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ORIGINAL ARTICLE

Screening and characterization of a non-insecticidal *Bacillus thuringiensis* strain producing parasporal protein with selective toxicity against human colon cancer cell lines

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Abstract Parasporins are a heterogenous group of Cry proteins produced by non-insecticidal *Bacillus thuringiensis* strains that specifically act on human cancer cells without affecting normal ones. This unique cytotoxic property has driven researchers to explore novel non-insecticidal *B. thuringiensis* strains possessing cancer cell-killing proteins. In this study, we isolated 65 non-clonal native isolates from 21 coastal soil samples and subsequently tested 28 strains which were non-haemolytic and non-insecticidal for their cytotoxicity against human colon cancer cell line HCT 116 and human cervical cancer cell line SiHa. Parasporal protein from a strain designated as *B.t.*LDC 501 showed significantly higher cytolytic activity on HCT 116 cells than on SiHa cells. The activated protein also exerted specific cell lethality against two other colon cancer cell lines, SW480 and SW620. However, it was notably non-toxic to normal cells, such as human peripheral blood leukocytes, human embryonic kidney cell line (HEK293) and human corneal epithelial cell line (HCEC) and showed only modest toxicity on a murine fibroblast cell line (NIH/3T3). The purified 20-kDa crystal protein obtained through gel filtration chromatography exhibited a markedly higher cytopathic effect than the unpurified protein. Liquid chromatography–tandem mass spectrometry analysis of the 20-kDa fragment revealed it to be an uncharacterized protein containing a tumor necrosis factor-like domain. The non-apoptotic mode of cell death, extensive membrane permeability and aminopeptidase N-dependent cytotoxicity suggests the pore-forming nature of the protein. Further characterization of the protein and the receptor will facilitate its use as a potential therapeutic drug against cancer.

Keywords Aminopeptidase N · Parasporin · Anticancer · Non-haemolytic · HCT 116

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Introduction

Bacillus thuringiensis is a Gram-positive bacterium capable of producing crystalline parasporal inclusions during sporulation. These inclusions are termed δ-endotoxins and have been grouped into two major classes, Cry and Cyt proteins (Crickmore et al. 1998). Cry protein is known for its specific insecticidal action against various insect orders, including Lepidoptera, Diptera, Coleoptera, Hymenoptera and nematodes (de Maagd et al. 2001). Cyt proteins exhibit in vivo toxicity against Dipterans, particularly mosquitoes and black flies (Butko 2003). They also show in vitro cytolytic activity

against insects and mammalian cells, such as erythrocytes, lymphocytes and fibroblasts (Thomas and Ellar 1983).

Worldwide screening of *B. thuringiensis* strains from various ecological niches has revealed the prevalence of many non-insecticidal strains (Ohba and Aizawa 1986; Hastowo et al. 1992). Studies on such strains has led to the identification of new anticancer proteins with selective action on human cancer cells (Mizuki et al. 1999), denoted as parasporins (PS). These proteins have been characterized as non-haemolytic and non-insecticidal (Mizuki et al. 2000), and to date, six types of PS (PS-1 to PS-6) have been reported (Okumura et al. 2010). Each type exhibits a different mode of action against different cancer cell lines (Mizuki et al. 2000; Ito et al. 2004; Yamashita et al. 2005; Okumura et al. 2006; Nagamatsu et al. 2010; Ekino et al. 2014). A few studies demonstrated that these parasporal proteins exert their effect on a specific cell line through specific receptors. An autophagy protein, Beclin-1, from the HeLa cell line has been identified as the receptor of PS-1 that demonstrated an apoptotic mode of action (Katayama et al. 2009). PS-2 is a pore-forming toxin that requires glycosylphosphatidylinositol (GPI)-anchored proteins for its oligomerization and pore formation on HepG2 cells (Kitada et al. 2005). This action is similar to that of insecticidal Cry proteins which mediate their toxicity by binding to aminopeptidase N (APN), a GPI-anchored protein (Knight et al. 1994).

Due to annual increase in the incidence of cancer and the limitations of the existing treatment methods, intensive research efforts are being made to identify new therapeutic agents, especially those from biological sources. Since PS are potential candidates for targeted anticancer therapy, it is essential to delineate their mode of action, which is probably through receptor mediation. An earlier study in our laboratory identified a potential cancer cell-specific cytotoxic strain isolated from inland soil samples (Poornima et al. 2010). As the marine environment is known to harbour many beneficial bacteria (Bernan et al. 1997), we report here our analysis of coastal soil for the presence of *B. thuringiensis* species expressing anticancer proteins. We also extended our study to investigate the role of APN (if any) in the cytocidal action.

Material and methods

Screening of *Bacillus thuringiensis*

Soil samples were collected from 21 coastal sites of Mandapam (9.28°N 79.12°E), Ramnad district, Tamil Nadu, India. *Bacillus thuringiensis* was isolated by the method of Ohba and Aizawa (1986). Briefly, 1 g of soil sample was suspended in 9 mL of phosphate buffered saline (PBS) and the suspension agitated constantly for 5 min, following which the upper layer of the suspension was removed and placed in a water bath at 80 °C for 15 min to kill any heat-sensitive organisms. Approximately 100 μL of the suspension was spread onto nutrient agar medium and incubated at 37 °C for 48 h. Colonies with *Bacillus*-like (fried egg) morphology were selected and observed under phase contrast microscope at 100× magnification (model DP12-CX41; Olympus, Tokyo, Japan). Strains with parasporal inclusions were denoted *Bacillus thuringiensis* and designated as *B.t.*LDC in a series starting with the number 500. The isolated strains were lyophilized (ALPHA1-2 LD Plus freeze dryer; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) for long-term storage. In this study, BGSC-4Q2 (*B. thuringiensis israelensis*) and BGSC-4D1 (*B. thuringiensis kurstaki*) obtained from Dr. Zeigler, Bacillus Genetic Stock Center (BGSC), Ohio State University, Ohio, USA were used as reference strains.

Solubilization and activation of spore crystal complex

The isolated strains were inoculated in Arret and Kirshbaum medium (sporulating agar) (Hi Media Laboratories Pvt. Ltd, Mumbai, India) and incubated at 28 °C for 3–4 days. When parasporal inclusions were visible, the spore-crystal mixture was harvested using 1 M NaCl followed by centrifugation (model 5810 R; Eppendorf AG, Hamburg, Germany) at 12000 *g* for 10 min at 4 °C. The pellet was first washed twice with distilled water supplemented with 1 mM phenyl methyl sulphonyl fluoride (PSMF) and then solubilized in 50 mM Na_2CO_3 buffer (pH 10.5) containing 10 mM DTT and 1 mM EDTA for 1 h at 37 °C (Mizuki et al. 1999). The solubilized protein in the supernatant was separated by centrifugation at 12000 *g* for 20 min at 4 °C and then digested with 10 μg/mL of proteinase K (Merck-Genei, Bengaluru, India) for 1.5 h at 37 °C to expose the active domain. Proteolytic action was stopped by the addition of 1 mM PMSF (Hi Media Laboratories). Protein concentration was estimated by the Bradford method using bovine serum albumin (BSA) as standard (Bradford 1976).

Haemolytic assay

Haemolytic activity of the proteinase K-treated proteins of all 65 isolates were tested using human erythrocytes (Uemori et al. 2007). Approximately 1 mL of human peripheral blood was collected in EDTA-coated tubes from healthy volunteers (who tested negative for both human immunodeficiency virus and hepatitis B virus). The blood samples were suspended in 9 mL PBS and centrifuged at 12,000 *g* for 5 min at 4 °C, following which the pellet was washed twice with PBS and resuspended to 1 % (v/v) in the same buffer. A 100 μL aliquot of the 1 % erythrocyte suspension was added to a round bottom 96-well plate. The activated protein at a concentration of 100 μg/mL was added to the erythrocyte

suspension in triplicate wells. Haemolysis was visually examined after 18 h incubation at 27 °C, with *B.thuringiensis israelensis* (BGSC-4Q2) as the positive control and PBS as the negative control. The strains with non-haemolytic activity were selected for further analysis.

