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MINI REVIEW

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Parasporin-4, A Novel Cancer Cell-killing Protein Produced by *Bacillus thuringiensis*

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Abstract *Bacillus thuringiensis* was isolated as a pathogen of the *sotto* disease of silkmoth larvae about a hundred years ago. Since then, this bacterium has attracted attentions of not only insect pathologists but also many other scientists who are interested in its strong and specific insecticidal activity. This has led to the recent worldwide development of *B. thuringiensis*-based microbial insecticides and insect-resistant transgenic plants, as well as a landmark discovery of parasporin, a cancer cell-specific cytotoxin produced by *B. thuringiensis*. In this review, we describe examination of interaction between inclusion proteins of *B. thuringiensis* and brush border membrane of insects using a surface plasmon resonance-based biosensor, identification and characterization of parasporin-4, the latest parasporin produced by the *B. thuringiensis* A1470 strain, and an effective method for preparing the parasporin-4 from inclusion bodies expressed in the recombinant *Escherichia coli* cells.

Keywords: *Bacillus thuringiensis*, bacterial insecticide, cytotoxic activity, inclusion body, parasporin

Introduction

Bacillus thuringiensis is a Gram-positive endospore-forming bacterium which produces large crystalline parasporal inclusion bodies in sporulating cells. It was isolated as a pathogen of the *sotto* disease of silkmoth larvae by a Japanese silkworm pathologist, Shigetane Ishiwata in 1901 (1). Later in 1915, the organism was described as *B. thuringiensis* by a German biologist, Ernst Berliner (2). The *B. thuringiensis* parasporal inclusion bodies often contain one or more proteins toxic to insects, and they are called δ-endotoxin. The δ-endotoxin proteins with hemolytic and non-hemolytic activities are called Cyt and Cry proteins, respectively (3). When the parasporal inclusion bodies are ingested by insect larvae, the proteins are solubilized and converted into toxins by midgut proteases of insects susceptible to δ-endotoxin (4). They are highly and specifically toxic to insect pests of Lepidoptera, Diptera, and Coleoptera (5), but are not pathogenic to mammals, birds, amphibians, or reptiles (6). This makes *B. thuringiensis* a promising microbial agent in the control of insect pests in agriculture, forestry, veterinary, and public health management (6). Presently, *B. thuringiensis* δ-endotoxins are classified and designated by the *B. thuringiensis* δ-endotoxin nomenclature committee (7) based on the identity of amino acid sequences.

On the other hand, non-insecticidal *B. thuringiensis* strains are more widely distributed than insecticidal ones (8). Mizuki *et al.* (9,10) have reported that human cancer cell-killing activity is associated with some non-insecticidal *B. thuringiensis* isolates, and have created a new category of protein, parasporin, which is defined as bacterial parasporal proteins that are capable of preferentially killing cancer cells (10). Parasporal inclusion protein of the *B. thuringiensis* A1470 strain also exhibit strong cytotoxicity against human leukemic T cells when it is activated by protease treatment, although it does not exhibits insecticidal or hemolytic activities (11). The A1470 strain was anticipated to produce multiple cytotoxic proteins with similar molecular masses (12). A cytotoxic protein was identified from the strain in 2005 (13). It was designated parasporin-4 by the committee of parasporin classification and nomenclature (14), and also designated Cry45Aa by the *B. thuringiensis* δ-endotoxin nomenclature committee.

In this review, we describe firstly a novel screening method using a surface plasmon resonance (SPR)-based optical biosensor (15). By the system, interaction between *B. thuringiensis* inclusion proteins and brush border membrane (BBM) vesicles of insects can be measured in real time. Secondly, we describe identification and characterization of parasporin-4 (13), and thirdly propose an effective method for purification of the cytotoxic protein (16).

A New Screening Method for δ-Endotoxins of *B. thuringiensis*

B. thuringiensis is well-known as an insecticidal bacterium, however, non-insecticidal *B. thuringiensis* strains are more widely distributed than insecticidal ones (8). Hence it is necessary to screen strains insecticidal against insect pests from various natural environments. Bioassay with target insects must be the method primarily taken for screening δ-endotoxins. However, the bioassay is difficult to be applied for the development of new *B. thuringiensis* insecticides, because breeding techniques for various insect pests have not yet been established. Thus, we examined to develop a novel screening method using a SPR-based optical biosensor (15). The SPR is used for an optical detection system that allows direct interaction analysis between a ligand immobilized on a sensor chip and a specific analyte in a continuous flow (17,18). In this system, one of the reactants is immobilized to an alkanethiol monolayer on

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the surface of a gold sensor chip. The angle of polarized light reflected from the surface of the chip changes according to total mass attached to the sensor chip. This change is detected by a photo-diode array. By linking this system to a personal computer, complex association and dissociation processes can be measured in real time. In this review, we introduce the SPR analysis of interaction between Cry1Ac, a δ-endotoxin produced by the *B. thuringiensis*, and a BBM prepared from its susceptible insect, diamondback moth, *Plutella xylostella* (15). These SPR-based interaction studies provide us with an insect-free rapid and large-scale screening system of *B. thuringiensis* insecticide proteins. The systems must be also useful for screening the parasporins.

