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Overexpression and Cytocidal Activity of Parasporin-1 from *Bacillus thuringiensis* against Human Cancer Cell Lines

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Abstract

Parasporin (PS) is a newly discovered family of cytocidal proteins isolated from *Bacillus thuringiensis* (Bt). Parasporin-1 gene was subcloned into pET-30a expression vector and overexpressed in *Escherichia coli* under the control of the T7 RNA polymerase promoter. The parasporin-1 gene was expressed in the strain *E. coli* BL21 (DE3). The purified, expressed, and trypsin-activated protein exhibited cytotoxic activity against three selected cancer cell lines (Vero, HeLa and Hep G2). Noticeable phenotypic changes, including cell morphology and cell-cell adhesion were observed in the culture after the treatment with recombinant parasporin. Further evidence of apoptosis includes the shrinkage of cells, cell bleb formation and cells detachment in the culture plates. This effect is probably due to the direct effect of activated parasporin on the cell oxidation system of cancer cell (Vero, HeLa and Hep G2). In conclusion, the study demonstrates good cytocidal activity exhibited of the expressed parasporin proteins by reducing the cell viability of the three selected cancer cell lines.

Keywords: Overexpression; Parasporin-1; HeLa; Hep G2; Vero

Introduction

Parasporins (PS) have been identified as Cry bacterial cytocidal protein isolated from *Bacillus thuringiensis* (Bt). Thirteen important PS proteins have been isolated from 11 strains of *Bacillus thuringiensis* (Bt). Of these, eight proteins are allied to PS1, PS2, PS3, PS4, PS5 and PS6. Certainly, a few genealogical relationships exist between the six PS families. The protein parasporin-1Aa1 (PS1Aa1), corresponding to Cry31Aa1, encoded by a gene 2,169 bp long, is a 723 amino acid peptide with a predicted molecular weight of ~82 Da. The PS1Aa1 has a typical three-domain type and contains five highly conserved blocks as that of the insecticidal three-domain Cry proteins. However, PS1Aa1 demonstrates a low sequence homology (<25%) to the existing classes of Cry and Cyt proteins. As reported, PS1Aa1 exhibits cytocidal activity only when digested with proteases; specific proteolytic processing is thus essential for the activation of Cry protoxins [1-5]. Subsequent to protease processing, the ~82 kDa precursor of PS1Aa1 generates an active heterodimer consisting of 15 and 56 kDa polypeptides which are toxic to cancer cells. The activation occurs by N-terminal processing of the ~82 kDa precursor protein. According to Ito et al., PS2Aa1 (Cry46Aa1) is a 338 amino acid peptide with a predicted molecular weight of 37,446 and encoded by 1,014 bp long gene. Unlike PS1Aa1, PS2Aa1 lacks the conserved domains of the insecticidal Cry proteins [6]. Thus, PS2Aa1 shares a low sequence homology only with Cry15Aa among the existing classes of Cry and Cyt proteins of *B. thuringiensis* [7,8]. Furthermore, Cry15Aa is highly homologous to mosquitocidal Mtx2 and Mtx3 proteins from *B. sphaericus* [9,10]. Interestingly, these mosquitocidal proteins show structural similarities with β-barrel-lined membrane pore-forming toxin, aerolysin from *Aeromonas hydrophila* [11-13]. The 37 kDa precursor cleaves into a 30 kDa active form through proteolytic processing at both N- and C-terminus and the activated toxin exhibits high cytotoxicity to cancer cells. PS3Aa1 comprises of 825 amino acid residues with a deduced molecular weight of 93,689 Da and shares low sequence homology with insecticidal Cry proteins. It is worth mentioning that the C-terminal sequence of PS3Aa1 is similar to hemagglutinin HA-33 of *Clostridium botulinum* [14-16]. As with other parasporins, proteolytic processing is indispensable for the toxin activity. The ~84 kDa precursor yields an active 64 kDa cytotoxic form by the proteolytic processing at both N- and C-terminus. The fourth parasporin, PS4Aa1 (Cry45Aa1), encoded by an 828 bp long gene, is a 275 amino acid peptide with a predicted molecular weight of 30 and 78 Da [17]. PS4Aa1 exhibits no homology to the three-domain Cry proteins and is lacking any of the five conserved blocks of the Cry proteins (including PS1Aa1 and PS3Aa1). Moreover, PS4Aa1 shares very low amino acid sequence homology (<30%) to the existing Cry and Cyt proteins of *B. thuringiensis* [18-20].