Insecticidal assay

The spore-crystal mixtures of the selected non-haemolytic strains were assayed for their insecticidal potential against Dipteran and Lepidopteran larvae using the one-dose toxicity assay (Ishii and Ohba 1993). Third instar larvae of *Aedes aegypti*, *Culex quinquefasciatus* (Diptera: Culicidae) and *Bombyx mori* (Lepidoptera: Bombycidae) were used as targets in this study. For *A. aegypti* and *C. quinquefasciatus*, ten third instar mosquito larvae of each species were placed in a separate plastic disposable cup containing of 7.7×10^7 CFU/mL spore-crystal mixture suspended in tap water. For *B. mori*, third instar silkworm larvae were fed with mulberry leaves coated with the spore-crystal mixture at a concentration of 2.5×10^8 CFU/mL. Mortality rate was scored after 96 h. The experiments were performed in triplicate using BGSC-4Q2 as the positive control for Dipteran larvae and BGSC-4D1 as control for Lepidopteran larvae. Respective larval species suspended in tap water without the addition of the spore-crystal mixture were observed as the negative control. Strains inflicting less than 30 % mortality were considered to be non-insecticidal.

Cytotoxicity testing using cancer cell lines

Growth and maintenance of cell lines

Normal and neoplastic cell lines for cytotoxicity testing were obtained from various sources. Cell lines HCT 116, SW620 (both human colon cancer cell lines), SiHa (human cervical cancer cell line), NIH/3T3 (normal murine fibroblast cell line) and HEK293 (normal human embryonic kidney cell line) were obtained from the National Centre for Cell Science, Pune, India. Cell line SW480 (human colon cancer cell line) was obtained from Madurai Kamaraj University, Madurai, India. Cell line HCEC (normal human corneal epithelial cell line) was obtained from the Aravind Medical Research Foundation, Madurai, India. All cells were cultured in media prescribed by the suppliers, usually supplemented with 10 % fetal bovine serum and 1 % antibiotic (100 IU/mL Penicillin and 10 μg/mL streptomycin) at 37 °C with 5 % CO_2 in a humidified incubator (model SCO10W; Sheldon Manufacturing Inc., Cornelius, OR). The only exception was the SW620 cell line which did not require CO_2.

Isolation of peripheral blood leukocytes

Peripheral blood was collected from a healthy volunteer and mixed with RPMI-1640 media containing 50 IU/mL of sodium heparin (Dykman et al. 1983). This mixture was slowly layered on HiSep Lymphocyte Separation Medium (Hi Media Laboratories) and centrifuged at 300 *g* for 20 min at 10 °C. The interface containing the nucleated cells was carefully aspirated and transferred to a fresh tube containing RPMI-1640 with 10 IU/mL of sodium heparin. After centrifugation, the cell pellet was resuspended in 1 mL of complete RPMI-1640 medium. The isolated cells were counted using a haemocytometer and subjected to cytotoxic assay.

One-dose cytotoxic assay

The one-dose cytotoxic assay was carried out by following the method of Mizuki et al. (1999). Cells of the adherent cell lines HCT 116, SW480, SW620, SiHa, HEK293, HCEC and NIH/3T3 were trypsinized, and 90 μL of cell suspension (2×10^5 cells) was seeded onto the 96-well tissue culture plates. The plates were then incubated for 16 h at 37 °C to achieve a confluent monolayer. The peripheral blood leukocytes serving as control were seeded onto the plate directly for the assay. The proteinase K-activated proteins (concentration of 100 μg/mL) from 28 non-haemolytic and non-insecticidal *B. thuringiensis* strains were tested against the HCT116, SiHa and NIH/3T3 cells and against normal peripheral blood leukocytes for initial screening. The activated proteins of potential strains were further checked on the SW480, SW620, HEK293 and HCEC cell lines. The activated proteins of BGSC-4Q2 were used as the positive control, and the solubilizing buffer containing proteinase K and PMSF served as the negative control. The cytopathic effect of parasporal proteins was observed after 16 h of incubation under an inverted microscope at a magnification of 100× (model CKX41; Olympus).

Dose–response study

The extent of cytotoxicity exhibited in the one-dose cytotoxic assay was measured using the 4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) assay (Heiss et al. 1997). Tenfold serial dilutions of the activated proteins (0.01, 0.1, 1, 10, 100 μg/mL) were added to the 96-well plate containing HCT 116, SW480, SW620, SiHa, NIH/3T3, HEK293 and HCEC cells and normal human peripheral blood leukocytes and incubated for 16 h at 37 °C. After incubation, 10 μL of MTT (5 mg/mL) solution was added to each well and the plate was kept in dark for 4 h. The supernatant was then discarded, and 100 μL of DMSO was added to each well to solubilize the formazan product. The absorbance was measured at 570 nm in the VersaMax™ ELISA Microplate

Reader (Molecular Devices, Sunnyvale, CA). The concentration that inflicted 50 % cell death was considered to be the median lethal dose (LC_{50}). Among the isolates screened, the potential strain with noteworthy cytocidal action was selected.

Purification of toxin protein and sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis

The parasporal inclusions of the selected *B.t.*LDC 501 strain were partially purified by the biphasic separation method (Goodman et al. 1967; Mohd-Salleh 1980). Approximately 50 mg of harvested spore-crystal mixture was added to 50 mL of the complete phase (66.8 g/L sodium dextran sulphate, 46.8 g/L PEG 6000, 35 g/L NaCl) in a 250-mL separating funnel, followed by the addition of 50 mL of fresh phase (0.4 g/L sodium dextran sulphate, 70.4 g/L PEG 6000, 17.6 g/L NaCl); the solution was then vigorously shaken for 2 min. After separation of the two phases, the upper phase was removed carefully, and a fresh upper phase was added to the funnel and the process repeated. The number of spores and crystals in the upper and lower phase was calculated by observation under a phase contrast microscope. Finally, both phases were combined and centrifuged at 20000 *g* for 30 min. The viscous pellet was collected and suspended in distilled water and kept at 4 °C overnight. The crystal-rich suspension was lyophilized and reconstituted with the solubilization buffer. The partially purified crystals were activated using proteinase K and purified further by passage through a gel filtration column (Sephacryl S-200; GE Healthcare Bio-Sciences, Pittsburgh, PA) fixed to an AKTA Prime plus purification system (Wipro GE Healthcare Pvt. Ltd., Bangalore, India). The column was equilibrated three times using 20 mM Tris and 150 mM NaCl. The proteinase K-activated protein was loaded onto the column and eluted with the same buffer. Fractions (approx. 1 mL) were collected at a flow rate of 1 mL/min and checked for cytotoxicity against the cancer cell lines (Amano et al. 2005).

The protein profile of *B.t.*LDC 501 was analysed by SDS-PAGE (Laemmli 1970). The solubilized, proteinase K-treated and purified cytotoxic protein fraction samples were resolved in a 5 % stacking gel and a 10 % separating gel at 80–100 V. The protein bands were visualized by silver staining.

