1, 3. *The lumen side of midgut epithelial tissue from control larvae of Helicoverpa assulta.*

Fig. 2, 4. *The lumen side of midgut epithelial tissue from* ***Helicoverpa assulta*** *larvae after ingestion of toxin. After 12h exposure to the toxin no microvilli remains, and fissures and microorganisms appear.*

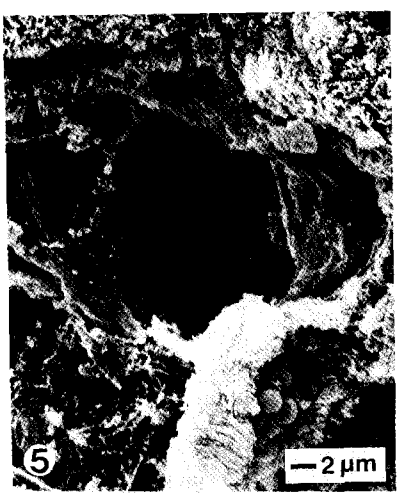

Fig. 5. *The magnification of pore in the gut wall of Bt-toxin ingested larvae. The pore size is estimated at about 12 μm. Some microvilli remain in the vicinity of the pore.*

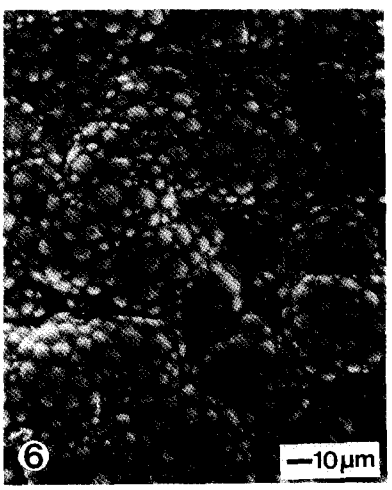

Fig. 6. *Fat body tissues from control larvae of* ***Helicoverpa assulta****. Many lipid granules are packed in cells.*

Control midguts are shown in Figs. 1 and 3. Toxin-treated midguts look a little different in shape from those of controls at the lowest magnification (Fig. 2). At the high magnification, however, it is found that the endotoxin caused extensive damage to the midgut. With sublethal doses of toxin, most of the microvilli disappeared and the epithelium completely eroded, leaving the fissures (Fig. 4). Moreover, bacteria and spores of unknown origin deposited on the midgut wall were observed. Probably these bacteria might cause the secondary effects of disruption. While, in control, the microvilli are closely packed together on the surface at epithelial cells (Fig. 3).

Toxin established an opening through the midgut cells (Fig. 5). Around the pore, a few of microvilli remained and spores were deposited. These morphological modifications in toxin-affected gut suggest that this might be a cytolytic effect, possibly due to colloid-osmotic lysis.

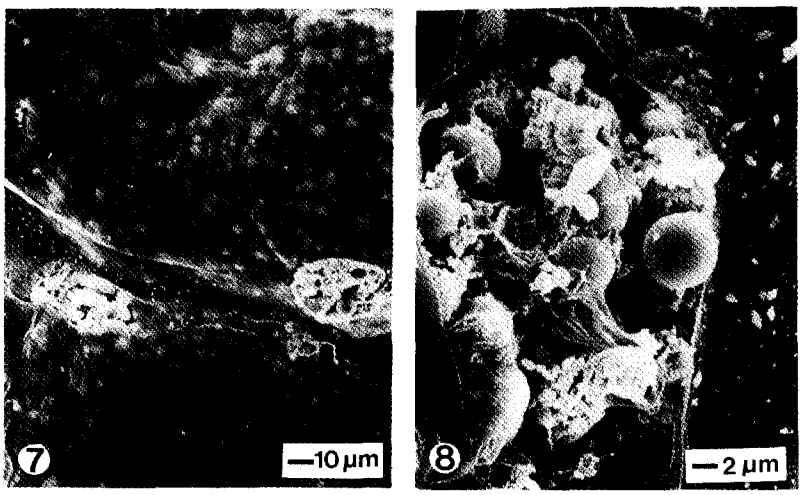

Fig. 7, 8. *Fat body tissues from* ***Helicoverpa assulta*** *after ingestion of toxin. At low magnification, two toxin-affected sites are found. Magnification of the damaged site reveals the protrusion of lipid granule through the tissue.*

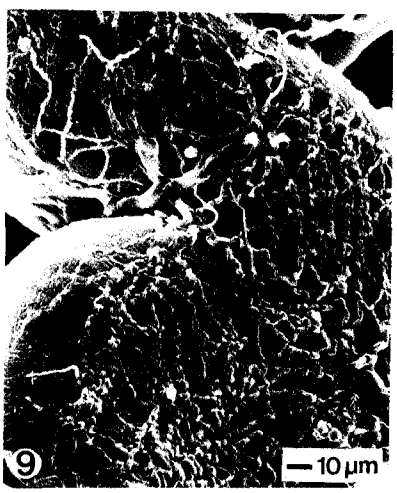

Fig. 9. *Malpighian tubule from control larvae of* ***Helicoverpa assulta****. Complicated surface structures are observed.*

Fig. 10. *Malpighian tubule from* ***Helicoverpa assulta*** *larvae after ingestion of toxin. The surface structure is eroded, leaving many furrows on the surface.*

The toxin may bind initially to a component of the cell membrane, for example a specific receptor, and generate pores (Knowles & Ellar, 1987). Since the toxin has a cumulative effect, the membranes may become leaky by the generation of pores, perhaps after binding of certain number of toxin molecules (Lane *et al*, 1989). Apparently, the mixing of the gut contents with the hemolymph might cause the toxin-affection to the nonmidgut tissues, and then lead for mortality.

