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Identification of a Novel Cytotoxic Protein, Cry45Aa, from *Bacillus thuringiensis* A1470 and Its Selective Cytotoxic Activity against Various Mammalian Cell Lines

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Parasporal inclusion proteins produced by *Bacillus thuringiensis* strain A1470 exhibit strong cytotoxicity against human leukemic T cells when activated by protease treatment. One of the cytotoxic proteins was separated by anion exchange chromatography and gel filtration chromatography and designated Cry45Aa. Its gene was then expressed in recombinant *Escherichia coli*, in which the Cry45Aa precursor was accumulated in an inclusion body. It was solubilized in sodium carbonate buffer and processed with proteinase K, and cytotoxic activities of the protein against various mammalian cell lines were evaluated using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide assay. The protein exhibited high cytotoxic activity against CACO-2, Sawano, MOLT-4, TCS, and HL60 cells and moderate activity against U-937 DE-4, PC12, and HepG2 cells. On the other hand, the EC_{50} values against Jurkat, K562, HeLa, A549, Vero, COS-7, NIH3T3, CHO, and four normal tissue cells (human primary hepatocyte cells, UtSMC, MRC-5, and normal T cells) were >2 μg/mL.

KEYWORDS: CACO-2; Cry protein; *δ*-endotoxin; *Escherichia coli*; inclusion protein; noninsecticidal; parasporin

INTRODUCTION

Bacillus thuringiensis is a Gram-positive endospore-forming bacterium, which produces large crystalline parasporal inclusions in sporulating cells. The parasporal inclusions often contain one or more proteins (protoxins) that are toxic to insects. The protoxins are ingested by insect larvae in the inclusion body and are solubilized and converted into toxins by midgut proteases of susceptible insects under alkaline conditions (*1*). The protoxins are highly and specifically toxic to insect pests of the Lepidoptera, Diptera, and Coleoptera (*2*) but are not pathogenic to mammals, birds, amphibians, or reptiles (*3*). This makes *B. thuringiensis* a promising microbial agent in the control of insect pests in agriculture, forestry, and veterinary and public health management (*3*). However, noninsecticidal *B. thuringiensis* strains are more widely distributed than insecticidal ones (*4*, *5*). We have previously reported that human cancer cell-killing activity is associated with some noninsecticidal *B. thuringiensis* isolates (*6*−*13*) and have created a new category of protein, parasporin, defined as bacterial parasporal proteins that are capable of preferentially killing cancer cells (*7*).

The parasporal inclusion of *B. thuringiensis* strain A1470 (previously designated as strain 89-T-34-22), which belongs to serovar *shandongiensis*, exhibits strong cytotoxicity against human leukemic T cells when activated by protease treatment, although it did not exhibit insecticidal or hemolytic activities (*10*). The native inclusion before proteinase K treatment consists of five major proteins with molecular masses of 160, 60, 34, 32, and 16 kDa (*10*). Also, a 28 kDa protein processed by the protease has been considered to be cytotoxic (*11*). However, we have recently revealed that A1470 produces multiple cytotoxic proteins with similar molecular masses of approximately 28 kDa (*14*). We isolated one of the genes for the cytotoxic proteins from A1470 (GenBank accession no. AB180980), and the protein expressed by the gene was designated as Cry45Aa by the *B. thuringiensis* *δ*-endotoxin nomenclature committee (http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/). The present study describes the identification of Cry45Aa and its selective cytotoxic activity against various mammalian cell lines.

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MATERIALS AND METHODS

Bacterial Strain and Harvesting of Parasporal Inclusions. Strain A1470 was isolated from a soil sample collected in the city of Hino, Tokyo, Japan (*10*). It was grown at 28 °C on nutrient agar (pH 7.6) consisting of meat extract (10 g), polypeptone (10 g), NaCl (2 g), agar (15 g), and distilled water (1000 mL). Sporulated cultures were harvested and washed three times in 1 M NaCl and then resuspended in distilled water. Parasporal inclusion bodies were purified by a biphasic separation technique (*15*).

