

## ***Cytotoxicity of the Bacillus thuringiensis Crystal Protein against Mammalian Cells***

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The crystal proteins produced by *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) and subsp. *coreanensis* A1519 strain were examined for the cytotoxicity against MOLT-4 and HeLa cells by MTT assay and LDH assay. The A1519 crystal proteins processed by proteinase K exhibited the specific cell-killing activity toward MOLT-4 with little damage to the cell membrane. On the other hand, the *Bti* crystal proteins processed by proteinase K caused the substantial damage to the cell membrane of both MOLT-4 and HeLa, leading to the cell lysis. The non-digested crystal proteins of both strains exhibited no cytotoxicity. These data suggested that while the *Bti* crystal proteins caused the colloid-osmotic swelling and cell lysis of MOLT-4 and HeLa, the proteinase K-digested A1519 crystal proteins induced the specific cell death of MOLT-4 through a mechanism other than that of *Bti*.

### **1. INTRODUCTION**

*Bacillus thuringiensis* produces crystalline inclusions consisting of highly specific insecticidal proteins called  $\delta$ -endotoxins during sporulation, which are toxic to the larvae of lepidopteran, dipteran, and coleopteran insects, and are currently classified into two families, Cry and Cyt proteins [7]. However it was also reported that non-insecticidal *B. thuringiensis* occurs in natural environments more widely than insecticidal ones [11,14]. In 1999 a novel activity, the cytotoxicity against leukemia T cells and other human cancer cells, was found in parasporal inclusions of non-insecticidal and non-haemolytic *B. thuringiensis* isolates [12]. *B. thuringiensis* subsp. *coreanensis* A1519 (90-K-14-20) strain is one of the strains whose parasporal crystal proteins are non-insecticidal and non-haemolytic but cytotoxic against human leukemic cells [12].

Among  $\delta$ -endotoxins, Cyt proteins also exhibit the cytotoxicity against mammalian cells and haemolytic activity in addition to the insecticidal activity against dipteran larvae. Therefore, the comparative study of the novel mammalian cell-recognizing crystal protein and the Cyt proteins for the cytotoxicity will give a lot of information about its mode of action. In this study the crystal proteins produced by *B. thuringiensis* subsp. *coreanensis* A1519 strain were examined for the functional properties regarding the cytotoxicity toward MOLT-4, the leukemic T cell, in comparison with those of *B. thuringiensis* subsp. *israelensis*.

### **2. MATERIALS AND METHODS**

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### 2.1 Bacterial strain and mammalian cells

The *B. thuringiensis* subsp. *coreanensis* A1519 strain (90-K-14-20, serotype H25) used in this study was isolated from the soil in Kyoto, Japan. The human cell lines used in this study were MOLT-4, a leukemic T cell, and HeLa, a uterus cervix cancer cell.

### 2.2 Preparation, solubilization, and digestion of crystal proteins

Purification of crystal proteins was done as described previously [13]. The crystal was solubilized in 100 mM Na<sub>2</sub>CO<sub>3</sub> (pH10.5)/ 10 mM DTT at 37°C for 1 hr. The solubilized crystal proteins were digested by proteinase K (Roche) or trypsin (Roche) at 37°C for 1 hr. At the end of incubation, PMSF, phenylmethylsulfonyl fluoride (Sigma) was added at a concentration of 1 mM.

### 2.3 Cytotoxicity assay

The cytotoxicity was estimated by MTT assay. Ninety microliters of a cell suspension (5×10<sup>5</sup> cells/ml) and 10 µl of the crystal proteins were placed in each well of a 96-well microplate, and incubated at 37°C for 3 hr. Then 0.5 µg/µl of MTT, 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (Sigma), was added and incubated for 3 hr. After centrifugation, the supernatant was removed, and the precipitate of the converted dye were solubilized with acidic isopropanol. The absorbance of the converted dye was measured at 570 nm and the survival rate of the cells was calculated. The average absorbance of mock-inoculated negative controls was taken as a high value (100% cell survival), and that of Triton-X100 (Nacalai Tesque)-inoculated positive controls was taken as a low value (0% cell survival).

