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Characterization of Crystal Proteins of *Bacillus thuringiensis* NT0423 Isolate from Korean Sericultural Farms

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A *Bacillus thuringiensis* designated NT0423, belonging to *B. thuringiensis* subsp. *aizawai* (H 7), was isolated from samples of dust and soil of sericultural farms. *B. thuringiensis* NT0423 having dualspecificity against Lepidoptera and Diptera produced bipyramidal inclusions consisting of two major polypeptides of approximately 130- and 70-kDa. Proteolytic processing by trypsin and gut juice of *Bombyx mori* yielded predominant proteins with molecular masses of about 66-kDa. The whole crystal protein of *B. thuringiensis* NT0423 immunologically was related to that of *B. thuringiensis* subsp. *aizawai*. PCR analysis showed that *B. thuringiensis* NT0423 has at least five crystal protein genes including *cryIA(a)*, *cryIA(b)*, *cryIC*, *cryID* and *cryIIA*, and southern blot was determined the location of each gene on intact and enzyme-digested plasmid DNA fragments. Except for *cryIA(a)* gene on the high molecular weight plasmid of 165-kb, all of four genes were located on the plasmid of 66-kb. The production of β-exotoxin from *B. thuringiensis* NT0423 was identified by the HPLC analysis. In addition, the β-exotoxin showed its ability to prevent pupation of treated larvae of house flies (*Musca domestica*) from developing into normal adults.

Key words : *Bacillus thuringiensis* NT0423, Dual specificity, Lepidoptera, Diptera, β-Exotoxin, *Bombyx mori*, *Musca domestica*

Introduction

Bacillus thuringiensis of gram-positive spore-forming soil bacterium produced insecticidal crystal proteins during the stationary phase of its growth cycle. Due to its ability to produce insecticidal toxins, *B. thuringiensis* have been used as bioinsecticides for the control of certain insects among the orders Lepidoptera, Diptera, and Coleoptera (Schnepf *et al.*, 1998). A large number of *B. thuringiensis* strains have been isolated from many habitats, including soil (Ohba and Aizawa, 1986; Kim *et al.*, 1998a), sericultural farm (Kim *et al.*, 1998c), stored-product dust (Delucca *et al.*, 1982; Kim *et al.*, 1998b), insects (Krieg *et al.*, 1983), and phylloplane (Smith and Couche, 1991). The relevant works on the screening and isolation are still being performed. During the past decade, the understanding of the gene expression, structure, and mode of action for *B. thuringiensis* crystal proteins was extensively developed. Recently, the miscellaneous researches for understanding of interaction between *B. thuringiensis* toxin and receptor protein of host cell is intensively popular. However, together with this work, the discovery and identification of novel toxin genes from *B. thuringiensis* isolates is also important.

For the screening and isolation of novel *B. thuringiensis* isolates having wide host range and strong insecticidal activity, we isolated *B. thuringiensis* NT0423 from samples of dust and soil of sericultural farms. This paper describes biochemical characteristics of *B. thuringiensis* NT0423 that appear to be related immunologically to the crystal proteins of *B. thuringiensis* subsp. *aizawai*, and genetic aspects on the presence and localization of crystal protein genes.

Materials and Methods

Bacterial strain and culture conditions

The samples of dust and soil were taken as possible source of bacteria in the sericultural farms in Kyonggi-do and

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isolated *B. thuringiensis* as described previously (Kim *et al.*, 1993). Among a large number of *B. thuringiensis* isolates, *B. thuringiensis* designated from NT0423 was selected. *B. thuringiensis* NT0423, *B. thuringiensis* subsp. *kurstaki* HD-1, and *B. thuringiensis* subsp. *aizawai* were used in this study. For purification of parasporal inclusions and for extraction of plasmid DNA of *B. thuringiensis*, GYS (Nickerson and Bulla, 1974) and SPY (Spizizen, 1958) media were used, respectively. Formation of spores and crystals were monitored by phase-contrast microscopy.

