Identification and characterization of a novel cytotoxic protein, parasporin-4, produced by *Bacillus thuringiensis* A1470 strain

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Abstract. In 1901, a unique bacterium was isolated as a pathogen of the sotto disease of the silkmoth larvae, and later in 1915, the organism was described as *Bacillus thuringiensis*. Since the discovery, this bacterium has widely attracted attention of not only insect pathologists but many other scientists who are interested in strong and specific insecticidal activity associated with inclusion bodies of *B. thuringiensis*. This has led to the recent worldwide development of *B. thuringiensis*-based microbial insecticides and insect-resistant transgenic plants, as well as the epoch-making discovery of parasporin, a cancer cell-specific cytotoxin. In the review, we introduce a detection study of interaction between inclusion proteins of *B. thuringiensis* and brush border membrane of insects using surface plasmon resonance-based biosensor, and then identification and cloning of parasporin-4, a latest cancer cell-killing protein produced by *B. thuringiensis* A1470 strain. Inclusion bodies of the parasporin-4 produced by recombinant *Escherichia coli* were solubilized and activated with a new method and purified by an anion-exchange chromatography. At last the characterization of the recombinant parasporin-4 was shown.

Keywords: *Bacillus thuringiensis*, brush border membrane vesicles, Cry1Ac, Cry protein, cytotoxic activity, δ-endotoxin, inclusion body, parasporin, *Plutella xylostella*, surface plasmon resonance.

Abbreviations

BBM	brush border membrane
BSA	bovine serum albumin
DTT	dithiothreitol
EC_{50}	50% effective concentration
EDTA	ethylenediaminetetraacetic acid
GalNAc	*N*-acetyl-D-galactosamine
HPA	hydrophobic association
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide
PC14	1,3-ditetradecylglycero-2-phosphocholine
PCR	polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPR	surface plasmon resonance.

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General introduction

In 1901, a Japanese silkworm pathologist, Shigetane Ishiwata, isolated a unique bacterium as a pathogen of the sotto disease of the silkmoth larvae [1]. Later in 1915, the organism was described as *Bacillus thuringiensis* by E. Berliner [2]. *B. 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 that are toxic to insects. The toxic proteins in the parasporal inclusions are called δ-endotoxin, and the δ-endotoxin with hemolytic and non-hemolytic activity are called Cyt and Cry protein, respectively. The parasporal inclusions are ingested by insect larvae and the proteins are solubilized and converted into toxins by midgut proteases of susceptible insects [3]. They are highly and specifically toxic to insect pests of the Lepidoptera, Diptera, and Coleoptera [4], but are not pathogenic to mammals, birds, amphibians, or reptiles [5]. This makes *B. thuringiensis*, a promising microbial agent in the control of insect pests in agriculture, forestry, veterinary, and public health management [5]. Now the *B. thuringiensis* toxins are classified and designated by the *Bacillus thuringiensis* δ-endotoxin nomenclature committee (http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/) based solely on amino acid identity.

Meanwhile non-insecticidal *B. thuringiensis* strains are more widely distributed than insecticidal ones [6]. Mizuki *et al*. have previously reported that human cancer cell-killing activity is associated with some non-insecticidal *B. thuringiensis* isolates [7,8], and have created a new category of protein, parasporin, defined as bacterial parasporal proteins that are capable of preferentially killing cancer cells [8]. Parasporal inclusion of *B. thuringiensis* A1470 strain (Fig. 1) also exhibits strong cytotoxicity against human leukemic T-cells (Fig. 2) when activated by protease treatment, although it did not exhibit insecticidal or hemolytic activities [9], and we anticipated the strain produces multiple cytotoxic proteins with similar molecular masses [10]. In 2005, the cytotoxic protein of the strain was identified [11] and designated parasporin-4 by the committee of parasporin classification and nomenclature (http://parasporin.fitc.pref.fukuoka.jp/). And it was also designated Cry45Aa by the *Bacillus thuringiensis* δ-endotoxin nomenclature committee.

To date, including the parasporin-4, 13 parasporins have been identified from non-insecticidal *B. thuringiensis* strains (Table 1). 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. And 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 parasporins.

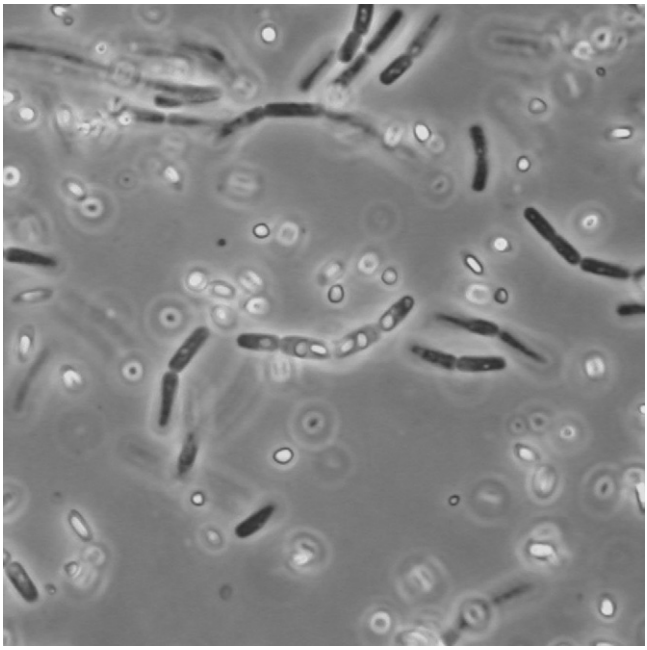

Fig. 1. *B. thuringiensis* A1470 strain.

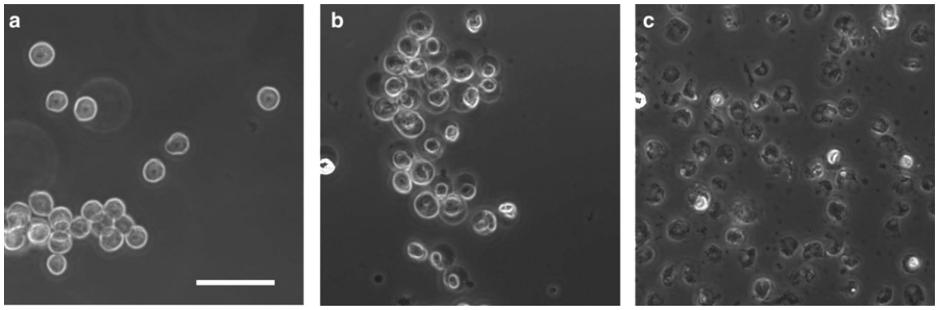

Fig. 2. Cytopathic effect of inclusion bodies produced by *B. thuringiensis* A1470 strain against MOLT-4 cells. The cells were incubated with the proteinase K-treated protein at 37°C for 0 h (A), 3 h (B), and 24 h (C) and visualized by phase-contrast microscopy. Bar indicates 50 μm.

Previously, the crystal proteins produced by *B. thuringiensis* strains have been attracting the attention only about their insecticidal activity. But in recent years, some new activities, anti-cancer cell activity, antitrichomonal activity [18], and lectin activity [19] were discovered in the crystal proteins of *B. thuringiensis* (Fig. 3). Especially, the anti-cancer cell activity should be

Table 1. The members of the parasporin family.

