J. Biochem. **137**, 17–25 (2005)
DOI: 10.1093/jb/mvi003

Parasporin-1, a Novel Cytotoxic Protein to Human Cells from Non-Insecticidal Parasporal Inclusions of *Bacillus thuringiensis*

Hideki Katayama[1], Haruo Yokota[2], Tetsuyuki Akao[1], Osamu Nakamura[1], Michio Ohba[3], Eisuke Mekada[4] and Eiichi Mizuki[1,*]

[1]Biotechnology and Food Research Institute, Fukuoka Industrial Technology Center, Kurume, Fukuoka 839-0861; [2]Department of Chemistry, Faculty of Science, Fukuoka University, Fukuoka 814-0180; [3]Graduate School of Agriculture, Kyushu University, Fukuoka 812-8581; and [4]Department of Cell Biology, Research Institute for Microbial Disease, Osaka University, Suita, Osaka 565-0871

Received September 13, 2004; accepted October 18, 2004

Pro-parasporin-1 is a parasporal inclusion protein of the non-insecticidal *Bacillus thuringiensis* strain A1190. Cytotoxic fragments, named parasporin-1, were generated from pro-parasporin-1 by trypsin digestion. Parasporin-1 was purified by a combination of chromatography procedures based on the cytotoxic activity to HeLa cells. Two different fragments of 15-kDa and 56-kDa were detected in the purified parasporin-1 fraction. These fragments were tightly associated with each other and could not be separated by chromatography under conditions that preserve cytotoxic activity, indicating that the active form of parasporin-1 is a heterodimer of the 15- and 56-kDa fragments. Amino acid sequencing and MALDI-TOF mass spectrometric analysis revealed that parasporin-1 is generated from pro-parasporin-1 by trypsin digestion at Arg 93 and Arg 231. Of 12 human cell lines tested, parasporin-1 showed strong cytotoxicity to four cell lines derived from cancer tissues, but low to no cytotoxicity to the other cell lines. The time-courses of cytotoxicity indicated that the mode of action of parasporin-1 to sensitive cells differs from that shown for previously isolated cytotoxic proteins from *Bacillus thuringiensis*, Cyt proteins, and other bacterial pore-forming toxins. Thus, parasporin-1 is a novel cytotoxic protein to human cancer cells produced by *B. thuringiensis*, and may be useful as a tool to recognize and destroy specific cancer cells.

Key words: *Bacillus thuringiensis*, cytotoxin, membrane permeability, molecular structure, purification and characterization.

Abbreviations: DTT, dithiothreitol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl-2*H*-tetrazolium bromide; MALDI, matrix-assisted laser desorption/ionization; TOF, time of flight; MS, mass spectrometer; MEM, minimum essential medium; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.

Bacillus thuringiensis, a Gram-positive bacterium, produces proteinaceous parasporal inclusions in sporangia. These parasporal inclusions often exhibit specific insecticidal toxicity against agriculturally and medically important insect pests (*1*). Thus, *B. thuringiensis* is used as a biological agent for pest control (*2–4*). The mechanism whereby insecticidal proteins from *B. thuringiensis* exert their toxicity has been extensively studied, and the generally accepted mechanism is as follows. In the insect midgut, the insecticidal parasporal proteins are solubilized under alkaline conditions and activated by proteases. The proteolytic fragments of the proteins then bind to specific receptors on the surface of the apical brush border membrane of epithelial columnar cells, form pores that disrupt cellular functions, and finally kill the insects (*5–12*).

Studies on *B. thuringiensis* strains isolated from a variety of natural environments have revealed that many strains of *B. thuringiensis* produce non-insecticidal inclusion proteins, and that such strains are more widely distributed than the strains producing the insecticidal proteins (*13–15*). Thus, the question arises as to what biological activities such non-insecticidal inclusion proteins exhibit. Although this question remains largely to be clarified, our previous study provided a partial answer, in that we found several strains of *B. thuringiensis* that produced non-insecticidal inclusion proteins with toxicities to human cells when the inclusion proteins were cleaved to proteolytic fragments by an appropriate protease (*16*). Each strain produced a different inclusion protein with a unique toxic spectrum (*16*). Interestingly, the inclusion proteins from some strains showed specific toxicity to cancer cells, raising the possibility that these proteins could be used for medical purposes (*16*). Based on the above observations, we proposed a new protein family, designated "parasporin," from *B. thuringiensis*, that exerts preferential cytotoxicity to human cancer cells (*17*).

Although most parasporins have not yet been characterized at the molecular level, partial characterization has been performed for the inclusion protein produced by the A1190 strain of *B. thuringiensis* (formerly 84-HS-1-

*To whom correspondence should be addressed. Tel: +81-942-30-6644, Fax: +81-942-30-7244, E-mail:emizuki@fitc.pref.fukuoka.jp

11). The A1190 strain produces non-insecticidal inclusions composed of a single 81-kDa protein (this protein and its proteolytically activated form are referred as pro-parasporin-1 and parasporin-1, respectively, hereafter). The primary amino acid sequence of pro-parasporin-1, which was deduced from the corresponding gene (*17*), indicates that it has low sequence similarity to known insecticidal inclusion proteins (<25%). Based on a comparison of the amino acid sequence similarities of pro-parasporin-1 with other inclusion proteins of *B. thuringiensis*, this protein was classified as a new class of Cry protein, Cry31Aa1 (*17*). In this paper, we describe the activation of pro-parasporin-1 by trypsin, and the purification, molecular structure and characterization of the cytotoxic activity of parasporin-1.

MATERIALS AND METHODS

Reagents and Bacterial Strains—Trypsin and proteinase K were purchased from Sigma. Polybuffer 96 and pharmalyte (broad range 8-10.5) were purchased from Amersham Pharmacia Biotech. The bacterial strains used in this study were the type strain of *B. thuringiensis* serovars *israelensis* H14 and the soil isolate A1190 (formerly, 84-HS-1-11) strain (*17*). The vector and *Escherichia coli* strains used in this study were described previously (*17*).

Cells and Culture Conditions—The following human and animal cell lines were purchased from the RIKEN Cell Bank: HeLa, HepG2, MOLT-4, HL60, A549, CACO-2, Vero, COS-7, Sawano, Jurkat, NIH3T3, and MRC-5. UtSMC cells originating from normal uterus smooth muscle and SmGM medium were obtained from BioWhittaker. HC cells originating from normal human hepatocytes and CS-C medium were purchased from Cell System.

HepG2, A549 and COS-7 cells were cultured in DMEM supplemented with 10% (v/v) FBS (ICN). HeLa, NIH3T3 and Vero cells were cultured in MEM supplemented with 10% (v/v) FBS. Sawano cells were cultured in MEM supplemented with 15% (v/v) FBS. CACO-2 cells were cultured in MEM containing 10% (v/v) bovine serum (ICN) and 1% (v/v) non-essential amino acids. MRC-5 cells were cultured in HF-RITC80-7 supplemented with 10% (v/v) FBS. MOLT-4, HL60 and Jurkat cells were cultured in RPMI1640 supplemented with 10% (v/v) FBS and 30 μg/ml kanamycin. UtSMC and HC cells were cultured in SmGM and CS-C medium, respectively. Normal human T cells isolated from peripheral blood were provided by the Fukuoka Red Cross Blood Center (Fukuoka, Japan) as described previously (*16*). All cells were cultured at 37°C in a humidified 5% CO_2 atmosphere.