Molecular methods and enzymes

Restriction enzymes used in this study were purchased from Bioneer Co. (Korea) and Takara Shuzo Co. (Shiga, Japan). All enzymes were used according to the instructions of the manufacturers. Plasmid DNA was prepared from *B. thuringiensis* by the improved procedure of alkaline lysis method (Kim *et al.*, 1998a) and further purified by using a Qiagen plasmid kit according to the manufacturers instructions (Qiagen GmbH, Hilden, Germany). Cultures were grown in 50 ml of Luria-Bertani (LB; Maniatis *et al.*, 1982) with shaking at 28°C overnight. The cultures were inoculated in 250 ml of LB or SPY medium with shaking at 28°C; cells were harvested at an optical density at 600 nm of 0.7. Plasmid profiles were determined for each strain by electrophoresis through 0.7% agarose gels.

In the hybridization experiments for the localization of five insecticidal crystal protein genes detected by PCR analysis, plasmid DNA purified from *B. thuringiensis* NT0423 were electrophoresed on a 0.7% horizontal agarose gel and performed southern blot using each PCR product as probes. Southern blot was performed against plasmid DNA purified from *B. thuringiensis* NT0423, *B. thuringiensis* subsp. *kurstaki*, *B. thuringiensis* subsp. *aizawai*, and *B. thuringiensis* NT0423 plasmid DNA digested with such restriction enzymes as *Hind*III, *Hinc*II, and *Pst*I. The gels were treated for 15 min in 0.25 N HCl, and transferred to Hybond N^+ filters (Amersham, Buckinghamshire, United Kingdom) in 0.4 N NaOH as a transfer buffer. The DNA fragments for probe were labeled with peroxidase by using the ECL direct nucleic acid labeling system (Amersham). Prehybridization, hybridization, washing, and detection procedures were followed as described by the manufacturer. The signal was visualized by using Hyperfilm-ECL (Amersham) exposed to the blot and reacted with the GBX developer (Kodak).

Preparation of H antisera and serological tests

H antisera to the reference strains of 33 serotypes (H 1-27) of *B. thuringiensis* were prepared as described by Kim *et al.* (1993). The bacteria were grown for 8 hrs in slowly shaken (50 rpm) Erlenmeyer flasks at 28°C, harvested by centrifugation at 3,000 rpm and resuspended in saline containing 0.25% formalin at 1×10^6 cell/ml. Fresh antigen was injected intravenously into mouse. The first injection of 250 μl was followed at an interval of 7 days by a series of four 0.5 m*l* injections. Mice were bled 3 days after the last injection, serum separated and stored at -20°C. H antigen studies were performed using 96 well microplates (U-bottom, Nunc) as described by Laurent *et al.* (1996). One hundred microlitres of the bacterial suspension was grown for 8 hrs at 28°C was mixed in each well with 0.1 ml of the H antiserum which had been diluted 50-fold with saline. Agglutination was assayed after inoculation at 37°C for 1 hr. The plates were examined for the development of the pellet of precipitation at the bottom. The presence of the pellet means positive results. Antiserum was raised in rabbit against the solubilized crystal proteins of *B. thuringiensis* NT0423 as previously described (Yu *et al.*, 1991). Double immunodiffusion test was performed on 1% agarose gel according to Ouchterlony (1968).

Protein analysis

Three liter of GYS medium was grown with shaking at 160 rpm at 28°C in 7 liter large-scale fermentor, until sporulation was complete, as determined by phase-contrast microscope (generally 4 days). The spores and crystals were harvested by transferring the culture into 500 ml centrifuge bottles on ice and pelletting the spores and crystals at 6000 - 7000 g for 5 - 10 min at 4°C. After centrifugation, sporangial debris and proteinase were removed from the pellet by washing with 1 M NaCl containing 0.01% Triton X-100 and ice-cold sterilized distilled water. The process was repeated up to 6 times until spores and crystals were free from debris by using phase-contrast microscopy. The final pellet was resuspended in sterilized distilled water. The spores and crystals were separated using differential ultracentrifugation through a discontinuous sucrose gradient, comprising 10 ml, 8 ml, 7 ml, 8 ml each of 67%, 72%, 79% and 87% (w/v) sucrose in sterilized distilled water. Centrifugation was carried out in a ultracentrifuge in a swing rotor operating at 80000 g for 14 hrs at 4°C (Thomas and Ellar, 1983). Crystals formed a major band at the interface between the 79% and 87% (w/v) sucrose, while the spore formed a discrete pellet at the bottom of the tube. The crystal band was removed and washed three times in sterilized distilled water by centrifugation at 15000 g for 5 min at 4°C, to remove the sucrose (Yamamoto, 1983). The final pellet was resuspended in sterilized distilled water and stored frozen at -20°C. The purity of parasporal inclusions was checked by phase-contrast microscopy. Solubilization of