Formation of the biosensor simulated a brush border membrane The first step of the action of δ-endotoxins is considered that the activated δ-endotoxin binds to BBM of the susceptible insect, and the toxin forms pores on the plasma membrane of midgut epithelial cells, which leads death of the insect. The interaction between the toxin and the sites with high toxin-binding affinity on the midgut epithelium of the susceptible insect species must be a major determinant for the specificity of the insecticidal proteins, and the midgut target sites was shown in the *in vitro* binding studies using insect midgut BBM (19). Recently, an SPR technique has been applied to examine interaction of *B. thuringiensis* toxins with both insect midgut BBM vesicles (17,20) and a purified toxin receptor (21). In the former case, the membrane vesicles were immobilized on the sensor chip via immobilized antibodies raised against either avidin or biotin. Binding of the toxin to receptor proteins embedded in a model membrane containing L-α-phosphatidylcholine prepared from egg yolk was also observed (22). Previously, it was demonstrated that multiple toxin-binding proteins were located on the midgut BBM of susceptible insect larvae, suggesting that the interaction between the toxin and receptor might be a complex process. Later, a biosensor system in which a simulated BBM monolayer of lepidopteran larvae was used as the membrane layer was constructed (15). The BBM vesicle of diamondback moth (*P. xylostella*) was isolated from midguts of late-instar larvae by selective magnesium precipitation (23), and the isolated BBM vesicle was diluted with a neutral charged artificial lipid, 1, 3-ditetradecylglycero-2-phosphocholine (PC14) (24) at a PC14:BBM protein ratio of 100:5 (w/w). The mixture was sonicated for 1 min 3 times and passed through a polycarbonate membrane filter (pore size: 100 nm). Then reconstruction of the monolayer was achieved on the hydrophobic association (HPA) chip using a SPR detector system, BIAcore 1000. The amount of the mixture bound to the chip showed the resonance response of 2,600 resonance unit (RU), followed by injection of bovine serum albumin (BSA) solution to assess that the chip surface was covered almost perfectly by BBM. When the BBM was loaded alone on the chip, it was almost washed out by injecting 4 mM sodium hydroxide in the last step (regeneration of the chip) in the measurement procedure, and the binding amount of BBM was only 200 RU. This suggested that the binding of BBM on the HPA chip was weak without the phospholipid PC14 and that the BBM binding was promoted greatly with PC14 (15). When observed with a transmission electron microscope, PC14 formed well-developed vesicles with diameters of 140-200 nm. In contrast, BBM from *P. xylostella* formed irregular-shaped aggregates with various sizes. When the BBM was mixed with PC14, vesicles with diameters of 80-130 nm were formed. The morphological observation showed that BBM was incorporated in the PC14 vesicles. The phospholipid content in BBM was considerably low, and the ratio of phospholipid to protein in BBM was approximately 1:1 (w/w). This low phospholipid content in BBM may be the cause of failure of BBM alone to form typical liposomal vesicles, and also may lead to instability of the BBM monolayer without PC14 on the HPA chip (15).

Interaction between δ-endotoxin and BBM by the SPR-based system Cry1Ac is an insecticidal protein produced by the *B. thuringiensis* strain HD-73 (25), the type strain of serovar *kurstaki*, which has an activity against the diamondback moth larvae. Now, the Cry1Ac gene is used for gene-modified corn or cotton (26) and transgenic rice containing Cry1Ac gene is also available from local markets in China (27). Inclusion bodies of this strain were solubilized in 50 mM sodium carbonate buffer (pH 10.5) and activated with proteinase K. The activated Cry1Ac toxin was delivered to the reconstructed PC14/BBM monolayer to determine the levels required for ligand saturation. The relative resonance response detected in the bio-sensor system increased with increasing the initial Cry1Ac toxin concentration ([toxin]), indicating that the amount of the toxin bound to the monolayer increased with an increase in the toxin concentration, [toxin]. The relative resonance response corresponding to the amount of the toxin bound on the sensor chip showed a Michaelis-Menten type saturation curve against [toxin]. The double reciprocal plot (1/[relative response] vs. 1/[toxin]) gave a straight line, and the affinity constant (K_d) of the toxin to the monolayer on the chip was determined to be 3.1 μM, and the maximum resonance response was 170 RU (15).