Expression of the Recombinant Cytotoxic Protein. *Escherichia coli* BL21 (DE3) cells transformed with a plasmid containing a gene for the full-length Cry45Aa protein (GenBank accession no. AB180980) were cultured in 400 mL of LB medium + kanamycin (20 μg/mL) for 24 h at 37 °C. This expression resulted in the production of the inclusion body. After the cells were harvested by centrifugation for 15 min at 8000*g*, the cells were left overnight at −20 °C and then suspended in 20 mL of 50 mM Tris·HCl buffer (pH 8.0) containing 25% sucrose and 1 mM ethylenediaminetetraacetic acid (EDTA). Then, 1 mL of 10 mg/mL lysozyme (Wako Pure Chemicals, Osaka, Japan; lot TLQ7574) was added, the mixture was incubated for 30 min at room temperature, and then, it was sonicated for 3 min in a Branson model 450D sonicator (Emerson Japan, Kanagawa, Japan). Then, 20 mL of detergent solution (0.2 M NaCl, 1% sodium deoxycholate, and 1% Nonidet P-40) was added to the sonicated sample solution and incubated for 30 min at room temperature. After inclusion bodies were harvested by centrifugation for 30 min at 30000*g*, they were washed two times in washing solution (1% Triton X-100 and 1 mM EDTA) and centrifuged for 15 min at 8000*g*. The pellet was washed two times in distilled water and was stored at −20 °C until use.

Solubilization and Proteolytic Processing of Inclusion Bodies Isolated from *B. thuringiensis* and *E. coli*. Inclusion bodies from *B. thuringiensis* A1470 and recombinant *E. coli* were solubilized in 50 mM sodium carbonate buffer (pH 10.5) at 37 °C for 1 h in the presence of 10 mM dithiothreitol and 1 mM EDTA. Solubilized proteins were treated with proteinase K (Wako Pure Chemicals; lot PAQ7953) at a final concentration of 50 μg/mL for 90 min at 37 °C. At the end of the treatment, phenylmethylsulfonyl fluoride (PMSF, Wako Pure Chemicals; lot WAL7386) was added to the solution at a final concentration of 1 mM to stop the proteolytic reaction.

Molecular masses of proteinase K-treated Cry45Aa from both *B. thuringiensis* A1470 and recombinant *E. coli* were determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Autoflex, Buruker Daltonics Inc., Billerica, MA). Sinapinic acid saturated in 33% (v/v) acetonitrile/67% (v/v) water containing 0.1% trifluoroacetic acid (TFA) was used as a matrix, and ubiquitin, myoglobin, trypsinogen, and bovine serum albumin (BSA) were used as molecular mass calibration standards.

Anion Exchange Chromatography and Gel Filtration Chromatography. For buffer exchange, a sample solution of the proteinase K-treated parasporal inclusions from *B. thuringiensis* A1470 was passed through an Econo-Pac 10DG gel chromatography column (Bio-Rad, Hercules, CA) against 50 mM Tris·HCl buffer (pH 9.0) containing 1 mM EDTA. The sample was then clarified by centrifugation at 15000*g* at 4 °C for 10 min. The supernatant was subjected to anion exchange chromatography on a Resource Q column (6.4 mm i.d., 30 mm length; Amersham Biosciences, Piscataway, NJ) at a flow rate of 1.0 mL/min at 4 °C. The protein was eluted with an increasing gradient of NaCl concentration from 0 to 1.0 M in the same buffer as above, and 1 mL of each fraction was collected. The fractions were concentrated from 1 mL to 100 μL by an ultrafiltration in a Vivaspin centrifugal filter (Sartorius AG, Goettingen, Germany) and subjected to gel filtration chromatography on a Superdex 75 HR 10/30 column (10 mm i.d., 300 mm length; Amersham Biosciences), in 50 mM Tris·HCl buffer (pH 9.0) containing 1 mM EDTA at a flow rate of 0.5 mL/min at 4 °C.