the purified inclusions was done in 50 mM Na_2CO_3 (pH 10.0) at 37°C for 1 hr in the presence of 10 mM dithiothreitol (DTT) and 1 mM EDTA. The solubilized inclusions were adjusted to pH 8.0 by 1 N HCl. For analysis of proteolytic processing, solubilized proteins (1 $mgml^{-1}$) were treated with the excess concentration (100 ug) of trypsin for 1- to 6 hrs and gut juice proteinases of *Bombyx mori* in 50 mM Na_2CO_3 (pH 10.0) at 37°C for 1- to 12 hrs. After incubation, the solutions were added with phenylmethylsulphonyl fluoride (Wako Pure Chem., Tokyo, Japan) to stop proteolytic processing and examined for SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) profiles.

SDS-PAGE was performed as described by Laemmli (1970) with gel 1.5 mm thick. Purified δ-endotoxins (2.5 μl - 10 μl) were heated (100°C, 5 min) in 5×sample buffer [0.6 ml of 1 M Tris-HCl (pH 6.8), 5 ml of 50% glycerol, 2 ml of 10% SDS, 0.5 ml of 2-mercaptoethanol, 1 ml of 1% bromophenol blue, 0.9 ml of H_2O] and then were centrifugated. The removed supernatants were subjected to 4% gels.

Sample preparation and PCR

B. thuringiensis strains were grown for 12 hrs to 16 hrs on a nutrient agar plate. A loopful of cells from a single colony was transferred to 0.15 to 0.2 ml of sterilized distilled water, and the mixture was vortexed and then boiled for 10 min to lyse the cells. The resulting cell lysate was spun down (20 s, 10,000 rpm), and 15 μl was transferred to a 0.2 ml PreMix™-TOP (Bioneer Co, Korea) premixed with *Taq* DNA polymerase and reaction mix, 10 to 30 pmoles each of the primers used in the reaction. The 19 primers (*cryIA(a), cryIA(b), cryIA(c), cryIB, cryIC, cryID, cryIE, cryIF, cryIG, cryIIA, cryIIIA, cryIIIB, cryIIIC, cryIIID, cryIVA, cryIVB, cryIVC, cryIVD* and *cryV)* used in this study and were synthesized as reported by Carozzi *et al.* (1991), Gleave *et al.* (1993) and Ceron *et al.* (1994, 1995). Amplification was performed with DNA Thermal Cycler (Perkin Elmer Cetus, USA) by using a single denaturation step (5 min at 95°C), followed by a 35-cycle program, with each cycle consisting of denaturation at 94°C for 1 min, annealing at 45 to 65°C for 1 min, and extension at 72°C for 1 min: a final extension step (72°C for 10 min) was also used. Following amplification the PCR product was analyzed by 3% NuSieve 3:1 agarose gel electrophoresis.

Purification of β-exotoxin and its toxicity

Cultures of *B. thuringiensis* NT0423 were harvested until the end of the logarithmic growth phase reached. The supernatants, its concentrates, and precipitates obtained from centrifugation with and without heat treatment at 121°C for 20 min, respectively were resuspended with distilled water or 50 mM KH_2PO_4 (pH 3.0). They were adjusted to pH 3.0 and incubated 4°C for 1 hr. The supernatants obtained with centrifugation were filtered with 0.2 μm microfilterfuge tube, and then the samples were analyzed with HPLC (high performance liquid chromatography). The fractions obtained were neutralized, and stored at -20°C until use. The exotoxin preparations applied on artificial diets were air dried, and tested the toxicity against 20 third instar larvae of *Musca domestica,* and 10 second instar larvae of *B. mori* and *Culex pipiens.* The bioassay against *M. domestica* was checked the number of morphological abnormalities and death during molting or pupation.