Mass spectrometry analysis

To analyse the peptide mixture of the activated proteins of *B.t.*LDC 501 by mass spectrometry (MS), we first resolved the protein samples in a 10 % polyacrylamide gel and stained the resulting bands using Coomassie Brilliant Blue G-250 for 6 h (Köcher et al. 2011). After destaining using distilled water, the protein bands were excised from the gel and subjected to sequencing (Clinbiocare Pvt Ltd, Chennai, India). Individual bands were digested with trypsin and analysed using liquid chromatography–tandem MS (Orbitrap Velos Pro Hybrid Ion Trap–Orbitrap Mass spectrometer, Thermo Fisher Scientific, Waltham, MA). Both fixed (carbamidomethyl) and variable (oxidation of methionine) modifications were considered with two missed cleavages by trypsin. The identities of the proteins were scrutinized by analysing the spectra against non-redundant UniProtKB/Swiss-Prot database using the Proteome Discoverer search engine (Greco et al. 2012). The search was restricted to *Bacillus thuringiensis* and was evaluated based on the score, intensity of peptide matches and molecular weight.

DNA fragmentation assay

To detect the mode of cell death exhibited by the *B.t.*LDC 501 protein, we performed a DNA fragmentation assay (Ahmad et al. 1997). SiHa and HCT 116 cells were seeded onto 12-well tissue culture plates at a density of 2×10^5 cells/mL and incubated for 16 h. After reaching 80 % confluence, 10 μg/mL of *B.t.*LDC 501 proteins was added to each well and the culture plates incubated further for 48 h. The cells were then washed twice with 1× PBS and suspended in cytoplasm extraction buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 0.5 % Triton X-100) on ice for 15 min. The cell pellet was collected by centrifugation at 14000 *g* at 4 °C and treated with DNA lysis buffer (10 mM Tris, pH 7.5, 400 mM NaCl, 1 mM EDTA, 1 % Triton X-100) for 20 min on ice. The collected supernatant was incubated with RNase (final concentration 0.2 mg/mL) at room temperature for overnight digestion, following which proteinase K (0.1 mg/mL) was added and incubated for further 2 h at 37 °C. The DNA of the respective cell lines was extracted with phenol:chloroform (1:1) and precipitated with 1:2 volumes of 95 % ethanol for 2 h at −80 °C. The DNA pellet was air dried and dissolved in 1× TE buffer. The extracted DNA was resolved in a 2 % agarose gel at 50 V/cm, and the bands were observed under a trans-UV illuminator (Gel Doc™ XR+ system; Bio-Rad, Hercules, CA).

Lactate dehydrogenase release assay

Cell lysis was assessed by measuring the amount of cytosolic lactate dehydrogenase (LDH) according to the method of Wang and McCarthy (1997). SiHa and HCT 116 cells at a density of 2×10^5/mL were seeded onto the 96-well cell culture plate and incubated for 16 h, following which the cells were treated with various concentrations (0.01, 0.1, 1, 10, 100 μg/mL) of *B.t.*LDC 501 protein for 16 h. After this last incubation, the plate was centrifuged at 120 *g* for 5 min. The standards used (S1–S6) were prepared as per the manufacturer's instructions (LDH Cytotoxicity Assay kit; Cayman Chemical Company, Ann Arbor, MI) and added to the wells (100 μL per well). The resultant supernatant from the protein-

treated cells and the prepared standards were transferred to a fresh 96-well plate. The LDH reaction solution was added to each well using a repeating pipette, and the plate was incubated at room temperature for 30 min with gentle shaking. Absorbance was read at 490 nm using the VersaMax™ ELISA Microplate Reader (Molecular Devices). LDH activity of the test was calculated by plotting the standard values, and the percentage of cytotoxicity was determined based on the LDH activity of the test, positive and negative control samples.

Whole cell APN inhibition assay

The whole cell APN inhibition assay was carried out to detect the role of APN in the cytopathy of parasporal protein on colon cancer cells. The APN inhibitor [half maximal inhibitory concentration (IC_{50}): 25 μM] (Santa Cruz Biotechnology, Inc., Dallas, TX) was serially diluted from 5 μM to 50 μM concentrations and added to the cells (triplicate samples). After 2 h of incubation, *B.t.*LDC 501 protein (final concentration 10 μg/mL) was added to both APN inhibitor-treated and APN inhibitor-untreated cells. Cells treated with APN inhibitor alone served as the negative control, and BGSC-4Q2 protein-treated cells served as the positive control. The quantum of cell death was observed visually through an inverted microscope (model CKX41; Olympus) after 24 h. The results were analysed by MTT assay as described in the Dose–response study section.

Statistical analysis

Data obtained for the MTT, LDH and APN inhibition assay values were presented as the mean ± standard error of the mean. Significant differences between the groups were tested by one-way analysis of variance using the SigmaStat 2.0 statistical software package (Systat Software Inc., San Jose, CA). When the level of significance was $p \leq 0.05$, then a posterori post hoc pairwise comparison was performed using the Tukey test.

Results

Screening of *B. thuringiensis*

A total of 785 *Bacilli* strains with a fried egg-like appearance were selected out of 1450 isolates from coastal soil samples. Of these, 65 isolates were identified as *B. thuringiensis* based on the presence of parasporal inclusions that distinguishes *B. thuringiensis* from *B. cereus* and *B. anthracis*. Almost 92 % of the isolates produced spherical shaped inclusions (Fig. 1). The frequency of *B. thuringiensis* distribution among the spore-formers was 8.28 % (Table 1).

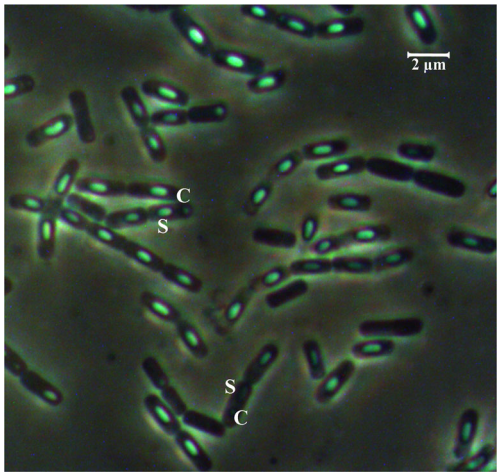

Fig. 1 Phase contrast view of *B.t.*LDC 501 parasporal inclusions. *B.t.*LDC 501 denotes a *Bacillus thuringiensis* strain (LDC 501) with parasporal inclusions that was isolated from the soil samples. *S* Spore, *C* Crystalline inclusion

Haemolytic and insecticidal assay

The haemolytic assay was performed on the proteinase K-activated proteins of the 65 isolates identified as *B. thuringiensis* in order to select the non-haemolytic strains. Strains that induced complete lysis of erythrocytes (matt formation) were considered to be haemolytic strains, whereas those exhibiting no lysis of erythrocytes (button formation) were considered to be non-haemolytic. Among the 65 isolates tested, 31 were non-haemolytic based on the haemolytic assay.

The insecticidal assay revealed that all of the 31 non-haemolytic strains were non-toxic against Dipteran larvae (*Aedes aegypti* and *Culex quinquefasciatus*), while the positive control strain (BGSC-4Q2; Dipteran specific) inflicted 100 % mortality within 1 h of toxin exposure. However, three of the non-haemolytic strains were toxic to Lepidopteran larvae (*Bombyx mori*). The positive control strain (BGSC-4D1; Lepidopteran specific) displayed 90 % mortality within 3 days. Based on these results, we eliminated the three insecticidal strains from further analysis and selected the remaining 28 isolates for the cytotoxic assay.

