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The cytotoxicity of Bacillus thuringiensis subsp. coreanensis A2316 strain against the human leukemic T cell

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Bacillus thuringiensis subsp. *coreanensis* A2316 is a newly isolated strain from Yonakunijima Island in Japan. It produces the proteinaceous inclusion body (crystal) which has no insecticidal and hemolytic activities. When the crystal proteins were digested by proteinase K, they exhibited the strong cytotoxicity against human leukemic T cell, MOLT-4. The proteinase K-digested A2316 crystal proteins have little damage upon the cell membrane of MOLT-4, suggesting that the cell death of MOLT-4 was induced through a mechanism other than the colloid-osmotic swelling and cell lysis as caused by hitherto known *B. thuringiensis* crystal proteins. The 29-kDa polypeptide proved to be an active component of the proteinase K-digested A2316 crystal proteins. EC_{50} of the purified 29-kDa polypeptide was 0.0579 μg/ml. The N-terminal amino acid sequence of the 29-kDa polypeptide was identical with that of p29 produced by *B. thuringiensis* A1519 strain and shared no significant homology with all the known proteins, suggesting that this polypeptide belong to a new family of *B. thuringiensis* crystal proteins.

1. INTRODUCTION

Bacillus thuringiensis produces crystalline inclusions consisting of highly specific insecticidal proteins called δ-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 [1]. However it was also reported that non-insecticidal *B. thuringiensis* occurs in natural environments more widely than insecticidal ones [2, 3]. 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 [4]. Among these isolates, the strain 84-HS-1-11 produces parasporal inclusions containing an 81-kDa protein which shows, upon activation by trypsin or proteinase K, a strong cytocidal activity against human leukemic T cells (MOLT-4) and human uterus cervix cancer cells (HeLa) but not against normal T cells. The *cry31Aa1* gene encoding this 81-kDa protein designated as parasporin was cloned and the

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deduced amino acid sequence contained the five conserved blocks, but shared very low homologies with the known classes of Cry and Cyt proteins [5]. Lee *et al.* reported that the 28-kDa polypeptide produced, upon proteinase K digestion, from the crystal protein of *B. thuringiensis* subsp. *shandongiensis* 89-T-34-22 strain exhibited the human leukemic cell-specific cytotoxicity [6, 7]. Furthermore, the 64-kDa major polypeptide produced, upon proteinase K digestion, from the crystal proteins of the *B. thuringiensis* isolate 89-T-26-17 exhibited cytotoxicity against MOLT-4 and HeLa cells, but showed no cytotoxicity to normal T cells [8].

In this study the crystal proteins produced by *B. thuringiensis* subsp. *coreanensis* A2316 strain were examined for the functional properties regarding the cytotoxicity toward MOLT-4, the leukemic T cell. It was suggested that the cytopathic effect by the A2316 crystal proteins with little damage to the cell membrane was different from the colloid-osmotic swelling and cell lysis induced by Cry or Cyt proteins of *B. thuringiensis*. We purified the 29-kDa active component from the proteinase K-digested A2316 crystal proteins, which is likely to be a member of a new family of *B. thuringiensis* crystal proteins.

2. MATERIALS AND METHODS

2.1 Preparation, solubilization, and digestion of crystal proteins

Purification of crystal proteins was done as described previously [9]. The crystal was solubilized in 100 mM Na_2CO_3 (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.

2.2 MTT assay

The cytotoxicity was estimated by MTT assay. Ninety microliters of a cell suspension (5×10^5 cells/ml) and 10 µl solution of the crystal proteins were placed in each well of a 96-well microplate, and incubated at 37°C for 3 hr. Then 10µl solution of 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 was 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).

2.3 Protein sequencing

The proteinase K-digested A2316 crystal proteins were fractionated in SDS-14%-PAGE and transferred to a PVDF membrane (Bio-Rad). The N-terminal amino acid residues of the 29-kDa polypeptide were sequenced using a model 491 protein sequencer of Applied Biosystems.

3. RESULTS

3.1 Processing of A2316 crystal proteins

The purified A2316 crystal proteins were processed *in vitro* by proteinase K and analyzed by SDS-PAGE (Fig. 1A). Through the proteinase K digestion, the 150-kDa band disappeared and bands of about 60 kDa were generated as the ratio of proteinase K to the crystal protein was increased. The other polypeptides were also processed into the smaller ones, and finally only polypeptides smaller than 30 kDa were detected at high ratios of proteinase K to the crystal protein. The cytotoxicity of the A2316 crystal proteins against MOLT-4 cells was analyzed by MTT assay, showing that the solubilized crystal proteins exhibited no detectable cytotoxicity (data not shown) and that the fully processed crystal proteins induced the cell death of MOLT-4 (Fig. 1B). We confirmed that proteinase K itself induced no detectable cell death of MOLT-4 at the concentrations indicated in Fig. 1 (data not shown).