Anticancer activity of parasporins

The PS proteins exhibited no hemolytic activity against sheep erythrocytes or *in vivo* insecticidal activity. *In vitro* cytotoxicity of PS1Aa1 against cultured insect cells was tested on the two cell lines NIASAeA1-2, derived from the mosquito (*Aedes albopictus*) and BM-N from the silkworm, *Bombyx mori* [1]. The results showed that these insect cells are not susceptible to PS1Aa1. Interestingly, a goldfish (*Carassius auratus*) cell line, GF-Scale, is also not sensitive to the three crude preparations of parasporal proteins containing PS1Aa1, PS2Aa1, and PS3Aa1, respectively [21,22]. The cytotoxicity spectra of the four PS proteins summarize the results obtained from recent investigations [23-26]. It involves 13 human cell lines originating from various tissues, nine from tumors, four non-cancer normal tissues and five non-human cells (two monkey and three rodent cell lines). Overall, the four PS proteins exhibited preferential cytocidal activities against human cancer cells.

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Interestingly, the four non-cancer normal cell lines were resistant to the PS proteins, two of them (UtSMC and MRC5) were sensitive to PS2Aa1 at low to moderate concentrations. PS4Aa1 showed a moderate level of cytotoxicity against PC12, a rat cancer cell line. Marked differences were evident in cytotoxicity spectra and the activity levels among the four PS families. Two low molecular weight proteins, PS2Aa1 and PS4Aa1, showed relatively broad cytotoxicity spectra, each inducing cell death in six out of nine human cancer cell lines. In addition, all the three leukemic cell lines (MOLT-4, HL60 and Jurkat) showed extreme sensitivity to PS2Aa1. PS3Aa1, a typical three domain-type Cry protein, exhibited moderate cytotoxic activities against only two cancer cell lines, HL-60 (leukemia) and HepG2 (liver cancer). Thus, PS3Aa1 showed the narrowest activity spectrum among the four PS proteins. It is also noteworthy that the three cancer cell lines (HeLa, TCS and Jurkat) are monosensitive to one of the three proteins, PS1Aa1, PS2Aa1 or PS4Aa1. The aim of the current study was to overexpress the parasporin in an expression vector and investigate the cytocidal activities of the purified protein against three selected cancer cell lines.

Materials and Methods

Cloning in expression vector and protein expression

A 2.3 kbp fragment of parasporin 1 was subcloned into the pET30a expression vector (Novagen) and overexpressed (≈ 30% of total protein) in *E. coli* BL21 (DE3) (Novagen). A single colony was used to inoculate 5 mL LB medium containing 100 µg/mL ampicillin and then overnight cultures were used to inoculate 100 mL LB broth. The cultures were grown at 37°C with vigorous shaking until the optical density (OD600) reached 0.6 and the same time IPTG (1 mM) was added. The cells were allowed to grow for an additional 4 h and were then harvested by centrifugation at 3000x g for 10 min at 4°C and froze.

Purification of recombinant protein

The protein purification was carried out at a large scale using nickel-nitrilotriacetic acid (Ni-NTA) batch chromatography under denaturing conditions [27]. The cell pellet was resuspended and lysed by sonication and centrifuged at high speed. Then, 4 mL of lysis buffer B (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 8.0) was added to the supernatant. The filtered supernatant was added to a Ni-NTA matrix (50%) and mixed gently for 1 h at room temperature. The lysate-resin mixture was batch-cleaned using 4 mL of washing buffer C (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 6.3) to remove unbound protein and added to a chromatography column. The 6-His-tagged fusion protein was eluted four times with 0.5 mL buffer D (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 5.9) followed by four times with buffer E (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 4.5). Finally, the fractions were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined by the BCA protein assay system (Sigma, St. Louis, MO, USA) using bovine serum albumin as the standard.