One-dose toxicity assay

Of the 28 isolates which were determined to be non-haemolytic and non-insecticidal, one strain, denoted as *B.t.*LDC 501, exerted significant cytotoxicity on all of the human colon cancer cell lines tested (HCT 116, SW480, SW620), although it showed less pronounced cytotoxicity on SiHa (human cervical cancer cell line) cells. This toxic action was not apparent in normal human peripheral blood leukocytes, HEK293 and HCEC cells (normal human embryonic kidney cell line and normal human corneal epithelial cell line, respectively) but there was a modest toxicity on NIH/3T3 (normal murine fibroblast cell line)

Table 1 Frequency of *Bacillus thuringiensis* distribution among the soil samples collected at Mandapam, Tamil Nadu, India

Soil sampling site	No. of soil samples tested per site	No. of samples with *B .thuringiensis*	No. of spore-formers obtained	No. of non-clonal *B. thuringiensis* strains
Thittakudi street	4	3	115	8
Sethukarai	4	4	120	9
Kunthukal	4	4	140	10
Vembar beach	5	5	210	21
Pamban Bridge	4	4	200	17
	Total = 21	Total = 20	Total = 785	Total = 65 (8.28 %)

cells. This cytopathic effect was readily observable within 1 h of toxin exposure in HCT 116 and SW480 cells, whereas it took 8 and 16 h for complete cell lysis in SW620 and SiHa cells, respectively. The morphological changes included—in the sequential order reported—cell swelling, detachment from the bottom of the flask, clumping and ultimately formation of cell debris (Fig. 2a, b). The positive control BGSC-4Q2 exerted drastic cell disruption without cell ballooning on all of the cell types tested.

MTT assay

The viability percentage obtained for each cell line after treatment with various protein concentrations is shown in Fig. 2c. The graph shows that the cytotoxic action of *B.t.*LDC 501-activated protein on cancer cell lines was dose-dependent. The LC_{50} of the activated protein on HCT 116, SW480, SW620 and SiHa cells was 1, 1.5, 5 and 10 μg/mL, respectively.

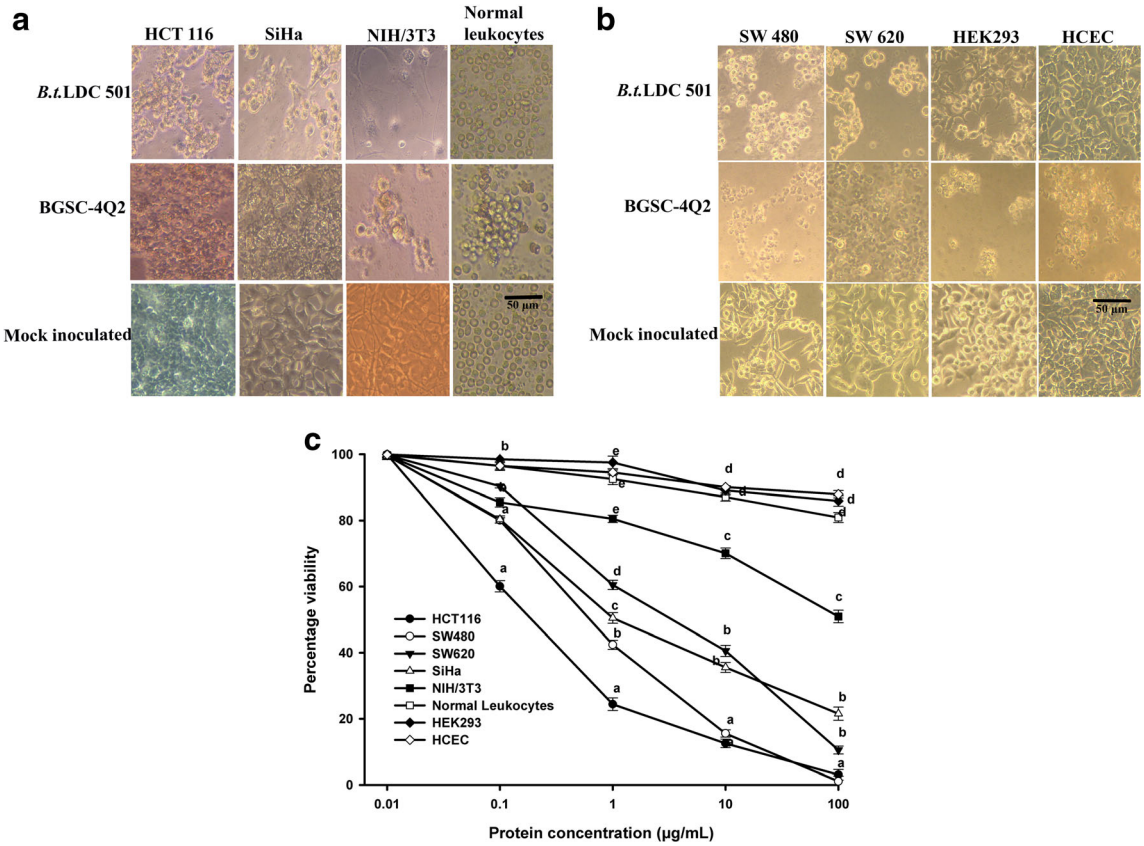

Fig. 2 **a** Cytotoxic effect of activated proteins from *B.t.*LDC 501, reference strain *B. thuringiensis israelensis* (*BGSC-4Q2*) and a mock (*Mock inoculated*; solubilization buffer + proteinase K)-inoculated sample against human cancer cell lines (*SiHa*, *HCT 116*), normal murine fibroblasts (*NIH/3T3*) and human peripheral blood leukocytes. **b** Cytopathic effect of activated proteins from *B.t.*LDC 501, BGSC-4Q2 and mock-inoculated sample against human colon cancer cell lines (*SW480*, *SW620*), normal human embryonic kidney (*HEK293*) and human corneal epithelial (*HCEC*) cell lines. **c** Dose–response curves of cytotoxic effect induced by *B.t.*LDC 501-activated proteins against HCT 116, SW480, SW620, SiHa, NIH/3T3, human peripheral blood leukocytes, HEK293 and HCEC. The level of cytotoxicity was assessed with the MTT assay at 16 h post inoculation. Each point represents mean ± standard error (SE) ($n = 3$). *Different lowercase letters above symbols* indicate statistical significance at $p \leq 0.05$ between the respective groups by the Tukey test

These results indicate that the human colon cancer cells used in this study were more susceptible to the cytotoxic activity of the protein than human cervical cancer cells. Although the highest concentration of the activated protein, i.e. 100 μg/mL, had only a modest toxicity of <30 % against NIH/3T3 cells, no such toxicity was observed on the other normal human cells tested, such as human peripheral blood leukocytes, HEK293 and HCEC cells.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis analysis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) of the alkali-solubilized protein of *B.t.*LDC 501 resulted in the separation of various protein bands, ranging from 5 to 217 kDa, with one major band at 89 kDa (Fig. 3). Proteinase K digestion resulted in the appearance of one prominent band at 66 kDa associated with cell-killing action on cultured cells. The prominent 20-kDa protein fraction partially purified from the column still retained significant cytotoxicity which was more than that of the activated protein mixture. The toxic action of the purified protein on HCT 116 and SiHa cells at different time intervals is shown in Fig. 4.