Name	Cry number[a]	Accession number	Authors[b]	Year[c]	Source strain
Parasporin-1Aa1	Cry31Aa1	AB031065	Mizuki *et al.* [8]	2000	A1190
Parasporin-1Aa2	Cry31Aa2	AY081052	Jung and Côté [12]	2002	M15
Parasporin-1Aa3	Cry31Aa3	AB250922	Uemori *et al.* [13]	2006	B195
Parasporin-1Aa4	Cry31Aa4	AB274826	Yasutake *et al.* [14]	2006	Bt 79–25
Parasporin-1Aa5	Cry31Aa5	AB274827	Yasutake *et al.* [14]	2006	Bt 92–10
Parasporin-1Ab1	Cry31Ab1	AB250923	Uemori *et al.* [13]	2006	B195
Parasporin-1Ab2	Cry31Ab2	AB274825	Yasutake *et al.* [14]	2006	Bt 31–5
Parasporin-1Ac1	Cry31Ac1	AB276125	Yasutake *et al.* [14]	2006	Bt 87–29
Parasporin-2Aa1	Cry46Aa1	AB099515	Ito and Kitada [15]	2004	A1547
Parasporin-2Ab1	Cry46Ab1	AB186914	Yamagiwa *et al.* [16]	2004	TK-E6
Parasporin-3Aa1	Cry41Aa1	AB116649	Yamashita *et al.* [17]	2005	A1462
Parasporin-3Ab1	Cry41Ab1	AB116651	Yamashita *et al.* [17]	2005	A1462
parasporin-4Aa1	Cry45Aa1	AB180980	Okumura and Saitoh [11]	2004	A1470

Source: The table was reprinted from a web site of the committee of parasporin classification and nomenclature (http://parasporin.fitc.pref.fukuoka.jp/).
[a]For reference, see a web site of the *Bacillus thuringiensis* δ-endotoxin nomenclature committee (http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/).
[b]Authors registered in GenBank.
[c]Year of registration in GenBank.

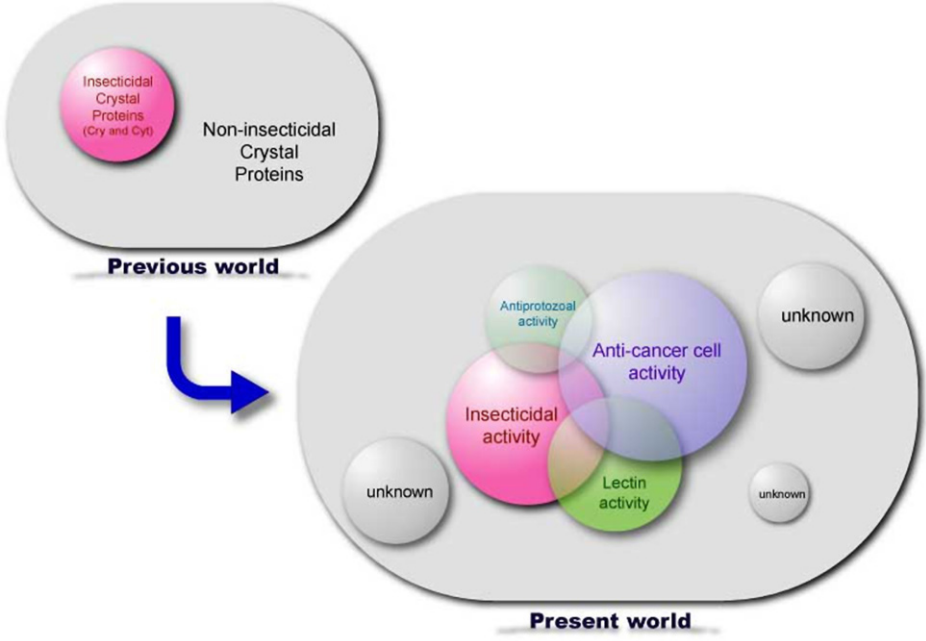

Fig. 3. A conceptual diagram of the present and previous *B. thuringiensis* crystal protein world. (The color version of this figure is hosted on Science Direct.)

highly potent, and more investigations are needed to apply parasporins to a cancer therapy or diagnosis.

In this overview, we first describe a novel screening method using the surface plasmon resonance (SPR)-based optical biosensor [20]. By the system, interactions between the inclusion proteins of *B. thuringiensis* and brush border membrane (BBM) vesicles of insects could be measured in real time as total mass changes. We then go on to show identification and cloning of the parasporin-4 [11] and an effective solubilization and purification method of the cytotoxic protein [21]. At last, we also show the characterization of the parasporin-4 [11,21]. The parasporin-4 revealed selective cytotoxic activities against various mammalian cell lines, but exhibited little or nothing against four normal tissue cells [11].

Interaction between a δ-endotoxin of *B. thuringiensis* and the artificial phospholipid monolayer incorporated with BBM vesicles of *Plutella xylostella* by SPR-based optical biosensor

B. thuringiensis is well-known as an insecticidal bacterium, however, non-insecticidal *B. thuringiensis* strains are more widely distributed than insecticidal ones [6]. Hence it is necessary to screen for insecticidal strains of *B. thuringiensis* against certain insect pests from various natural environments. Bioassay with target insects would be the primary method for screening of *B. thuringiensis* toxins. However, it is often difficult to apply the bioassay to the development of a new *B. thuringiensis* insecticide, because breeding techniques for many pests have not yet been established. Thus a novel screening method using an SPR-based optical biosensor was proposed [20]. 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 [22]. In this system, one of the reactants is immobilized to a monolayer of alkane thiol on 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. These changes are detected by a diode array. By linking this system to a personal computer, complex association and dissociation can be measured in real time.

Interactions between the toxins and high-affinity binding sites on the midgut epithelium of the susceptible insect species are a major determinant for the specificity of the insecticidal proteins. The importance of midgut target sites was shown in the *in vitro* binding studies using BBM of the insect midgut [23]. Recently the SPR technique has been applied to examine interactions of *B. thuringiensis* toxins with both insect midgut BBM vesicles [22,24] and a purified toxin receptor [25]. In the former case, the membrane vesicles were immobilized on the sensor chip using immobilized antibodies against either avidin or biotin. The binding of the toxin to a receptor protein embedded in a model membrane environment containing egg yoke

L-α-phosphatidylcholine was also reported [26]. The previous studies have demonstrated that multiple toxin-binding proteins exist in the midgut BBM of susceptible insect larvae, and the interaction between the toxin and the receptor is a complex process.

In this section, we introduce the SPR analysis of interactions between *B. thuringiensis* Cry1Ac δ-endotoxin and a BBM from its susceptible insect, diamondback moth, *Plutella xylostella* [20]. The monolayer of the BBM and an artificial phospholipid was spontaneously reconstructed on the hydrophobic surface of the sensor chip HPA. And the amount of the toxin binding to the BBM showed a saturation curve of the Michaelis-Menten type against toxin concentration. It is demonstrated that the monolayer newly introduced in this study provides us with an insect-free rapid and large-scale screening system of *B. thuringiensis* insecticide proteins by the aid of the SPR-based biosensor technology. And the reconstituted monolayer system will be also useful for screening of the parasporins.

Formation of the reconstructed monolayer

The BBM vesicle of diamondback moth (*P. xylostella*) was isolated from midguts of late-instar larvae by selective magnesium precipitation [27]. The isolated BBM vesicle was diluted with a neutral-charged artificial lipid, 1,3-ditetradecylglycero-2-phosphocholine (PC14, phase transition temperature = 25°C) [28] at a PC14/BBM protein ratio of 100:5 (w/w). The mixture was sonicated for 1 min three times and passed through polycarbonate membrane filter (pore size: 100 nm). And reconstruction of the monolayer was achieved on the hydrophobic association (HPA) chip using an SPR detector system, BIAcore 1000. The amount of the mixture bound to the chip showed the resonance response of (2,620 ± 75) RU. Followed by the binding of the mixture, bovine serum albumin (BSA) solution was injected to assess the extent of coverage of the surface. The resonance response of the BSA solution increased by (112 ± 6) RU, and was an adequate small amount to the coverage. When the BBM was loaded alone on the chip, it was almost washed out by the 4 mM NaOH injection of the regeneration step, and the final binding amount measured was only 200 RU. This suggested that the BBM was considerably unstable on the HPA chip. For the examination on the interaction of the activated Cry1Ac toxin to the BBM, the PC14/BBM monolayer reconstructed on the HPA chip was used as the sample chip.