Protein Determination and Analysis. The protein concentration was measured by the method of Bradford (*16*) with BSA as the standard. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed as described by Laemmli (*17*), using 4% stacking and 15% resolving gels stained with a Silver Stain II kit or a Quick CBB kit (Wako Pure Chemicals). The molecular mass of the protein was estimated against molecular standards (Bio-Rad). Immunoblotting was performed as described previously (*6*) with rabbit antiserum raised against Cry45Aa protein from the recombinant *E. coli*.

Table 1. Cytotoxic Activity of Recombinant Cry45Aa against Various Cultured Mammalian Cells[a]

cell line	origin	EC_{50} (μg/mL)
MOLT-4	leukemic T cell, human	0.472
Jurkat	leukemic T cell, human	>2
HL60	promyelocytic leukemia cell, human	0.725
K562	myelogenous leukemia cell, human	>2
U-937 DE-4	lymphoma cell, human	0.980
T cell	normal T cell, human	>2
HeLa	uterus cervix cancer, human	>2
TCS	uterus cervix cancer, human	0.719
Sawano	uterus cancer, human	0.245
UtSMC	normal uterus, human	>2
HepG2	hepatocyte cancer, human	1.90
HC	normal hepatocyte, human	>2
A549	lung cancer, human	>2
MRC-5	normal embryonic lung fibroblast, human	>2
CACO-2	colon cancer, human	0.124
Vero	kidney cell, monkey	>2
COS-7	kidney cell, monkey	>2
PC12	pheochromocytoma, rat	1.78
NIH 3T3	embryo cell, NIH Swiss mouse	>2
CHO	ovary cell, chinese hamster	>2

[a] EC_{50} values (at 20 h) were calculated from the data shown in **Figure 5** by probit analysis.

Cells and Culture Conditions. Mammalian cell lines used in this study and tissues of origin are listed in **Table 1**. MOLT-4, Jurkat, HL60, Sawano, HepG2, HeLa, TCS, A549, MRC-5, CACO-2, COS-7, K562, CHO, PC12, U-937 DE-4, Vero, and NIH3T3 cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan), UtSMC (normal uterine smooth muscle cell) was purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, WA), and human primary hepatocyte cells (HC) were purchased from Applied Cell Biology Research Institute (Kirkland, WA). All cells were maintained under conditions recommended by the suppliers. Normal T cells were isolated from buffy coats supplied from Fukuoka Red Cross Blood Center (Fukuoka, Japan) by a method previously described (*6*) and were cultured in RPMI1640 medium (Nissui Pharma. Co. Ltd., Tokyo, Japan) containing 10% fetal bovine serum and kanamycin (30 μg/mL).

Cytotoxicity Assay and Dose−Response Study. The cytopathic effect to the cells was monitored over 20 h after administration of the protein under a phase contrast microscope, and the degree of the cytopathic effect was classified on the basis of the population of damaged cells, as described previously (*8*).

The cytotoxic activity of the proteinase K-treated recombinant Cry45Aa was measured by the MTT [3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide] method (*18*, *19*), using a Premix WST-1 kit (Takara Bio Inc., Shiga, Japan) for normal T cells or in CellTiter96 AQueous One Solution Reagent (Promega, Madison, WI) for all other cells. Each well of the 96 well microtest plate received 90 μL of cell suspension containing 9×10^5 cells (normal T cells) or 2×10^4 cells (all other cells) and incubated at 37 °C for 24 h. Next, 10 μL of the cytotoxic protein solution in 2-fold serial dilutions in PBS was added to each well and the cells were further incubated at 37 °C for 20 h. Then, 10 μL of the MTT reagent was added to each well, followed by further incubation at 37 °C for 4 h. Finally, the absorbance at 450 (Premix WST-1 kit) or 492 nm (CellTiter96 AQueous One Solution Reagent) was measured on a Multiskan Bichromatic microplate reader (Labsystems Oy., Helsinki, Finland) with a control wavelength of 690 nm. The survival rate was determined by comparing the absorbance value with that in the control without cytotoxic proteins. The 50% effective concentration (EC_{50}) values were decided by probit analysis (*20*).