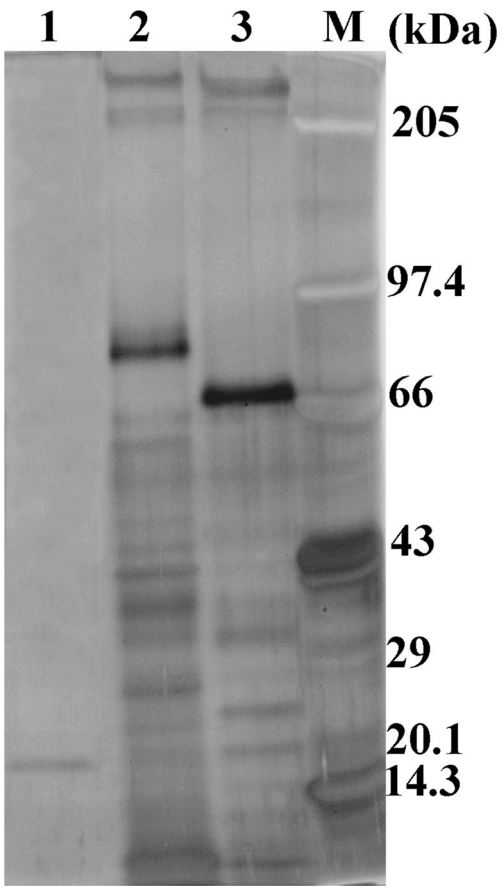

Fig. 3 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis profile analysis of *B.t.*LDC 501 parasporal protein. *Lanes 1* Purified cytotoxic protein fraction, *2* solubilized proteins, *3* proteinase K-activated proteins, *M* molecular weight of standard protein markers

Mass spectrometry analysis

The one-dimensional SDS-PAGE protein fragments of the activated protein mixture were analysed by mass spectrometry and the results are tabulated in Table 2. No similarities to Cry or the parasporin moiety were found. However, the two major peptide fragments of 20 and 66 kDa were homologous to the category of uncharacterized protein under the tumour necrosis factor (TNF)-like domain.

DNA fragmentation assay

The DNA damage induced by *B.t.*LDC 501 protein was analysed in the DNA fragmentation assay. Absence of DNA ladder formation in toxin-treated HCT 116 and SiHa cells confirmed that the cytocidal action was not apoptotic. The cancer cells treated with cisplatin, an anticancer drug inducing DNA fragmentation was used as the positive control (Fig. 5a, b).

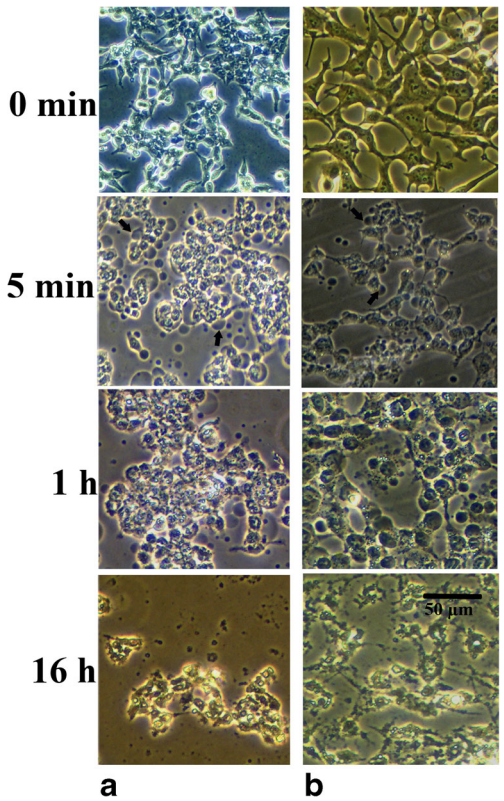

Fig. 4 Cytopathic effect of purified protein on the **a** HCT 116 and **b** SiHa cell lines at different (0 min, 5 min, 1 h, 16 h) time intervals. *Arrows* indicate the blebs formed after toxin exposure

Table 2 Identification of peptides from activated proteins of *B.t.*LDC 501[a] using mass spectrometric analysis

Protein identified	Accession	Domain	Sequence	Observed molecular weight (kDa)	Experimental molecular weight (kDa)	pI	Score	Coverage (%)	No. of peptides identified
Uncharacterized protein	EEM47994.1	TNF-like domains	VLFQNEQFDLANEYNPVTSIFTPK	66	21.5 (trimer)	7.97	84.85	40.31	5
Stage 0 sporulation protein A	AJG78136.1	CheY-like superfamily	VcLVDDNKELVSmLESYVAAQ DDMEVIGTAYNGQEcLNLLK	30	30.7	8.31	20.90	21.01	3
Uncharacterized protein	AJH08277.1	TNF-like domains and C1q domain	NTVNQNVPANTFVK	20	19.9	7.69	2.72	31.46	4

TNF, Tumour necrosis factor

[a] *B.t.*LDC 501 refers to a *Bacillus thuringiensis* strain with parasporal inclusions which was determined to be both non-haemolytic and non-insecticidal and to show cytotoxicity on all of the human colon cancer cell lines tested

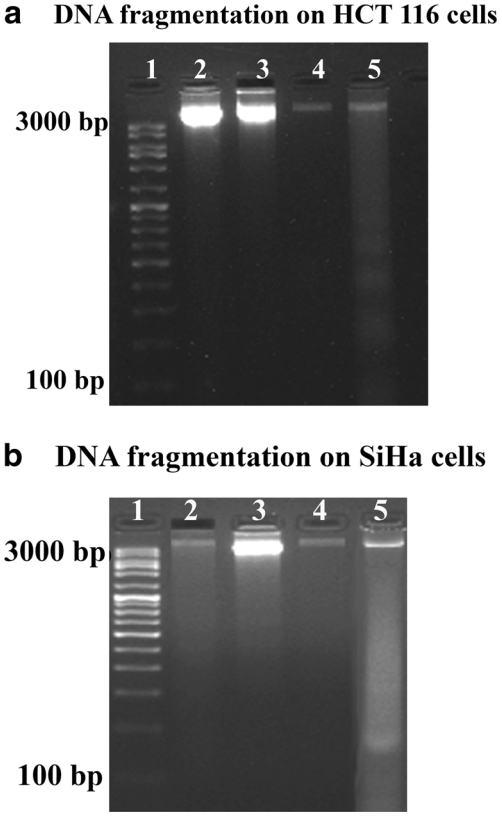

Fig. 5 **a** Agarose gel showing DNA fragmentation analysis of toxin-treated HCT 116 cells. *Lanes*: *1* 100- to 3000-bp DNA marker, *2* *B.t.*LDC 501, *3* BGSC-4Q2, *4* negative control (cells without treatment), *5* positive control (cisplatin treated). **b** Agarose gel showing DNA fragmentation analysis of toxin-treated SiHa cells. *Lanes*: *1* 100- to 3000-bp DNA marker, *2* *B.t.*LDC 501, *3* BGSC-4Q2, *4* negative control (cells without treatment), *5* positive control (cisplatin treated)

LDH release assay

The LDH release assay was performed to study the effect of *B.t.*LDC 501 protein on membrane permeability. The toxin induced prominent LDH leakage in HCT 116 cells but had a lower effect on SiHa cells. The percentage of LDH efflux increased with increasing protein concentration, associated with significant cell death. The anionic detergent Triton X-100, which causes invariable damage to the experimental cell lines, was used as the positive control. The total amount of LDH released after toxin treatment was measured and the percentage of cytotoxicity was tabulated (Table 3).

Whole cell APN inhibition assay

The APN inhibition assay was performed in order to identify the role of APN in the cytotoxicity of *B.t.*LDC 501 parasporal protein on HCT 116 cells. We found that the addition of an APN inhibitor to the HCT 116 cells hindered the cytotoxic action of *B.t.*LDC 501 parasporal protein. There was no detectable cell death in the cells exposed to different

Table 3 Percentage of cytotoxicity based on lactate dehydrogenase release in HCT 116 and SiHa cell lines at various protein concentrations

Sample no.	Cell line[a]	Percentage of cytotoxicity at various protein concentrations				
		0.01 μg/mL	0.1 μg/mL	1 μg/mL	10 μg/mL	100 μg/mL
1.	HCT 116	13.88 ± 1.5	46 ± 0.67	72.21 ± 0.99	89.05 ± 0.67	98.09 ± 0.5
2.	SiHa	3.06 ± 1.56	6.23 ± 0.45	14.41 ± 1.05	38.09 ± 0.69	55.71 ± 0.85

Values in table are presented as the mean ± standard error

[a] HCT 116, Human colon cancer cell line; SiHa, human cervical cancer cell line

concentrations of APN inhibitor and protein (Fig. 6a). This effect was verified by the cell viability assay (Fig. 6b). In contrast, cells exposed to *B.t.*LDC 501 protein without APN inhibitor showed complete cell lysis. The cells treated with proteins from the haemolytic strain BGSC-4Q2 exhibited drastic cell disruption irrespective of the APN inhibitor treatment.