Surprisingly, the fat bodies of toxin-ingested larvae showed the disintegration in part (Fig. 7). In control larvae, fat body tissues showed many lobes which were composed of lipid granule-packed cells (Fig. 6). On intoxication of Bt, the membrane covering fat body tissues was eroded, resulting the rupture in the several sites. At high magnification, it was found that large lipid granule was protruded from the tissues. In the vicinity of the fissure, few spores were dispersed (Fig. 8). In fat bodies, res-

ponse to the toxin was not homogeneous as damage to some cells was more advanced than in adjacent cells.

In case of Malpighian tubules, the toxin seems to dissolve the surface structures. In control, many reticular surface structure, which might appear to be trachea and other connective tissues, was observed (Fig. 9). Whereas, in the toxin-treated tissue, the surface structure was eroded homogenously and yet no opening was observed (Fig. 10). Probably higher concentration of toxin seems to be necessary to cause the fissures or opening.

Estes and Faust (1964) suggested that δ-endotoxins dissolve the extracellular cell cementing substance. Preliminary *in vitro* studies with the insect Malpighian tubule have indicated that the binding of the 27Kd δ-endotoxin var. *isrealensis* to the plasma membrane also cause changes that ultimately permit leakiness (Lane *et al.*, 1987; Maddrell *et al.*, 1988).

The toxin also caused the rupture of testis. The control testis showed oval-shape, and the surface was shiny and smooth (Fig. 11). In contrast, toxin-treated testis showed the groove on the surface and was broken to pieces. But we could not observe the swelling phenomenon as seen in *H. cunea* (data not shown).

The phenomenon observed during our investigation of pathological significance is the erosion and disruption of tissues. This is very common in all tissues tested. Several studies have been conducted on the toxin receptor of nonmidgut cells, along with midgut cells. The membrane components with which the *B. thuringiensis* var. *kurstaki* interacts apparently do not occur on all insect cell surfaces because the toxin does not bind to or lyse *P. brassicae* hemocytes (Hofmann and Lüthy, 1986). In contrast, the mosquitocidal 27Kd δ-endotoxin derived from *B. thuringiensis* var. *isrealensis* recognizes and binds to all cells so far tested including hemocytes (Thomas and Ellar, 1983), which corroborates to our results. Further, our results on the *in vivo* binding of crystal proteins to insect tissue sections are supported by the results obtained by immunolocalization of *B. thuringiensis* var. *kurstaki* exposed tissues (Ryerse *et al.*, 1990).

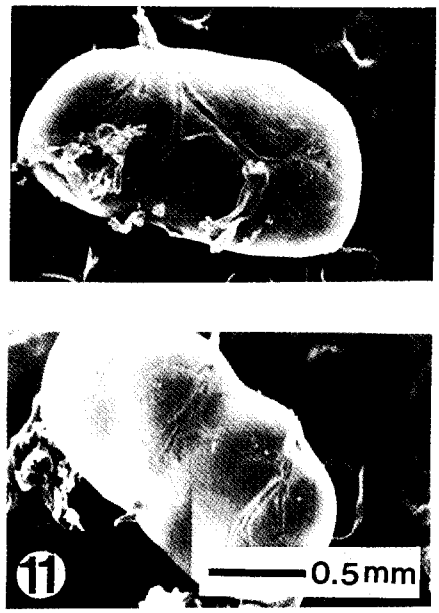

Fig. 11. *Testis from control larvae of Helicoverpa assulta. The surface of testis appears smooth and shiny.*

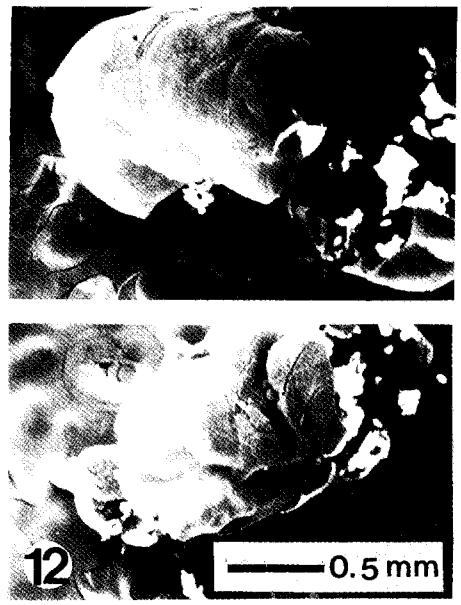

Fig. 12. *Testis from Helicoverpa assulta larvae after ingestion of toxin. The testis is completely broken.*

This could possibly be explained by the specificity of antibodies (Bravo *et al.*, 1992) and by the specificity of intoxicated insects.

The present results indicate the wide distribu-

tion of "receptors" for the *B. thuringiensis* var. *kurstaki* endotoxin on the surface of various cells. However, they provide no clues as to the molecular characteristics of these components. Further studies on the cellular distribution of receptors may require the development of specific antibody probes coupled with immunolocation studies at the ultrastructural level.

적 요

Bacillus thuringiensis var. *kurstaki*의 내독소단백질을 섭취한 *Helicoverpa assulta*의 유충에서 각 조직의 표면구조변화를 주사전자현미경으로 조사하였다. *Bt* 내독소단백질은 조사된 모든 조직 표면에서 부식과 파괴현상을 유발시켰다. 이러한 결과들로 볼 때 독소분자들은 세포막에 대한 뚜렷한 특이성이 없이 독소에 노출된 모든 종류의 세포막에 결합하는것으로 보인다.

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