SDS-PAGE and western blotting analysis

Protein analysis was performed on 10% SDS-PAGE. Protein samples were reduced by boiling for 5 min in a loading buffer containing 5% β-mercaptoethanol then centrifuged at 10,000x g for 3 min and directly loaded onto the gel. Electrophoresis was performed in vertical sub-cells (Bio-Rad, Hercules, CA, USA) at 80 V using 10% (w/v) resolving gel and 5% stacking gel concentrations of polyacrylamide. Detection was performed with Coomassie brilliant blue R-250 and silver staining. The gels were destained for 3 h in 5% methanol and 7% acetic acid in distilled water [28]. For immunodetection, the resolved proteins were transferred onto a PVDF membrane (Millipore, Billerica, MA, USA) in a semidry blotter (Bio-Rad) for 1 h at constant 100 V using transfer buffer (14.42 g/L glycine, 2.90 g /L Tris base). The membranes were blocked with milk-based blocking buffer (5% w/v non-fat milk, 0.5% Tween 20, 100 mL TBS, pH 7.4) for 1 h at room temperature, then probed with the 6-His-tagged antibody (1:1000 dilution in blocking buffer) indicated primary antibodies for 4 h at room temperature. After extensive washing with TBS (10 mM Tris base, 0.9% NaCl; 50 mL, 15 min per wash), the membranes were incubated with a 1:10,000 dilution of HRP-conjugated goat IgG (Pierce Biotechnology, Rockford, IL) or horse anti-mouse IgG (Cell Signaling, Beverley, MA) at 1:10,000 dilution for 2 h at room temperature and washed with TBS (50 mL, 15 min per wash) again. The alkaline phosphate (100 mM Tris base, 100 mM sodium chloride, 25 mM MgCl2, pH 9.5) activities were determined using a Nitro Blue Tetrazolium (NBT) Tablet Western Blotting Detection Kit (Sigma; Cat. No. N5514). Of an NBT stock solution, 330 mL was mixed with 10 mL of substrate buffer; 33 mL of 5-bromo-4-chloro-3-indolyl phosphate stock solution was then added and incubated for 1-10 min. This substrate system produced an insoluble end product that was determined using an ECL kit from Amersham Pharmacia (Uppsala, Sweden) [29-32]. The solubilized proteins (1.5 mg/mL) were treated with different concentration of trypsin (2, 1.75, 1.5, 1, 0.75, 0.5, 0.25 and 0.1 mg/mL) and examined for cytocidal activity against HeLa, Hep G2 and Vero cell lines.

Anticancer activity and selectivity

Cell culture: The human cells used were HeLa (uterus cervix cancer cell), Vero cell line (lung cancer cell line), and Hep G2 (human liver carcinoma cell line). They were obtained from American Type Culture Collection (ATCC). The cells were cultured and maintained in RPMI1640 or DMEM medium (Lonza) with 10% FBS (Lonza) and 1% penicillin/streptomycin (Lonza). The cells were cultured at 37°C in humid air with 5% CO_2. The cells were given at least two days to attach before refreshing the medium and were passaged at 80-90% confluence. The cells were washed, detached with 0.25% trypsin-EDTA (Lonza) and re-suspended in the complete medium. About 2.5×10^4 cells were plated in triplicate in a 96-well plate (Santa Cruze) and incubated overnight at 37°C in humid air with 5% CO_2. These three cell lines were incubated with eight varying treatment concentrations (5-15 µg/mL) for 24 h at 37°C and the viability of the cells was measured using cell counting kits 8 (CCK-8) (Sigma). The Cell Counting Kit-8 (CCK-8) allows sensitive colorimetric assays for the determination of the number of viable cells in the proliferation and cytotoxicity assays. The level of cytotoxic activity was determined by a cell proliferation test, MTT assay (BioMatik).

Cytotoxicity assays: A Cell Counting Kit-8 (CCK-8) assay (Sigma Chemical Co., St. Louis, MO) was used to measure the cytotoxicity of the eight different treatments on HeLa, Hep G2 and Vero cell line [33]. The cells (5×10^4 cells/mL) were seeded in 96-well plates and treated with eight different treatments over a range of concentrations (5-15 µg/mL). After 24 h, the cells were washed, and the extent of cell growth was assessed using CCK-8 solution (10 µL per well). After incubation for 2 h at 37°C, the absorption at 450 nm of each well was measured using a microplate reader. Cell viability was expressed in percentage normalized to the control (untreated) cells. For each concentration of treatment, mean values of the mean absorbance rates from eight wells were calculated.

MTT assay for cell proliferation: Cell proliferation was determined by MTT assay according to Meky et al. [34]. Approximately, 1×10^4

cells/mL (HeLa cells, Hep G2 and Vero) in their exponential growth phase were seeded in a 96-well polystyrene coated plate and were incubated for 24 h at 37°C in a 5% CO_2 incubator. After 24 h of the incubation, 10 µL of MTT reagent was added to each well and was further incubated for 4 h. Formazan crystals formed after 4 h in each well were dissolved in 150 µL of dimethyl sulfoxide (DMSO) and the plates were read immediately in a microplate reader at 490 nm. The effect of different treatments on the growth inhibition was assessed as the percentage of inhibition in cell growth. The background absorbance of the medium in the absence of cells was subtracted. Percent viability was calculated as [value of drug-treated group (A)/control group (A)] × 100%. Each assessment was performed in triplicate and the results were expressed as the mean (± SEM).