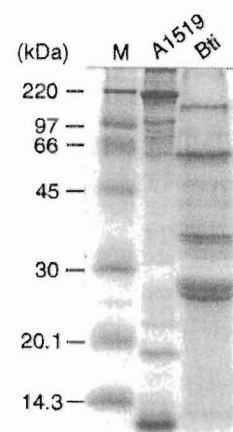
### 2.4 Evaluation of the cell membrane damage

The damage of cell membrane was estimated by measuring the activity of LDH, lactate dehydrogenase, leaked out of the damaged cells. One hundred and eighty microliters of a cell suspension containing 1×10<sup>5</sup> cells and 20 µl of the crystal proteins were placed in each well of a 96-well microplate, and incubated at 37°C for 3 hr. Then, the microplate was centrifuged at 250 × g for 10 min and 100 µl of the supernatant from each well were transferred to a new microplate. The activity of LDH in the supernatant was estimated by measuring the absorbance at 490 nm using LDH cytotoxicity detection kit (Takara). The average absorbance of mock-inoculated negative controls was taken as a low value (0% of damage), and that of Triton-X100 (Nacalai Tesque)-inoculated positive controls was taken as a high value (100% of damage).

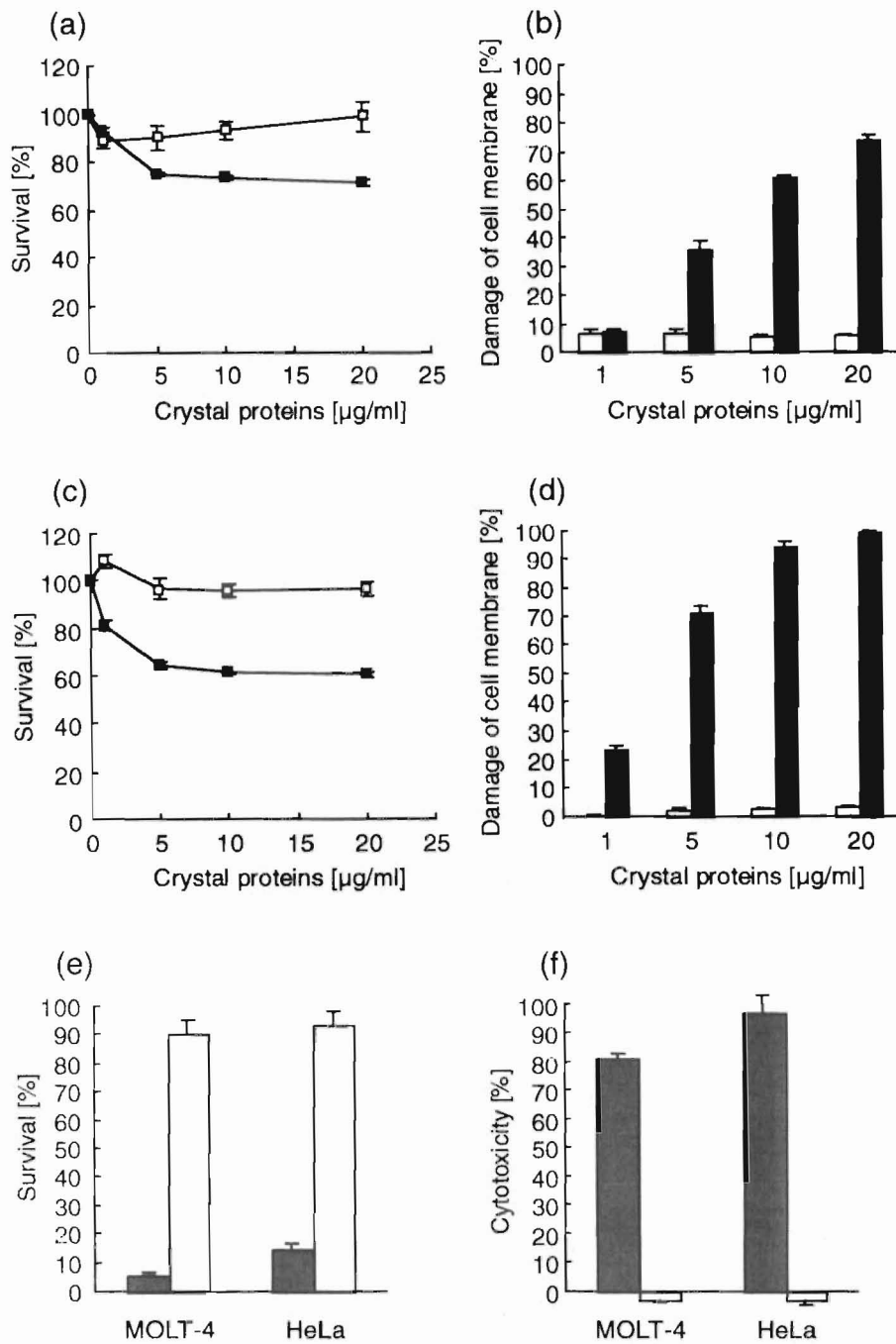
## 3. RESULTS

### 3.1 Protein composition of the crystal

The purified crystal produced by A1519 strain contained a variety of proteins ranging from 150 kDa to 10 kDa in size (Fig. 1). The major components detected in SDS-14% PAGE were polypeptides of about 150 kDa, 90 kDa, 70kDa, 20 kDa, and 10 kDa. The crystal produced by *B. thuringiensis* subsp. *israelensis* consisted of Cry4A and Cry4B of 130 kDa, Cry11A of 70 kDa, and Cyt1A of 28 kDa, all of which were observed in Fig. 1. Among them, Cyt1A, the most abundant polypeptide, is known to have non-specific cytotoxicity.



**Fig. 1** Protein composition of the crystals from A1519 strain and *Bti*. Ten micrograms of the solubilized crystal proteins were analyzed by SDS-14% PAGE followed by CBB staining. M, molecular size markers.



**Fig. 2 The damage of cell membrane induced by the proteinase K-digested *Bti* crystal proteins.