When observed with an electron microscope stained with uranyl acetate, amphiphile PC14 formed well-developed vesicles with a diameter of 140–200 nm (Fig. 4C). In contrast, BBM from *P. xylostella* formed large irregular-shaped aggregates with various sizes (Fig. 4A). When the BBM was mixed with PC14, vesicles with a diameter of 80–130 nm were formed (Fig. 4B). The morphological observations revealed that the BBM were

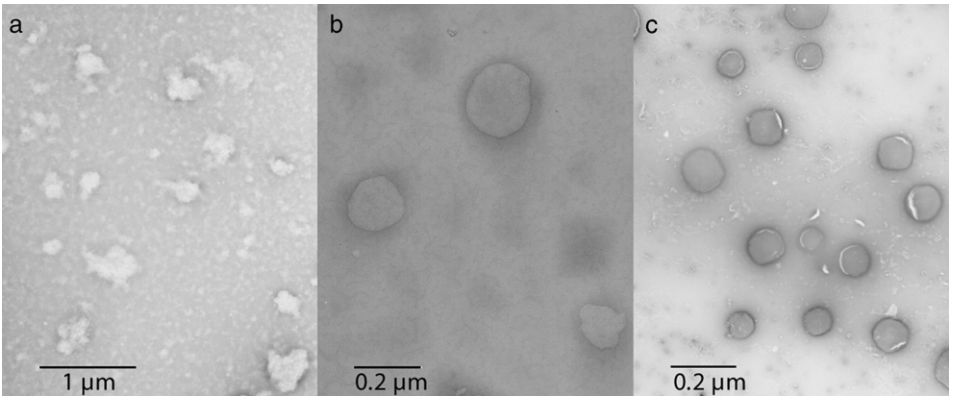

Fig. 4. Electron micrographs of the BBM vesicles. (A) BBM of the diamondback moth, (B) the mixture of PC14 and BBM, and (C) PC14. The samples were stained with uranyl acetate and subjected to electron microscopic examination. (Reprinted from reference [20].)

incorporated in the phospholipid PC14 vesicles. The ratio of [phospholipid]/[protein] in the BBM was approximately 1:1 (w/w) because of this low value of the phospholipid ratio against the BBM protein may account for the failure of BBM to form typical liposome vesicles. This may lead to instability of the BBM monolayer on the HPA chip.

Binding of the Cry1Ac toxin to the reconstructed monolayer

Cry1Ac is an insecticidal protein produced by *B. thuringiensis* strain HD-73, the type strain of serovar *kurstaki*, which has an activity against the diamondback moth larvae. Inclusion body of the strain was solubilized in 50 mM sodium carbonate buffer (pH 10.5) and activated with proteinase K. And the activated Cry1Ac was delivered to the reconstructed PC14/BBM monolayer to determine the levels required for ligand saturation. The relative resonance response increased with increasing the initial Cry1Ac toxin concentration ([toxin]), indicating that the amount of the toxin bound ([relative response]) to the reconstructed monolayer increased with an increase in [toxin]. The relative resonance response meaning the amount of the toxin binding showed a saturation curve of the Michaelis-Menten type against [toxin] (Fig. 5A). The double reciprocal plot ($[\text{toxin}]^{-1}$ vs. $[\text{relative response}]^{-1}$) showed a straight line, and the affinity constant of the toxin to the monolayer was evaluated to be 3.1 μM, and the maximum resonance response was 170 RU (Fig. 5B).

Subsequently, *N*-acetyl-D-galactosamine (GalNAc) was examined for its ability to reduce the binding of the Cry1Ac toxin to the reconstructed monolayer. GalNAc inhibited the toxin binding to the reconstructed

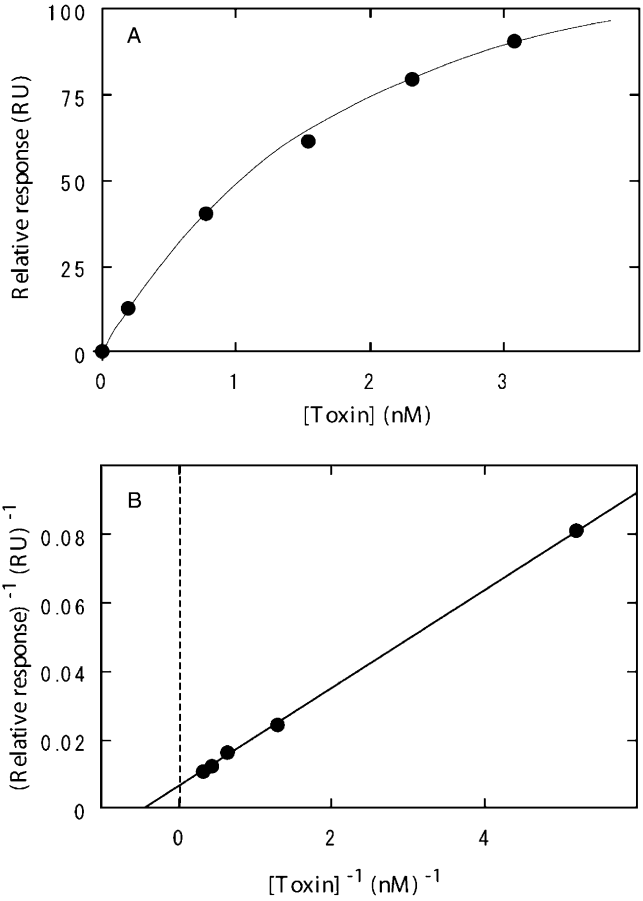

Fig. 5. Relationship between the Cry1Ac toxin concentration bound to the reconstituted monolayer and the initial concentration of the Cry1Ac toxin. (A) The Michaelis-Menten type relationship between the resonance responses at the saturated level obtained at 600 s and the initial toxin concentration, [toxin]. The relative resonance response is proportional to the concentration of the toxin bound onto the reconstituted monolayer, [relative response]. (B) The double reciprocal plot between [relative response] and [toxin]. The affinity constant between the toxin and the monolayer was evaluated to be 3.1 μM and the maximum value of the resonance response was 170 RU. (Reprinted from reference [20].)

monolayer. The inhibition was dose-dependent, and 89% of the toxin binding was inhibited at 80 mM GalNAc. The inhibitor constant of GalNAc against the monolayer was determined to be 8 mM. The results obtained in our present detecting system are in good agreement with the previous observations that the Cry1Ac toxin molecules bind to receptors on the lepidopteran epithelium cell membrane through GalNAc [25,26,29].

Analysis of the SPR data

It has been reported that Cry toxins generate pores in cell membrane by electrophysiological and biochemical studies [30]. The action of the toxin includes two steps; namely, the binding of the toxins to the receptors on the cell membrane, and the subsequent penetration of the toxins into the cells. Thus, the sensorgrams with the two-state reaction model was analyzed. Data for the binding of the Cry1Ac, corrected for bulk reflective index changes, fitted well to the model; the standard errors for kinetic constants calculated gave an overall χ^2 of 1.8 for the simultaneously fitting of five binding curves. The binding of the toxin occurred with an affinity constant (K_1) of 0.51 μM, followed by a second slower event with a constant (K_2) of 0.47. The latter was a uni-molecular event that is concentration-independent, 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.

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 [31–34]. This difference is apparently due to the difference in the methods used; most of the other studies have been done with the competitive binding assay using radiolabeled Cry1Ac toxin. In SPR experiments, an affinity constant (K_d) of 7 nM was reported previously with the Cry1Ac toxin and diamondback moth BBM vesicles [24], and this value is 34-fold smaller than that obtained in the introducing study. At present, the cause of this difference is uncertain. It should be noted, however, that the K_d value (0.24 μM) observed here is on the level of those (94.5–346 nM) reported by the previous workers in SPR studies with the Cry1Ac toxin and BBM vesicles of other lepidopterans, *Manduca Sexta*, and *Heliothis virescens* [25,26,29]. It was reported that when analyzed with a two-state reaction algorithm, the interaction between the Cry1Ac toxin and a lepidopteran receptor involved two steps, the second step having an affinity constant (K_2) of 2.79×10^{-2} [26]. This value is significantly lower than that obtained in the introducing study. The second step, the penetration of toxins into membranes, might be affected by the property of lipid. Thus, it is likely that the lower affinity observed in the introducing study might be caused by the use of an artificial lipid, PC14.