Protease Treatment of Pro-Parasporin-1—Parasporal inclusions were prepared by a biphasic separation method as described previously (*17*). The inclusions were incubated in alkaline solution (50 mM Na_2CO_3, 2 mM DTT, 1 mM EDTA) at 37°C for 60 min. After centrifugation (18,000 × *g*, 4°C, 10 min) to remove insoluble material, the protein concentration of the supernatant was determined and adjusted to 1 mg/ml with the alkaline solution. After adjustment of the pH to 8.3 ± 0.1 with 1 N HCl, the solubilized proteins were treated with trypsin (final concentration, 0.3 mg/ml) or proteinase K (final concentration, 0.1 mg/ml) at 37°C for 90 min. Phenylmethylsulfonyl fluoride (PMSF) in isopropanol was added (final concentration, 1 mM) to stop the protease digestions.

Assay of Cytotoxicity—Each well of a 96-well plate received 90 μl of a cell suspension containing 2.0×10^4 cells. After preincubation for 20 h at 37°C in a CO_2 incubator, 10 μl of sample, which had been filtered through a 0.45 μm membrane filter and diluted with the appropriate buffer, was added to each well of the plate. After incubation for 20 h at 37°C, the cytopathic effect (CPE) was monitored under a phase-contrast microscope, and the cytotoxic activity was quantified by the MTT conversion assay using a Cell Titer 96™ Non-Radioactive Cell Proliferation Assay Kit (Promega). The MTT assay was performed according to the manufacturer's instructions. One unit of cytocidal activity was defined as the amount of protein that caused a 50% decrease in the viability of 2×10^4 cells in the MTT assay.

Purification of Parasporin-1 by Column Chromatography—All chromatographic procedures were performed at 4°C. Pro-parasporin-1 was solubilized and trypsinized as described above, and then centrifuged (18,000 × *g*, 4°C, 20 min) to remove aggregated material. The supernatant was applied to a gel filtration column, Superdex 75 pg (1.6 × 65 cm; Amersham Pharmacia Biotech) equilibrated with Buffer A (25 mM diethanolamine-HCl, pH 9.5). The cytotoxic activity of each fraction against HeLa cells was measured, and the fractions showing cytotoxicity were pooled. The pooled cytotoxic fractions were then applied to a Mono P HR 5/20 chromatofocusing column (Amersham Pharmacia Biotech) equilibrated with Buffer A. After washing the column with 2.5 bed volumes of Buffer A, proteins bound to the resin were eluted by a pH gradient from 9 to 7 of Buffer F [5.2% (v/v) Polybuffer96, 1% (v/v) Pharmalyte8-10.5, HCl, pH 7.0], and fractions showing cytotoxicity were pooled. Finally, for replacement of the buffer, the pooled fractions were dialyzed against Buffer B (150 mM NaCl, 25 mM Tris-HCl, pH 8.8) for 2 days.

For the production of epitope-tagged pro-parasporin-1, a recombinant plasmid for *B. thuringiensis* with a His6-tag at the C-terminal was constructed. The recombinant plasmid was transformed into *B. thuringiensis* acrystalliferous mutant strain BFR1 (*17*). Production and preparation of parasporal inclusions of the recombinant pro-parasporin-1 were performed as described previously (*17*). The recombinant inclusions were solubilized in 50 mM Na_2CO_3 solution and trypsinized by the same method described above. The trypsinized sample was clarified by centrifugation (18,000 × *g*, 4°C, 20 min), and the supernatant was applied to a Ni^{2+}-chelating column (Amersham Pharmacia Biotech) equilibrated with Buffer C (150 mM NaCl, 25 mM Tris-HCl, pH 8.0). Unbound proteins were removed by washing the column with Buffer C. The recombinant protein was eluted by a stepwise gradient of imidazole at various concentrations (0, 25, 250 mM) in Buffer C. Cytotoxic fractions were dialyzed against Buffer B for buffer replacement.

Purified wild-type and recombinant parasporin-1 were divided into aliquots and stored at –80°C until use. The protein concentration of each fraction was measured by

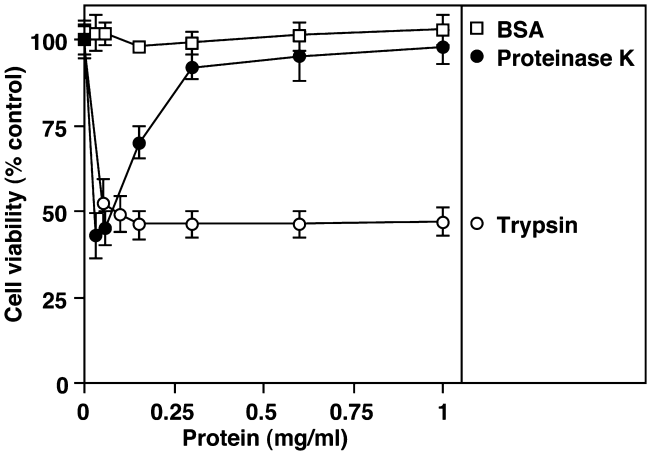

Fig. 1. **Activation of pro-parasporin-1 by proteases.** Solubilized pro-parasporin-1 was treated with various concentrations of trypsin or proteinase K as described under "EXPERIMENTAL PROCEDURES." BSA was also treated with trypsin as a control. Protease-treated pro-parasporin-1 or BSA was added to HeLa cells. After incubation for 20 h, the cell viability was determined by the MTT assay using the average absorbance value at 590 nm of cell samples incubated with alkaline buffer as a control (100%). Each value is the mean ± SD of three experiments.

the method of Bradford (*18*) with bovine serum albumin as the standard.

Molecular Analysis of Purified Parasporin-1—SDS-PAGE using pre-cast 10–20% gradient acrylamide gels, READY GELS J (Bio-Rad), was performed as described by Laemmli (*19*). Protein bands were detected by silver staining as described by Oakley *et al.* (*20*). The proteins resolved by SDS-PAGE were transferred onto a PVDF membrane (Bio-Rad), and their N-terminal amino acid sequences were determined with a Model 473A automatic protein sequencer (Applied Biosystems). Mass determinations of parasporin-1 were carried out with a Voyager DE-STR MALDI-TOF MS instrument (PE-Biosystems). The sample was dialyzed against 10 mM trimethylamine-CO_2, pH 8.5, and mixed with an equal volume of acetonitrile and a 4-fold volume of 2-(4-hydroxyphenylazo)-benzoic acid (HABA) solution [10 mg/ml in 50% (v/v) acetonitrile] as the matrix. The droplet on the sample plate was allowed to dry at room temperature before introduction into the mass spectrometer. MALDI-TOF MS measurement was performed in the positive linear mode with bovine serum albumin as the external standard.