Subsequently, *N*-acetyl-D-galactosamine (GalNAc) was examined for its ability to reduce the binding of the Cry1Ac toxin to the reconstructed monolayer, and GalNAc was shown to inhibit the binding. The degree of the inhibition was dose-dependent, and 89% of the toxin binding was inhibited at 80 mM GalNAc. The inhibitor constant of GalNAc against the Cry1Ac binding to the monolayer was determined to be 8 mM (15). This result was in good agreement with the previous observations that Cry1Ac binds to the receptors on the lepidopteran epithelium cell membrane with the support of GalNAc (21,22,28).

Comparison the methods for interaction of Cry toxins with the receptors Cry toxins were reported to generate pores in cell membranes by electrophysiological and biochemical studies (29). Their action includes two steps; namely, binding of the toxins to the receptors on the cell membrane, and subsequent penetration of the toxins into the cells to form pores. Thus, the data obtained by the SPR system was analyzed using a 2-state reaction model. Data for the binding of Cry1Ac were corrected for bulk reflective index changes, and fitted well to the theoretical

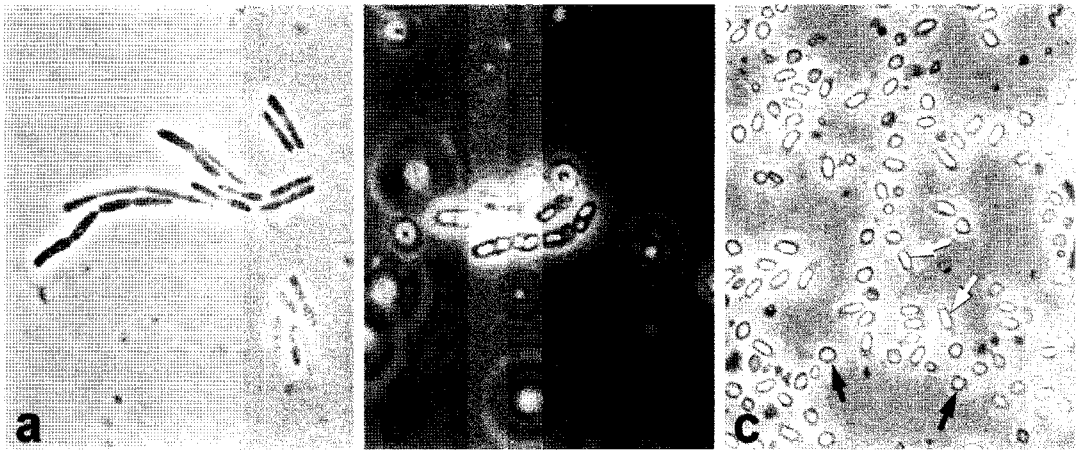

Fig. 1. *Bacillus thuringiensis* A1470 strain. (a) Vegetative cells, (b) cells at a late stage of spore formation, (c) spores (white arrow) and parasporal inclusion bodies (black arrow).

equation. The toxin binding to the receptors was occurred with an affinity constant (K_1) of 0.51 μM in the first step, followed by a second slower event with an affinity constant (K_2) of 0.47. The latter step is a uni-molecular event, which is concentration-independent, and thus the equilibrium constant is dimensionless. The overall affinity constant (K_d) defined as the product of K_1 and K_2, was calculated to be 0.24 μM (15).

The affinity constants obtained in the assay system by the BBM/PC14 were 8.8-120-fold greater than those reported by the other workers (30-33). This difference is considered due to the difference in the methods used; most of the other studies have been done with the competitive binding assay using the radiolabeled Cry1Ac toxin. In another SPR experiment, an affinity constant (K_d) of 7 nM was reported in the binding of Cry1Ac with the diamondback moth BBM vesicles (20). This value is 34-fold smaller than that obtained in the present study. The cause of these differences is uncertain. It should be noted, however, that the K_d value (0.24 μM) observed here is on the level of those (95-350 nM) reported by the previous workers in SPR studies with Cry1Ac and BBM vesicles of other lepidopterans, *Manduca sexta* and *Heliothis virescens* (21,22,28). It was reported that when analyzed with a 2-state reaction algorithm, the interaction between Cry1Ac and a lepidopteran receptor was well analyzed by 2-step mechanism, and the affinity constant (K_2) of the second step was reported to be 2.8×10^{-2} (22). This value is significantly lower than that obtained in the present study. The second step or the penetration of toxins into membranes might be affected by the property of lipid. Therefore, it is likely that the lower affinity observed here might be given by the use of an artificial lipid PC14.