Results

As a first step towards cloning of Parasporin1 gene, the polymerase chain reaction (PCR) based screening was performed for the detection of Parasporin1 in genomic DNA of BT. The parasporin gene sequence was submitted to GenBank (Accession no.: KJ576792). The Parasporin1 gene was cloned into the pGEMT easy vector and followed by the expression in the pET-30a vector. The 84 kDa cloned protein gene was expressed with a high-level expression under the T7 RNA polymerase promoter (Figure 1). Western blot analysis of the protein transferred to the membrane showed that antibody reacted positively with a protein of the expected size, which confirmed that the expressed protein was parasporin (Figure 2A). The specificity of the resultant antiserum was evaluated using protein extracts of purified protein and recombinant *E. coli* (Figure 2B). The antiserum reacted with the protein of about 84 kDa presented in the induced culture of *E. coli* harboring plasmid pET-30a and also with the purified protein. Parasporin was released from the inclusion bodies at pH 10. The remnant insoluble fraction was collected by centrifugation and an aliquot of that was resolved on SDS-PAGE (Figure 2A). Further, the purified protein was activated with various concentrations of trypsin which processed resulted in a major band of about 64 kDa in all the eight treatments (Figure 3).

Anticancer activity and selectivity

The solubilized and the trypsin activate human cancer cell lines, HepG2, HeLa and Vero. HepG2 cells were treated with different doses of 0, 5 and 15 µM for 24 h. Cytotoxicity assays CCK-8 results are reported as relative cell viability (%). All data were normalized to the control group which was considered to be 100%. The proliferation of HepG2, HeLa and Vero cells was inhibited in a dose- and time-dependent manner ($p<0.05$ and $p<0.01$ versus the control group (0 µM) (Table 1 and Figures 4 and 5). Two-way ANOVA followed by Tukey's post hoc test was performed for the analysis (Tables 1-3 and Figures 4 and 5). HeLa, HepG2 and Vero cells were treated with different doses of 0, 5 and 15 µM for 24 h. The cell viability was evaluated by the MTT assay and the results are reported as relative cell viability (%). All data were normalized to the control group which was considered to be 100%. The MTT assay sho wed a dose-dependent effect of protein on cell viability ($P<0.05$ and $P<0.01$ versus the control group (0 µM, two-way ANOVA followed by Tukey's post hoc test (Tables 4-6, Figures 6 and 7). A significant decrease in cell number percentage compared to control after 24 h of the treatment was observed with all the eight different proteins and except for the treatment for number 6 (B), which showed no significant effect in lower doses.

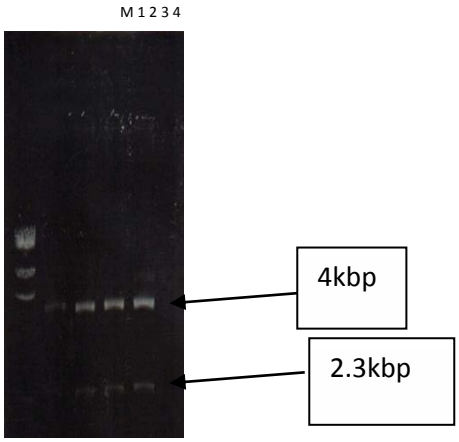


Figure 1: Ethidium bromide stained agarose gel resolving double digestion using BamH1/note 1. Lanes 1, 2, 3 and 4, lane 1: in both A and B are λ hind III marker.

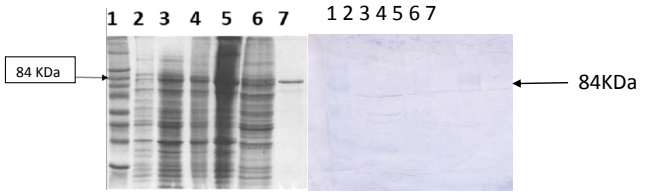


Figure 2: A: Expression and purification of parasporin1. Lane 1: wide range molecular weight protein marker; lanes 2-6: recombinant BL21 (DE3) cells at 0, 1, 2, 4 and 6 h after induction with IPTG; lane 7: affinity-purified parasporin using Ni^{2+} column. B: Immunoblot of parasporin1 protein expressed in *E. coli*. Lanes 2, 3, 4, 5, and 6 are BL21 (DE3) recombinant cells after 0, 1, 2, 4 and 6 h of IPTG induction. Lane 7: affinity-purified parasporin using Ni^{2+} column. Lane 1: broad range protein marker.