RESULTS

Optimal Conditions for Protease Treatment—Although proteinase K was used to activate pro-parasporin-1 in preceding studies (*17*), we investigated more optimal conditions for the protease activation of pro-parasporin-1. Pro-parasporin-1 was solubilized in alkaline buffer and treated with various concentrations of proteinase K (0-1 mg/ml) or trypsin (0–1 mg/ml). The cytotoxic activity of the samples was assessed by the MTT assay using HeLa cells (Fig. 1). Although proteinase K treatment at a concentration of 60 μg/ml gave the maximum cytotoxic activity against HeLa cells, this sharply decreased at the higher concentrations. In contrast, trypsin treatment at 50 μg/ml led to maximal cytotoxic activity and the cytotoxic activity was maintained at trypsin concentrations above 50 μg/ml (Fig. 1). Each sample was analyzed by SDS-PAGE. Whereas the high molecular weight protein bands disappeared with higher concentrations of proteinase K, they remained after treatment with trypsin at high concentrations (data not shown). Because of the broad substrate specificity of proteinase K, it is considered that parasporin-1, which is generated from pro-parasporin-1 by proteinase K treatment, is further digested to lose its cytotoxicity. Thus, trypsin was used for the activation of parasporin-1 in this study.

Purification of Parasporin-1—Trypsinized pro-parasporin-1 was chromatographed on a Superdex 75 gel filtration column. The cytotoxic activity against HeLa cells was detected as a single peak (Fig. 2A). The toxic fractions were pooled and then chromatographed on a Mono P chromatofocusing column, and the toxic activity eluted at pH 7.8–7.6 (Fig. 2B). The chromatofocusing using the Mono P column indicated that the pI of parasporin-1 is approximately 7.8–7.6. SDS-PAGE analysis of samples from each step of the purification revealed that two major protein bands of 15- and 56-kDa were specifically concentrated by the purification (Fig. 2C). Densitometric analysis indicated that the 15- and 56-kDa protein bands in lane 5 in Fig. 2C correspond to 98% of the total proteins loaded. The purification steps are summarized in Table 1. The cytotoxic activity was purified by approximately 20-fold with a recovery of 1.8%.

To identify and characterize the active fragment(s) of pro-parasporin-1, a recombinant pro-parasporin-1 with a His6-tag at the C-terminal was produced, solubilized and activated with trypsin. The trypsin-activated recombinant pro-parasporin-1 was purified by Ni^{2+}-chelating affinity chromatography. In the affinity chromatography, the cytotoxic activity against HeLa cells was eluted with 250 mM imidazole as a single peak (data not shown).

Table 1. **Summary of parasporin-1 purification.**

Purification step	Total protein (mg)	Total activity[a] (units)	Specific activity (unit/μg protein)	Purification (fold)
Trypsin digestion	14.3	70,777	4.97	1
Gel filtration				
Superdex 75 pg	2.45	44,899	18.3	3
Chromatofocusing				
Mono P HR	0.297	28,828	96.9	19
Gel filtration				
Superdex 75 HR	0.255	24,837	97.4	20

[a]One unit is the amount of protein that decreases HeLa cell viability (2×10^4 cells) by 50% in the MTT assay.

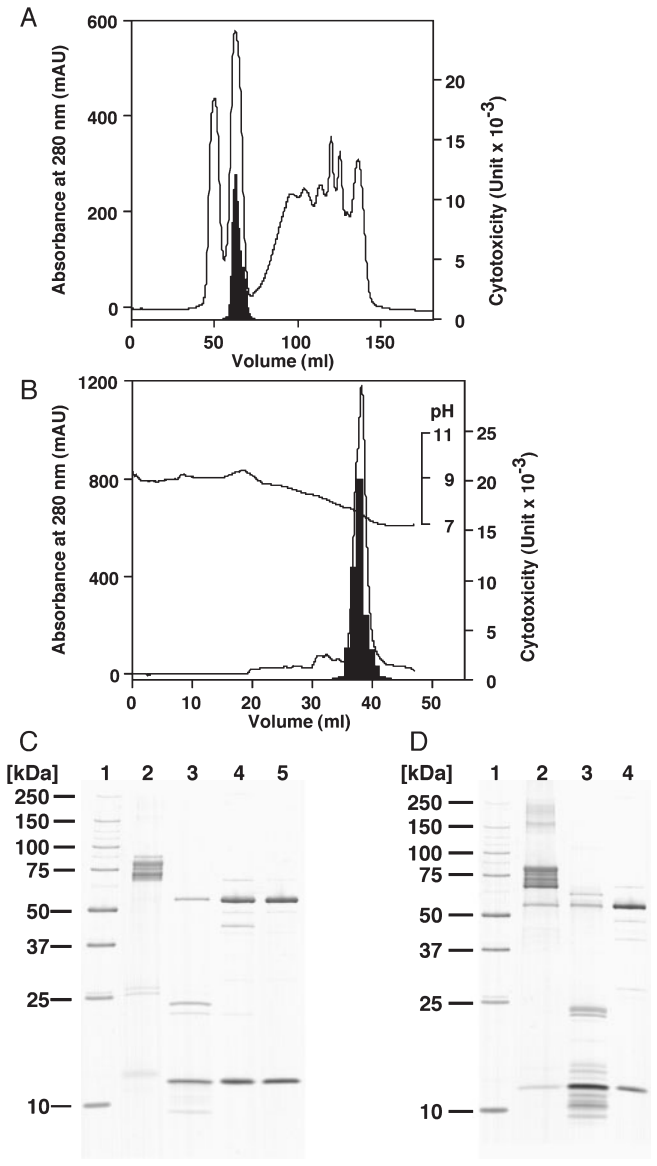

Fig. 2. **Purification of parasporin-1 by column chromatography.** A: Gel filtration chromatography of trypsin-digested pro-parasporin-1 using Superdex 75 pg (1.6 × 60 cm). B: Chromatofocusing using Mono P HR 5/20. Column eluates were monitored for absorbance at 280 nm (solid lines), and the pH gradient is indicated by the dashed line. The cytotoxic activity of each fraction was determined by the MTT assay and is indicated by the solid bars. One unit of cytocidal activity was defined as the amount of protein that decreased the viability of 2×10^4 cells by 50% in the MTT assay. Fractions containing the cytotoxic activity from the gel filtration chromatography were applied to a chromatofocusing column. In the chromatofocusing, proteins were eluted by a pH gradient, and the cytotoxic activity was detected at pH 7.8–7.6. *C*, SDS-PAGE profiles of the parasporin-1 fractions in each purification step using a 10–20% gradient polyacrylamide gel. Lane 1, molecular markers; Lane 2, solubilized crude pro-parasporin-1; lane 3, crude pro-parasporin-1 treated with trypsin; lane 4, parasporin-1 partially purified by gel filtration chromatography; lane 5, parasporin-1 purified by chromatofocusing. D: SDS-PAGE profiles of the epitope-tagged recombinant parasporin-1 in each purification step using a 10–20% gradient polyacrylamide gel. Lane 1, molecular markers; Lane 2, solubilized recombinant pro-parasporin-1; lane 3, recombinant pro-parasporin-1 treated with trypsin; lane 4, recombinant parasporin-1 purified by Ni^{2+}-chelating affinity chromatography. Each lane contained 0.5 μg of protein preparation. Protein bands were detected by silver staining. Molecular weights (in kDa) are shown on the left.

