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The cytotoxicity of *Bacillus thuringiensis* subsp. *coreanensis* A1519 strain against the human leukemic T cell

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Abstract

A novel cytotoxic protein was isolated from the crystal produced by *Bacillus thuringiensis* subsp. *coreanensis* A1519 strain. Upon treatment of the crystal proteins by proteinase K, the significant cytotoxicity toward the leukemic T cell, MOLT-4, was exhibited. The microscopic observation indicated that the cell death was accompanied by no extensive rupture of the cell membrane. It was, therefore, suggested 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 A1519 crystal proteins. EC_{50} of the purified 29-kDa polypeptide was 0.078 μg/ml. The N-terminal amino acid sequence of the 29-kDa polypeptide shared no significant homology with all the known proteins, suggesting that this polypeptide belong to a new family of *B. thuringiensis* crystal proteins. In the ligand blotting analysis, specific binding proteins for the 29-kDa polypeptide were detected from the cell membrane of MOLT-4.

Keywords: *Bacillus thuringiensis*; Cytotoxicity; MOLT-4; Crystal protein; T cell

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 of an 81-kDa protein which shows, upon activation by trypsin or proteinase K, 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 its 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].

These mammalian cell-recognizing crystal proteins have formed a new protein family of δ-endotoxin produced by *B. thuringiensis*. However, among δ-endotoxins, Cyt proteins also exhibit the cytotoxicity against mammalian cells

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and haemolytic activity in addition to the insecticidal activity against dipteran insect larvae. Therefore, the comparative study of the mammalian cell-recognizing crystal protein and the Cyt proteins for the cytotoxicity will give a lot of information about the mode of action against mammalian cells. *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 cytocidal against human leukemic cells [4]. No further biochemical analysis has been done to date.

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. It was suggested that the cytopathic effect by the A1519 crystal proteins was different from that of *B. thuringiensis* Cry or Cyt proteins inducing the colloid-osmotic swelling and cell lysis. We purified the 29-kDa active component from the proteinase K-digested A1519 crystal proteins, which is likely to be a member of a new family of *B. thuringiensis* crystal proteins. Furthermore, we found a few specific binding proteins for the 29-kDa cytotoxic polypeptide in the membrane fraction of MOLT-4.

2. Materials and methods

2.1. Bacterial strain and human cell lines

The *B. thuringiensis* subsp. *coreanensis* A1519 strain (90-K-14-20, serotype H25) used in this study was isolated from 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. Normal human T cells were prepared from buffy coats obtained from Okayama Red Cross Blood Center (Okayama, Japan). T cells were separated from lymphocyte cells using a Collect™ Human T Cell kit (Biotex Laboratories Inc., Alberta, Canada) and were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum and kanamycin (30 μg/ml) at 37 °C.

2.2. Preparation, solubilization, and digestion of crystal proteins

Purification of crystal proteins was done as described previously [9]. Briefly, A1519 strain was cultured in Schaeffer medium until the cells were lysed completely, resulting in the lysate containing the spores and crystals. The crystal was purified from the cell lysate by ultracentrifugation through a stepwise sucrose gradient. The crystal was solubilized in 100 mM Na_2CO_3 (pH 10.5)/10 mM DTT at 37 °C for 1 h. The solubilized crystal proteins were digested by proteinase K (Roche) or trypsin (Roche) at 37 °C for 1 h. At the end of incubation, phenylmethylsulfonyl fluoride (Sigma), PMSF, was added at a concentration of 1 mM.

2.3. 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 h. Then 10 μl solution of 0.5 μg/μl of MTT, 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2*H*-tetrazolium bromide (Sigma), was added and incubated for 3 h. 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).

2.4. Purification and digoxigenin-labeling of the 29-kDa active polypeptide

The 29-kDa active polypeptide was purified with the gel filtration chromatography using the superdex 200 pg (Amersham Pharmacia Biotech) column at the flow rate of 0.5 ml/min. The elution buffer was 100 mM Na_2CO_3 (pH 10.5). Labeling of the 29-kDa polypeptide with DIG, digoxigenin, was done as described previously [10].

2.5. Protein sequencing

The proteinase K-digested A1519 crystal protein was 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.