The receptor protein recognized by Cry1Ac is identified as aminopeptidase N and/or cadherin-like protein that are bound on the midgut-cell membrane of lepidoptera. Aminopeptidase N is a metallopeptidase containing a zinc atom essential for the enzyme activity. It participates in the processing of peptide hormones in mammalian cells, and is also noticed as an immunological cell marker, CD13. The structural homology between the aminopeptidase N enzymes of insects and mammals were reported, although the physiological roles of the insect enzyme are not well revealed. The SPR-based biosensor method applied for investigating the interaction between the insecticidal proteins and the BBM monolayer was shown also to be applicable for investigating the proteolytic properties of aminopeptidase N. This method might also provide us with a suitable tool to study the structure-function relationship of this enzyme veiled in mystery, and enables us to discuss this enzyme in comparison with the well-studied metallo-proteinases, thermolysin (34,35) and matrix metallo-proteinases (36,37).

Identification and Characterization of Parasporin-4

The *B. thuringiensis* A1470 strain (Fig. 1) belongs to serovar *shandongiensis*. In 2000, Lee *et al.* (11) reported that the parasporal inclusion of the strain exhibited strong cytotoxicity against human leukemic T cells when activated by protease treatment. They suggested that a 28 kDa protein was cytotoxic (38). In 2004, it was confirmed that the strain produced at least 2 kind of cytotoxic proteins with similar molecular masses of 28 kDa (12), and the 2 cytotoxic proteins were identified separately (13). One of them was designated as parasporin-4 and the other was parasporin-2-like protein. Parasporins are classified and named based on the identity of the amino acid sequence in the same naming system as that of *B. thuringiensis*, Cry proteins, and a new accession number composed of 2 numerals and 2 letters indicating 4 ranks in the sequence identity is assigned to a novel parasporin protein as parasporin1Aa1 (39). Approximately 95, 78, and 45% sequence identities are the borders of the four ranks. In present, 13 parasporins are registered, and the number of the primary rank of parasporin is only 4 (Table 1). Parasporin-4 is the latest one in the primary rank. In this review, we describe identification of 2 cytotoxic proteins produced by the A1470 strain and characterization of one of them that is named parasporin-4.

Identification of two cytotoxic proteins of the *B. thuringiensis* A1470 strain The *B. thuringiensis* A1470 strain was isolated from a soil sample collected in Hino city, Tokyo, Japan (11). It had been concluded that the cytotoxic activity of inclusions from *B. thuringiensis*

Table 1. List of parasporins[1)]

Name	Cry number[2)]	Authors[3)]	Year[4)]	Accession number	Source strain
Parasporin-1Aa1	Cry31Aa1	Mizuki *et al.* (10)	2000	AB031065	Bt A1190
Parasporin-1Aa2	Cry31Aa2	Jung & Côté (40)	2002	AY081052	Bt M15
Parasporin-1Aa3	Cry31Aa3	Uemori *et al.* (41)	2006	AB250922	Bt B195
Parasporin-1Aa4	Cry31Aa4	Yasutake *et al.* (42)	2006	AB274826	Bt 79-25
Parasporin-1Aa5	Cry31Aa5	Yasutake *et al.* (42)	2006	AB274827	Bt 92-10
Parasporin-1Ab1	Cry31Ab1	Uemori *et al.* (41)	2006	AB250923	Bt B195
Parasporin-1Ab2	Cry31Ab2	Yasutake *et al.* (42)	2006	AB274825	Bt 31-5
Parasporin-1Ac1	Cry31Ac1	Yasutake *et al.* (42)	2006	AB276125	Bt 87-29
Parasporin-2Aa1	Cry46Aa1	Ito & Kitada (43)	2004	AB099515	Bt A1547
Parasporin-2Ab1	Cry46Ab1	Yamagiwa *et al.* (44)	2004	AB186914	Bt TK-E6
Parasporin-3Aa1	Cry41Aa1	Yamashita *et al.* (45)	2005	AB116649	Bt A1462
Parasporin-3Ab1	Cry41Ab1	Yamashita *et al.* (45)	2005	AB116651	Bt A1462
parasporin-4Aa1	Cry45Aa1	Okumura & Saitoh (13)	2004	AB180980	Bt A1470

[1)]The table was reprinted from a web site of the committee of parasporin classification and nomenclature (14).
[2)]For reference, see a web site of the *Bacillus thuringiensis* δ-endotoxin nomenclature committee (7).
[3)]Authors registered in GenBank.
[4)]Year of registration in GenBank.