Results

Among a large number of *B. thuringiensis* isolates from various natural environments like soil, granary, sericultural farm and forestry, we selected a *B. thuringiensis* isolate, designated from NT0423, toxic for the larvae of Lepidoptera and Diptera tested, i.e., *Plutella xylostella, Heliothis assulta, Hypantria cunea, B. mori, Spodoptera exigua, C. pipiens. B. thuringiensis* NT0423 produced typical bipyramidal inclusions with various size under phase-contrast microscopy (Fig. 1). as described previously (Kim *et al.*, 1993). But, no cuboidal inclusions were observed. These bipyramidal inclusions consisting of two predominant parasporal components of approximately 130- and 70-kDa polypeptides were easily solubilized under alkaline condition (Fig. 2A, lane 1), and activated by treatment of proteolytic enzyme such as trypsin to about 66 kDa (Fig. 2A, lane 2, 3). After 6 hr incubation, the toxic protein was resistant to trypsin without non-spe-

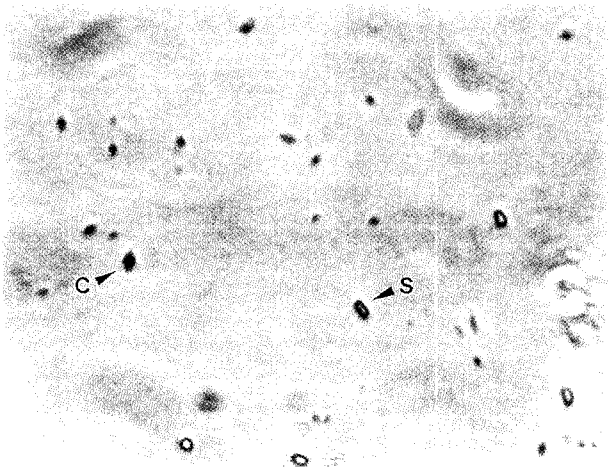


Fig. 1. Phase-contrast micrograph of a sporulating culture of *B. thuringiensis* NT0423. C, crystal; S, spore.

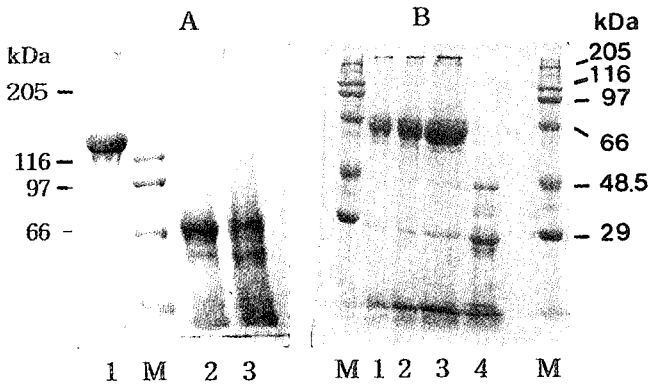


Fig. 2. Activation patterns of *B. thuringiensis* NT0423 crystals by treatment of proteolytic enzymes. (A) 7.5% Crystals trypsinization. Lane 1, *B. thuringiensis* NT0423 crystals intact (1 mg) after 1 hr incubation at 37, 50 mM Na_2CO_3 (pH 10.0); Lane 2, after 1 hr incubation at 37in the presence of 100 μg of trypsin; Lane 3, after 6 hr incubation in the presence of 100 μg of trypsin. (B) 10% polyacrylamide gel. Lane 1- 3, after 1, 6 and 12 hrs incubation, respectively, in the presence of *Bombyx mori*-gut juices; Lane 4, *B. mori*-gut juices.

cific or specific degradation. Also, the protoxins of *B. thuringiensis* NT0423 were digested by gut juice proteinases of *B. mori* to less than 66 kDa "resistant core" (Fig. 2B, lane 1, 2, 3). In the comparison of the protein patterns digested by trypsin and larval gut juice, the toxic moiety converted by gut proteases produced smaller polypeptides of less than 66 kDa. These results indicate that the parasporal inclusions of *B. thuringiensis* NT0423 could well function as active toxins by two processes of solubilization and activation.