It should be noted that the receptor protein of the *B. thuringiensis* insecticidal proteins is considered to be aminopeptidase N and/or cadherin-like protein that are bound on the midgut-cell membrane. The amino-peptidase N is a metallopeptidase containing a zinc atom essential for the enzyme activity. Aminopeptidase N of mammalian cells is known to participate in the processing of peptide hormones, and recently it is noticed as an immunological cell marker, CD13. The structural homology between aminopeptidase N of insects and mammals were reported, but the physiological roles of the insect enzyme are not well revealed. The SPR-based

biosensor method proposed in the present study for investigating the interaction between the insecticidal proteins and the BBM monolayer could be applicable to examine the proteolytic properties of the 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 metalloproteinases, thermolysin [35,36], and matrix metalloproteinases [37,38].

Identification and cloning of two cytotoxic proteins produced by the *B. thuringiensis* A1470 strain

B. thuringiensis A1470 strain (Fig. 1) belongs to serovar *shandongiensis*. Lee *et al*. reported that the parasporal inclusion of the strain exhibits strong cytotoxicity against human leukemic T-cells when activated by protease treatment, however, it did not exhibit insecticidal or hemolytic activities [9]. The native inclusion before proteinase treatment consists of five major proteins with molecular masses of 160, 60, 34, 32, and 16 kDa [9]. And proteinase K-treated inclusion consists of three major proteins with molecular masses of 43, 30, and 28 kDa [39]. They were subjected to anion-exchange chromatography and cytotoxic activity of each fraction was determined. And then, a 28-kDa protein processed by the protease has been considered to be cytotoxic [39]. Thereafter, we demonstrated that the strain produces at least two kinds of cytotoxic proteins with similar molecular masses of 28 kDa against human cancer cells [10]. In this section, we introduce the identification of two cytotoxic proteins produced by the A1470 strain [11]. As a result of homology searching for databases, one of them was certificated as a novel cytotoxic protein, and it was designated as Cry45Aa by the *Bacillus thuringiensis* δ-endotoxin nomenclature committee, and parasporin-4 by the committee of parasporin classification and nomenclature.

Purification of two cytotoxic proteins of B. thuringiensis *A1470 strain*

B. thuringiensis A1470 strain was isolated from a soil sample collected in the city of Hino, Tokyo, Japan [9]. It had been 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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [39]. As a result of SDS-PAGE stained with Coomassie brilliant blue R250, a proteinase K-treated inclusion protein of the A1470 strain consisted of three major proteins with molecular masses of 40, 30, and 26 kDa. However, a slightly stained band was also observed at a little above the 26-kDa band. Then silver-staining clearly showed three distinct bands of 26, 27, and 28 kDa in that area (Fig. 6B,

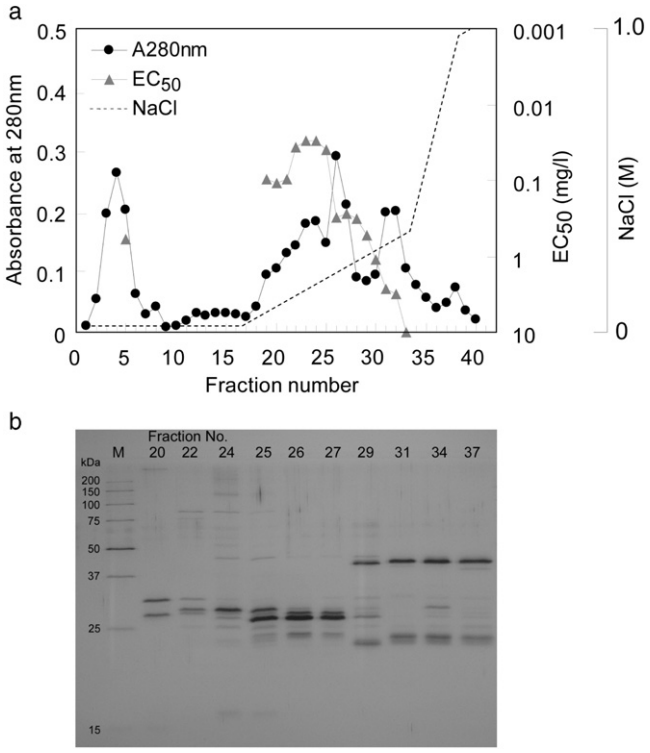

Fig. 6. (A) Anion-exchange chromatography of the proteinase K-treated parasporal inclusion proteins from the *B. thuringiensis* A1470 strain. (B) SDS-PAGE of proteins in the fractions of anion-exchange chromatography. Each lane was provided with 100 ng of proteins. Lane M indicates molecular size marker. Proteins were stained with silvers.

fraction No. 24–26). The fraction 24 contained 26 kDa band mainly, fraction 25 contained 26 kDa and 28 kDa bands, and fraction 26 contains 27 kDa and 28 kDa bands. The 27- and 28-kDa proteins in the fraction 26 were separated from each other by gel-filtration chromatography on Superdex 75 HR 10/30, and the 27-kDa protein only revealed the cytopathic effect against MOLT-4 cells. In the next place, the fraction 24, which mainly contained 26 kDa protein, was applied to gel-filtration chromatography. The 26-kDa protein was isolated and it exhibited cytotoxicity against both MOLT-4 and Jurkat cells. After this manner, two cytotoxic proteins have been confirmed.

Identification of a novel cytotoxic protein, parasporin-4

At first, identification of the 27-kDa cytotoxic protein was attempted. Using N-terminal and internal amino acid sequences, the gene encoding the

protein was obtained from the strain. The gene cloned was 828 bp long, encoding a polypeptide of 275 amino acid residues with a predicted molecular weight of 30,078, and the nucleotide sequence obtained in the study has been deposited in the GenBank database (accession number AB180980). Neither typical promoter nor ribosome binding site was identified upstream. The inverted repeat sequence, presumably capable of forming a stem-loop structure ($\Delta G = -67.8$ kJ/mol), was identified 136 bp downstream from the stop codon. This structure may act as a transcriptional terminator. When deduced from the gene sequence, amino acid sequence from the position 3 to 11 of the N terminus of the peptide was identical to that (IINLANELA) of the protein. The internal sequence (ADIAGH) of the protein was found in the deduced peptide sequence at positions 173 through 179.

The cloned gene was expressed in recombinant *E. coli*, in which the protein was accumulated as an inclusion body. The recombinant protein showed strong cytotoxicity to MOLT-4 and CACO-2 cells at a final concentration of 2 μg/ml. The 27-kDa protein and the recombinant protein could be judged to be the same substance by the method of SDS-PAGE, Western blotting, and MALDI-TOF MS spectrometry.

The amino acid sequence of the protein was compared with those of the other known proteins in Swiss-Prot and PIR-Prot databanks. No significant homologies (<30%) were observed on the sequence between the protein and the existing proteins including *B. thuringiensis* Cry and Cyt proteins. Thus the protein was designated as Cry45Aa by the *Bacillus thuringiensis* δ-endotoxin nomenclature committee, and parasporin-4 by the committee of parasporin classification and nomenclature.

Proteolytic processing of the parasporin-4

The gene for full-length pro-parasporin-4, which is the precursor of the activated protein, encodes a polypeptide of 275 amino acid residues with a predicted molecular mass of 30,078. The pro-parasporin-4 produced by the recombinant *E. coli* had no apparent cytocidal activity without proteinase K-treatment, and the native parasporal inclusions of *B. thuringiensis* A1470 also only exhibited cytotoxic activity when activated by protease treatment [9]. Thus, proteolytic processing was essential for activation of the cytotoxic protein. MALDI-TOF MS analysis of the proteinase K-treated recombinant parasporin-4 estimated the molecular mass as 26,808. The N-terminal amino acid sequence of the protein was AIINLANELA and was completely identical to Ala2–Ala11 of the pro-parasporin-4. It is likely that the proteinase K-treated parasporin-4 corresponds to amino acid residues Ala2 to Arg246 of the precursor, as a predicted molecular mass of such a protein 26,828 is close to that measured for proteinase K-treated parasporin-4 26,808.