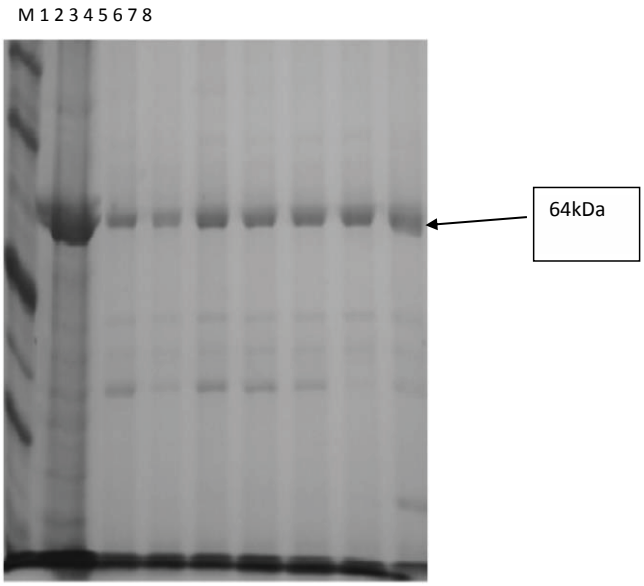


Figure 3: SDS-PAGE after eight different treatments of trypsin, lane 1: 2 mg/mL, lane 2: 1.75 mg/mL, lane 3: 1.5 mg/mL, lane 4: 1 mg/mL, lane 5: 0.75 mg/mL, lane 6: 0.5 mg/mL, lane 7: 0.25 mg/mL, Lane 8: 0.1 mg/mL.

Hep G2 cell line (CCK-8)										
Dose (24 h)		Control (C)	1	2	3	4	5	6	7	8
5 µg/mL	Mean	100.0	74.10	53.69	53.30	96.74	77.68	96.16	57.96	73.23
	SE	0.0	15.08	14.49	10.98	5.029	6.725	12.01	11.38	4.627
	P-value		P>0.05	P<0.05	P<0.05	P>0.05	P>0.05	P>0.05	P<0.05	P>0.05
15 µg/mL	Mean	100.0	31.78	26.14	19.76	51.84	27.29	66.49	17.94	44.33
	SE	0.0	11.32	3.384	2.988	11.35	6.684	12.40	1.435	5.968
	P value		P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	P<0.05	P<0.01	P<0.01

Table 1: Cytotoxicity assays (CCK-8) for Hep G2 cells.

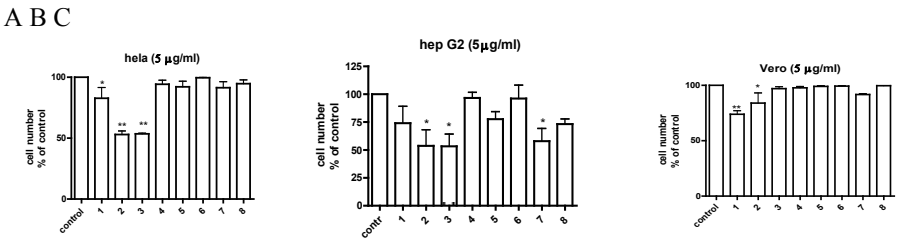


Figure 4: A: There was a significant decrease in Hep G2 cell number percentage, P>0.05 after treatment with 5 µg/mL of no. 2, 3 and 7. The treatment with no. 4 and 6 has no significant effect. B: There was a significant decrease in HeLa cell number percentage, P>0.05 after treatment with 5 µg/mL of no. 1. While no. 2 and 3 produced a significant decrease in HeLa cell number percentage. P>0.01 (almost 50%). The treatment with 4, 5, 6, 7 and 8 had no significant effect. C: There was a significant decrease in Vero cell number percentage, P>0.05 after treatment with 5 µg/mL of no. 2. The treatment with no. 1 resulted in a decrease in cell number percentage, P>0.01. The treatment with no. 3, 4, 5, 6, 7 and 8 had no significant effect.