SDS-PAGE analysis revealed that the purified fractions showing cytotoxic activity against HeLa cells also contained the 15- and 56-kDa proteins (Fig. 2D). These results indicate that the 15- and 56-kDa proteins are fragments derived from pro-parasporin-1 by trypsin treatment, and that the cytotoxic activity resides in either or both of these fragments.

The recombinant pro-parasporin-1 had a His6-tag at the C-terminus, but both fragments were co-eluted by Ni^{2+}-chelating affinity chromatography. Furthermore, these fragments were not separated by either anion exchange chromatography or chromatography on a Superdex 75 HR 10/30 column, even in the presence of non-ionic detergents or 8M urea (data not shown), suggesting that parasporin-1 is a tightly associated complex of the 15- and 56-kDa fragments.

Molecular Characterization of Parasporin-1—To define the primary structure of parasporin-1, the N-terminal amino acid sequence and molecular mass of purified parasporin-1 were determined by an amino acid sequencer and MALDI-TOF MS, respectively. The N-terminal amino acid sequences of the 15- and 56-kDa fragments were STVTVPSFSNQFDPIK and MAEPPSTGVITQFRIL, respectively. These sequences are completely identical to Ser94–Lys109 and Met232–Leu247 of pro-parasporin-1, respectively (Fig. 3). Analysis of the molecular masses of the 15- and 56-kDa fragments by MALDI-TOF MS indicated that they are 15,365.9 and 54,808.3 Da, respectively. These mass values are consistent with the predicted values of the 15- and 56-kDa fragments corresponding to amino acid residues 94 to 231 and 232 to 723 of pro-parasporin-1 (Table 2). Taking all these results together, we conclude that parasporin-1 is composed of two fragments consisting of Ser94 to Arg231 and Met232 to Ser723 of pro-parasporin-1, as illustrated in Fig. 3.

Cell Specificity of the Parasporin-1 Cytotoxic Activity—The cytotoxic activity of purified parasporin-1 to 15 cell lines was examined. Fig. 4 shows the dose-response curves of parasporin-1 to cell lines originating from the uterus (A), liver (B) and hematopoietic cells (C). Parasporin-1 showed cytocidal toxicity against all cancer cell lines tested except for Jurkat cells (Fig. 4C). However, the protein showed no cytotoxic activity against normal uterus smooth muscle cells (UtSMC), normal hepatocyte cells (HC), or normal peripheral blood T cells. The LC_{50} values for parasporin-1 against various cell types are summarized in Table 3.

The cytotoxic effect of parasporin-1 was also examined under a phase-contrast microscope. Fig. 5 shows the morphologies of four sensitive cell lines (HeLa, HepG2, HL60 and MOLT-4) after treatment with parasporin-1 for 20 h. HepG2 and HeLa cells were detached and swollen, HL60 cells were fragmented, and MOLT-4 cells were swollen but not fragmented after 20 h treatment with parasporin-1.

Time-Course of Parasporin-1 Toxicity Is Different from That of the Cyt Toxin from B. thuringiensis Serovar Israelensis—The time-dependent decrease in the viability of HeLa cells by parasporin-1 was monitored by the MTT assay. Parasporin-1 decreased cell viability gradually, and required about 10 h for complete toxicity (Fig. 6A). The concentration of parasporin-1 used here (10 μg/ml) corresponds to about 80-fold the LC_{50} value for HeLa

Table 2. **Analysis of the 15- and 56-kDa subunits of parasporin-1 by MS spectrometry.**

15-kDa protein			56-kDa protein		
Position in pro-parasporin-1	Cleavage site[a]	Theoretical[b] (Da)	Position in pro-parasporin-1	Cleavage site[a]	Theoretical[b] (Da)
94–231	Arg231	15,336.7	232–723	No	54,810.4
94–223	Lys223	14,316.6	232–709	Lys709	53,346.8
94–220	Arg220	13,990.2	232–706	Arg706	53,003.4
Measured mass[c] (Da)		15,365.9			54,808.3

[a]The number indicates the amino acid residue number in pro-parasporin-1. [b]The cleavage sites were estimated from the substrate specificity of trypsin. The molecular mass of each fragment was calculated by the DNAsis program. [c]The molecular masses of the 15- and 56-kDa proteins of parasporin-1 were determined by MALDI-TOF mass spectrometry.

Table 3. **LC_{50} of purified parasporin-1 to various cell lines.**

Origin	Cell line	Characteristics	LC_{50} (μg/ml)[a,b]
Human	HeLa	Uterus cervix cancer	0.12 ± 0.037
	Sawano	Uterus gland cancer	>10
	UtSMC	Normal uterus smooth muscle	>10
	HL60	Promyelocytic cell	0.32 ± 0.04
	MOLT-4	Leukemic T cell	2.2 ± 0.1
	Jurkat	Leukemic T cell	>10
	T cell	Normal peripheral blood T cell	>10
	HC	Normal hepatocyte	>10
	HepG2	Hepatocyte cancer	3.0 ± 0.4
	A549	Lung cancer	>10
	MRC5	Normal embryonic lung fibroblast	>10
	CACO-2	Colon cancer	>10
Monkey	Vero	Kidney	>10
	COS-7	Kidney	>10
Mouse	NIH3T3	Embryo	>10

[a]The LC_{50} was calculated from the data of the dose-response curve of cytotoxicity against each cell line. [b]Data represent the mean ± SD, $n = 5$.

cells. Even at such a high concentration, no cytotoxic effect of parasporin-1 was observed within 1 h of incubation.

When HeLa cells were treated with parasporin-1 and observed under a microscope, the cytotoxic effect seemed to occur in three phases. In the first phase (from 0.5–2 h), the outline of the nuclei in the toxin-treated cells became clear, and bright granules appeared in the nuclei (Fig. 6B; arrow a in B). Subsequently, the cells detached from the bottom of the dish in the second phase (from 4–12 h; Fig. 6B; arrow b in E). In the final phase, the cells swelled (Fig. 6B; arrow d in G) with subsequent granulation of the cytoplasm (Fig. 6B; arrow c in F).