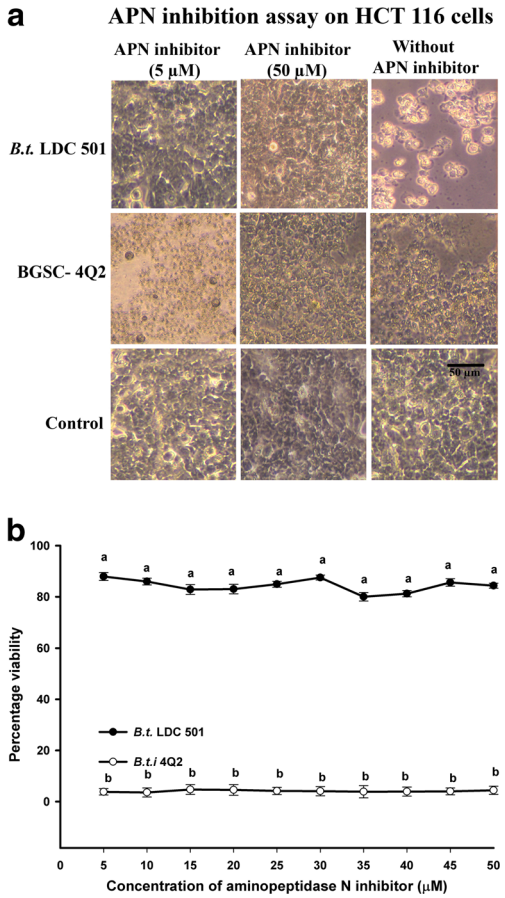

Fig. 6 **a** Inhibition of cytopathic effect of *B.t.*LDC 501 parasporal protein on HCT 116 cells at aminopeptidase N (APN) inhibitor concentrations of 5 and 50 μM. No APN inhibition was observed in cells treated with activated proteins from BGSC-4Q2. **b** Cell viability assay showing approximately 85 % viable HCT 116 cells following treatment with APN inhibitor at various concentrations (5–50 μM) and *B.t.*LDC 501 protein; treated proteins from BGSC-4Q2 displayed only approximately 4 % of cell viability, independent of the APN inhibitor concentration. Each point represents mean ± SE (n = 3). *Different lowercase letters above symbols* indicate statistical significance at $p \leq 0.05$ between the respective groups by the Tukey test

Discussion

In our study we found the distribution frequency of *B. thuringiensis* strains in the marine soil samples to be 8.28 % (Table 1), which is notably higher than that reported in soil samples from Saudi Arabia (0.25 %; El-kersh et al. 2012), India (0.42 %; Poornima et al. 2010), Vietnam (1.6 %; Yasutake et al. 2006) and Syria (4.6 %; Ammouneh et al. 2011). The spherically shaped parasporal inclusion of many isolates (data not shown) was consistent with earlier reports (Poornima et al. 2010). Other groups of inclusion morphology, such as irregular shapes and oval shapes, were also found. We also found a higher proportion of spherical crystals (92 %) compared to that reported in Colombian (88 %; Jara et al. 2006), Vietnamese (58 %; Yasutake et al. 2006) and Saudi Arabian soil isolates (56 %; El-kersh et al. 2012). Our observation suggests the richness of spherically shaped crystals in the strains isolated from soil samples. One of our isolates *B.t.*LDC 501 produced spherical crystals which were much smaller than the spores (Fig. 1).

We eliminated the haemolytic and insecticidal strains from subsequent analyses, selecting only those isolates with non-haemolytic and non-insecticidal activity for further study because these two properties are the characteristic features of earlier reported parasporins (Mizuki et al. 2000; Poornima et al. 2010). Such strains accounted for approximately 45 % of the total *B. thuringiensis* isolates from our coastal soil samples, which is in contrast to previous studies where abundant proportions of non-insecticidal strains were reported (Ohba and Aizawa 1986; Hastowo et al. 1992). Among the 28 non-insecticidal isolates we screened for cytotoxicity, only one strain, which we denoted *B.t.*LDC 501, exhibited cell-killing action on the colon cancer cells tested, such as HCT 116, SW480 and SW620 cells. However, the cell-killing effect of *B.t.*LDC 501 protein was much less profound in the cervical cancer cell line SiHa. It had a slightly toxic effect on the normal murine cell line NIH/3T3 for unknown reasons, but it had no toxic effect on the normal control cell types,

such as human peripheral blood leukocytes, HEK293 and HCEC cell lines (Fig. 2a, b). This result implies that *B.t.*LDC 501 protein is able to discriminate cancer cells from normal ones (Mizuki et al. 1999; Ito et al. 2004).

We observed that the action of activated proteins of *B.t.*LDC 501 action was less pronounced on SW620 cells (5 μg/mL) and more pronounced on SW480 cells (1.5 μg/mL). This disparity may be due to the transformation stage at which the two types of human colon cancer cells were established. It is a known fact that cancer cells acquire more resistant features as they progress from a primary stage to a more aggressive metastatic stage (Hewitt et al. 2000). Among the known PS, PS2, PS4 and PS5 are the only types to have an action similar to that of *B.t.*LDC 501 protein, as evidenced by their toxic effect on colon cancer cell types such as Caco-2 (Kitada et al. 2006; Ekino et al. 2014). Therefore, our results support the notion that *B.t.*LDC 501 protein may have a larger effect on some colon cancer cell types than on others.

It is interesting to note that the cytopathic activity was realized only upon proteinase K digestion of the solubilized proteins extracted from *B.t.*LDC 501. This result is similar to those reported from previous studies on PS and insecticidal Cry proteins where the proteins had to be cleaved to bring about toxicity (Thomas and Ellar 1983; Knowles 1994; Mizuki et al. 2000). In the present study, proteolytic cleavage of the solubilized 89-kDa protein exposed a 66-kDa by-product along with a 20-kDa minor polypeptide (Fig. 3). This feature is similar to that observed for PS-3, where protease digestion of a 81-kDa precursor yielded an active 64-kDa protein (Yamashita et al. 2005).

Due to non-availability of the whole genome sequence of cytotoxic *Bacillus thuringiensis* strains (Huang et al. 2012), peptide fragments resolved by mass spectrometry were searched against general proteins of *B. thuringiensis* strains. As *B.t.*LDC 501 is a non-insecticidal strain, there was no specific hit against Cry and Cyt proteins. Furthermore, the identified peptides did not match with any of the available PS sequences in the database. Our scrutiny of the prominent 66-kDa protein band by mass spectrometry revealed smaller subunits of 21.5-kDa that were joined together; a subsequent homology search revealed that these subunits resembled an uncharacterized protein category belonging to the TNF-like domain (Table 2). It is interesting to note that TNF plays a major role in mediating cytotoxicity through cell-surface receptors. The other smaller peptide of about 20 kDa also showed a high homology to the TNF-like domain, suggesting that the bacteria harbours a large 89-kDa precursor that breaks down into approximately 20-kDa subunits. Li et al. (2012) also obtained an uncharacterized 20-kDa protein in the open reading frame of the CryB1 5′ region during the spore and crystal formation stages. The 30-kDa peptide fragment obtained was scored as stage 0 sporulation protein A, which is essential for spore formation regulatory network (Rang et al. 2015).