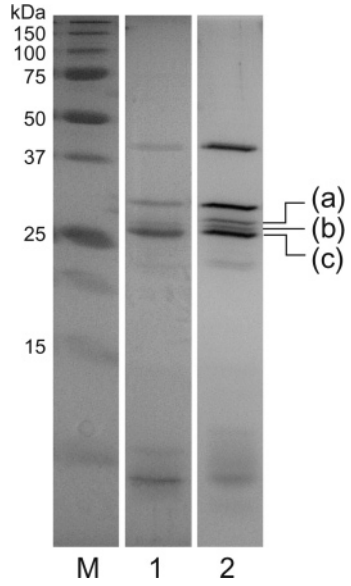

Figure 1. SDS–PAGE of the proteinase K-treated parasporal inclusion proteins from *B. thuringiensis* A1470. Lane 1: Proteins were stained with CBB R250. Protein loading was 1.5 μg. Lane 2: Proteins were stained with silver. Protein loading was 150 ng. The letters a–c indicate the candidates for cytotoxic proteins against MOLT-4 cells. Lane M indicates molecular size markers.

RESULTS

Three Candidates of Cytotoxic Proteins from *B. thuringiensis* Strain A1470. Figure 1 shows results of SDS−PAGE of proteinase K-treated inclusion bodies of *B. thuringiensis* A1470 as stained with Coomassie brilliant blue (CBB) R250 (lane 1) or silver (lane 2). CBB R250 staining showed three obvious bands at 26, 30, and 40 kDa. A slightly stained band was also observed at 27 kDa, a little above the 26 kDa band, but we were uncertain as to whether it was a distinct band or not. However, the silver staining clearly showed two distinct bands in that area (lane 2). Thus, there were five bands altogether, ranging between 25 and 40 kDa. Lee et al. previously estimated that a 28 kDa protein could be the cytotoxic substance (*11*), but we have shown that that fraction contains the three proteins designated a, b, and c in **Figure 1**. They are therefore considered to be candidates of cytotoxic proteins.

The proteinase K-treated parasporal inclusions were fractionated by anion exchange chromatography with an NaCl gradient, and fraction 26 contained two major bands with a molecular mass of approximately 26 kDa (**Figure 2B**, lane 6), which seem to correspond with proteins b and c in **Figure 1**. They were separated from each other by gel filtration chromatography (**Figure 2A**). We examined the cytopathic effect of each fraction against MOLT-4 cells and clearly saw that the cytotoxic activity was expressed mostly by protein b (**Figure 2B**). Using N-terminal and internal amino acid sequences, we cloned the gene for protein b. The protein was designated Cry45Aa by the *B. thuringiensis* δ-endotoxin nomenclature committee.

Cry45Aa Protein Expressed in *E. coli*. The *Cry45Aa* gene was expressed in recombinant *E. coli*, in which the protein was accumulated as an inclusion body. **Figure 3A** compares SDS−PAGE profiles of protease K-treated inclusion proteins of the recombinant *E. coli* with those of *B. thuringiensis* A1470. The 31 kDa proteins observed in the proteinase K-treated inclusion bodies from both recombinant *E. coli* (lane 2) and *B. thuringiensis* (lane 1) were presumed to be proteinase K, because they did not react with the antiserum against the Cry45Aa protein