Cry46Aa-like toxin of A1470 strain

And then, identification of the cytotoxic 26-kDa protein was attempted. Its N-terminal amino acid sequence was determined to be QSTTDVIREY. This sequence perfectly corresponded to the sequence from Gln48 to Tyr57 of cytotoxic protein from the *B. thuringiensis* A1547 strain [15], which has been designated as parasporin-2. To compare the 26-kDa protein of the A1470 strain with the parasporin-2, a polymerase chain reaction (PCR) was performed from total plasmids of A1470 as the template with the primers to clone the precursor of the parasporin-2. A 1,000-bp fragment was amplified by PCR. Sequence analysis verified that this amplified DNA fragment differed from the parasporin-2 by 8 bp and the deduced amino acid sequence from this amplified fragment also differed by 4 amino acid residues. As just described, the 26-kDa protein of A1470 strain and the parasporin-2 were almost identical. It was also confirmed that the 26-kDa protein of the A1470 reacted with antiserum against the parasporin-2 by Western blotting analysis.

Efficient solubilization, activation, and purification of recombinant parasporin-4 of the *B. thuringiensis* A1470 strain expressed as inclusion bodies in *E. coli*

Parasporin-4 is one of the cytotoxic proteins produced by *B. thuringiensis* A1470 strain. A gene coding for the pro-parasporin-4 was expressed in recombinant *E. coli*, in which the protein was accumulated as an inclusion body. It was solubilized in 50 mM sodium carbonate buffer, pH 10.5, containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM dithiothreitol (DTT), and the 30-kDa precursor protein was degraded to approximately 27 kDa by proteinase K-treatment. The processed protein exhibited high cytotoxic activity against MOLT-4 and CACO-2 cells. However, the protein concentration of the processed parasporin-4 by the preparing method above was up to 20 μg/ml [21]. Thus, higher concentration has been required to improve the study of the protein.

There were no reports except for a report of Koller *et al*. [40] demonstrating that the native inclusion bodies of *B. thuringiensis* were solubilized in an acidic or a neutral solution. Digestive juice of most insects is under an alkaline condition [3], and thus the native parasporal inclusions are generally solubilized in an alkaline buffer or insect gut extracts *in vitro* [5]. Additionally, inclusion bodies of recombinant *B. thuringiensis* insecticidal proteins in *E. coli* were also solubilized in alkaline conditions. Generally, high-level expression of recombinant proteins in *E. coli* often results in the formation of insoluble and inactive aggregate known as inclusion bodies [37]. They occur by deposition of misfolding or partially folded polypeptides through the expression of hydrophobic patches and the consequent intermolecular interactions [41]. Therefore, the purified inclusion bodies

must be solubilized by strong denaturants such as 6 M guanidine hydrochloride or 8 M urea, which promote the disruption of intermolecular interactions and the complete unfolding of the protein [42]. And, consequently, the solution is removed excess denaturants by dilution or a buffer exchange step [43] due to renaturation of denatured protein. Insecticidal proteins of *B. thuringiensis* were also expressed in *E. coli*, and often resulted in formation of inclusion bodies. They were usually fed to susceptible insect larvae directly for their bioassay and not necessary to be solubilized in this case. However, they were also solubilized in alkaline conditions if need arose, and they often revealed their own activity without denaturation and renaturation steps [44,45]. As just described, in most studies regarding *B. thuringiensis* the inclusion bodies of either native or in *E. coli* have been solubilized in alkaline solutions.

In this section, we introduce an efficient solubilization and activation method of the recombinant pro-parasporin-4 [21]. The inclusion body of the protein was solubilized in a hydrochloric acid solution and 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 were substantially equal. This enables us to prepare the purified parasporin-4 in higher yield in comparison with that by the solubilization in alkaline pH and proteinase K-treatment.

Solubility of the recombinant pro-parasporin-4 in various solutions

Inclusion bodies of the pro-parasporin-4 produced by the recombinant *E. coli* BL21 (DE3) cells were mixed with each buffer solution and incubated at room temperature for 30 min. Figure 7 shows protein concentrations of the saturated solution of the pro-parasporin-4 in various buffer solutions. Solubility of the protein tended to be higher in both alkaline and acidic regions, and lower in the neutral pH region. In 50 mM sodium carbonate buffer (pH 10.5) containing 10 mM DTT and 1 mM EDTA (designated as buffer A), which has been used in many previous study to dissolve the inclusion bodies of the *B. thuringiensis*, the protein was solubilized at the highest concentration in the buffers examined except for 10 mM and 100 mM HCl. The solubility was approximately half when DTT was removed from the buffer A.

A solubility of the protein in HCl was determined in a broad range depending on HCl concentration. In 1 M HCl, the solubility was as low as that in the buffers at neutral pH. On the other hand, both in 100 mM and 10 mM HCl, the solubility was greatly improved. Especially, in 10 mM HCl, the solubility was approximately 10.8 mg/ml, being 25 times higher than that in the buffer A.

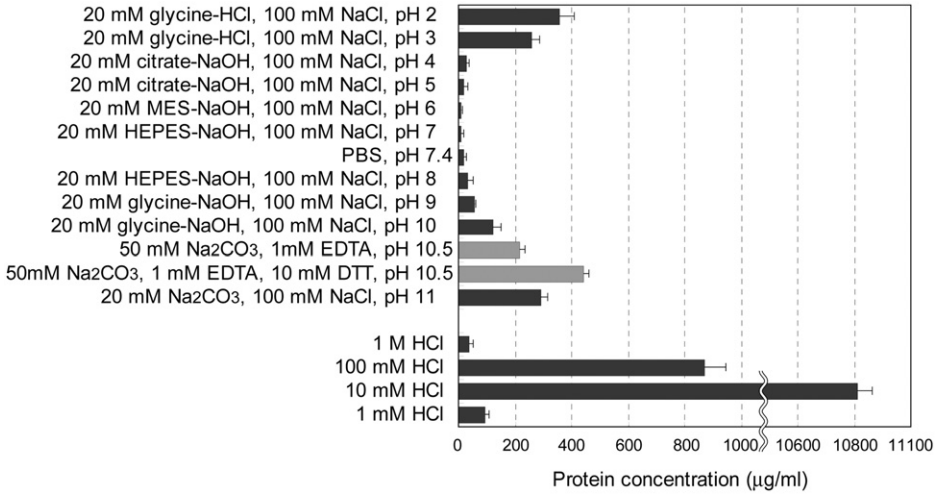

Fig. 7. Solubility of the recombinant pro-parasporin-4 in various buffer solutions. Purified inclusion bodies of the pro-parasporin-4 expressed in *E. coli* were saturated in each buffer. The protein concentration of the solution was determined by the BCA protein assay method except for the solution containing with dithiothreitol, which was determined by the method of Bradford. Shaded bars represent the solubility in the buffers generally used in the studies to dissolve inclusion bodies of the *B. thuringiensis*. (Reprinted from reference [21].)

Koller *et al.* previously reported about the effect of pH on the solubilization of the native parasporal inclusion protein of *B. thuringiensis* var. *san diego* [40]. The native crystals from the strain was dissolved well in the universal buffers at above pH 10 and below pH 4, but it was almost remained in crystal form between pH 5 and pH 9.5. The pH-dependence of the solubility profile of the native inclusion protein of the strain was similar to that of the recombinant pro-parasporin-4 in *E. coli.*

Cytotoxic activity of the recombinant parasporin-4 processed by pepsin or proteinase K

Activated parsporin-4 was prepared in an alkaline or an acidic condition, and their activities were compared. In alkaline condition, the inclusion bodies were solubilized in 50 mM sodium carbonate buffer, pH 10.5, containing 1 mM EDTA and 10 mM DTT, and solubilized pro-parasporin-4 was treated by proteinase K at a final concentration of 50 μg/ml for 90 min. In an acidic condition, they were solubilized in 10 mM HCl, and were treated with pepsin 1:10,000 (Wako Pure Chemicals) at a final concentration of 200 μg/ml. Figure 8A shows dose-response curve against CACO-2 cells of the parasporin-4 activated by proteinase K (closed circles) and by pepsin (open circles). The cytotoxic activities of the samples were measured by the MTT