The 20-kDa peptide isolated from the column was subjected to detailed characterization using the HCT 116 and SiHa cell lines (Fig. 4). The cytotoxic effect of this fractionated 20-kDa protein on HCT 116 was comparable to that of the 30-kDa PS-2 on the liver cancer cell line HepG2 (Kitada et al. 2009). Following toxin exposure, HCT 116 and SiHa cells formed characteristic blebs within 5 min (Fig. 4). HCT 116 cells were more sensitive to the protein, displaying significant cell clumping and detachment from the surface, whereas these changes were not observed in SiHa cells. These differences in the level of cytotoxicity and mode of action demonstrate the target-specific nature of the protein. Consequently, we performed LDH and DNA fragmentation assays of the protein using HCT116 and SiHa cells in order to gain an understanding of its toxic action at the molecular level.

The absence of apoptotic DNA fragments revealed that the mode of action of the proteins was non-apoptotic in nature, similar to PS-2 (Fig. 5a, b). The increase in LDH release revealed that the toxin damages the plasma membrane of HCT 116 cells to a much higher degree than it does SiHa cells (Table 3). This finding is in accordance with the action of PS-2 on HepG2 cells, which requires GPI-anchored proteins (Kitada et al. 2006, 2009). The clear cytocidal action of the protein on the target HCT 116 cells suggests the presence of a specific receptor. In addition, the target-specific action, non-apoptotic mode of cell death and increase in membrane permeability, similar to that of insecticidal Cry proteins, provided a scheme to investigate the role of APN, a GPI-anchored protein which is present in the most susceptible cell line, HCT116.

In the APN inhibition assay, the toxic effect of *B.t.*LDC 501 protein on HCT 116 cells was blocked by the APN inhibitor (Fig. 6a), suggesting that APN is essential for the cytopathic action of this protein. Katayama et al. (2009) found that the beclin-1 polypeptide inhibitor suppressed the apoptotic cell death caused by PS-1 on HeLa cells, asserting the fact that beclin-1 was the receptor of PS-1. Previous authors had claimed APN as a putative receptor for insecticidal Cry proteins (Knight et al. 1994; Gill et al. 1995; Rajagopal et al. 2003). More recently, the involvement of APN isoforms in the Cry protein toxin action on *Ostrinia nubilalis* was studied (Crava et al. 2013).

The study reported here is the first to delineate the role of APN in mediating the cytotoxic effect of non-insecticidal parasporal proteins on human cancer cells. The mode of toxin action and the requirement of GPI-anchored proteins suggests that *B.t.*LDC 501 protein might be a variant of PS-2, a pore-forming toxin. Earlier studies conducted in our laboratory identified the cytotoxic proteins from *B.t.*LDC 391 strain which immunologically cross-reacted with 81-kDa PS-1 (Poornima et al. 2010). This finding highlights the fact that PS are

distributed widely in diverse geographical locations (Gonzalez et al. 2011). Further studies on immunophenotyping using a labeled ligand or APN antibody could help to verify the expression of APN on the cancer cells. The current investigation provides insight on the receptor involved in the cytotoxic action of parasporal protein. The target-specific action of *B.t.*LDC 501 cytocidal protein might qualify it as a potential therapeutic molecule for human colon carcinoma in future.

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References

Ahmad N, Feyes DK, Agarwal R, Mukhtar H, Nieminen AL (1997) Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. J Natl Cancer Inst 89:1881–1886. doi:10.1093/jnci/89.24.1881

Amano H, Yamagiwa M, Akao T, Mizuki E, Ohba M, Sakai H (2005) A novel 29-kDa crystal protein from *Bacillus thuringiensis* induces caspase activation and cell death of Jurkat T cells. Biosci Biotechnol Biochem 69:2063–2072. doi:10.1271/bbb.69.2063

Ammouneh H, Harba M, Idris E, Makee H (2011) Isolation and characterization of native *Bacillus thuringiensis* isolates from Syrian soil and testing of their insecticidal activities against some insect pests. Turk J Agric For 35:421–431. doi:10.3906/tar-1007-1117

Bernan VS, Greenstein M, Maiese WM (1997) Marine microorganisms as a source of new natural products. Adv Appl Microbiol 43:57–90. doi:10.1016/S0065-2164(08)70223-5

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72:248–254. doi:10.1016/0003-2697(76)90527-3

Butko P (2003) Cytolytic toxin Cyt1A and its mechanism of membrane damage: data and hypotheses. Appl Environ Microbiol 69:2415–2422. doi:10.1128/AEM.69.5.2415-2422.2003

Crava CM, Bel Y, Jakubowska AK, Ferré J, Escriche B (2013) Midgut aminopeptidase N isoforms from Ostrinia nubilalis: Activity characterization and differential binding to Cry1Ab and Cry1Fa proteins from Bacillus thuringiensis. Insect Biochem Mol Biol 43:924–935. doi:10.1016/j.ibmb.2013.07.009

Crickmore N, Zeigler DR, Feitelson J, Schnepf ESCHERICHIA, Van Rie J, Lereclus D, Dean DH (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol Mol Biol Rev 62:807–813, **1092-2172/98/$04.0010**

de Maagd RA, Bravo A, Crickmore N (2001) How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. Trends Genet 17:193–199. doi:10.1016/S0168-9525(01)02237-5

Dykman TR, Cole JL, Iida KYOKO, Atkinson JP (1983) Structural heterogeneity of the C3b/C4b receptor (Cr 1) on human peripheral blood cells. J Exp Med 157:2160–2165. doi:10.1084/jem.157.6.2160

Ekino K, Okumura S, Ishikawa T, Kitada S, Saitoh H, Akao T, Mizuki E (2014) Cloning and characterization of a unique cytotoxic protein parasporin-5 produced by *Bacillus thuringiensis* A1100 Strain. Toxins 6:1882–1895. doi:10.3390/toxins6061882

El-kersh TA, Al-sheikh YA, Al-akeel RA, Alsayed AA (2012) Isolation and characterization of native *Bacillus thuringiensis* isolates from Saudi Arabia. Afr J Biotechnol 11:1924–1938

Gill SS, Cowles EA, Francis V (1995) Identification, isolation, and cloning of a *Bacillus thuringiensis* CryIAc toxin-binding protein from the midgut of the lepidopteran insect *Heliothis virescens*. J Biol Chem 270:27277–27282. doi:10.1074/jbc.270.45.27277

Gonzalez E, Granados JC, Short JD, Ammons DR, Rampersad J (2011) Parasporins from a Caribbean Island: evidence for a globally dispersed *Bacillus thuringiensis* strain. Curr Microbiol 62:1643–1648. doi:10.1007/s00284-011-9905-5

Goodman NS, Gottfried RJ, Rogoff MH (1967) Biphasic system for separation of spores and crystals of *Bacillus thuringiensis*. J Bacteriol 94:485

Greco TM, Miteva Y, Conlon FL, Cristea IM (2012) Complementary proteomic analysis of protein complexes. In: Hoopler S, Vize PD (eds) Xenopus Protocols, 2nd edn. Humana Press, New York, pp 391–407

Hastowo S, Lay BW, Ohba M (1992) Naturally occurring *Bacillus thuringiensis* in Indonesia. J Appl Microbiol 73:108–113. doi:10.1111/j.1365-2672.1992.tb01695.x

Heiss P, Bernatz C, Burchelt G, Senekowitsch-Schimidtke R (1997) Cytotoxic effect of immunoconjugate composed of glucose-oxidase coupled to an anti-ganglioside (G_{D2}) antibody on spheroids. Anticancer Res 17:3177–3178. doi:10.1016/j.biocontrol.2008.08.020

Hewitt RE, McMarlin A, Kleiner D, Wersto R, Martin P, Tsokos M, Stamp GW, Stetler-Stevenson WG (2000) Validation of a model of colon cancer progression. J Pathol 192:446–454