** The *Bti* crystal proteins were solubilized and digested by proteinase K, and analyzed by MTT assay for the cytotoxicity toward MOLT-4 (a) and HeLa (c). Cell survival in the presence of the solubilized (open box) and the proteinase K-digested (solid box) crystal proteins were indicated. The damage of cell membrane of MOLT-4 (b) and HeLa (d) was also analyzed by LDH assay. The damage of cell membrane was indicated as the activities of LDH leaked out of the cell in the presence of the solubilized (open box) and the proteinase K-digested (solid box) crystal proteins. MTT assay (e) and LDH assay (f) were done in the presence of 100 µg/ml *Bti* crystal proteins. Results with the proteinase K-digested *Bti* crystal proteins (shaded box) and with proteinase K-digested BSA (open box) are shown.

### 3.2 The cytotoxic properties of *Bti* crystal proteins

To investigate the cytotoxic effect of *Bti* crystal proteins on MOLT-4 cells, both MTT assay and LDH assay were done. In Fig. 2a and c are shown the results of MTT assay with MOLT-4 cells and HeLa cells, respectively. These results suggested that neither the solubilized nor the proteinase K-digested *Bti* crystal proteins induced the effective cell death of MOLT-4 and HeLa, i.e. most cells survived from the treatment with the *Bti* crystal proteins. However, the results of LDH assay indicated that the proteinase K-digested *Bti* crystal proteins caused the significant damage of cell membrane of both MOLT-4 and HeLa and, again, the solely solubilized crystal was non-cytotoxic (Fig. 2b and 2d), suggesting that the major consequence by the *Bti* crystal proteins was the cell membrane damage. Thus, it was strongly suggested that the cytotoxic effects shown above were caused by the non-specific cytolysin Cyt1A. We also confirmed the cell death and membrane damage of both MOLT-4 and HeLa cells in the presence of a higher concentration (100 µg/ml) of *Bti* crystal proteins (Fig. 2e and f).

