Parasporal Cry Protein Parasporin-2 Produced by Bacillus thuringiensis Has in Vitro Toxicity on Human Cancer Cells (HepG2) under the Action of Proteinase K

Xuan Zhou
School of Biochemistry and molecular biology, University of Western Australia, Perth, WA6009, Australia

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Abstract: Liver cancer is one of the malignant tumors with the fastest increasing morbidity and mortality and the greatest threat to people's health and life. This article investigates whether parasporin-2 produced by the hydrolytic non-hemolytic Bacillus thuriensis can recognize liver cancer cells and have cytotoxicity to them. In this paper, Bacillus thurinensis parasporal Cry protein—parasporin genotypes were determined in this strain using the PCR amplification. Then, parasporin-2 was separated by SDS-PAGE and purified. The same concentration of cultured HepG2 (human liver cancer cells) and L-O2 (normal human liver fibroblasts) was added to the plate and divided into two groups. Then, 3 solutions contain parasporin-2 and protease K was added to the plate. The damage to the cells was observed under a microscope and graded the degree of cell damage (CPE), and MTT then determined cytotoxicity (CT). Analysis the SDS-page and following conclusions may be drawn by comparing CPE and CT in each group. First, through horizontal comparison, the data of HepG2 and L-O2 cells in each group were compared, to determine whether parasporin-2 is toxic to liver cancer cells but not to ordinary liver fibroblasts. Besides, longitudinal comparison is the situation of CPE and CT in different groups, whether Parasporin-2 plus Protease K can produce toxicity on HepG2 of liver cancer cells. Bacillus thuringiensis can produce parasporin-2, and after the decomposition of protease K protein, parasporin-2 can produce recognition and cytotoxicity to liver cancer cells. analysis the degree of cell destruction and toxicity, after dealing with the proteinase k, cut parasporin – 2 toxicity is activated. Conclude that parasporin - 2 which hydrolyzed by protease k has a recognition on the liver cancer cells and it's toxic to cancer cells but will not produce toxicity to normal liver cells.

1 INTRODUCTION

Shigetane Ishiwata, a Japanese, first discovered *Bacillus thuringiensis* in Japan in 1901 and described the dying state of the larvae in *Bacillus thuringiensis* (E. Hough, 1989). In 1905, from his experiments, he realized that the poisoning appeared to be caused by a sort of poison and occurred before the multiplication of bacillus. His incomplete identification led to the German Ernst Berliner's first morphologically valid description and the successful isolation and naming of *Bacillus thuringiensis* from Anagasta Kuehniella in the Mediterranean (Sansinenea, 2012).

Bacillus thuringiensis is an aerobic, gram-positive, spore-forming facultative bacterial pathogen. Under conditions of adequate nutrition and environment, spores germinate and then produce vegetative cells, which grow and reproduce by fission and produce a variety of nutrients. Bacterial spores consist mainly of one or more insecticidal proteins in the form of crystalline inclusions, known as insecticidal crystal proteins (ICP) or δ-endotoxin (Kim, 2000). The insecticidal crystal proteins of *Bacillus thuringiensis* mainly consist of CRY crystal protein and CYT cytolytic protein.

Bacillus thuringiensis, as a soil bacterium, is known to be used as a biological insecticide and mosquito control in agriculture and forestry. Today, the use of biological insecticides is one of the most important components of integrated pest management and has been recognized by countries around the world. However, bacillus thuringiensis was shown in 1999 to have a new cytotoxic effect on human cancer cells (Akao, Mizuki, Yamashita, Saitoh, and Ohba, 1999).

Parasporin-2 is a new Cry crystal protein that can be isolated from Bacillus thuringiensis. Through research, the n-terminal region of Parasporin-2 can be

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cleared to effectively activate the toxin activity, while C-terminal digestion can also lead to cell damage (Akiba, 2009); (Kitada, 2006). This paper investigates whether the human liver cells HepG2 can be killed by Parasporin-2— a kind of crystal protein of Bacillus thurinensis in vitro. This study predict that parasporin-2 can induce toxicity on liver cancer cells (HepG2) under the action of protease K.

2 METHODS AND MATERIALS

Bacteria cultivation- The soil isolates of Bacillus subtilis HepG2 were grown on AGAR at 28 ° C for 8 days, and an appropriate amount of beef powder and polypeptide were added, along with a small amount of NaCl, and the PH was maintained at 7.6.

Human cells cultivation- In this study, we used HepG2 (human liver cancer cells) and L-O2 (human normal liver fibroblasts) cell lines and purchased normal T cells in the blood center to separate from lymphocytes. Cells were maintained in RPMI 1640 and 10% fetal BSA and 30uL kanamycin were added at 37℃, and prepare normal human red blood cells (Mizuki, Ohba, Akao, Yamashita, Saitoh, and Park, 1999).