Huang S, Ding X, Sun Y, Yang Q, Xiao X, Cao Z, Xia L (2012) Proteomic analysis of *Bacillus thuringiensis* at different growth phases by using an automated online two-dimensional liquid chromatography-tandem mass spectrometry strategy. Appl Environ Microbiol 78:5270–5279

Ishii T, Ohba M (1993) Diversity of *Bacillus thuringiensis* environmental isolates showing larvicidal activity specific for mosquitoes. J Gen Microbiol 139:2849–2854. doi:10.1099/00221287-139-11-2849

Ito A, Sasaguri Y, Kitada S, Kusaka Y, Kuwano K, Masutomi K, Ohba M (2004) A *Bacillus thuringiensis* crystal protein with selective cytocidal action to human cells. J Biol Chem 279:21282–21286. doi:10.1074/jbc.M401881200

Jara S, Maduell P, Orduz S (2006) Diversity of *Bacillus thuringiensis* strains in the maize and bean phylloplane and their respective soils in Colombia. J Appl Microbiol 101:117–124. doi:10.1111/j.1365-2672.2006.02901.x

Katayama H, Kusaka Y, Mizuki, E (2009) Parasporin-1 receptor and use thereof. U.S. Patent Application 12/935,513. U.S. Patent and Trademark Office, Alexandria, VA

Kitada S, Abe Y, Ito A, Osamu K, Akao T, Mizuki E, Ohba M (2005) Molecular identification and cytocidal action of parasporin, a protein group of novel crystal toxin targeting human cancer cells. In: Proc 6th Pacific Rim Conference on the Biotechnology of *Bacillus thuringiensis* and its Environmental Impact. National Sciences and Engineering Research Council of Canada, Ottawa, pp. 23–27.

Kitada S, Abe Y, Shimada H, Kusaka Y, Matsuo Y, Katayama H, Ito A (2006) Cytocidal actions of parasporin-2, an anti-tumor crystal toxin from *Bacillus thuringiensis*. J Biol Chem 281:26350–26360. doi:10.1074/jbc.M602589200

Kitada S, Abe Y, Maeda T, Shimada H (2009) Parasporin-2 requires GPI-anchored proteins for the efficient cytocidal action to human hepatoma cells. Toxicology 264:80–88. doi:10.1016/j.tox.2009.07.016

Köcher T, Swart R, Mechtler K (2011) Ultra-high-pressure RPLC hyphenated to an LTQ-Orbitrap Velos reveals a linear relation between peak capacity and number of identified peptides. Anal Chem 83: 2699–2704. doi:10.1021/ac103243t

Knight J, Crickmore N, Ellar J (1994) The receptor for *Bacillus thuringiensis* CrylA (c) delta-endotoxin in the brush border membrane of the lepidopteran *Manduca sexta* is aminopeptidase N. Mol Microbiol 11:429–436. doi:10.1111/j.1365-2958.1994.tb00324.x

Knowles BH (1994) Mechanism of action of *Bacillus thuringiensis* insecticidal δ-endotoxins. Adv Insect Physiol 24:275–308. doi:10.1016/S0065-2806(08)60085-5

Laemmli K (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685. doi:10.1007/s00018-003-3072-1

Li X, Ding X, Xia L, Sun Y, Yuan C, Yin J (2012) Proteomic analysis of *Bacillus thuringiensis* strain 4.0718 at different growth phases. Sci World J. doi:10.1100/2012/798739

Mizuki E, Ohba M, Akao T, Yamashita S, Saitoh H, Park S (1999) Unique activity associated with non-insecticidal *Bacillus thuringiensis* parasporal inclusions: in vitro cell-killing action on human cancer cells. J Appl Microbiol 86:477–486. doi:10.1046/j.1365-2672.1999.00692.x

Mizuki E, Park S, Saitoh H, Yamashita S, Akao T, Higuchi K, Ohba M (2000) Parasporin, a human leukemic cell-recognizing parasporal protein of *Bacillus thuringiensis*. Clin Diagn Lab Immunol 7:625–634. doi:10.1128/CDLI.7.4.625-634.2000

Mohd-Salleh MB (1980) Effect of crystals, spores, and exotoxins of six varieties of *Bacillus thuringiensis* on selected corn insects. PhD thesis. Iowa State University of Science and Technology, Ames

Nagamatsu Y, Okamura S, Saitou H, Akao T, Mizuki E (2010) Three cry toxins in two types from *Bacillus thuringiensis* strain M019 preferentially kill human hepatocyte cancer and uterus cervix cancer cells. Biosci Biotechnol Biochem 74:494–498. doi:10.1271/bbb.90615

Ohba M, Aizawa K (1986) Distribution of *Bacillus thuringiensis* in soils of Japan. J Invertebr Pathol 47:277–282. doi:10.1016/0022-2011(86)90097-2

Okumura S, Saitoh H, Wasano N, Katayama H, Higuchi K, Mizuki E, Inouye K (2006) Efficient solubilization, activation, and purification of recombinant Cry45Aa of *Bacillus thuringiensis* expressed as inclusion bodies in *Escherichia coli*. Protein Expr Purif 47:144–151. doi:10.1016/j.pep.2005.10.011

Okumura S, Ohba M, Mizuki E, Crickmore N, Côté JC, Nagamatsu Y, Kitada S, Sakai H, Harata K, Shin T (2010) Parasporin nomenclature. Available at http://parasporin.fitc.pref.fukuoka.jp/

Poornima K, Selvanayagam P, Shenbagarathai R (2010) Identification of native *Bacillus thuringiensis* strain from South India having specific cytocidal activity against cancer cells. J Appl Microbiol 109:348–354. doi:10.1111/j.1365-2672.2010.04697.x

Rajagopal R, Agrawal N, Selvapandiyan A, Sivakumar S, Ahmad S, Bhatnagar RK (2003) Recombinantly expressed isozymic aminopeptidases from *Helicoverpa armigera* midgut display differential interaction with closely related Cry proteins. Biochem J 370:971–978. doi:10.1042/BJ20021741

Rang J, He H, Wang T, Ding X, Zuo M, Quan M, Xia L (2015) Comparative analysis of genomics and proteomics in *Bacillus thuringiensis* 4.0718. PLoS One 10(3):e0119065. doi:10.1371/journal.pone.0119065

Thomas WE, Ellar DJ (1983) *Bacillus thuringiensis* var. *israelensis* crystal delta-endotoxin: effects on insect and mammalian cells in vitro and in vivo. J Cell Sci 60:181–197

Uemori A, Maeda M, Yasutake K, Ohgushi A, Kagoshima K, Mizuki E, Ohba M (2007) Ubiquity of parasporin-1 producers in *Bacillus thuringiensis* natural populations of Japan. Naturwissenschaften 94:34–38. doi:10.1007/s00114-006-0153-7

Wang SW, McCarthy WJ (1997) Cytolytic activity of *Bacillus thuringiensis* CryIC and CryIAc toxins to Spodoptera sp. midgut epithelial cells in vitro. In Vitro Cell Dev Biol Anim 33:315–323. doi:10.1007/s11626-997-0053-y

Yamashita S, Katayama H, Saitoh H, Akao T, Park YS, Mizuki E, Ito A (2005) Typical three-domain Cry proteins of *Bacillus thuringiensis* strain A1462 exhibit cytocidal activity on limited human cancer cells. J Biochem 138:663–672. doi:10.1093/jb/mvi177

Yasutake K, Binh ND, Kagoshima K, Uemori A, Ohgushi A, Maeda M, Mizuki E, Yu M, Ohba M (2006) Occurrence of parasporin-producing *Bacillus thuringiensis* in Vietnam. Can J Microbiol 52: 365–372. doi:10.1139/w05-134