DISCUSSION

B. thuringiensis parasporal inclusion proteins currently form two categories, namely Cyt and Cry proteins, according to the *Bacillus thuringiensis* Toxin Nomenclature Com-

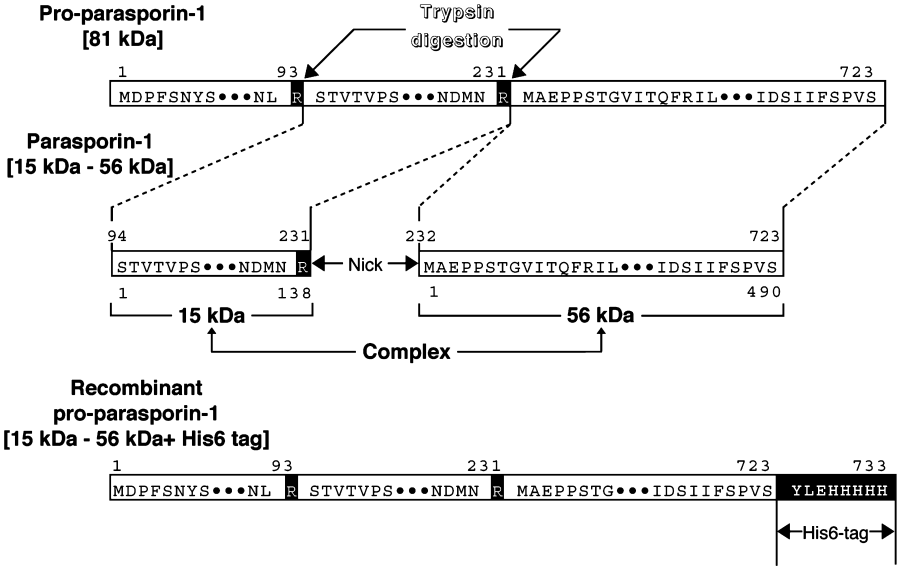

Fig. 3. **Schematic structure of parasporin-1.** Pro-parasporin-1 is cleaved by trypsin at two sites and converted into an active form. The 15- and 56-kDa proteins form a complex. The recombinant pro-parasporin-1 has a His6-tag at the C-terminus.

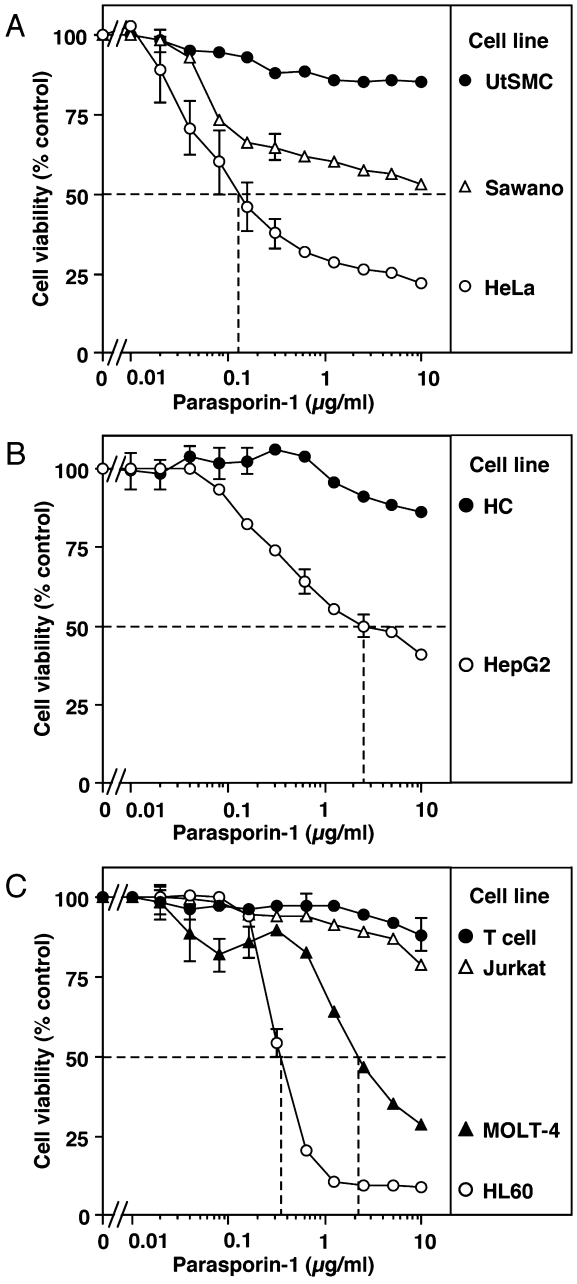

Fig. 4. **Dose-dependent cytotoxic activity of parasporin-1 to various cell lines.** A: Cytotoxicity against three cell lines originating from the uterus (HeLa, Sawano and UtSMC). B: Cytotoxicity against two cell lines derived from the liver (HepG2 and HC). C: Cytotoxicity against four hematopoietic cell lines (HL60, MOLT-4, Jurkat and normal T cell). Cells were treated with the indicated concentrations of purified parasporin-1. After incubation for 20 h, cells were subjected to the MTT assay to determine cell viability. The average absorbance of mock-treated negative controls (treated with Buffer B) was used as a blank value. Cell viability was calculated from the MTT assay on the basis of the relative absorbance value at 590 nm to the blank value (100%). Each value is the mean ± SD of three independent experiments.

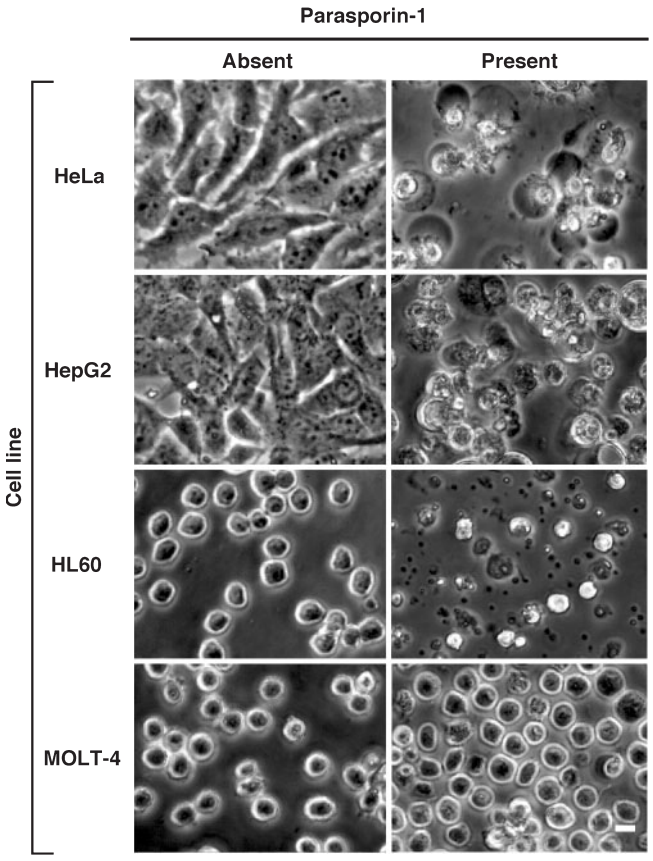

Fig. 5. **Cytopathic effect of parasporin-1 on cancer cells.** HeLa, HepG2, HL60 and MOLT-4 cells were incubated with or without parasporin-1 (10 μg/ml) for 20 h at 37°C. After incubation, the cells were observed under a phase-contrast microscope. Bar, 10 μm.

mittee (see web site; http://www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html). The Cyt protein category represents broad-spectrum cytolysins active on a wide range of invertebrate and vertebrate cells, including insect cells and mammalian erythrocytes (*5*). The Cry protein category is a phylogenetically heterogeneous group. Historically, it has long been believed that the Cry proteins are insect-specific toxins. However, the current classification scheme of the Cry protein group also includes non-insecticidal parasporal inclusion proteins (see web site described above). Recently, we reported the occurrence of a unique functional protein group among Cry proteins, designated parasporin, defined as the *B. thuringiensis* and related bacterial parasporal proteins that are non-hemolytic but capable of preferentially killing cancer cells (*17*, *21–24*). To date, we have identified four parasporins (Table 4) from non-insecticidal *B. thuringiensis* strains. In this paper, the structure, molecular characterization, cell specificity and partial characterization of the cytotoxic activity of parasporin-1 are described.