2.6. Preparation of the membrane fraction of cells

One gram in wet weight of the cells was washed with PBS and suspended in 10 ml of MET buffer (300 mM mannitol/5 mM EGTA/17 mM Tris–HCl, pH 7.5). After sonication (15 s, three times), 10 ml of 24 mM $MgCl_2$ was added and incubated for 15 min on ice. Then the mixture was centrifuged at $2500 \times g$ for 15 min at 4 °C, then the supernatant was ultracentrifuged at $100{,}000 \times g$ for 30 min at 4 °C. This procedure was repeated once again. The final pellet, the membrane fraction of cells, was suspended in PBS, and stored at −80 °C.

2.7. Ligand blotting

Five micrograms of membrane proteins was separated by SDS-14% PAGE, and transferred to a PVDF membrane. The membrane was washed in PBS for 10 min and blocked with the blocking buffer (10 mM Tris–HCl, 150 mM NaCl, 0.05% Tween 20, 2% BSA) for 1 h. Then the membrane

was incubated with the DIG-labeled 29-kDa polypeptide and the competitor for 1 h at room temperature. After the membrane was washed with the blocking buffer for 15 min three times, the DIG-labeled 29-kDa polypeptide bound to the membrane proteins was detected with anti-DIG antibody conjugated with horseradish peroxidase (Boehringer Mannheim) using ECL western blotting detection reagents (Amersham Pharmacia Biotech).

3. Results

3.1. Protein composition of the crystal

The purified crystal produced by A1519 strain contained a variety of proteins ranging from 150 to 10 kDa in size (Fig. 1). The major components detected in SDS-14% PAGE were polypeptides of about 150, 90, 70, 20, and 10 kDa. Protein bands of 130, 70, and 28 kDa were observed in the crystal produced by *B. thuringiensis* subsp. *israelensis*, corresponding to the molecular weight of the Cry4A and Cry4B, Cry11A, and Cty1A, respectively. The composition of A1519 crystal proteins was quite different from that of *B. thuringiensis* subsp. *israelensis* crystal proteins.

3.2. Processing of A1519 crystal proteins

The purified A1519 crystal proteins were processed in vitro by proteinase K and analyzed by SDS-PAGE (Fig. 2A).

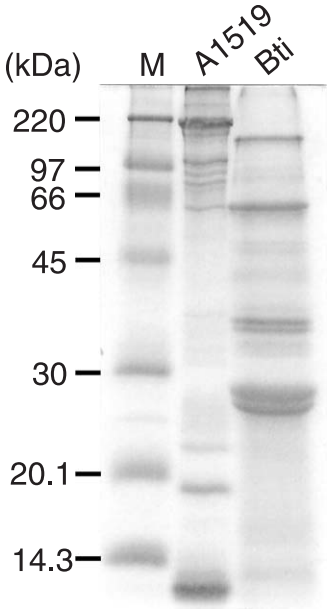

Fig. 1. Protein composition of the crystal from A1519 strain. Ten micrograms of the solubilized crystal proteins were analyzed by SDS-14% PAGE followed by CBB staining. M, molecular size markers. Bti, the crystal proteins of *B. thuringiensis* subsp. *israelensis*.

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 only polypeptides of 30 kDa or smaller were detected at high ratios of proteinase K to the crystal protein. On the other hand, in the tryptic digestion, the band profile remained almost unchanged even at the trypsin/crystal ratio of 10^{-2} (Fig. 2C). The bands of about 60 kDa were observed even at the ratio of 10 (Fig. 2C). These results indicated that the A1519 crystal proteins were, perhaps not unexpectedly, processed much more efficiently by proteinase K than by trypsin. The cytotoxicity of the A1519 crystal proteins against MOLT-4 cells was analyzed by MTT assay, showing that the solubilized crystal proteins exhibited no detectable cytotoxicity and that the proteinase K-processed crystal proteins induced the cell death of MOLT-4 (Fig. 2B and D). We confirmed that proteinase K itself induced no detectable cell death of MOLT-4 at the concentrations indicated in Fig. 2 (data not shown). According to the results shown in Fig. 2, we established that activation of the A1519 crystal proteins was accomplished by incubating the solubilized crystal with proteinase K at the ratio of 10^{-1}.