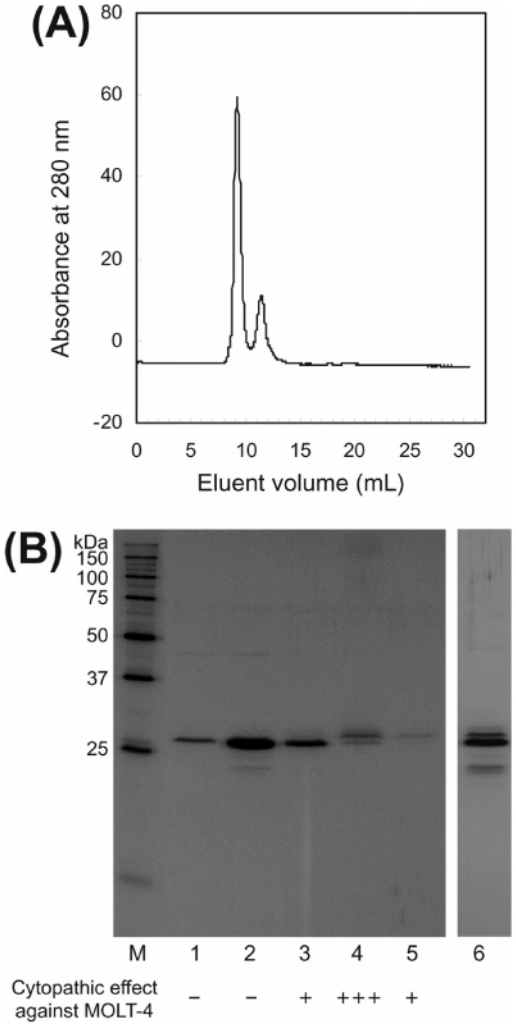

Figure 2. (**A**) Gel filtration chromatography of fraction 26 of anion exchange chromatography. The sample solution was concentrated from 1 mL to 100 μL by an ultrafiltration centrifugal filter and applied to a Superdex 75 HR 10/30 gel filtration chromatography column. (**B**) SDS–PAGE profile of proteins in fractions 8–12 of gel filtration chromatography (lanes 1–5) and fraction 26 of anion exchange chromatography before fractionation (lane 6). The cytopathic effect against MOLT-4 cells is shown under each lane: +++, high; ++, moderate; +, low; and –, no cell damage. Lane M indicates molecular size marker. One hundred nanograms was applied to each lane; stained with silver.

of the recombinant *E. coli* (**Figure 3B**). Except for proteinase K, the inclusion proteins of the recombinant *E. coli* contained only 27 kDa protein (**Figure 3A**, lane 2), which seemed to be protein b from its mobility. Moreover, molecular masses of the cytotoxic proteins of the recombinant *E. coli* and *B. thuringiensis* A1470 were determined by MALDI-TOF MS as 26808 and 26798, respectively. Accordingly, the recombinant Cry45Aa and protein b from wild type could be judged to be the same substance.

Previously, we partially purified protein a by anion exchange chromatography and gel filtration chromatography, and it exhibited cytotoxicity against MOLT-4 and Jurkat cells (*14*). It was difficult to separate Cry45Aa from another cytotoxic protein a of *B. thuringiensis* A1470; therefore, the cytotoxic activity of Cry45Aa was examined by use of the recombinant Cry45Aa.

Morphological Changes in Cultured Cells by the Recombinant Cry45Aa Protein. Figure 4 shows morphological changes in cultured cells induced by the addition of the

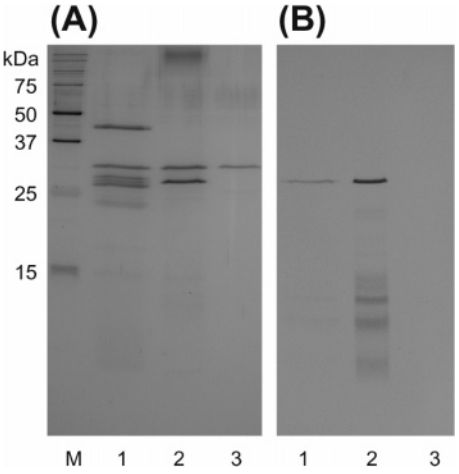

Figure 3. Protein profiles of proteolytic processed inclusion bodies from *B. thuringiensis* A1470 and recombinant *E. coli.* (**A**) The proteinase K-treated inclusion bodies were subjected to SDS–PAGE and stained with silver; 150 ng was applied to lanes 1 and 2, and 20 ng was applied to lane 3. (**B**) Immunoblotting with rabbit antiserum against Cry45Aa protein of the recombinant *E. coli.* Lane 1, proteinase K-treated inclusion proteins of *B. thuringiensis* A1470; lane 2, those of recombinant *E. coli*; lane 3, proteinase K; and lane M, molecular size marker proteins.

proteinase K-treated recombinant Cry45Aa at a final concentration of 2 μg/mL. The protein showed strong cytotoxicity in the early phase to both MOLT-4 and CACO-2 cells. Within 10 min after administration of the cytotoxic protein, nuclear condensation started to occur (data not shown) and appeared distinctly within 1 h. Ballooned cells appeared 2 h after exposure to the cytotoxic protein (**Figure 4**, arrows) and burst within 24 h to lead to the cell death. No morphological change was observed in resistant cells (data not shown).