A1470 was attributed to a 28 kDa protein in a fraction separated by anion-exchange chromatography (38). However, the cytotoxic fraction contained 3 proteins with molecular masses of 26-28 kDa. These proteins were separated from each other by gel-filtration chromatography on Superdex 75 HR 10/30. It was confirmed that two of them exhibited cytopathic effect against MOLT-4 cells, and they were discriminated on the basis of their N-terminal and internal amino acid sequences (13).

The parasporin-4 gene (accession number **AB180980**), 828-bp long, encodes a polypeptide of 275 amino acid residues with a predicted molecular weight of 30,078. Neither typical promoter nor ribosome binding site was identified upstream of the gene. The cloned gene was expressed in recombinant *Escherichia coli*, in which the protein was accumulated as inclusion bodies. The recombinant protein also showed strong cytotoxicity to MOLT-4 and CACO-2 cells. The recombinant protein was proved to be identical with the native proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF MS). Its amino acid sequence was compared with those of other proteins in Swiss-Prot and PIR-Prot databanks. No significant sequence homology (<30%) was detected with known proteins including other insecticidal inclusion proteins of *B. thuringiensis*. Comparing the amino acid sequence with other parasporins, this protein was classified as parasporin-4, which belongs to a new primary rank of parasporin (13).

Another cytotoxic protein was discovered from the *B. thuringiensis* A1470 strain, and its N-terminal amino acid sequence corresponded to the sequence from Gln48 to Tyr57 of parasporin-2, a cytotoxic protein produced by the *B. thuringiensis* A1547 strain (43). The deoxyribonucleic acid (DNA) sequence of the protein of the A1470 strain was compared with that of parasporin-2. A 1,000-bp fragment of the A1470 strain was amplified by polymerase chain reaction (PCR). Sequence analysis verified that this amplified fragment is different from the parasporin-2 DNA by 8 bp, and the deduced amino acid sequence from this amplified fragment differed from parasporin-2 by 4 amino acid residues. The cytotoxic protein of the A1470 strain was shown to react with antiserum raised against parasporin-2 by Western blotting analysis. The two parasporins produced by the 1470 strain is still now an only strain that produces multiple parasporins belonging to different primary ranks.

Morphological changes in cultured cells by the recombinant parasporin-4 protein Figure 2 shows morphological changes in MOLT-4 cells induced by the administration of the proteinase K-treated recombinant parasporin-4 observed by a phase contract microscope. Parasporin-4 showed strong cytotoxicity in the early phase. Within 10 min after administration, nuclear condensation was occurred. The condensation was observed distinctly within 1 hr, and ballooned cells were appeared. The cells were burst within 24 hr to lead to the cell death. No morphological change was observed in resistant cells (13).

Cytotoxic activity of parasporin-4 against various mammalian cells Dose-response of various cultured mammalian cells to the proteinase K-treated recombinant parasporin-4 was monitored by MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-dipheny-2H tetrazolium bromide] assay. The 50% effective concentration (EC_{50}) values for parasporin-4 and other parasporins are shown in Table 2. Parasporin-4 showed high cytotoxicity to CACO-2, Sawano, MOLT-4, TCS, and HL60 cells, with EC_{50} values in the range of 0.1-0.8 μg/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. However, this result does not necessarily imply that parasporin-4 is not toxic to normal tissues, because it showed weak cytotoxicity to UtSMC and normal T cells at the concentration of 2 μg/mL (13).