3.3. Specific cytotoxicity of the A1519 crystal proteins against MOLT-4

The proteinase K-digested A1519 crystal proteins showed strong cytocidal activity against MOLT-4, the human leukemic T cell. To investigate the cytocidal specificity of the A1519 crystal proteins, the cytotoxicity against human normal T cells and human uterus cervix cancer cells (HeLa) was assessed. The survival rate of MOLT-4 cells was about 40% at 1 μg/ml of the proteinase K-digested A1519 crystal proteins, and 0% at 5 μg/ml, whereas about 80% of the normal T cells were not affected even at the 20 μg/ml (Fig. 3). A weak cytotoxicity against HeLa cells was found, but 60% was still alive at 20 μg/ml (Fig. 3). It was, therefore, strongly suggested that the A1519 crystal proteins recognized and killed MOLT-4 cells specifically.

3.4. The cytopathic effect of A1519 crystal proteins toward MOLT-4 cells

The cytopathic effect of the proteinase K-digested A1519 crystal proteins toward MOLT-4 cells was observed under a light microscopy (Fig. 4). The proteinase K-digested A1519 crystal proteins caused the nuclear condensation and the cell swelling 3 h after the toxin administration, but cell lysis was not observed even after the prolonged incubation for 18 h. It was suggested that the cytopathic effect of the A1519 crystal proteins was different from that of so far known *B. thuringiensis* Cry or Cyt toxins causing the colloid-osmotic swelling and cell lysis.