Cytotoxic Activity of Cry45Aa against Various Mammalian Cells. The dose−response of various cultured mammalian cells to the proteinase K-treated recombinant Cry45Aa protein was monitored by MTT assay (**Figure 5**), and the EC_{50} values obtained 20 h after administration are shown in **Table 1**. Cytotoxicity varied greatly among the different cells. The recombinant protein was highly cytotoxic to CACO-2, Sawano, MOLT-4, TCS, and HL60 cells, with EC_{50} values in the range of 100−800 ng/mL. It was moderately cytotoxic to U-937 DE-4, PC-12, and HepG2 cells. On the other hand, Jurkat, K562, normal T, HeLa, UtSMC, HC, A549, MRC-5, Vero, COS-7, NIH3T3, and CHO cells were resistant. The EC_{50} values for all normal tissues were >2 μg/mL. Although we found no general rule for specificity of the cells and no common characteristics of sensitive or resistant cells, some cells derived from tumor tissues seemed more sensitive to the protein than those derived from normal tissues, as shown by the difference in cytotoxicity between Sawano and UtSMC.

DISCUSSION

Lee et al. concluded that the cytocidal activity of inclusions from *B. thuringiensis* A1470 was resided a 28 kDa protein based on the experimental data obtained by anion exchange chromatography and SDS−PAGE (*11*). In this paper, we show that the A1470 strain produces three proteins: a, b, and c, all with molecular masses of approximately 28 kDa (**Figure 1**, lane 2, silver staining). Proteins a and b were not sufficiently stained by CBB, and their expression in A1470 was much lower than that of protein c. The majorly expressed protein c showed no cytotoxic activity against MOLT-4 cells (**Figure 2B**). The cytotoxicity of *B. thuringiensis* A1470 against MOLT-4 depends on proteins a (data not shown) and b (**Figure 2**).

The gene for full-length Cry45Aa, which is the precursor of the activated protein, encodes a polypeptide of 275 amino acid residues with a predicted molecular mass of 30078. The Cry45Aa precursor of the recombinant *E.* coli had no apparent cytocidal activity without proteinase K treatment (data not shown). The native parasporal inclusions of *B. thuringiensis* A1470 also only exhibited cytotoxic activity, when activated by protease treatment (*10*). Thus, proteolytic processing was essential for activation of the cytotoxic protein. MALDI-TOF MS analysis of the proteinase K-treated recombinant Cry45Aa estimated the molecular mass as 26808. The N-terminal amino acid sequence of the protein was AIINLANELA (data not shown) and was completely identical to Ala2-Ala11 of the Cry45Aa precursor. It is likely that the proteinase K-treated Cry45Aa corresponds to amino acid residues Ala2 to Arg246 of the precursor, as a predicted molecular mass of such a protein 26828 is close to that measured for proteinase K-treated Cry45Aa 26808.

Cry45Aa had cytotoxic activity against various mammalian cells with markedly divergent target specificity (**Figure 5** and **Table 1**), preferentially killing colonic, uterine, and blood cancer cells. Strict cytotoxic specificity toward target cells suggests the presence of a receptor-like protein or lipid at the surface of the sensitive cells. Thus, identification of the cell receptor might provide some insight into the mechanism of target specificity and cytotoxicity of Cry45Aa.

In this study, the EC_{50} values in all of the normal cells (HC, UtSMC, MRC-5, and normal T cells) were >2 μg/mL (**Table 1**). However, this result does not imply that Cry45Aa is not cytotoxic to normal tissues; for example, 2 μg/mL Cry45Aa was weakly cytotoxic to UtSMC and normal T cells (**Figure 5**). A higher Cry45Aa concentration would be required to determine the EC_{50} values of these cells, but we cannot produce a high enough concentration.