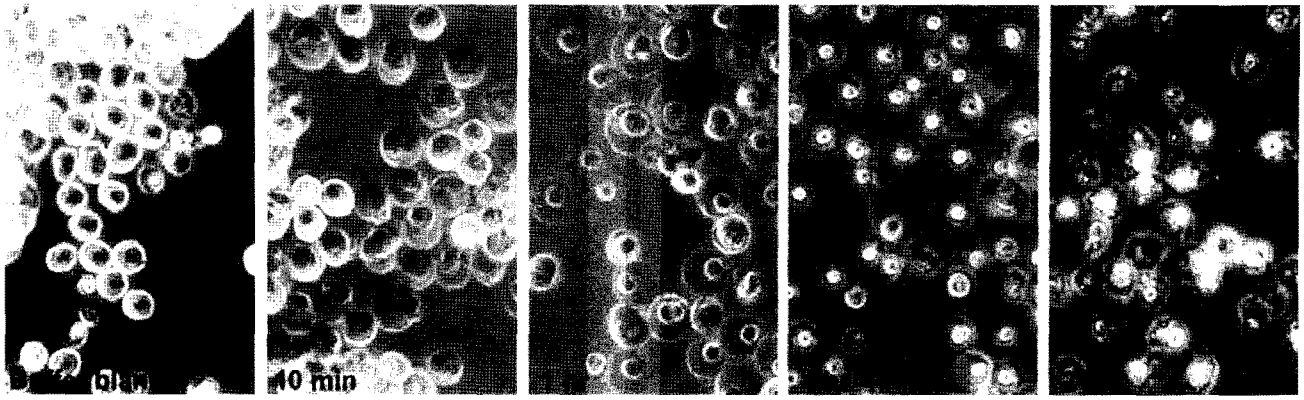

Fig. 2. Cytopathic effect of the recombinant parasporin-4 on MOLT-4 cells. The cells were incubated with the purified protein at a final concentration of 2 μg/mL for the indicated times and visualized by phase-contrast microscopy. Bar indicates 20 μm.

The 4 parasporins are different from each other in target spectrum against mammalian cells. This cytotoxicity specificity toward target cells suggests that the interaction of parasporin proteins with receptor-like proteins or lipids at the surface of the sensitive cells plays significant roles in the cytotoxicity. Thus, identification of the cell receptors for each parasporins might provide some insights into the mechanism of their target specificity and cytotoxicity. Parasporin-4 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 parasporin-4 to CACO-2 cells suggests that it may be toxic to normal colon cells. Therefore, it is needed to be confirmed whether parasporin-4 acts on normal colon cells or intestinal epithelial cells.

Other toxins from microorganisms that act on CACO-2 cells were previously reported: the CytK toxin of *Bacillus*

Table 2.The EC_{50} values of parasporins against various cultured mammalian cells

Name of cell lines	Origin	EC_{50} (μg/mL)[1]			
		PS1Aa1[2]	PS2Aa1[3]	PS3Aa1[4]	PS4Aa1[5]
Cells derived from normal human tissues					
T cell	Normal T cell	>10	0.15	>10	>2
UtSMC	Normal uterus	>10	9.3	>10	>2
HC	Normal hepatocyte	>10	>10	>10	>2
MRC-5	Normal embryonic lung fibroblast	>10	7.2	>10	>2
Cell lines derived from human hematologic malignancy					
MOLT-4	Leukemic T cell	2.2	0.044	>10	0.47
U-937 DE-4	Diffuse histiocytic lymphoma cell	ND[6]	ND	ND	0.98
Jurkat	Leukemic T cell	>10	0.015	>10	>2
HL60	Promyelocytic leukemia cell	0.32	0.066	1.3	0.73
K562	Myelogenous leukemia cell	ND	ND	ND	>2
Cell lines derived from other human neoplasm					
HeLa	Uterus cervix cancer	0.12	>10	>10	>2
TCS	Uterus cervix cancer	ND	>10	>10	0.72
Sawano	Uterus cancer	>10	0.041	>10	0.25
HepG2	Hepatocyte cancer	3.0	0.023	1.9	1.9
A549	Lung cancer	>10	>10	>10	>2
CACO-2	Colon cancer	>10	4.9	>10	0.12
Cell lines derived from mammalians beyond human					
Vero	Kidney cell, monkey	>10	ND	>10	>2
COS-7	Kidney cell, monkey	>10	ND	>10	>2
PC12	Pheochromocytoma, rat	ND	ND	ND	1.8
NIH 3T3	Embryo cell, NIH Swiss mouse	>10	ND	>10	>2
CHO	Ovary cell, chinese hamster	ND	ND	ND	>2

[1]Cell viabilities of intoxication were determined based on the metabolically active cells using MTT assay.
[2]-[5]Previously reported values (41, 38, 40, 12) are presented in this table; PS is abbreviation for parasporin.
[6]Not determined.

cereus which causes severe food poisoning (46), a thermostable hemolysin of *Vibrio parahaemolyticus* (47), and a vulnificolysin-like cytolysin of *Vibrio tubiashii* (48). Most of them are derived from virulent microorganisms, hence the studies of these toxins were aimed mainly to elucidate the mechanism of pathogenesis. Generally, *B. thuringiensis* is considered non-pathogenic (6). Recently, however, hemolytic (49) and non-hemolytic (GenBank accession no. **AB099298**) enterotoxins have been found in *B. thuringiensis* and *B. cereus* (50). It is noted that *B. thuringiensis* is indistinguishable from *B. cereus*, a common food pathogen, except for the production of parasporal inclusion proteins (51). Therefore, whether the *B. thuringiensis* A1470 strain is pathogenic or not would be an interesting research subject.