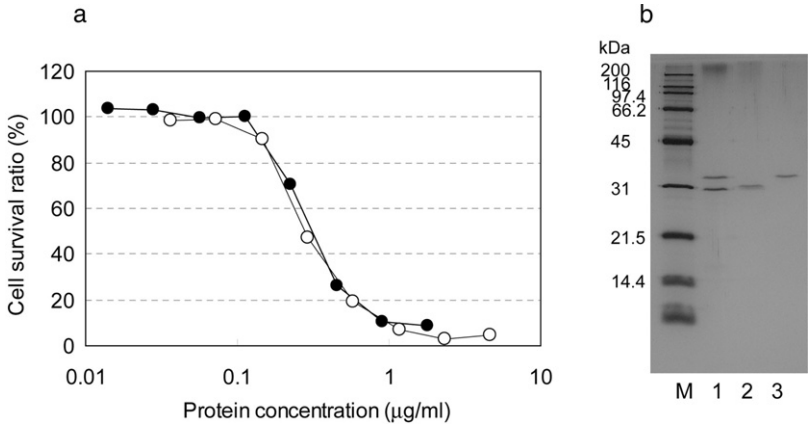

Fig. 8. Cytotoxic activities and SDS-PAGE profiles of the recombinant parasporin-4 processed by pepsin or proteinase K. Inclusion bodies of parasporin-4 were solubilized in sodium carbonate buffer (pH 10.5) and activated by proteinase K (A: closed circles, B: lane 1), or they were solubilized in 10 mM HCl and activated by pepsin (A: open circles, B: lane 2). Cell survival rates against CACO-2 cells of both the processed samples were determined by MTT assay (A). The samples were subjected to SDS-PAGE and stained with silver (B). 150 ng was applied to lanes 1 and 2. Lane 3 indicates blank sample of lane 1 containing proteinase K at an identical dilution ratio with lane 1. Blank of lane 2 is not shown because pepsin was not analyzed by the SDS-PAGE. Lane M indicates molecular size marker. Protein concentration of the solution was determined by the method of Bradford. (Reprinted from reference [21].)

[3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide] method [46,47], and 50% effective concentration (EC_{50}) for cytotoxicity was decided by probit analysis [48]. The EC_{50} values obtained at 20 h after the administration of the parasporin-4 activated by proteinase K and by pepsin were 0.369 and 0.331 μg/ml, respectively. Both cytotoxic activities were substantially equal. The cytotoxic activity of the protein solubilized in 10 mM HCl and then activated by proteinase K was also determined. In the case, the solution was adjusted to pH 9.4 with 50 mM sodium carbonate before activation, because proteinase K does not activate at acidic pH. The EC_{50} value of the protein was 0.351 μg/ml, which was almost identical to those of two proteins aforementioned.

Figure 8B shows SDS-PAGE profiles of the proteinase K-treated parasporin-4 and the pepsin-treated one. The pepsin-treated protein included only one major band because pepsin was not analyzed by the SDS-PAGE. The bands of the two kind of the processed parasporin-4 located in approximately 27 kDa together, but the band of the pepsin-treated protein was a little upper than that of the proteinase K-treated one. Molecular masses of the pepsin-treated and the proteinase K-treated parasporin-4 were determined by MALDI-TOF mass spectrometer as 27,466 (Fig. 9B) and

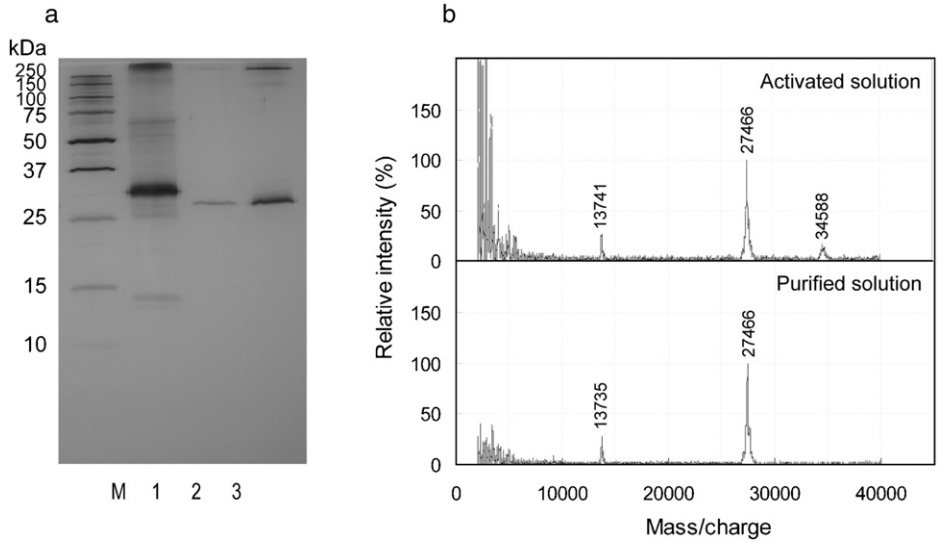

Fig. 9. Protein profiles of samples in each step of purification of the pepsin-treated parasporin-4. (A) Samples were subjected to SDS-PAGE and stained with silver. 150 ng was applied to all lanes. Lane 1, the inclusion bodies of pro-parasporin-4 in *E. coli* solubilized in 10 mM HCl; Lane 2, the proteins which was solubilized in 10 mM HCl and processed with pepsin (activated solution); Lane 3, the pepsin-treated parasporin-4 purified by cation-exchange chromatography (purified solution); Lane M, molecular size marker proteins. (B) Results of MALDI-TOF MS analysis of the activated and the purified solutions. Relative intensities to the peak of parasporin-4 (27,466) were represented. (Reprinted from reference [21].)

26,808 [11], respectively. However, the difference of 658 between molecular masses of these proteins had no effect on their cytotoxic activities against CACO-2 cells. The N-terminal amino acid sequence of the pepsin-treated protein was AIINLANELA. It was completely identical to that of the proteinase K-treated protein [11]. They are also identical to Ala2–Ala11 of the pro-parasporin-4 [11]. It is likely that the pepsin-treated parasporin-4 corresponds to amino acid residues Ala2 to Glu252 of the precursor, as a predicted molecular mass of such a protein 27,469 is close to that measured for the protein 27,466, which was 6 amino acids residues longer than the proteinase K-treated protein.

Purification of the pepsin-treated parasporin-4 from inclusion bodies in E. coli

Then the recombinant parasporin-4 activated in an acidic condition mentioned above was purified by Resource S cation-exchange column (size: 6.4 mm inner diameter × 30 mm length: GE Healthcare). The activated parasporin-4 was subjected to the column, and non-cytotoxic proteins or peptide fragments by

pepsin-treatment were eluted with an increasing gradient of NaCl. Then the running buffer was changed from 20 mM glycine buffer (pH 3.0) to 10 mM 2-aminoethnol buffer (pH 10.8) and the fraction of the major peak appeared at 20 ml in elution volume after the buffer change only revealed cytotoxic activity. The purification procedure was summarized in Table 2. Relative activity of the purified protein was approximately 32 and 3.9-fold higher than the solubilized solution and the activated solution, respectively. The concentration of the purified protein was 539 μg/ml (Table 2), which was 27-fold higher than that of the activated parasporin-4 by the previous method.

Figure 9A shows result of SDS-PAGE of the samples of each purification step. Although the activated solution (lane 2) seemed to include one band in SDS-PAGE, many peaks were included by the analysis using MALDI-TOF MS (Fig. 9B, upper stand), and the highest relative intensity attained 1,500% to a 27,466 peak of the pepsin-treated parasporin-4. It was considered that the many intensive peaks in the region up to 5,000 mass/charge would be short peptide fragments generated by activation. Although most of them were removed by the step of cation-exchange chromatography, they could not be eliminated entirely from the sample.