Parasporin-1 was generated by tryptic digestion of pro-parasporin-1. Experiments including purification by a combination of column chromatography and SDS-PAGE analysis, Ni-chelating affinity chromatography of epitope-tagged recombinant pro-parasporin-1, N-terminal amino acid sequencing and mass spectrometry analysis revealed that parasporin-1 comprises 15- and 56-kDa fragments of pro-parasporin-1 (Fig. 2C). The 15- and 56-kDa fragments could not be separated under ordinary conditions

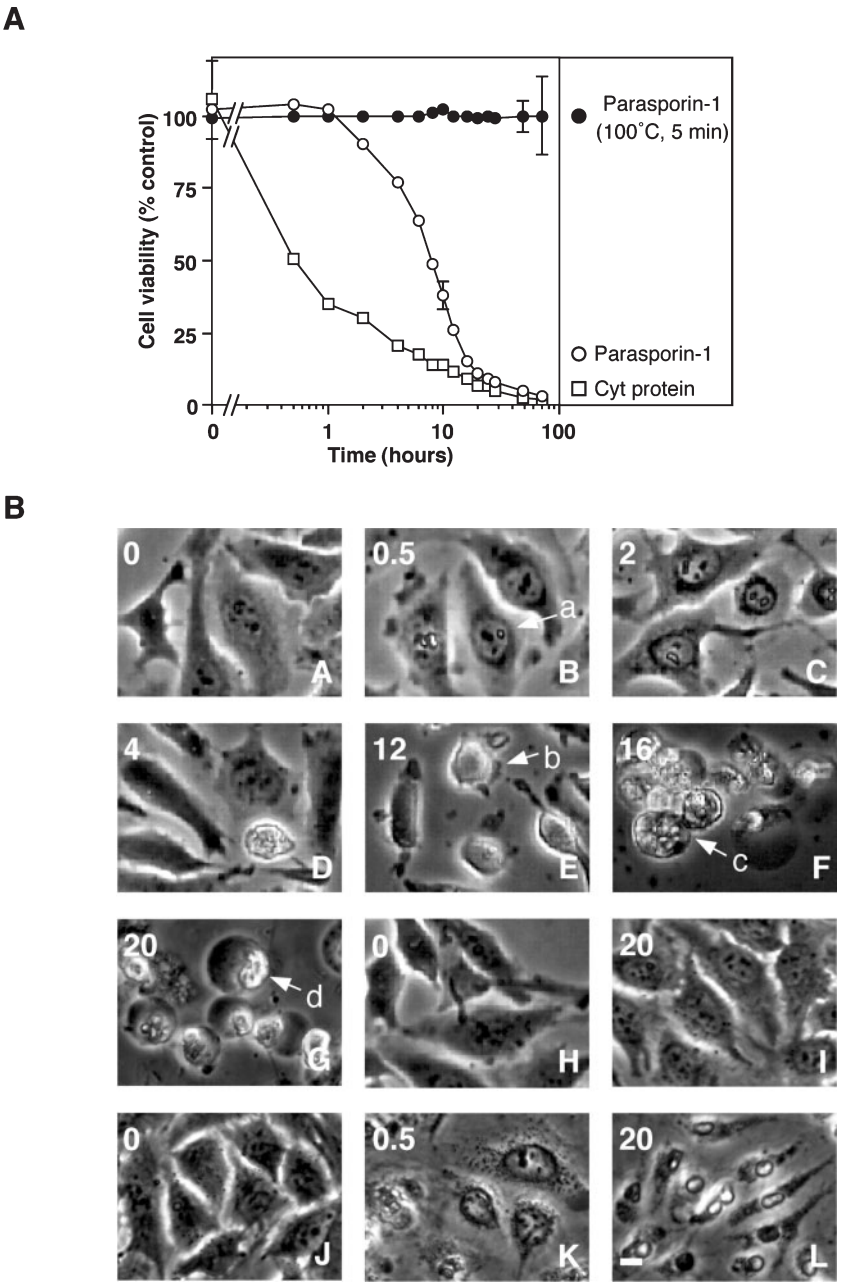

Fig. 6. **Time-courses of the cytotoxic effect of parasporin-1.** A: Time-courses of the cell viability of HeLa cells incubated with parasporin-1, heat-inactivated parasporin-1 or Cyt protein of *B. thuringiensis* serovar *israelensis*. Cell viability was determined by the MTT assay. B: Time-dependent morphological changes in HeLa cells treated with parasporin-1. HeLa cells were treated with 10 μg/ml of parasporin-1 at 37°C. Cells were observed under a phase-contrast microscope at the indicated times. The numbers in the upper left corner of each photograph indicate the incubation time. A–G, cells incubated with parasporin-1; H and I, control cells incubated with Buffer B only; J–L, cells incubated with crude Cyt toxin. Bar, 10 μm.

for chromatography, even in the presence of a detergent or denaturing agent [*c.f.* 1% (v/v) Triton X-100, 0.1% (v/v) CHAPS, 0.1% (v/v) CHAPSO, 1% (w/v) octyl glucoside or 8 M urea]. Thus, the 15- and 56-kDa fragments were not separated under conditions that preserve cytotoxic activity, and only 0.1% (w/v) SDS treatment with heat denaturing was able to dissociate the fragments. These results suggest that the 15- and 56-kDa fragments form a tight complex. Recently, the three-dimensional structure of recombinant parasporin-1 was analyzed by X-ray crystal diffraction, and the study revealed that parasporin-1 is composed of 15- and 56-kDa proteins at a molecular ratio of 1:1 in the heterodimer (Akiba, T. and Harata, M., unpublished data). It has been reported that Cry4A and Cry11A, insecticidal *B. thuringiensis* toxins, are cleaved by proteases to form active toxins composed of two different molecules (*25*, *26*). Parasporin-1 is also a heterodimer comprising the 15- and 56-kDa fragments. Although there is no evidence for the requirement of both fragments for cytotoxic activity, the heterodimeric structure of parasporin-1 is reminiscent of the common A-B structure of bacterial protein toxins.