An Efficient Preparation Method for Parasporin-4

Digestive juice of insects gives alkaline pH in most of the cases (4), and thus the native parasporal inclusions are solubilized in an alkaline buffer or insect gut extracts *in vitro* (6). When *B. thuringiensis* insecticidal proteins were expressed in *E. coli*, inclusion bodies were formed. For bioassay of the insecticidal activity, the inclusion bodies were fed to susceptible insect larvae without dissolving. They are insoluble in acidic or neutral conditions (52), but when they are needed in a soluble form, they are solubilized in alkaline conditions. Inclusion bodies of *B. thuringiensis* parasporal proteins formed in *B. thuringiensis* naturally or in *E. coli* as recombinant proteins can be solubilized in alkaline solutions. Parasporin-4 is also produced in inclusion bodies from a recombinant *E. coli* strain (13). Initially, it was solubilized in alkaline buffer and processed by proteinase K. Although the processed protein was highly cytotoxic, their concentration was fairly low as only 20 μg/mL (16), and thus higher concentration has been required. We have improved the method and introduced an efficient method for solubilization and activation of recombinant parasporin-4 (16). We found that the inclusion bodies could be solubilized in a hydrochloric-acid solution, and they were activated by pepsin. The pepsin-treated parasporin-4 was purified in one-step purification by cation-exchange chromatography. Cytotoxic activities of the pepsin-treated and the proteinase K-treated parasporin-4 preparations were substantially the same. This enables us to prepare the purified parasporin-4 in higher yield in comparison with that prepared by solubilization in alkaline pH and proteinase K-treatment.

Solubility of the recombinant pro-parasporin-4 in various solutions Inclusion bodies of parasporin-4 produced by the recombinant *E. coli* cells were treated under various conditions, and the protein concentrations of the solutions were measured. In the Good buffers having pH ranging from pH 2 to 11, the solubility of the protein was shown to be high at alkaline and acidic pH regions, and low at neutral pH region. The solubility was examined in various concentration of hydrochloric acid. The solubility at 1 M hydrochloric acid was as low as that at neutral pH, but it was greatly improved at 100 and 10 mM hydrochloric acid. At 10 mM hydrochloric acid, the protein concentration of 11 mg/mL was obtained, indicating that the solubility increases up to 25 times higher than that in the alkaline buffer (16). The native parasporal inclusions of *B. thuringiensis* var. *san diego* were dissolved in the universal buffers at pH above 10 and below 4, whereas crystal forms of the inclusions were remained at pH between 5 and 9.5 (52). The pH-dependence of the solubility of the native inclusion protein was shown similar to that of the recombinant parasporin-4.

Cytotoxic activity of the recombinant parasporin-4 processed by pepsin or proteinase K The cytotoxic activity of the activated parsporin-4 samples prepared under alkaline or acidic conditions was examined. In the alkaline activation, the inclusion bodies were solubilized in 50 mM sodium carbonate buffer (pH 10.5) containing 1 mM ethylene diamine tetraacetic acid (EDTA) and 10 mM dithiothreitol, and then treated by proteinase K. In the acidic activation, they were solubilized in 10 mM hydrochloric acid, and then treated with pepsin. The EC_{50} values of parasporin-4 prepared in the alkaline activation and acidic activation were 0.37 and 0.33 μg/mL, respectively. The protein was also solubilized in 10 mM hydrochloric acid, and then the pH of the solution was adjusted to pH 9.4 with 50 mM sodium carbonate buffer, followed by the treatment with proteinase K. In this case, the EC_{50} value was determined to be 0.35 μg/mL. The cytotoxic activities of the parasporin-4 preparations obtained by 3 different activation processes were substantially the same. By SDS-PAGE, a band of parasporin-4 obtained by pepsin treatment was a little upper than that obtained by proteinase K treatment. Molecular masses of the parasporin-4 proteins obtained by treatment with pepsin and proteinase K were determined by MALDI-TOF MS to be 27,466 Da (16) and 26,808 Da (13), respectively. The 658 Da difference had no effect on the cytotoxic activity. The N-terminal amino acid sequences of these proteins were identical to that of Ala2-Ala11 of the parasporin-4 precursor (13). Based on the molecular mass difference, the cleavage sites of the parasporin-4 precursor protein were estimated to be the peptide bonds immediately after Glu252 by proteinase K and Arg246 by pepsin.