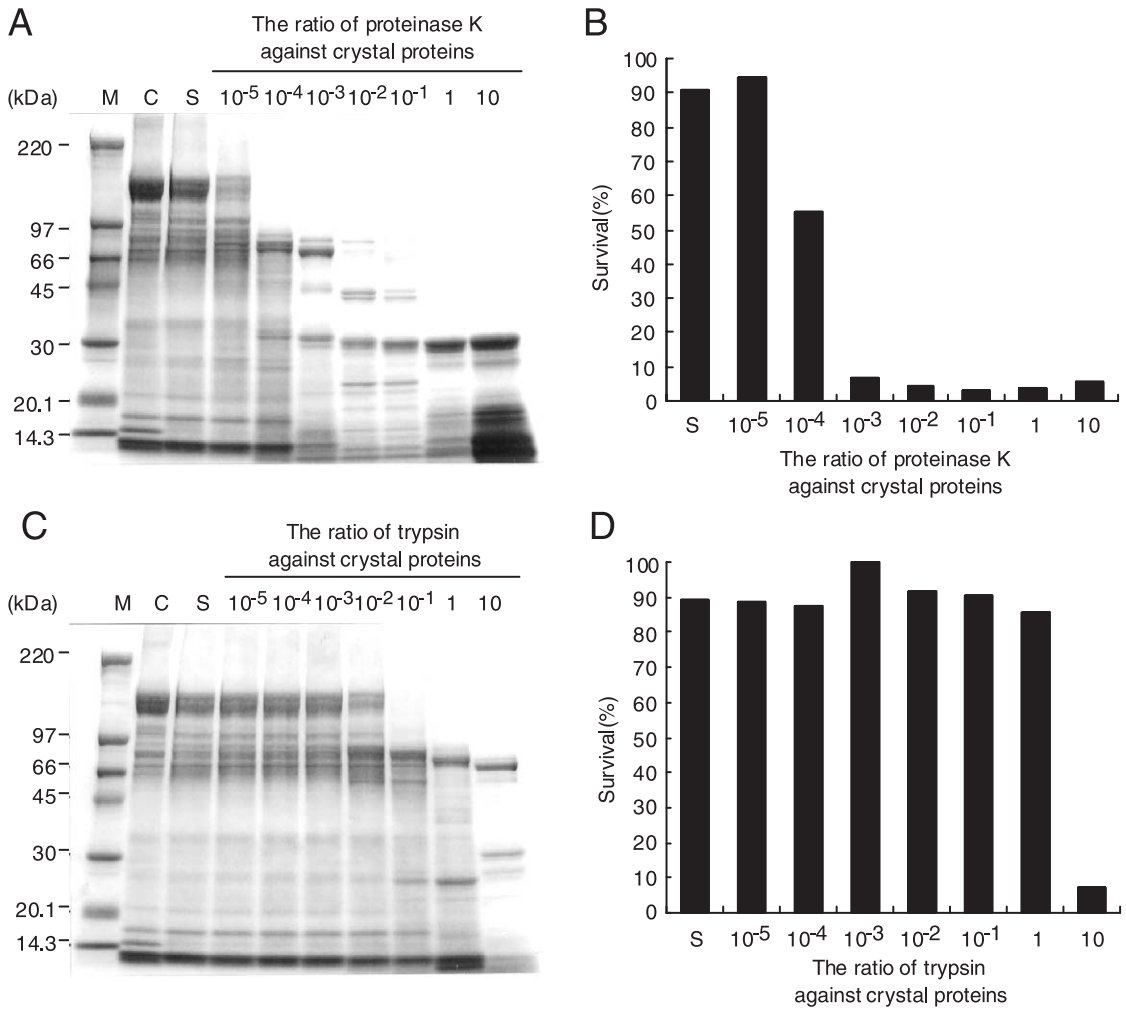

Fig. 2. The processing and the cytotoxicity of the A1519 crystal proteins. The purified A1519 crystal proteins were solubilized and processed by proteinase K (A) or trypsin (C) at the indicated enzyme concentrations. Ten micrograms of each digested crystal protein were analyzed by SDS-PAGE through a 5–20% gradient gel followed by CBB staining. M, molecular size marker; C, 10 μg of the purified crystal proteins; S, 10 μg of the solubilized crystal proteins. The cytotoxicity against MOLT-4 of the proteinase K-digested (B) or the trypsinated (D) crystal proteins was estimated by MTT assay.

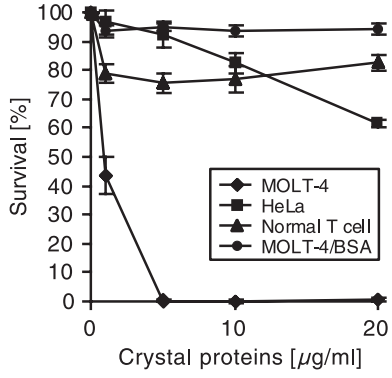

Fig. 3. Specific cell death of MOLT-4 induced by the proteinase K-digested A1519 crystal proteins. The A1519 crystal proteins were solubilized and digested by proteinase K at the ratio of 10^{-1}, and then the cytotoxicity against MOLT-4, Normal T cells and HeLa cells was tested by MTT assay. The cytotoxicity of the proteinase K-digested BSA against MOLT-4 was also analyzed as a negative control. Data represent means for three independent experiments and error deviations are shown.

3.5. Purification of the active component from the proteinase K-digested A1519 crystal proteins

To identify the active component responsible for the cytotoxicity against MOLT-4 cells, the gel filtration chromatography of the proteinase K-digested A1519 crystal proteins was done, and the cytotoxicity of each fraction was monitored by MTT assay with MOLT-4 cells (Fig. 5A). The fractions that contained the cytotoxic activity were found and analyzed by SDS-PAGE, manifesting a band corresponding to a 29-kDa polypeptide (Fig. 5B). The N-terminal amino acid sequence of the 29-kDa polypeptide was proved DVIREYLMFNELSALSSSPE, which shared no homology with those of all the known proteins including Cry or Cyt proteins of *B. thuringiensis*. The EC_{50} of the purified 29-kDa polypeptide to MOLT-4 cells was estimated to be 0.078 μg/ml by the Probit method (Fig. 5C).

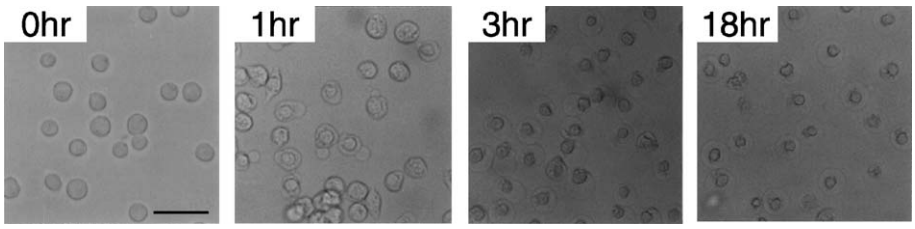

Fig. 4. The cytopathic effect of the proteinase K-digested A1519 crystal proteins at the ratio of 10^{-1} against MOLT-4 cells. MOLT-4 cells were incubated in the presence of 20 μg/ml of the proteinase K-digested A1519 crystal proteins. Under this condition, all the cells were killed 3 h after administration of the crystal proteins. The cytopathic effect on the cells was observed under a light microscopy 1, 3, or 18 h after applying the crystal proteins. The length of a bar corresponding to 50 nm, and all the panels are shown in the same magnification.