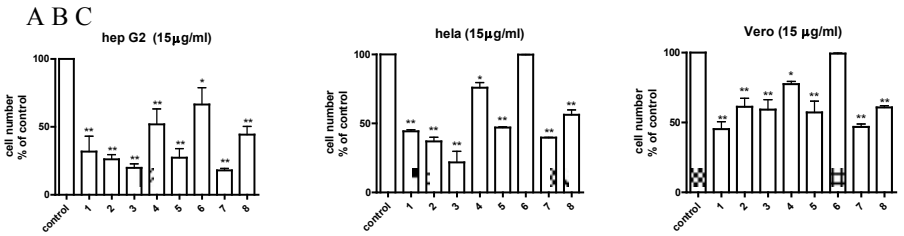


Figure 5: A: There was a significant decrease in Hep G2 cell number percentage. P>0.05 after treatment with 15 µg/mL of no. 6. While the treatment with the rest 15 µg/mL produced significant decrease in Hep G2 cell number percentage, P>0.01 (cell number less than 50%). B: There was a significant decrease in HeLa cell number percentage, P>0.05 after treatment with 15 µg/mL of no. 4. While treatment with all the rest 15 µg/mL produced a significant decrease in HeLa cell number percentage, P>0.01 (cell number less than 50% except no. 8). The treatment with no. 6 had no significant effect. C: There was a significant decrease in Vero cell number percentage, P>0.05 after treatment with 15 µg/mL of no. 4. While the treatment with 1, 2, 3, 5, 7 and 8 at 15 µg/mL produced a significant decrease in Vero cell number percentage, P>0.01 (cell number less than 50% with 1, 5 and 7). The treatment with no. 6 had no significant effect.

HeLa cell line (CCK-8)										
Dose (24 Hrs)		Control (C)	1	2	3	4	5	6	7	8
5 µg/mL	Mean	100.0	82.75	52.94	53.56	94.22	92.14	99.50	91.41	94.74
	SE	0.0	8.792	2.916	0.5200	3.338	4.539	0.2887	4.959	3.038
	P value		P<0.05	P<0.01	P<0.01	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05
15 µg/mL	Mean	100.0	44.31	37.00	21.76	75.89	46.95	99.88	39.71	56.33
	SE	0.0	1.075	2.980	7.947	3.705	0.6298	0.06657	0.1789	3.431
	P value		P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	P>0.05	P<0.01	P<0.01

Table 2: Cytotoxicity assays (CCK-8) for HeLa cells.

Vero cell line (CCK-8)										
Dose(24 h)		Control (C)	1	2	3	4	5	6	7	8
5 µg/mL	Mean	100.0	73.87	83.97	97.00	97.75	99.00	99.25	91.70	99.73
	SE	0.0	3.264	9.257	1.780	1.315	0.7071	0.4787	0.8265	0.1560
	P value		P<0.01	P<0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05
15 µg/mL	Mean	100.0	45.33	61.30	59.19	77.48	57.25	99.25	46.87	60.80
	SE	0.0	5.252	5.988	7.159	2.008	8.067	0.4787	2.073	1.255
	P value		P<0.01	P<0.01	P<0.01	P<0.05	P<0.01	P>0.05	P<0.01	P<0.01

Table 3: Cytotoxicity assays (CCK-8) for Vero cells.

Vero cell line MTT										
Dose (24 h)		Control (C)	1	2	3	4	5	6	7	8
5 µg/mL	Mean	100.0	33.29	63.54	56.12	76.26	67.51	96.26	75.63	84.40
	SE	0.0	0.4340	3.420	0.6584	1.184	5.631	2.202	4.150	4.197
	P value		P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	P<0.05	P<0.01	P<0.01
15 µg/mL	Mean	100.0	10.53	54.19	38.02	39.97	47.24	83.28	38.74	39.94
	SE	0.0	0.3547	0.8028	3.107	0.1154	5.903	0.2492	0.7779	2.285
	P value		P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01

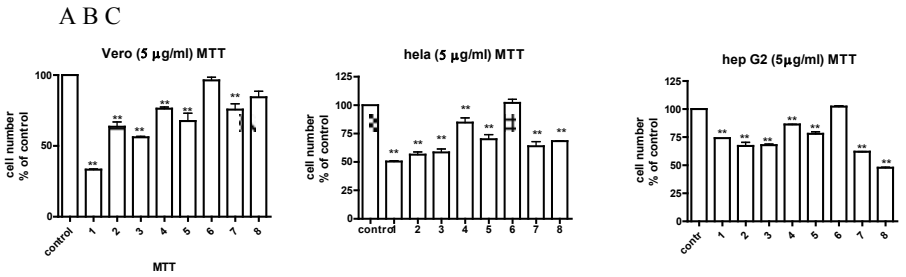
Table 4: Determination of anti-proliferation activity against Vero cells.