For classification of *B. thuringiensis* NT0423, the H agglutination assay on the basis of flagella serotypes and double immunodiffusion assay of crystal serotypes were performed. In the H agglutination assay using the fresh cultures of *B. thuringiensis* NT0423, this isolate was reacted the serotype 7 of subsp. *aizawai* among H antisera of 33 *B. thuringiensis* prepared (Table 1). And the antibody preparation of *B. thuringiensis* NT0423 crystals formed a precipitation line with *B. thuringiensis* subsp. *aizawai* crystals in the double immunodiffusion assay (Fig. 3). These results found that the flagella and crystal antigenecities of *B. thuringiensis* NT0423 have a correlation with those of *B. thuringiensis* subsp. *aizawai*.

In our previous report (Kim *et al.*, 1993), *B. thuringiensis* NT0423 had at least nine plasmids ranged from 2.3- to 110-MDa, and the plasmid profile was different from that of *B. thuringiensis* subsp. *aizawai*. To examine the content of the insecticidal crystal protein genes within *B. thuringiensis* NT0423, PCR analysis was performed using primer sets detectable the specific genes encoded the Lepidopteran-, Dipteran-, and Coleopteran-active δ-endotoxin (Table 2). We designed oligonucleotide sequences covering *cryI, II, III, IV,* and *V* genes, and determined the size of DNA fragments by enzyme digestion for accurate identification. *B. thuringiensis* NT0423 produced five PCR products corresponding to the sizes of *cryIA(a), cryIA(b), cryIC, cryID* and *cryIIA* genes (Fig. 4). Each of five PCR products was reconfirmed by enzyme digestion, which produced DNA fragments of the expected size as

Table 1. H agglutination test of *B. thuringiensis* NT0423

H serotype[a]	Serovar	NT0423
1	*Thuringiensis*	-
2	*Finitimus*	-
3a	*Alesti*	-
3a3b	*Kurstaki*	-
4a4b	*Sotto*	-
4a4c	*Kenyae*	-
5a5b	*Galleriae*	-
6	*Entomocidus*	-
7	*Aizawai*	+
8a8b	*Morrisoni*	-
8a8c	*Ostriniae*	-
8b8d	*Nigeriensis*	-
9	*Tolworthi*	-
10	*Darmstadiensis*	-
11a11b	*Toumanoffi*	-
11a11c	*Kyushuensis*	-
12	*Thompsoni*	-
13	*Pakistani*	-
14	*Israelensis*	-
15	*Dakota*	-
16	*Indiana*	-
17	*Tohokuensis*	-
18	*Kumamotoensis*	-
19	*Tochigiensis*	-
20a20b	*Yunnanensis*	-
20a20c	*Pondicheriensis*	-
21	*Colmeri*	-
22	*Shandongiensis*	-
23	*Japonensis*	-
24	*Neoleonensis*	-
25	*Coreanensis*	-
26	*Silo*	-
27	*Mexicanensis*	-

[a] 33 H antisera were tested.