3.2 The cytopathic effect of A2316 crystal proteins toward MOLT-4 cells

The cytopathic effect of the proteinase K-digested A2316 crystal proteins toward MOLT-4 cells was observed under a light microscopy (Fig. 2). The proteinase K-digested A2316 crystal proteins caused the nuclear condensation and the cell swelling 1 hr after the toxin administration, but cell lysis was not observed even after the prolonged incubation for 24 hr. It was suggested that the cytopathic effect of the A2316 crystal proteins was different from the colloid-osmotic swelling and cell lysis caused by so far known *B. thuringiensis* Cry or Cyt toxins.

3.3 Purification of the active component from the proteinase K-digested A2316 crystal proteins

To identify the active component responsible for the cytotoxicity against MOLT-4 cells, gel filtration chromatography of the proteinase K-digested A2316 crystal proteins was done, and the cytotoxicity of each fraction was monitored by MTT

assay with MOLT-4 cells. The fractions that contained the cytotoxic activity were found and analyzed by SDS-PAGE, manifesting a band corresponding to a 29-kDa polypeptide (Fig. 3A and 3B).

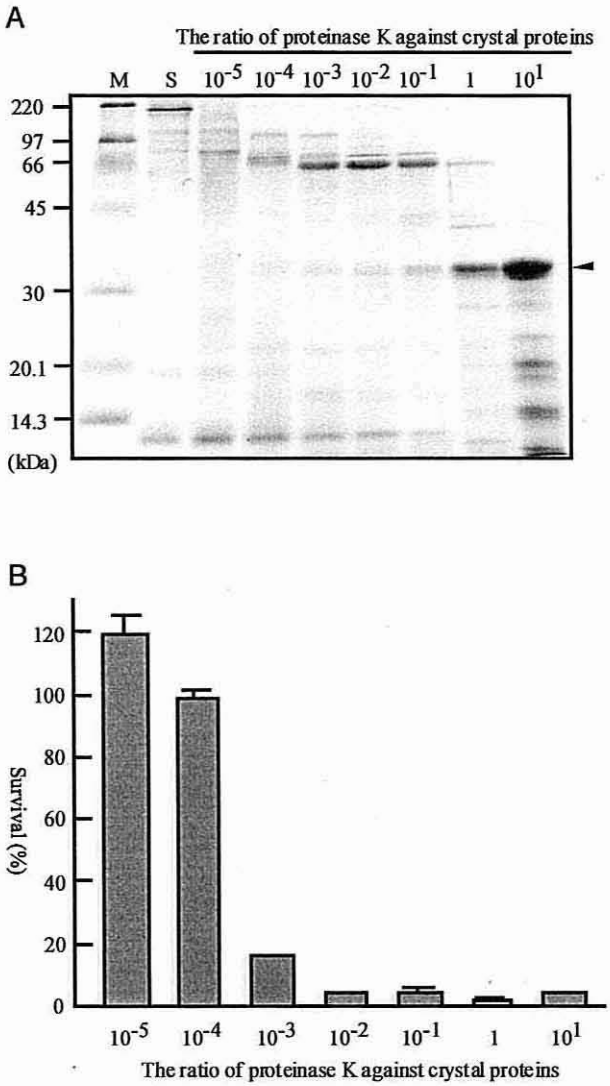

Fig. 1 The processing and the cytotoxicity of the A2316 crystal proteins.
(A) The purified A2316 crystal proteins were solubilized and processed by proteinase K at the indicated ratio of proteinase K to the crystal proteins. Ten micrograms of each digest were analyzed by SDS-14%-PAGE followed by CBB staining. The arrowhead indicates proteinase K. M, molecular size marker; S, the solubilized crystal proteins. (B) The cytotoxicity against MOLT-4 of the proteinase K-digested crystal proteins was estimated by MTT assay.

The N-terminal amino acid sequence of the 29-kDa polypeptide was proved DVIREYLMFNELS ALSS(T/S)PE, which was identical with that of p29 produced by A1519 strain [10] and shared no homology with those of all the known proteins including Cry or Cyt proteins of *B. thuringiensis*. Based on the obtained data of MTT assay, EC_{50} of the purified 29-kDa polypeptide to MOLT-4 cells was estimated to be 0.0579 μg/ml (95% confidence limits; 0.0557-0.0602) by Probit analysis.

4. DISCUSSION

In this study, we investigated the cytotoxicity against human leukemic cells of the crystal proteins produced by *B. thuringiensis* subsp. *coreanensis* A2316 strain. We found that the proteinase K-digested crystal proteins induced the specific cell death of MOLT-4 through a mechanism other than the colloid-osmotic swelling and cell lysis that were caused by hitherto known Cry or Cyt proteins of *B. thuringiensis*, and that the 29-kDa fragment of the proteinase K-digested crystal proteins was the active component against MOLT-4. The purified 29-kDa polypeptide had a cytotoxic activity similar to p29 from A1519 strain; EC_{50} of the 29-kDa polypeptide against MOLT-4 was 0.0579 μg/ml, whereas that of p29 was 0.078 μg/ml [10]. The N-terminal 20-amino acid sequence of the 29-kDa polypeptide was identical with that of p29 produced by A1519 strain [10] and shared no significant homology with all the known proteins including Cry or Cyt toxins.