A feature of parasporin-1 is its cell type-specific toxicity. Parasporin-1 showed strong toxicity to HeLa, MOLT-4 and HL60 cells, and moderate toxicity to Sawano and HepG2 cells, but it was almost non-toxic to UtSMC, HC, MRC-5 and peripheral blood T cells. These results suggest that a specific receptor is involved in the toxicity of parasporin-1, since specific receptors mediate the toxicities of other bacterial toxins (*27*–*32*), although the possible existence of a specific intracellular target in the sensitive cell lines could not be excluded. For Cry toxins of *B.*

Table 4. **The members of the parasporin family.**

Protein	*Bacillus thuringiensis* Strain	Precursor (kDa)	Active form (kDa)	Cry protein class*	Remarks	Reference
Parasporin-1	A1190	81	15+56	Cry31Aa1	Three domain type, activated by N-terminal digestion.	This study and Mizuki *et al.* (*17*)
Parasporin-2	A1547	37	30	Cry46Aa1	Non-three domain type, activated by N and C-terminal digestion.	Ito *et al.* (*24*)
Parasporin-3	A1462	88	64	Cry41Aa1	Three domain type, activated by N-terminal digestion.	Yamashita *et al.* (*23*)
Parasporin-4	A1470	31	27	Cry45Aa1	Non-three domain type, activated by C-terminal digestion.	Lee *et al.* (*21*)

thuringiensis, which show specific insecticidal activity, receptors have been identified in many insect species (*33–39*). In contrast, a class of Cyt toxins shows toxicity to a broad spectrum of cell lines including insect and human cells.

Time-course studies of the effect of parasporin-1 on HeLa cells indicated that the morphological changes in HeLa cells occurred in three phases, and that the decrease in cell viability was quite slow compared with the effect of the Cyt toxin from *B. thuringiensis* serovar *israelensis*. In contrast, the Cyt toxin, which primarily has affinity for phospholipids of the cell membrane (*40–42*), causes drastic cell lysis. It is also well established that bacterial membrane pore-forming toxins, including *B. thuringiensis* insecticidal toxins, induce marked morphological changes, increase membrane permeability and cause cell lysis of target cells in a short time (*39*, *43*). In conclusion, parasporin-1 exerts its cytotoxicity to mammalian cells by a different mechanism from that observed for previously characterized insecticidal proteins and hemolytic proteins from *B. thuringiensis*.

The results of the present study indicate that parasporin-1 is highly toxic to some cell lines derived from human cancers (for example, HeLa and HL60), while it is almost non-toxic to cells obtained from normal tissues. Thus, parasporin-1 may be useful for the diagnosis and treatment of particular types of cancer cells. Furthermore, we have reported that additional parasporins produced by other *B. thuringiensis* strains (Table 4) also show cytotoxic activity to human cells after proteolytic digestion, similar to parasporin-1 (*16*, *21–24*). These parasporins differ in molecular weight, cell specificity and efficient concentration for killing human cancer cells. Thus, parasporins would be a good source of substances for analyzing normal and cancer cells. Our results also suggest that the parasporal inclusion proteins of *B. thuringiensis* may provide a novel tool for developing new diagnostic or anti-cancer agents in addition to their use as insecticides.

We thank Dr. S. Ando (Fukuoka University) for the mass spectrometric analysis. This manuscript was kindly reviewed by Dr. C. Ponzetto (Turin University). This study was supported by Special Coordination Funds for the Promotion of Science and Technology (Leading Research Utilizing Potential of Regional Science and Technology) of the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government.

REFERENCES

1. Höfte, H. and Whiteley, H.R. (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* **53**, 242–255
2. Cannon, R.J.C (1996) *Bacillus thuringiensis* use in agriculture: a molecular perspective. *Bio. Rev.* **71**, 561–636
3. Lambert, B. and Peferoen, M. (1992) Insecticidal promise of *Bacillus thuringiensis*. Facts and mysteries about a successful biopesticide. *BioSicence* **42**, 112–122
4. Federici, B.A. (1995) The future of microbial insecticides as vector control agents. *J. Amer. Mosquito Contr. Ass.* **11**, 260–268
5. Gill, S.S., Cowles, E.A., and Pietrantonio, P.V. (1992) The mode of action of *Bacillus thuringiensis* endotoxins. *Annu. Rev. Entomol.* **37**, 615–636
6. Knowles, B.H. (1994) Mechanism of action of *Bacillus thuringiensis* insecticidal (δ-endotoxins. *Adv. Insect Physiol.* **24**, 275–308
7. Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., and Dean, D.H. (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **62**, 775–806
8. Bravo, A., Jansens, S., and Peferoen, M. (1992) Immunocytochemical localization of *Bacillus thuringiensis* insecticidal crystal proteins in intoxicated insects. *J. Invertebr. Pathol.* **60**, 237–246
9. Hofmann, C., Vanderbruggen, H., Höfte, H., Van Rie, J., Jansens, S., and Van Mellaert, H. (1988) Specificity of *Bacillus thuringiensis* δ-endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. *Proc. Natl Acad. Sci. USA* **85**, 7844–7848
10. Soberón, M., Perez, R.V., Nuezñez-Valdéz, M.E., Lorence, A., Gómez, I., Sánchez, J., and Bravo, A. (2000) Evidence for intermolecular interaction as a necessary step for pore-formation activity and toxicity of *Bacillus thuringiensis* Cry1Ab toxin. *FEMS Microbiol. Lett.* **191**, 221–225
11. Lorence, A., Darszon, A., Díaz, C., Liévano, A., Quintero, R., and Bravo, A. (1995) δ-endotoxins induce cation channels in *Spodoptera frugiperda* brush border membranes in suspension and in planar lipid bilayers. *FEBS Lett.* **360**, 217–222
12. Schwartz, J.L., Garneau, L., Masson, L., Brousseau, R., and Rousseau, E. (1993) Lepidopteran-specific crystal toxins from *Bacillus thuringiensis* form cation- and anion-selective channels in planar lipid bilayers. *J. Membr. Biol.* **132**, 53–62
13. Meadows, M.P., Ellis, D.J., Butt, J., Jarrett, P., and Burges, H.D. (1992) Distribution, frequency, and diversity of *Bacillus thuringiensis* in an animal feed mill. *Appl. Environ. Microbiol.* **58**, 1344–1350
14. Ohba, M. and Aizawa, K. (1978) Serological identification of *Bacillus thuringiensis* and related bacteria isolated in Japan. *J. invertebr. Pathol.* **32**, 303–309
15. Roh, J.Y., Park, H.W., Jin, B.R., Kim, H.S., Yu, Y.M., and Kang, S.K. (1996) Characterization of novel non-toxic *Bacillus thuringiensis* isolates from Korea. *Lett. Appl. Microbiol.* **23**, 249–252
16. Mizuki, E., Ohba, M., Akao, T., Yamashita, S., Saitoh, H., and Park, Y.S. (1999) Unique activity associated with non-insecti-