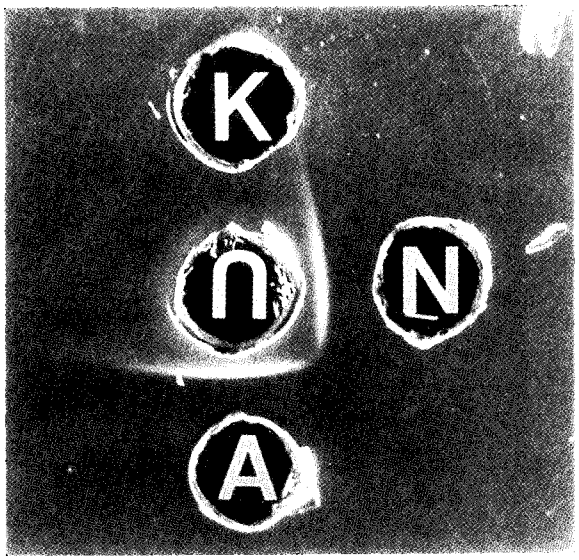


Fig. 3. Double immunodiffusion assay using a crystal antibody (n) against crystal proteins of *B. thuringiensis* NT0423. K, N, and A indicate parasporal antigens of *B. thuringiensis* subsp. *kurstaki* HD-1, NT0423, and subsp. *aizawai*, respectively.

Table 2. Characterization of insecticidal crystal protein genes as determined by PCR and identification of PCR products with several enzymes[a]

Gene type	PCR products (bp)	Digested enzyme	Digested size (bp)
cryIAa	786	*Eco*RI	521, 265
cryIAb	238	*Sau3*AI	137, 101
cryIAc	486	*Acc*I	338, 148
cryIB	830	*Hinc*II	487, 339
cryIC	288	*Sau3*AI	187, 101
cryID	465	*Sac*I	332, 133
cryIE	882	*Eco*RI	766, 116
cryIF	368	*Sau3*AI	267, 101
cryIG	235	*Sau3*AI	181, 54
cryIIA	1070	*Sau3*AI	990, 828, 322, 242
cryIIIA	1964	*Acc*I	1487, 476
cryIIIB	1359	*Pst*I	1213, 144
cryIIIC	1074	*Hinc*II	615, 458
cryIIID	1135	*Pst*I	529, 381
cryIVA	1032	*Acc*I	527, 504
cryIVB	2610	*Acc*I	2105, 504
cryIVC	2040	*Acc*I	1271, 768
cryIVD	1932	*Acc*I	1174, 757
cryV	2174	*Acc*I	1771, 410

[a]Each of PCR products was identified the digested size by treatment of the specified enzymes

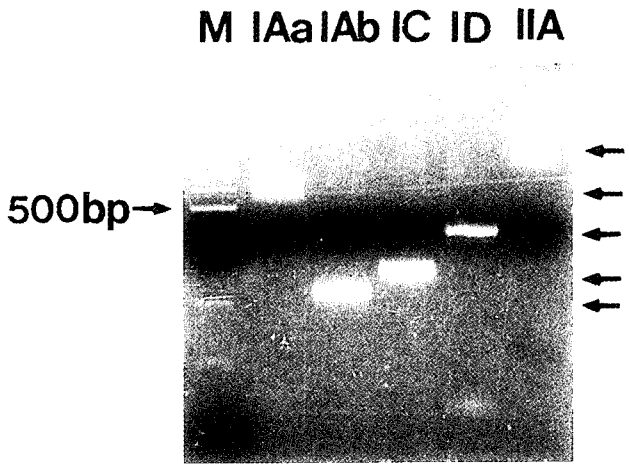


Fig. 4. Profiles of crystal protein genes encoded in a *B. thuringiensis* NT0423. IAa: *cryIA(a)* gene, 786 bp PCR product, IAb: *cryIA(b)* gene, 238 bp, IC: *cryIC* gene, 288 bp, ID: *cryID* gene, 465 bp, IIA: *cryIIA* gene, 1070 bp, M: size marker of 100 bp ladders.

described in Table 2 (data not shown). This fact indicates that at least five insecticidal crystal protein genes are related to express parasporal inclusion bodies within *B. thuringiensis* NT0423 exist.