3.6. The MOLT-4 cell membrane proteins that specifically bind to the 29-kDa polypeptide

Because the results shown in Fig. 3 suggested that the A1519 crystal proteins were specifically active against MOLT-4 cells, a ligand blotting analysis with the MOLT-4 cell membrane was done to detect the specific binding protein for the 29-kDa active polypeptide. The purified 29-kDa polypeptide was labeled with digoxigenin (DIG), and was confirmed to be biologically as active as the non-labeled 29-kDa polypeptide against MOLT-4 cells (data not shown). Upon ligand blotting experiments, a few bands corresponding to molecular masses from 40 to 45 kDa were detected, indicating that some MOLT-4 cell membrane proteins, whose sizes are from 40 to 45 kDa, bound to the DIG-labeled 29-kDa polypeptide. And the association was

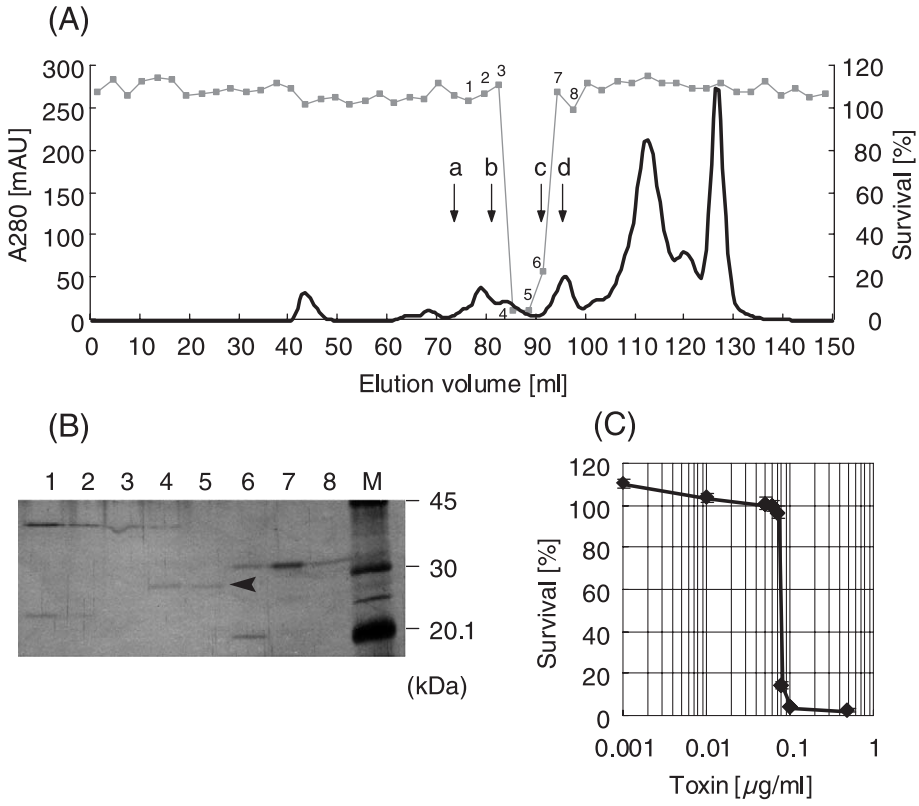

Fig. 5. Purification of the 29-kDa polypeptide active toward the MOLT-4 cells. (A) The gel filtration chromatography of proteinase K-digested A1519 crystal proteins was done using the superdex 200 pg (Amersham Pharmacia Biotech) column. The eluate from the column was pooled in 3-ml aliquots, and was analyzed by MTT assay for the cytotoxicity against MOLT-4 cells. The thick line indicates A_{280} in milliabsorbance unit. The gray line with dots indicate the rate of cell survival in percentage. The positions of elution of the molecular size markers are indicated by downward arrows: a, albumin of 67.0 kDa; b, ovalbumin of 43.0 kDa; c, chymotrypsinogen A of 25.0 kDa; d, ribonuclease A, 13.7 kDa. The fractions from 1 to 8 were analyzed with SDS-PAGE in B. (B) The fractions exhibiting the cytotoxicity were analyzed with SDS-14% PAGE followed by silver staining. The arrowhead indicates the 29-kDa polypeptide cytotoxic against MOLT-4 cells. M, molecular size marker. (C) The cytotoxicity of the purified 29-kDa polypeptide against MOLT-4 cells was analyzed with MTT assay, demonstrating that EC_{50} was estimated as 0.078 μg/ml (95% confidence limits; 0.043–0.140) by the Probit method. The assay was replicated at least three times and representative data and error deviation are shown.