cidal *Bacillus thuringiensis* parasporal inclusions: in vitro cell-killing action on human cancer cells. *J. Appl. Microbiol.* **86**, 477–486
17. Mizuki, E., Park, Y.S., Saitoh, H., Yamashita, S., Akao, T., Higuchi, K., and Ohba, M. (2000) Parasporin, a human leukemic cell-recognizing parasporal protein of *Bacillus thuringiensis*. *Clin. Diagn. Lab. Immunol.* **7**, 625–634
18. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
19. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
20. Oakley, B.R., Kirsch, D.R., and Morris, N.R. (1980) A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* **105**, 361–363
21. Lee, D.W., Akao, T., Yamashita, S., Katayama, H., Maeda, M., Saitoh, H., Mizuki, E., and Ohba, M. (2000) Noninsecticidal parasporal proteins of a *Bacillus thuringiensis* serovar *shandongiensis* isolate exhibit a preferential cytotoxicity against human leukemic T cells. *Biochem. Biophys. Res. Commun.* **272**, 218–223
22. Kim, H.S., Yamashita, S., Akao, T., Saitoh, H., Higuchi, K., Park, Y.S., Mizuki, E., and Ohba, M. (2000) In vitro cytotoxicity of non-Cyt inclusion proteins of a *Bacillus thuringiensis* isolate against human cells, including cancer cells. *J. Appl. Microbiol.* **89**, 16–23
23. Yamashita, S., Akao, T., Mizuki, E., Saitoh, H., Higuchi, K., Park, Y.S., Kim, H.S., and Ohba, M. (2000) Characterization of the anti-cancer-cell parasporal proteins of a *Bacillus thuringiensis* isolate. *Can. J. Microbiol.* **46**, 913–919
24. Ito, A., Sasaguri, Y., Kitada, S., Kusaka, Y., Kuwano, K., Masutomi, K., Mizuki, E., Akao, T., and Ohba, M. (2004) A *Bacillus thuringiensis* crystal protein with selective cytocidal action to human cells. *J. Biol. Chem.* **279**, 21282–21286
25. Yamagiwa, M., Esaki, M., Otake, K., Inagaki, M., Komano, T., Amachi, T., and Sakai, H. (1999) Activation process of dipteran-specific insecticidal protein produced by *Bacillus thuringiensis* subsp. *israelensis*. *Appl. Environ. Microbiol.* **65**, 3464–3469
26. Yamagiwa, M., Ogawa, R., Yasuda, K., Natsuyma, H., Sen, K.M., and Sakai, H. (2002) Active form of dipteran-specific insecticidal protein Cry11A produced by *Bacillus thuringiensis* subsp. *israelensis*. *Biosci. Biotechnol. Biochem.* **66**, 516–522
27. Mekada, E., Okada, Y., and Uchida, T. (1988) Identification of diphtheria toxin receptor and a nonproteinous diphtheria toxin-binding molecule in Vero cell membrane. *J. Cell Biol.* **107**, 511–519
28. Mekada, E., Senoh, H., Iwamoto, R., Okada, Y., and Uchida, T. (1991) Purification of diphtheria toxin receptor from Vero cells. *J. Biol. Chem.* **266**, 20457–20462
29. Naglich, J.G., Metherall, J.E., Russel, D.W., and Eidels, L. (1992) Expression cloning of a diphtheria toxin receptor: Identify with a heparin-binding EGF-like growth factor precursor. *Cell* **69**, 1051–1061
30. Yahiro, K., Niidome, T., Kimura, M., Hatakeyama, T., Aoyagi, H., Kurazono, H., Imagawa, K., Wada, A., Moss, J., and Hirayama, T. (1999) Activation of *Helicobacter pylori* VacA toxin by alkaline or acid conditions increases its binding to a 250-kDa receptor protein-tyrosine phosphatase ß. *J. Biol. Chem.* **274**, 36693–36699
31. Nelson, K.L., Raja, S.M., and Buckley, J.T. (1997) The glycosylphosphatidylinositol-anchored surface glycoprotein Thy-1 is a receptor for the channel-forming toxin aerolysin. *J. Biol. Chem.* **272**, 12170–12174
32. Diep, D.B., Nelson, K.L., Raja, S.M., Pleshak, E.N., and Buckley, J.T. (1998) Glycosylphosphatidylionositol anchors of membrane glycoproteins are binding determinants for the channel-forming toxin aerolysin. *J. Biol. Chem.* **273**, 2355–2360
33. Gill, S.S., Cowles, E.A., and Francis, V. (1995) Identification, isolation, and cloning of a *Bacillus thuringiensis* CryIAc toxin-binding protein from the midgut of the lepidopteran insect *Heliothis virescens*. *J. Biol. Chem.* **270**, 27277–27282
34. Knight, P.J.K., Knowles, B.H., and Ellar, D.J. (1995) Molecular cloning of an insect aminopeptidase N that serves as a receptor for *Bacillus thuringiensis* CryIA(c) toxin. *J. Biol. Chem.* **270**, 17765–17770
35. Masson, L., Lu, Y., Mazza, A., Brousseau, R., and Adang, M.J. (1995) The CryIA(c) receptor purified from *Manduca sexta* displays multiple specificities. *J. Biol. Chem.* **270**, 20309–20315
36. Masson, L., Mazza, A., Brousseau, R., and Tabashnik, B. (1995) Kinetics of *Bacillus thuringiensis* toxin binding with brush border membrane vesicles from susceptible and resistant larvae of *Plutella xylostella*. *J. Biol. Chem.* **270**, 11887–11896
37. Yaoi, K., Kadotani, T., Kuwana, H., Shinkawa, A., Takahashi, T., Iwahana, H., and Sato, R. (1997) Aminopeptidase N from *Bombyx mori* as a candidate for the receptor of *Bacillus thuringiensis* Cry1Aa toxin. *Eur. J. Biochem.* **246**, 652–657
38. Nagamatsu, Y., Toda, S., Yamaguchi, F., Ogo, M., Kogure, M., Nakamura, M., Shibata, Y., and Katsumoto, T. (1998) Identification of *Bombix mori* midgut receptor for *Bacillus thuringiensis* insecticidal CryIA(a) toxin. *Biosci. Biotechnol. Biochem.* **62**, 727–734
39. Nagamatsu, Y., Koike, T., Sasaki, K., Yoshimoto, A., and Furukawa, Y. (1999) The cadherin-like protein is essential to specificity determination and cytotoxic action of the *Bacillus thuringiensis* insecticidal CryIAa toxin. *FEBS Lett.* **460**, 385–390
40. Knowles, B.H., Blatt, M.R., Tester, M., Horsnell, J.M., Carroll, J., Menestrina, G., and Ellar, D.J. (1989) A cytolytic δ-endotoxin from *Bacillus thuringiensis* var. *israelensis* forms cation-selective channels in planar lipid bilayers. *FEBS Lett.* **244**, 259–262
41. Li, J., Koni, P.A., and Ellar, D.J. (1996) Structure of the mosquitocidal δ -endotoxin CytB from *Bacillus thuringiensis* sp. *kyushuensis* and implications for membrane pore formation. *J. Mol. Biol.* **257**, 129–152
42. Thomas, W.E. and Ellar, D.J. (1983) Mechanism of action of *Bacillus thuringiensis* var. *israelensis* insecticidal δ -endotoxin. *FEBS Lett.* **154**, 362–368
43. Petit, L., Gibert, M., Gillet, D., Laurent-Winter, C., Boquet, P., and Popff, M.R. (1997) *Clostridium perfringens* epsilon-toxin acts on MDCK cells by forming a large membrane complex. *J. Bacteriol.* **179**, 6480–6487