A 34,588 peak of MALDI-TOF MS analysis in Fig. 9B seemed to be pepsin. The pepsin cannot be sufficiently analyzed by SDS-PAGE, so the band of the pepsin was not observed in the activation sample (Fig. 9A, lane 2). Most of the pepsin was considered to be removed by the cation-exchange chromatography step according to the result of MALDI-TOF MS analysis, although the analysis is not a quantitative one by nature. Furthermore, by the analysis the molecular mass of the pepsin-treated parasporin-4 was 27,466 (Fig. 9B). The peak of approximately 13,740 also seemed to be the parasporin-4 which is ionized bivalent. The SDS-PAGE analysis of the purified solution showed two obvious bands at 27 kDa and above 250 kDa (Fig. 9A, lane 3). The band above 250 kDa would be an aggregate of the pepsin-treated parasporin-4, because it reacted with rabbit antiserum against parasporin-4 by Western blotting. The aggregate was not observed by gel-filtration chromatography, therefore, it was considered to be produced during the procedure of the SDS-PAGE analysis.

The solubilized native parasporal inclusions from *B. thuringiensis* A1470 without proteolytic process was non-cytotoxic [9], however, the pro-parasporin-4 showed weak activity ($EC_{50} = 17.7$ μg/ml) against CACO-2 cells in the study (Table 2, solubilized solution). It seemed to be because of a higher concentration of the pro-parasporin-4 by an effective solubilization and processing by the endogenous proteinase.

The EC_{50} value of the pepsin-treated parasporin-4 against CACO-2 cells was 0.555 μg/ml (Table 2) by the BCA protein assay method [49]. On the other hand, that of the proteinase K-treated protein was reported to be 0.124 μg/ml [11] by the method of Bradford [50]. Although the EC_{50} values of

Table 2. Purification of the pepsin-treated recombinant parasporin-4 of *B. thuringiensis* expressed in *E. coli*.

Purification step	Volume (ml)	Protein concentration (μg/ml)	Protein (mg)	EC_{50} (μg/ml)	Relative activity (fold)	Recovery (%)
Solubilized solution	10.0	1180	11.8	17.7	1.00	100
Activated solution	10.2	920	9.02	2.15	8.22	76
Cation-exchange chromatography	3.5	539	1.89	0.555	31.9	16

Source: Reprinted from reference [21].

Note: The protein was obtained from 40 mg (wet weight) of purified inclusion body in *E. coli*, and solubilized in 10 mM HCl at 37°C for 1 h (solubilized solution) and processed with pepsin 1:10,000 (Wako Pure Chemicals) at a final concentration of 200 μg/ml for 90 min at 37°C (activated solution). The solution was subjected to cation-exchange chromatography on a Resource S column (GE Healthcare), and then the eluted parasporin-4 was collected (cation-exchange chromatography). Protein concentration was measured by the BCA protein assay method with BSA as the standard. The cytotoxic activity of each sample against CACO-2 cells was determined by the MTT method, and EC_{50} values were decided by probit analysis.

the parasporin-4 were different depending on the protease-activation method, the cytotoxic activities of both the pepsin-treated and the proteinase K-treated parasporin-4 were considered substantially equal under the same experimental conditions (Fig. 8).

Generally, it is considered that inclusion bodies in *E. coli* are the results from misfolding or partially folding through the expression [41], thus they are inactive [51] and must need a renaturation step [43]. On the other hand, although *B. thuringiensis* insecticidal proteins are expressed in native parasporal inclusion bodies by nature, they keep their activities [5]. In many cases, inclusion bodies of insecticidal proteins expressed in *E. coli* were also active without a renaturation step [44,45]. The inclusion body of recombinant parasporin-4 in *E. coli* also keeps its activity as they are.

Characterization of a novel cytotoxic protein, parasporin-4, produced by the *B. thuringiensis* A1470 strain

At present, 13 parasporins are reported, but the number of primary rank is only 4 (Table 1), and the parasporin-4 is the latest primary rank of all parasporins. In the nomenclature scheme of the parasporin, the same system of numbers and letters for Cry proteins of *B. thuringiensis* [52] is adopted so that a novel parasporin protein is assigned to a new class incorporating four ranks such as parasporin1Aa1. Currently, approximately 95%, 78%, and 45% sequence identities are the borders of the four ranks. Each parasporin has a specific and distinct target spectrum against mammalian cells. And selective cytotoxic activity of the parasporin-4 was also determined [11]. In this section, we introduce the characterization of the parasporin-4 excerpt from two studies, references [11] and [21].

Morphological changes in cultured cells by the recombinant parasporin-4 protein

Morphological changes in two cultured cells, MOLT-4 and CACO-2, induced by the addition of the proteinase K-treated recombinant parasporin-4 were observed by a phase contract microscope. The parasporin-4 showed strong cytotoxicity in the early phase to both cells. Within 10 min after administration of the cytotoxic protein, nuclear condensation started to occur, and appeared distinctly within 1 h. Ballooned cells appeared 2 h after exposure to the cytotoxic protein, and burst within 24 h to lead to the cell death. No morphological change was observed in resistant cells [11].

Cytotoxic activity of parasporin-4 against various mammalian cells

The dose-response of various cultured mammalian cells to the proteinase K-treated recombinant parasporin-4 protein was monitored by MTT assay,

Table 3. Cytotoxic activity of the recombinant parasporin-4 against various cultured mammalian cells. EC_{50} values (at 20 h) were calculated by probit analysis.

Name	Origin	Medium	EC_{50} (μg/ml)
Cell lines derived from normal human tissues			
T cell	Normal T cell	RPMI1640 + 10% FBS	>2
UtSMC	Normal uterus	Sm-BM + 5% FBS	>2
HC	Normal hepatocyte	CS-C + 10% FBS	>2
MRC-5	Normal embryonic lung fibroblast	MEM + 10% FBS	>2
Cell lines derived from human neoplasm			
MOLT-4	Leukemic T cell	RPMI1640 + 10% FBS	0.472
U-937 DE-4	Lymphoma cell	RPMI1640 + 10% FBS	0.980
Jurkat	Leukemic T cell	RPMI1640 + 10% FBS	>2
HL60	Promyelocytic leukemia cell	RPMI1640 + 10% FBS	0.725
K562	Myelogenous leukemia cell	RPMI1640 + 10% FBS	>2
HeLa	Uterus cervix cancer	MEM + 10% FBS	>2
TCS	Uterus cervix cancer	MEM + 10% FBS	0.719
Sawano	Uterus cancer	MEM + 15% FBS	0.245
HepG2	Hepatocyte cancer	DMEM + 10% FBS	1.90
A549	Lung cancer	DMEM + 10% FBS	>2
CACO-2	Colon cancer	MEM + 20% FBS + 1% NEAA	0.124
Cell lines derived from mammalians beyond human			
Vero	Kidney cell, monkey	MEM + 10% FBS	>2
COS-7	Kidney cell, monkey	DMEM + 10% FBS	>2
PC12	Pheochromocytoma, rat	DMEM + 10% FBS + 10% HS	1.78
NIH 3T3	Embryo cell, NIH Swiss mouse	DMEM + 10% FBS	>2
CHO	Ovary cell, Chinese hamster	DMEM + 10% FBS	>2

and the EC_{50} values obtained 20 h after administration are shown in Table 3. Cytotoxicity varied greatly amongst 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 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. 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 the parasporin-4.

The EC_{50} values in all of the normal cells (HC, UtSMC, MRC-5, and normal T-cells) were $>2\,\mu g/ml$ (Table 3). However, this result does not imply that the parasporin-4 is not cytotoxic to normal tissues. For example, $2\,\mu g/ml$ parasporin-4 was weakly cytotoxic to UtSMC and normal T-cells [11].

The 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 the parasporin-4 to CACO-2 cells may imply that it is toxic to normal colon cells. Therefore, it needs to be confirmed that the parasporin-4 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 [53], a thermostable hemolysin of *Vibrio parahaemolyticus* [54], and a vulnificolysin-like cytolysin of *Vibrio tubiashii* [55]. 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 [5]. However, different enterotoxins, including hemolytic [56] and non-hemolytic (GenBank accession no. AB099298) enterotoxins, have been found in *B. thuringiensis* or *B. cereus*. Additionally, *B. thuringiensis* is indistinguishable from *B. cereus*, a common food pathogen, except for the production of parasporal inclusion proteins [57]. Therefore, whether *B. thuringiensis* A1470 strain is pathogenic or not would be a subject of an interesting study.