Purification of parasporin-4 from inclusion bodies in *E. coli* by the treatment with pepsin Recombinant parasporin-4 prepared in the acidic activation mentioned above was purified on a Resource S cation-exchange column (GE Healthcare UK Ltd., Chalfont, England). The parasporin-4 sample prepared in the acidic activation procedure was subjected to the column. Non cytotoxic proteins or peptide fragments formed by the pepsin treatment were eluted with increasing the sodium chloride concentration in the elution buffer (20 mM glycine buffer, pH 3.0). Then the elution buffer was changed to 10 mM 2-aminoethnol buffer (pH 10.8). The major peak appeared at 20 mL in elution volume after the buffer change solely exhibited cytotoxic activity. The activity of the purified protein was approximately 32 and 3.9 times higher than the solubilized solution and the activated sample prepared in the acidic activation procedure, respectively. The concentration of the purified protein was 540 μg/mL, which was 27-fold higher than that of the activated parasporin-4 by the method reported previously (16). Although the activated sample was judged to contain

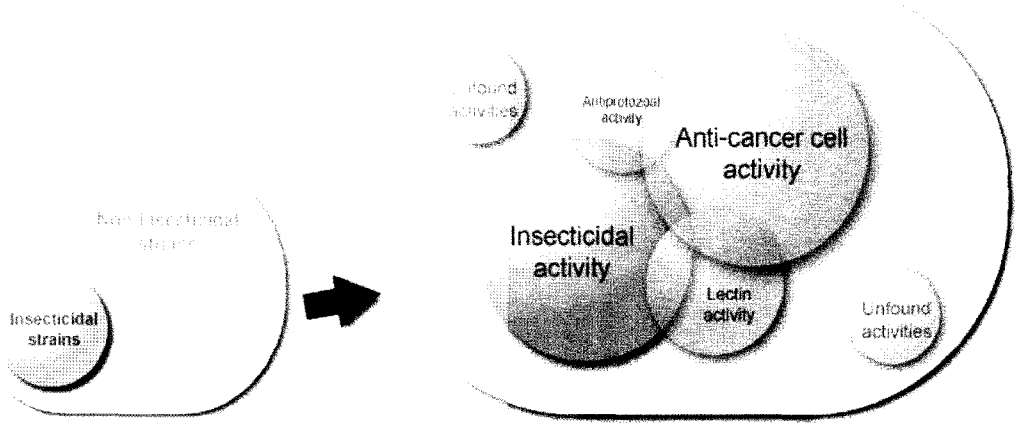

Fig. 3. A conceptual diagram of the *B. thuringiensis* crystal protein world. From the past to the present.

one band by SDS-PAGE, many peaks were detected by MALDI-TOF MS. Many intensive peaks were observed in the region up to 5,000 mass/charge, and they were considered to be short peptide fragments generated by activation. Most of them were removed by the cation-exchange chromatography, although some of them were not eliminated.

It is considered that inclusion bodies formed in periplasmic region of *E. coli* are resulted from misfolding or partial-folding of the foreign proteins expressed in the recombinant cells (53). The proteins are inactive (54), and thus a renaturation step is needed for their activation (55). On the other hand, *B. thuringiensis* insecticidal proteins are natural proteins expressed in native parasporal inclusion bodies by nature, and they show their activities without activation process (6). Interestingly, inclusion bodies of insecticidal proteins expressed in *E. coli* were also active in many cases, and the renaturation step is not needed (56, 57). In fact, the inclusion bodies of the recombinant parasporin-4 formed in *E. coli* keep its activity as the inclusion bodies formed in *B. thuringiensis* do.

Future Research

B. thuringiensis has attracted attention for its insecticidal activity. However, recently, new activities such as anti-cancer activity, anti-trichomonal activity (58), and lectin activity (59), were discovered in the crystal proteins of the *B. thuringiensis*. Presently, the crystal protein world is significantly changing from the insecticide to the assembled mass of new activities (Fig. 3).

B. thuringiensis is a spore-forming bacterium, so isolation and preservation of the strains are comparatively facile. They are widely distributed in nature, and have been isolated from various natural environments, especially from soil. About 10 years ago, it was estimated that almost 50,000 *B. thuringiensis* strains have been kept in various collections in the world (60). It might required to isolate and screen much more strains in step-by-step for new parasporins and activities. In screening for an insecticide, parasporal inclusions are fed to susceptible insect larvae directly for their bioassay. In screening for a parasporin, solubilization, and activation are essentially needed. Actually, all parasporins were discovered after the process of solubilization and activation under an alkaline condition. However, parasporin-4 keeps its cytotoxic activity even in the activation under an acidic condition. This finding shows a possibility that novel parasporins would be discovered by an activation procedure under an acidic condition. To date, 13 parasporins including parasporin-4 have been identified (Table 1). These parasporins differ in molecular weight, cell specificity, and efficient concentration for killing human cancer cells. It is very likely that each parasporin has a specific receptor molecule on susceptible cancer cells. Most parasporins have not yet been characterized at the molecular level, and future studies will attempt to identify their receptors and clarify the mode of action of the parasporins. The parasporins would be a good source of substances for developing new diagnostic or anti-cancer agents.

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