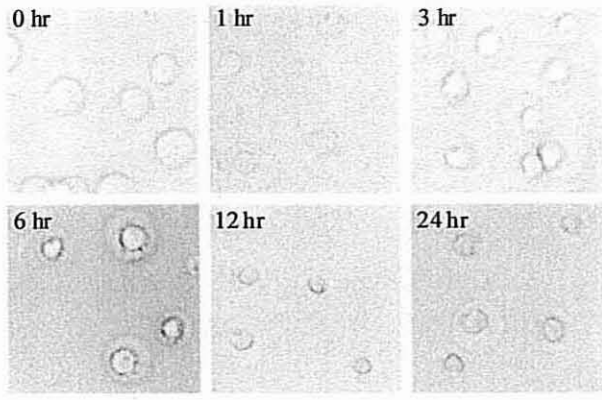

Fig. 2 The cytopathic effect of the proteinase K-digested A2316 crystal proteins against MOLT-4.
MOLT-4 cells were incubated in the presence of 10 μg/ml of the proteinase K-digested A2316 crystal proteins. The cytopathic effect on the cells was observed under a light microscopy 1 hr, 3 hr, 6 hr, 12 hr or 24 hr after applying the crystal proteins.

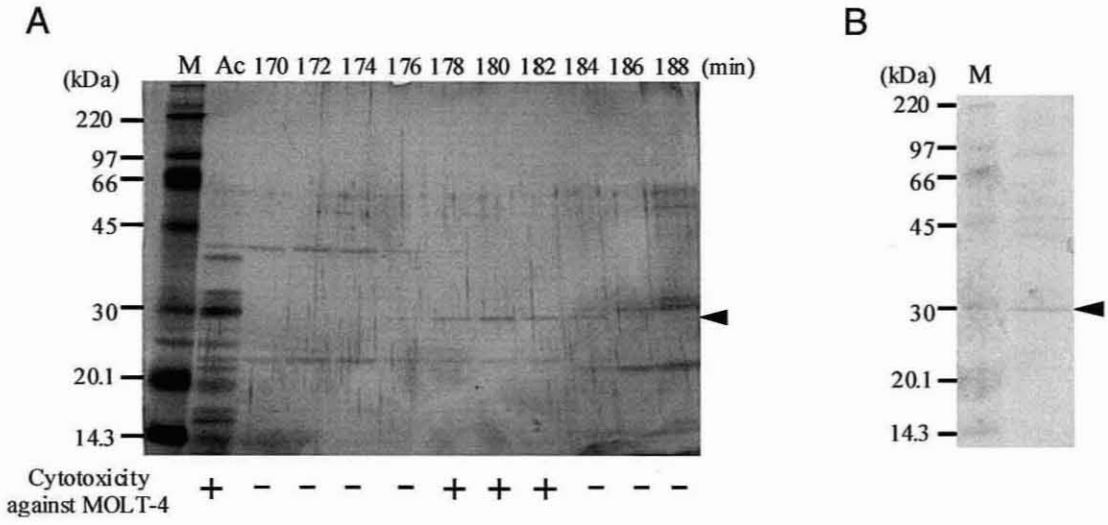

Fig. 3 Purification of the 29-kDa polypeptide active toward the MOLT-4 cells.
(A) Gel filtration chromatography of the proteinase K-digested A2316 crystal proteins was done using the superdex 200 pg (Amersham Pharmacia Biotech) column. The eluate from the column was pooled in 2-ml aliquots, and was analyzed by MTT assay for the cytotoxicity against MOLT-4 cells. The arrowhead indicates the 29-kDa polypeptide cytotoxic against MOLT-4 cells. +, cytotoxic against MOLT-4; -, not cytotoxic against MOLT-4. M, molecular size marker; Ac, the proteinase K-digested A2316 crystal proteins. (B) One microgram of the purified 29-kDa polypeptide was analyzed by SDS-14% PAGE followed by silver staining. M, molecular size marker.

Since the 29-kDa polypeptide from A2316 strain had the same N-terminal amino acid sequence as p29 from A1519 strain, it was suggested that both the polypeptides were very similar to each other. Recently we have found that p29 from A1519 strain induce apoptosis in Jurkat, another human leukemic T cell line (manuscript in preparation). Therefore, it is reasonable to assume that the 29-kDa polypeptide from A2316 strain would also induce the apoptotic cell death.

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