HeLa cell line MTT										
Dose (24 Hrs)		Control (C)	1	2	3	4	5	6	7	8
5 µg/ml	Mean	100.0	50.33	56.46	58.35	84.67	70.02	100	63.90	68.40
	SE	0.0	0.8003	2.333	3.156	4.140	4.048	1.104	4.024	0.2328
	P value		P>0.01	P>0.01	P>0.01	P>0.01	P>0.01	P<0.05	P>0.01	P>0.01
15 µg/ml	Mean	100.0	10.53	54.19	38.02	39.97	47.24	83.28	38.74	39.94
	SE	0.0	0.3547	0.8028	3.107	0.1154	5.903	0.2492	0.7779	2.285
	P value		P<0.01	P<0.01	P<0.01	P<0.05	P<0.01	P>0.01	P<0.01	P<0.01

Table 5: Determination of anti-proliferation activity against HeLa cells.

Hep G2 cell line MTT										
Dose (24 h)		Control(C)	1	2	3	4	5	6	7	8
5 µg/mL	Mean	100.0	74.21	67.14	67.81	86.27	78.00	99.3	62.08	47.72
	SE	0.0	0.0051	3.222	1.013	0.091	1.812	0.776	0.0784	0.413
	P value		P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	P>0.05	P<0.01	P<0.01
15 µg/mL	Mean	100.0	64.10	44.50	46.97	55.16	56.37	78.88	44.13	27.96
	SE	0.0	2.968	0.3359	1.544	1.865	1.294	1.658	3.265	1.788
	P value		P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	P<0.05	P<0.01	P<0.01

Table 6: Determination of anti-proliferation activity against Hep G2 cells.



*P<0.05 and ** P<0.01

Figure 6: A: There was a significant decrease in Vero cell number percentage, P>0.01 after treatment with 5 µg/mL of no. 1, 2, 3, 4, 5 and 7. The treatment with no. 6 and 8 had no significant effect. No. 1 produced less than 50%. B: There was a significant decrease in HeLa cell number percentage, P>0.01 after the treatment at 5 µg/mL of no. 1, 2, 3, 4, 5, 7 and 8. No. 2 and 3 produced 50% decrease. The treatment with no. 6 had no significant effect. C: There was a significant decrease in Hep G2 cell number percentage, P>0.01 after treatment with 5 µg/mL of no. 1, 2, 3, 4, 5 7 and 8. No. 8 produced 50% decrease. The treatment with no. 6 had a significant effect.

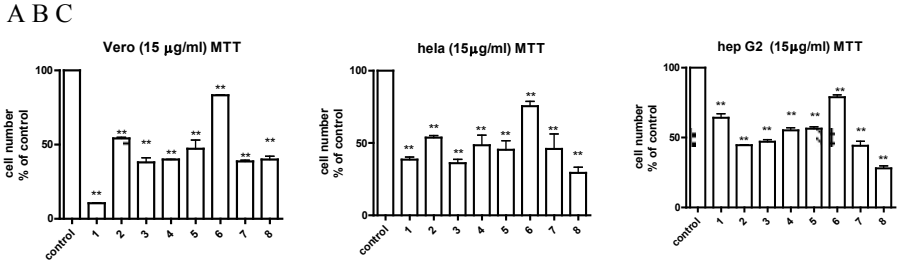


Figure 7: A: The treatment with 1, 2, 3, 4, 5, 6, 7 and 8 at 15 µg/mL produced a significant decrease in Vero cell number percentage. P>0.01 (cell number less than 50% with 2, 3, 4, 5, 7 and 8) While no 1 produced 80% decrease. B: There was a significant decrease in HeLa cell number percentage, P>0.01 after treatment with 15 µg/mL of all of the different treatments. While treatment with the rest at 15 µg/mL produced a significant decrease in HeLa cell (cell number less than 50% except for no. 6). C: There was a significant decrease in Hep G2 cell number percentage P>0.01 after the treatment at 15 µg/mL of all the different types (cell number less than 50% with no. 2, 3 and 7 while 8 produced 70%).