For the presence and localization of each gene on the plasmid and/or chromosomal DNA, based on the presence of five genes by the PCR analysis, southern blot was analyzed with each probe specific to five insecticidal crystal protein genes. Except that *cryIA(a)* gene was localized on the species of 165-kDa plasmid DNA, all of *cryIA(b), cryIC, cryID* and *cryIIA* genes were positively reacted on the 66-kDa plasmid DNA (Table 3). On the plasmid DNAs digested with several restriction enzymes, the positive reactions of each gene showed various sizes of DNA fragments. This analysis may be contributed to clone the insecticidal crystal protein genes within *B. thuringiensis* NT0423. Above results indicate that all of five insecticidal crystal protein genes are localized on the plasmid DNA.

The existence of a second potent toxin, β-exotoxin produced by *B. thuringiensis* NT0423 was analyzed by the HPLC technique (Fig. 5). Several presumptive exotoxin peaks in the analysis of HPLC were obtained, and each of fractions was tested the toxicity against third instar larvae of *M. domestica*. The fourth peak marked by arrow contained pure exotoxin preparations and had stronger toxicity than others (Fig. 5), indicating the production of ß-exotoxin from *B. thuringiensis* NT0423.

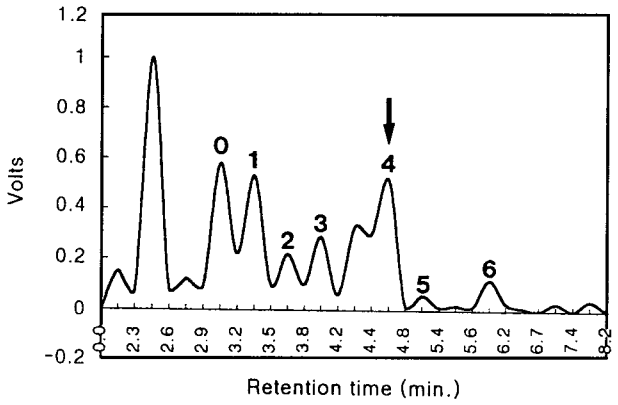
Discussion

The presence of multiple δ-endotoxin genes in *B. thur-*

Table 3. Localization of five crystal protein genes in *B. thuringiensis* NT0423 by the southern blot analysis

Gene	plasmid DNA	*B. thuringiensis* NT0423 plasmid DNA digested with[a]						
		*Eco*RI	*Cla*I	*Hinc*II	*Hind*III	*Pst*I	*Xba*I	*Bam*HI
cryIA(a)	165 kb	1.25 kb	-	11 kb 4 kb	-	23 kb (15 kb)	-	23 kb
cryIA(b)	66 kb (12 kb)[b]	14 kb	3.3 kb	3.5 kb	6.6 kb 4.5 kb 0.7 kb	16.2 kb	1.4 kb	-
cryIC	(66 kb) 12 kb	14 kb	1.5 kb	-	2.0 kb 0.7 kb	-	1.4 kb	-
cryID	66 kb (12 kb)	14 kb 1.3 kb	1.5 kb	-	5.8 kb 0.58 kb	1.3 kb	1.5 kb 0.7 kb	-
cryIIA	66 kb (8.5 kb)	13 kb 8 kb	1.8 kb	3.6 kb	6.6 kb 4.3 kb	8.6 kb	8.5 kb	-

[a]Plasmid DNA of *B. thuringiensis* NT0423 undigested and digested with *Cla*I, *Eco*RI, *Hinc*II, *Hind*III, *Pst*I, and *Xba*I were transferred onto Hybond N^+ filters and hybridized with labeled with peroxidase by using the ECL direct nucleic acid labeling system. Probes were used each of five PCR products.
[b]indicates faint bands detected in hybridization experiments.



(A)

Peak number	No. larvae killed*
0	1
1	0
2	0
3	0
4	5
5	1
6	1

* out of 20 larvae

(B)

Fig. 5. Identification of putative exotoxin peak on HPLC samples (μl) of autoclaved culture supernatant were injected after pH adjustment. (A) The arrow indicates the position of the peak suspected to contain the exotoxin. (B) Insecticidal activity of each fraction obtained by HPLC against third instar larvae of *Musca domestica*.