Cry45Aa exhibited the highest cytotoxic activity against CACO-2, which is derived from a human colonic adenocarcinoma cell line. CACO-2 is generally used as a substitute for human colon cells in various studies. The cytotoxicity of Cry45Aa to CACO-2 may imply that it is toxic to normal colon cells. Therefore, it needs to be confirmed that the Cry45Aa toxin would not act on normal colon cells or normal human intestinal epithelial cells.

Other toxins from microorganisms that act on CACO-2 cells were previously reported: the CytK toxin of *Bacillus cereus*, which causes severe food poisoning (*21*), a thermostable hemolysin of *Vibrio parahaemolyticus* (*22*), and a vulnificolysin-like cytolysin of *Vibrio tubiashii* (*23*). Most of these toxins are derived from virulent microorganisms; hence, the purposes of those studies were mainly to elucidate the mechanism of pathogenesis. Generally, it is considered that *B. thuringiensis* is not pathogenic (*3*). However, different enterotoxins, including hemolytic (*24*) and nonhemolytic (GenBank accession no. AB099298) enterotoxins, have been found in *B. thuringiensis*. Additionally, *B. thuringiensis* is indistinguishable from *B. cereus*, a common food pathogen, except for the production of parasporal inclusion proteins (*25*). Therefore, whether *B. thuringiensis* strain A1470 is pathogenic or not would be a subject of an interesting study.

We have created a new category of protein—parasporins—a functional protein group defined as parasporal proteins from *B. thuringiensis* and related bacteria that are nonhemolytic but

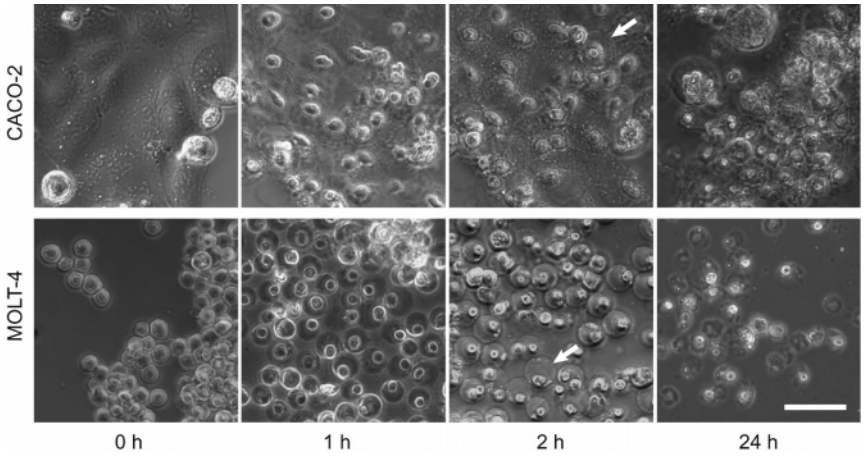

Figure 4. Cytopathic effect of recombinant Cry45Aa protein on MOLT-4 and CACO-2 cells. The cells were incubated with proteinase K-treated recombinant Cry45Aa at 37 °C for the indicated times and visualized by phase contrast microscopy. The final concentration of the cytotoxic protein was 2 μg/mL. Arrows indicate ballooned cells caused by administration of the cytotoxic protein. The bar indicates 10 μm.