Stabilities and physical properties of the pepsin-treated parasporin-4

The $200\,\mu g/ml$ solution of the purified parasporin-4 with distilled water was prepared from the lyophilized protein powder. The solution was diluted into the same buffers used in the solubility study (Fig. 7). Figure 10A shows relative EC_{50} values of the pepsin-treated parasporin-4 in the buffers at various pH after 3 days at 4°C. The cytotoxic activity of the protein was decreased a little by preservation in the buffer of pH 2, 7.4, and 8. However, significant inactivation was not observed in the buffers examined. The activity of the lyophilized protein solubilized in 20 mM citrate · NaOH buffer (pH 4.0) and 20 mM glycine · NaOH buffer (pH 9.0) contained with 100 mM NaCl were determined over the long term (Fig. 10B). Each half-life of the activity was calculated by each regression line to be 365 and 152 days, respectively. The thermal stability of the protein from 30°C to 60°C was examined (Fig. 10C). The protein kept 97% of its activity at 30°C for 360 min. On the other hand, it was fully inactivated at 50°C for 150 min or 60°C for 10 min.

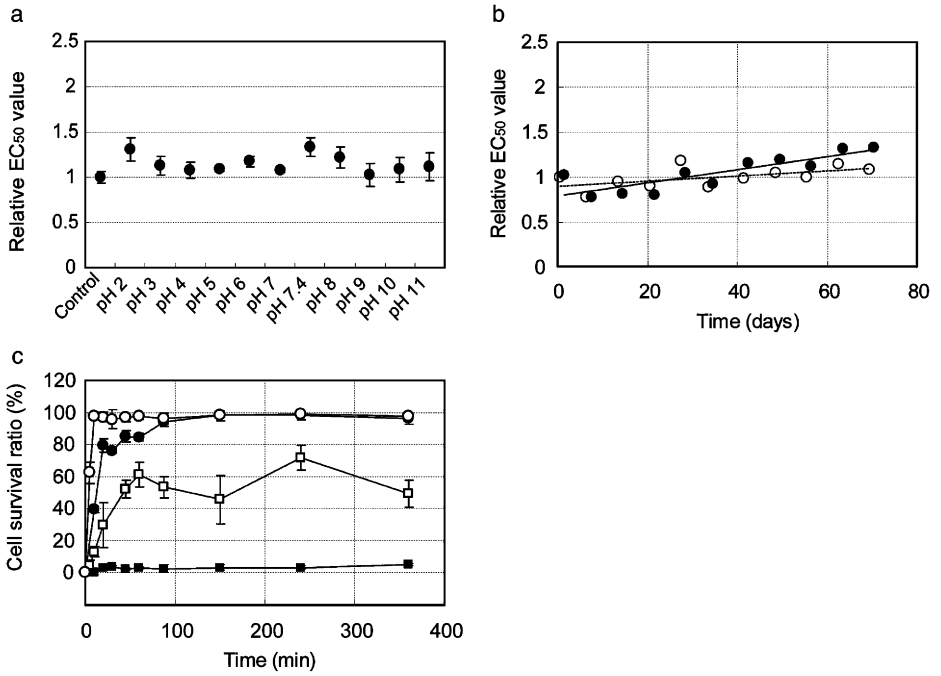

Fig. 10. pH and thermal stabilities of the pepsin-treated parasporin-4. (A) The lyophilized pepsin-treated parasporin-4 was dissolved in buffers prepared, and preserved for 3 days at 4°C. Then the cytotoxic activities of samples against CACO-2 cells were determined. Relative EC_{50} values to the lyophilized protein dissolved in distilled water were represented. (B) Cytotoxic activity of the protein solutions stored at 4°C was determined once a week for 70 days. The lyophilized powder was solubilized in 20 mM citrate · NaOH buffer (pH 4.0) contained with 100 mM NaCl (open circles and broken line) or in 20 mM glycine · NaOH buffer (pH 9.0) with 100 mM NaCl (closed circles and unbroken line). Relative EC_{50} values to that of each sample on the first day were represented. Unbroken and broken lines represent the regression line of each. (C) Thermal stability of the protein at 30 (closed squares), 40 (open squares), 50 (closed circles), and 60°C (open circles) was examined. The purified protein was diluted into 20 μg/ml with distilled water. And the protein solutions were incubated from 5 to 360 min in a water bath, and were immediately cooled with iced water. 10 μl of the sample solution was added to CACO-2 cells prepared in a 96-well microtiter plate with 90 μl medium. Cell survival rate was determined by the MTT method. (Reprinted from reference [21].)

The physical properties of the protein were determined. Isoelectric point (pI) of the pepsin-treated parasporin-4 was determined as 7.7 by electrofocusing, and the value was close to calculated theoretical pI of 7.1 by the method of Bjellqvist *et al.* [58]. Absorbance at 280 nm of the 1 mg/ml pepsin-treated protein prepared from lyophilized powder was 1.80 and a molecular extinction coefficient at 280 nm (ε280) of the protein was

calculated to be $4.94 \times 10^4\,M^{-1}\,cm^{-1}$. It was close to the predicted value of $4.41 \times 10^4\,M^{-1}\,cm^{-1}$, which could be forecasted from the number of tyrosine and tryptophan residues [59] present in the protein.

Conclusion

The binding of Cry1Ac, an insecticidal protein of *B. thuringiensis*, to a BBM isolated from midguts of the diamondback moth *P. xylostella* was examined by SPR-based biosensor. The BBM was mixed with PC14, a neutral-charged artificial lipid, and was reconstructed to a monolayer on a hydrophobic chip for the biosensor. Amounts of the toxin bindings to the monolayer showed a saturation curve of the Michaelis-Menten type against toxin concentration. And the binding of the Cry1Ac to the reconstructed monolayer was analyzed by a two-state binding model, and it was shown that Cry1Ac bound to the monolayer in the first step with an affinity constant (K_1) of 508 nM, followed by the second uni-molecular step with an equilibrium constant (K_2) of 0.472. The overall affinity constant K_d was determined to be 240 nM. The binding was markedly inhibited by GalNAc ($K_i = 8$ mM). The monolayer was shown to retain a high affinity to Cry1Ac, providing an insect-free system for rapid and large-scale screening of *B. thuringiensis* insecticidal proteins by the SPR-based biosensor technology.

Parasporal inclusion proteins produced by *B. thuringiensis* A1470 strain exhibit strong cytotoxicity against human leukemic T-cells when activated by protease treatment. Two cytotoxic proteins were separated by anion-exchange chromatography and gel-filtration chromatography, and they were identified. One of them was confirmed as a novel cytotoxic protein, and designated parasporin-4. The other was determined to be parasporin 2-like protein, which is the cytotoxic protein produced by the *B. thuringiensis* A1547 strain. Pro-parasporin-4 gene was cloned and expressed in recombinant *E. coli* BL21(DE3), in which the protein was accumulated in an inclusion body. It was solubilized in sodium carbonate buffer and processed with proteinase K, and the activated recombinant parasporin-4 revealed cytotoxic activity against human leukemic T-cells.

The efficient solubilization and activation method of the recombinant parasporin-4 from its inclusion body expressed in *E. coli* was introduced. The pro-parasporin-4 was solubilized in 10 mM HCl and activated by pepsin, and it revealed cytotoxic activity against CACO-2 cells equal to that of the protein prepared by conventional method. There are no other reports that the inclusion bodies of *B. thuringiensis* protein were solubilized in an acidic solution for its purification. By the new method, a concentration of the purified parasporin-4 was 27-fold higher than that of the protein by the previous method. The improvement of preparation method of the parasporin-4 might be useful for the other studies on the *B. thuringiensis* or recombinant proteins.

Cytotoxic activities of the parasporin-4 produced by recombinant *E. coli* against various mammalian cell lines were evaluated using the MTT 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 $>2\,\mu g/ml$. The cytotoxic activity of the purified protein was stable in broad pH region (pH 2.0–11.0) for 3 days, and 97% cytotoxic activity was remained after incubation at 30°C for 360 min. The p*I* of the protein was determined as 7.7, and a molecular extinction coefficient at 280 nm of the protein was to be $4.94 \times 10^4\,M^{-1}\,cm^{-1}$. The molecular mass of the pepsin-treated parasporin-4 was 2.75×10^4.

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