ingiensis may be closely related to the its availability and host range spectrum, so the gene content of each strains was preferentially searched. The analysis of southern blot was confirmed the localization of five insecticidal crystal protein genes existing in *B. thuringiensis* NT0423. The fact that four genes are contained on the 66-kb plasmid DNA except for *cryIA(a)* gene was different from those of *B. thuringiensis* subsp. *kurstaki* HD-1 and *B. thuringiensis* subsp. *aizawai*. In particular, *B. thuringiensis* NT0423 was identified to possess *cryIIB* gene together with *cryIIA* gene by the analysis of identification of *cryII* genes in *B. thuringiensis* isolates by the use of oligonucleotide DNA primers (Asano *et al.*, 1993). It had been reported that a few *B. thuringiensis* strains possess multiple δ-endotoxin genes (Sanchis *et al.*, 1988; Visser *et al.*, 1988), especially Chestukhina *et al.* in 1994 described that *B. thuringiensis* contains five or six structure genes encoding insecticidal crystal proteins on chromosomal DNA. *B. thuringiensis* NT0423 used in this study showed a unique characteristic that all of five δ-endotoxin genes are localized on not the chromosome but high molecular weight plasmid DNA.

β-exotoxin production is not a general attribute of the species *B. thuringiensis* (Faust, 1975). β-exotoxin has been reported to be highly active against Diptera, for example *M. domestica* or *Aedes aegypti*, its activity may be contributed to increase the toxicity of δ-endotoxin. But the host spectrum of β-exotoxin affect not only invertebrates but also vertebrates and even microorganisms (McConnell and Richards, 1959). Therefore, the production and toxicity of β-exotoxin from *B. thuringiensis* NT0423 were analyzed. As it is excreted during the logarithmic growth phase into the medium, the supernatants, its concentrates, and precipitates obtained from centrifu-

gation with and without heat treatment, respectively, were tested the toxicity against *M. domestica* larvae. Only supernatants of non-treated samples showed very low toxicity, and the heat-treated samples had high toxicity for the supernatants and precipitates. In the case of the samples adjusted to pH 3.0, they showed no toxicity for loss of β-exotoxin function (data not shown). After identified the production of the exotoxin from *B. thuringiensis* NT0423, the preparation obtained by the HPLC technique was tested the toxicity. But the exotoxin preparations were active against *M. domestica*, and inactive against *B. mori* and *C. pipiens* (data not shown). Possibly, the exotoxin from *B. thuringiensis* NT0423 will be affected its activity against insects. But the formulation using *B. thuringiensis* NT0423 should be considered the activity of the exotoxin. To date, several strains including especially subsp. *kurstaki*, *aizawai*, *kenyae*, and *galleriae* have been reported as *B. thuringiensis* strain displaying dual-specificity against Lepidoptera and Diptera. They commonly contained *cryII*-type gene (Höfte and Whiteley, 1989; William and Whiteley, 1989). Comparison of the antigenecity, morphology of parasporal inclusions and δ-endotoxin gene content between *B. thuringiensis* NT0423 and these strains having dual-specificity suggested that i) *B. thuringiensis* NT0423 has the same antigenecity with *B. thuringiensis* subsp. *aizawai*. ii) the gene content of *B. thuringiensis* NT0423 is the same with those of subsp. *aizawai* IPL and juroi including *cryIIA* gene (Asano *et al.*, 1993). iii) *B. thuringiensis* NT0423 also produced bipyramidal crystal proteins, but had no cuboidal crystal proteins. iv) the dual-specificity of *B. thuringiensis* NT0423 may be not derived from cuboidal crystal proteins.

Isolation of novel *B. thuringiensis* strains have been reported from various natural environments, so it is important to study the intensive, extensive screening and isolation. These works would be contributed to provide solutions that protect the appearance of resistant pests and develop the *B. thuringiensis* formulations. Therefore, in this study we had isolated a *B. thuringiensis* NT0423 from sericultural farm, and analyzed its characteristics in the biochemical and genetic aspects. The understanding of further information on the *B. thuringiensis* NT0423 will provide its availability as a biopesticide. *B. thuringiensis* NT0423 crystals consisted of multiple crystal protein genes may be less likely to lead to resistant insects.

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