### 3.3 The cytotoxic properties of A1519 crystal proteins

Similar experiments using the A1519 crystal proteins were also done with MOLT-4 and HeLa cells. The proteinase K-digested A1519 crystal proteins were toxic to MOLT-4 cells. Almost all the cells were killed at 5 µg/ml of the proteinase K-digested crystal proteins, although the non-processed crystal proteins exhibited no detectable cytotoxicity against MOLT-4 cells even at a concentration of 20 µg/ml (data not shown). Interestingly, the results of LDH assay showed that the mortality of the MOLT-4 cells was only 40% even in the presence of 20 µg/ml proteinase K-processed crystal proteins, and that the merely solubilized crystal proteins exhibited, again, no detectable cytotoxicity (data not shown). This suggested that the cell death of MOLT-4 occurred through some mechanisms other than the damage of cell membrane with pore formation caused by so far known  $\delta$ -endotoxins. We also investigated the cytotoxic effect of A1519 crystal proteins on HeLa cells. The result of MTT assay with HeLa cells suggested that both the solubilized and the proteinase K-digested crystal proteins exhibited very weak cytotoxicity against HeLa cells (data not shown). Moreover, neither the solubilized nor the proteinase K-digested crystal proteins caused detectable membrane damage of the HeLa cell as indicated by LDH assay (data not shown). These results suggested that the A1519 crystal proteins were specifically active against human leukemic T cell, MOLT-4. It was confirmed that proteinase K itself and the proteinase K-digested BSA exhibited no detectable cytotoxicity against MOLT-4 cells at the concentrations employed in this study (data not shown). Furthermore, we investigated the cytotoxicity of the proteinase K-digested A1519 crystal proteins against normal T cells, proving that it caused little cytotoxicity (data not shown). Therefore it is suggested, again, that the A1519 crystal proteins were specifically active against the human leukemic T cell.

## 4. DISCUSSION

In this study, we investigated the cytotoxicity of the crystal proteins produced by *Bacillus thuringiensis* subsp. *israelensis* and subsp. *coreanensis* A1519 strain. They are proved cytotoxic against the human leukemic cells. We found that the proteinase K-digested *Bti* crystal proteins caused the colloid-osmotic swelling and cell lysis of MOLT-4 and HeLa, but the proteinase K-digested A1519 crystal proteins induced the specific cell death of MOLT-4 through a mechanism other than that of *Bti*.

*Bti* crystal proteins consist of Cry4A (130 kDa), Cry4B (130 kDa), Cry11A (70 kDa), and Cyt1A (28 kDa) [1, 7, 8], which exhibited non-specific cytotoxic activities against insect and mammalian cells in addition to the dipteran-specific insecticidal activity [15]. The cytotoxicity of *Bti* crystal proteins was attributed to Cyt1A, a broad-spectrum cytolysin, active on a variety of vertebrate and invertebrate cells [4, 15]. Currently eleven Cyt  $\delta$ -endotoxins are reported, and are believed to adopt a similar structure because they share a high degree of sequence similarity [5]. The structure of Cyt2Aa was revealed in aqueous solution [10]. Based on the structural

information of Cyt2Aa together with the characterization of Cyt1Aa mutants [16] and the proteolysis of membrane-bound Cyt1Aa and Cyt2Aa [6], it is suggested that Cyt toxin form a  $\beta$ -barrel pore via insertion of  $\beta 5$ ,  $\beta 6$ , and  $\beta 7$  into the membrane. On the other hand, Butko *et al.* reported that the effect of Cyt1A is a general and detergent-like perturbation of the membrane surface rather than creation of a proteinaceous channels or pores [2, 3]. No matter which incident, formation of the pore or detergent-like perturbation effect on the membrane, the Cyt toxin causes, the consequence of the Cyt toxin action is the cell membrane damage leading to the colloid-osmotic cell lysis [9]. The data obtained in this study also strongly suggested that the proteinase K-digested *Bti* crystal proteins, major active component of which is Cyt1A, caused membrane damage of mammalian cells (Fig. 2). However, the proteinase K-digested A1519 crystal proteins exhibited the strong cell-killing activity not accompanied with the cell membrane damage. It is, therefore, suggested that the major effect of A1519 crystal proteins is not the cell membrane damage, but some other incident such as apoptosis. But we cannot rule out the possibility that the A1519 crystal proteins cause the pore formation or a detergent-like perturbation effect on the membrane. One of the most plausible interpretation for the mode of action of A1519 crystal proteins is as follows; a receptor on the membrane of susceptible cells for the A1519 crystal proteins was by chance a cell-death inducing membrane protein, so the binding of the A1519 crystal proteins to such a membrane protein set on the cell-death inducing (apoptotic) signal. The cytotoxic effect through membrane damage by the A1519 crystal proteins is, if any, negligible at the concentration employed in this study.

In this study we have reported the distinctive biological activity of A1519 crystal proteins. Further investigation is in progress to reveal what the active component of A1519 crystal proteins is, what the receptor molecule of mammalian cells for the A1519 crystal proteins is, and how A1519 crystal proteins induce the death of the mammalian cells.

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