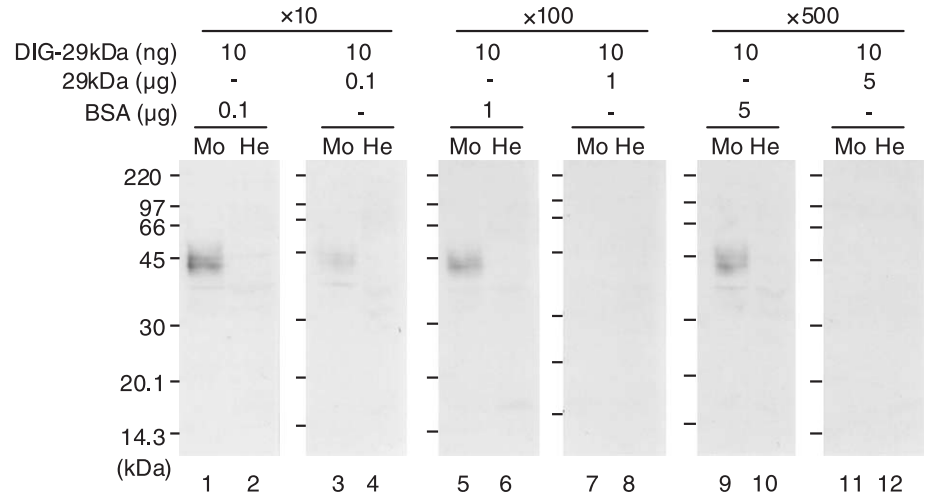

Fig. 6. Detection of the membrane proteins from MOLT-4 cells that binds specifically to the 29-kDa polypeptide. Five micrograms of membrane fraction of MOLT-4 or HeLa cells were separated with SDS-14% PAGE and blotted onto a PVDF membrane, on which the digoxigenin-labeled 29-kDa polypeptide was overlaid together with the indicated amount of the non-labeled 29-kDa polypeptide or BSA as the competitor. After washing the membrane to remove the unbound toxin, the bands corresponding to the specific binding proteins were detected using anti-digoxigenin antibody conjugated with horseradish peroxidase (Roche) and ECL western blotting detection reagents (Amersham Pharmacia Biotech). Mo, membrane fraction of MOLT-4 cells; He, membrane fraction of HeLa cells. Lanes 1 and 2, a 10-fold amount of BSA was added; Lanes 3 and 4, a 10-fold amount of the non-labeled 29-kDa polypeptide was added; Lanes 5 and 6, a 100-fold amount of BSA was added; Lanes 7 and 8, a 100-fold amount of the non-labeled 29-kDa polypeptide was added; Lanes 9 and 10, a 500-fold amount of BSA was added; Lanes 11 and 12, a 500-fold amount of the non-labeled 29-kDa polypeptide was added. The experiments were replicated at least twice, and representative results are shown.

blocked in the presence of more than 100-fold concentration of the non-labeled 29 kDa (Fig. 6). Therefore, it was strongly suggested that they were the specific binding proteins for the 29-kDa polypeptide from the A1519 crystal. On the contrary, we detected no specific bands in the ligand blotting analysis with the HeLa cell membrane (Fig. 6). These results suggested that the specific binding proteins in the MOLT-4 cell membrane were involved in the specific cytotoxicity of the purified 29-kDa polypeptide against MOLT-4 cells (Fig. 3).