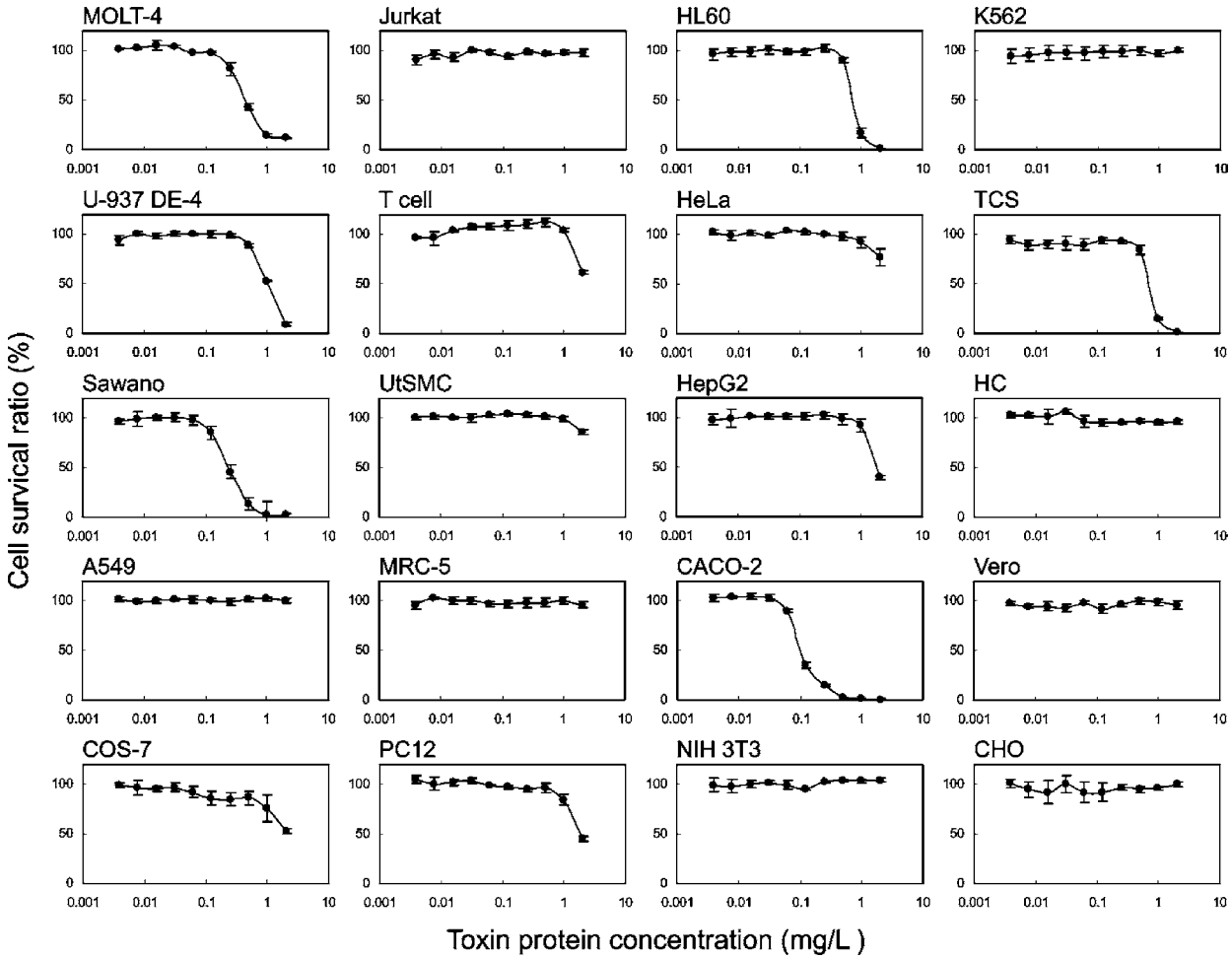

Figure 5. Cytotoxic activity of proteinase K-treated recombinant Cry45Aa protein to cultured mammalian cells. To cells preincubated at 37 °C for 24 h, we added the cytotoxic protein (final concentrations, 4 ng/mL to 2 μg/mL) and further incubated them at 37 °C for 20 h. Cell proliferation was assayed by the MTT assay.

capable of preferentially killing cancer cells (*7*). To date, we have identified four parasporins from noninsecticidal *B. thuringiensis* strains (*26*). Cry45Aa belongs to this category and was designated Parasporin-4. Each parasporin has a specific and distinct target spectrum against mammalian cells. This raises the possibility that we could screen novel proteins cytotoxic to specific cancer cells. These proteins could be useful for therapy and diagnosis of cancers.

ABBREVIATIONS USED

PMSF, phenylmethylsulfonyl fluoride; TFA, trifluoroacetic acid; BSA, bovine serum albumin; UtSMC, normal uterine smooth muscle cell; HC, human primary hepatocyte cell; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide; EC_{50}, 50% effective concentration; CBB, Coomassie brilliant blue.

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