Discussion

The present study demonstrated significant cytotoxic activity against all the three selected cancer cell lines (Vero, HeLa, and HepG2) in a dose-dependent manner. The higher concentrations of the protein showed a more prominent reduction in the cell viability and cytotoxicity. Some treatments, such as number 4 and 6, had a minute effect, while the other produced a greater effect up to 50-80%. In contrast, PS1Aa1 induced cytopathic effects within 8-10 h post-administration [1,35,36]. The expression and crystallization of Cry65Aa require C-terminus, revealing a novel evolutionary strategy of Cry proteins in *B. thuringiensis*. These findings, together with the facts that the four PS proteins are genealogically and structurally unrelated to each other, suggest that the cell-killing mechanism of PS1Aa1 is substantially different from those of the three other PS proteins. Recently, Katayama et al., have observed that unlike the insecticidal Cry proteins, PS1Aa1 is not a membrane pore-forming cytotoxin [37]. This is further supported by the findings such as the absence of lactate dehydrogenase (LDH) release and lack of penetration of propidium iodide in HeLa cells as well as no alteration in the membrane potential of the cells treated with Parasporin-1Aa1. (i) Neither lactate hydrogenase release nor penetration of propidium iodide occurred in PS1Aa1-treated HeLa cells, as well as (ii) there was no alteration in the membrane potential of the cells treated with Parasporin-1Aa1. Parasporin-1 treatment is marked by the overall reduction in the levels of cellular protein and DNA synthesis and rapid increase in intracellular calcium ions. Furthermore, it has been shown that suramin, an inhibitor of the heterotrimeric G-protein signaling, suppresses both Ca^{2+} influx and the cytotoxic activity of PS1Aa1. There is strong evidence that PS1Aa1 induces apoptosis in HeLa cells. Briefly, the cytocidal activity of PS1Aa1 is suppressed by synthetic caspase inhibitors. PS1Aa1 treatment leads to the degradation of apoptosis-related proteins, procaspase-3 and poly (ADP-ribose) polymerase in HeLa cells. Yet another, anti-cancer Cry protein, PS2Aa1, has been intensively investigated in terms of the mechanism of cell-killing activity [24,38]. Unlike PS1Aa1, PS2Aa1 increases plasma membrane permeability of the susceptible cells, most of the cytoplasmic lactate hydrogenase leaks from the intoxicated HepG2 cells, while the extracellular propidium iodide enters the cytoplasm. In addition, PS2Aa1 does not form pores in the mitochondrial membrane and endoplasmic reticula [39-41]. The initial step in the cytocidal action of PS2Aa1 is the specific binding of the toxin to a putative receptor in the lipid raft of the plasma membrane of the susceptible cells followed by the formation of oligomers (>200 kDa) of PS2Aa1 in plasma membranes leading to pore formation and cell lysis. The oligomerization occurs in the presence of membrane proteins, lipid bilayer, and cholesterols. It is noteworthy that PS2Aa1 exhibit a substantial sequence homology with epsilon toxin from *C. perfringens* whose cell-killing mechanism involves the toxin oligomerization in lipid rafts and pore formation in the plasma membrane [42,43]. Relatively little is known about PS3Aa1, which is a typical three-domain-type Cry protein and conformationally closely related to the insecticidal Cry proteins. It may be hypothesized that, by an analogy to insecticidal cry proteins, PS3Aa1 may act as a pore-forming toxin on the plasma membrane of cancer cells. This is supported, in part, by the fact that PS3Aa1 increases plasma membrane permeability of the target cells [44-46]. It is also well known that the insecticidal Cry1A proteins induce cell death through pore formation in plasma membrane after initial binding to the GPI-anchored receptors, aminopeptidases and cadherin-like proteins in the midgut epithelial cells of the susceptible lepidopteran insects [47]. Further, PS4Aa1 is different from the three other parasporins in many aspects. Thus, it is conceivable that the mode of action of this protein also differs from those of the others. At present, however, not much information is available for understanding the mechanism of preferential activity associated with PS4Aa1. PS proteins are also preferentially active on sliced cancer tissues *in vitro*. Recently, Ito et al., reported that PS2Aa1 is selectively cytotoxic to cancer cells but not to non-neoplastic cells, chronic inflammatory cells or blood vessels, when tested on cultured slices of liver and colon cancer tissues prepared immediately after a surgical resection [6]. The results of the present study are in good agreement with the above observations as PS2Aa1 showed an extremely high cytotoxicity against HepG2, a liver cancer cell line; however, it exhibited a little activity against HC, a normal hepatocyte cell line. A similar preferential activity was also found to be associated with PS1Aa1 when tested on tissue slices of colon and liver cancers [48].

Conclusion

The expressed protein parasporin showed a good cytotoxic effect in all the three selected cancer cell lines with moderate cytotoxicity. Noticeable phenotypic changes, including cell morphology and cell–cell adhesion were also observed in the culture after the treatment with parasporin expressed protein. The early stages of apoptosis characterized by the shrinkage of cells, cell bleb formation, and cells detachment in the culture plates were also noted. This is probably a direct effect of parasporin on the cell oxidation system. A significant dose-dependent cytotoxicity of all the eight treatments on all three cancer cell lines (Vero, HeLa, and Hep G2) was observed in the study. Most of the treatments showed 50-80% effect, while treatments 4 and 6 showed very little effect on cell viability. This suggested that the treatments can inhibit the growth and reduce the viability of these cells.

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