4. Discussion

In this study, we investigated the cytotoxicity against human leukemic cell of the crystal proteins produced by *B. thuringiensis* subsp. *coreanensis* A1519 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 EC_{50} of the purified 29-kDa polypeptide against MOLT-4 was 0.078 μg/ml. The N-terminal amino acid sequence of the 29-kDa polypeptide shared no significant homology with all the known proteins including Cry or Cyt toxins. It is reported that the polypeptide of similar molecular size (28 kDa) produced by proteinase K digestion also exhibited the human leukemic cell-specific cytotoxicity [6,7]. The 28-kDa polypeptide was produced by proteinase K-digestion of the 32-kDa protein of the *B. thuringiensis* subsp. *shandongiensis* A1470 strain. Its EC_{50} against MOLT-4 was 0.23 μg/ml and it induced necrosis-like cytotoxicity against MOLT-4. The 28-kDa polypeptide and the 29-kDa fragment in this study are different, because of two reasons; (1) the N-terminal amino acid sequences of both polypeptides were completely different and (2) the 28-kDa polypeptide induced necrosis-like cytotoxicity against MOLT-4 cells, while the observation with a fluorescence microscopy suggested that the 29-kDa fragment induced the apoptotic cell death of MOLT-4 (data not shown).

Generally, a proteolytic processing by proteases is indispensable for the δ-endotoxins to exhibit their activities, and so are the A1519 crystal proteins. The solubilized and non-digested A1519 crystal proteins exhibited no detectable cytotoxicity (Fig. 2). Although the insecticidal Cry toxins are well processed by trypsin, the A1519 crystal proteins were not efficiently processed by trypsin (Fig. 2C and D). The major components of A1519 crystal detected in SDS-14% PAGE were polypeptides of 150, 90, 70, 20, and 10 kDa (Fig. 1). It is reasonable that one of these polypeptides is the protoxin of the 29-kDa fragment. However, since it was suggested that the 28-kDa polypeptide was derived from the 32-kDa protoxin [7], we cannot rule out a possibility that a protoxin for the 29-kDa polypeptide is one of the minor components with molecular sizes from 30 to 40 kDa that is hardly visible in Fig. 1.

In the ligand blotting analysis with cell membrane proteins of MOLT-4 and HeLa, it was suggested that the specificity of the 29-kDa cytotoxic fragment was closely

correlated with the presence of the specific binding proteins with molecular sizes from 40 to 50 kDa in the cell membrane of MOLT-4 (Fig. 6). The mode of action of the 29-kDa polypeptide against MOLT-4 cells is unclear yet, but it is plausible that apoptosis-like cell death is induced by association of the 29-kDa polypeptide to the specific binding proteins or receptors in the membrane of MOLT-4. Thus, the 29-kDa polypeptide may recognize and bind a cell death-inducing membrane protein of MOLT-4, setting on the death signal. Yet, we cannot rule out the possibility of the pore formation or the detergent-like perturbation of membrane by the 29-kDa polypeptide. However, the cytotoxic effect through membrane damage by the 29-kDa polypeptide would, if any, be negligible as shown in Fig. 4.

Observation of the MOLT-4 cells stained with PI and Annexin V-FITC under a fluorescence microscopy suggested that the proteinase K-activated A1519 crystal proteins induced the apoptotic cell death of MOLT-4 (data not shown). At present, therefore, we assume that the A1519 crystal proteins induce specifically the apoptosis of MOLT-4.

Interestingly, it is reported that the α-toxin produced by *Staphylococcus aureus* binds to specific as yet unidentified cell surface receptors and produced small heptameric pores that selectively facilitate the release of monovalent ions, resulting in DNA fragmentation and apoptosis [11,12]. It remains to be seen if the 29-kDa fragment displays a similar mode of action.

Identification and characterization of the specific binding proteins with molecular sizes from 40 to 50 kDa in the cell membrane of MOLT-4 may bring us a significant insight into the mode of action of the 29-kDa polypeptide and other mammalian-cell-recognizing crystal protein family of δ-endotoxins.

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