DNA isolation and PCR amplification- PCR was used to test the gene of parasporin-2. Total genomic DNA was used as PCR template by parasporin pure bacterial DNA purification equipment isolated from parasporal protein. Heat circulator was used to prepare a reaction mixture containing 50-100 ng total genome DNA of Bacillus thuringiensis with 19× L PCR buffer (10 mM TRis-HCl). PH value 9.0, 50mm KCl, 1.5mm $MgCl_2$), dNTPs 75×M each, primers 0.2×M each (Table 1), Taq DNA polymerase 1.5U. Template DNA preheated at 94℃ for 2 minutes. Denaturation at 94℃ for 1 min, primer annealing for 45 s, PCR amplification at 72℃ for 1 min. PCR detection was performed for 30 cycles. PCR was analyzed under 1.2% agarose gel, and then stained with UV irradiation (Sansinenea, 2012).

DNA sequencing- PCR products were purified using PCR purification kit (Bolotin, 2016).

Isolation Parasporin from parasporal crystals- Separation spores of Bacillus thuringiensis strains cells with distilled water three times, and broken in distilled water, using two-way separation purification spores by parasposal crystal, the parasposal Cry protein in the 50 mM, ph10 $NaCO_3$ dissolve 1 h, then add 1 mM phenylmethylsulfonyl fluoride and EDTA to stop. The protein solution of PH10.0 was treated with protease K and incubated at 37°C for 90min, and PMSF is used to stop protease digestion. Combining on ion exchange column, with 50 mm NaCl elution toxin protein parasporin 20 mM Tris HCl buffer, in pH 8.0. The active component is then treated and gel filtration (Ito, 2004).

SDS-PAGE AND WESTERN BLOTTING- Membrane-enriched or cytosolic fractions were suspended in SDS sample buffer for SDS-PAGE. The separated proteins were transferred electrophoretically to nitrocellulose membranes, and immunodetection was carried out using antibodies against parasporin-2. Binding of the primary antibody was visualized using a horseradish peroxidase-labeled anti-rabbit parasporin-2 secondary antibody and Lumilight plus. Chemiluminescence need to use FluorS-MultImager.

Cytotoxicity assay and Hemolytic assay- prepare some microplates each containing the same amount and concentration of HEPG2 and L-O2 cell solutions in each well. The measured solution was divided into six groups as below (Table 2), group 1&2 were the negative control group, and the solution was 100% absorbent cell suspension. Group 3&4 was parasporin-2 solution actioned with protease K. Group 4&5 are parasporin-2 solution after separation and purification. At the same time, Drop three solutions, each solution into an HEPG2 plate and an L-O2 plate. Then mark microplates. Measure their absorbance and repeat the experiment more than three times. The degree of CPE was graded based on the proportion of damaged cells (Table 3).

Table 1: Solution and cell composition tables for each group of microplates. Contain (✓).

	Buffer	Parasporin-2	Protease K	HepG2	L-O2
Group 1	✓		✓	✓	
Group 2	✓		✓		✓
Group 3	✓	✓	✓	✓	
Group 4	✓	✓	✓		✓
Group 5	✓	✓		✓	
Group 6	✓	✓			✓

Table 2: The expression of percentage of cells destroyed.

	< 5%	5%-10%	10%-30%	30% -60%	60%-90%
The proportion of damaged cells (CPE)	-	+	++	+++	++++

The experiment was repeated for more than three times. Cytotoxicity was determined by MTT [3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2h Tetrazolium Bromide] (Mizuki, 2000). Each of the microplates contained 90 microliters of cell suspension, each containing a certain number of cells. Measurements were made using a nonradioactive cell test system. The number of cell proliferations was measured after incubation for 16 hours at 37 ° c and 24h after administration. Calculate survival rate. The average absorption properties of the sample had 100% cell survival as a negative control. The degree of cytotoxicity (CT) was graded on the basis of the relative value of absorbance. (Table 4)

Table 3: The expression of the degree of cytotoxicity.

	> 0.70	0.60-0.90	0.30-0.60	0.10-0.30	< 0.10
The degree of cytotoxicity (CT)	-	+	++	+++	++++

Statistical analysis- All of the numerical data were analyzed through one-way ANOVA method All of the mean multiple comparisons were conducted using Tukey's Post Hoc test. P≤0.05 was considered a significant difference. Graphs were prepared using Microsoft Office Excel.

Prime sequence	Annealing (°C)	Gene	Ampli-con size (bp)
F: TAGTCTTTATACCAATTTCCCAACAC	50	*capA*	1178
R: GTGCTTTTAACCGCTTGTGGTAATAC			
F: ATCAAGAATTTTCCGATAATC	50	*ps1*	1136
R: CCAAAAGTGCCAGAATG			
F: TGTTGGGACTGTTCAGTACGT	56	*ps2*	503
R: CGTCACGGTACCTCTTAGTGT			
F: GGAATCCAGGTGCACTGCT	67	*ps3*	701
R: GTCCCGGATCATACGTTGGA			
F: AGTGGTCTCCAGGCTCATACTGG	59	*ps4*	681
R: TGATATTCCCGAACCTGCCC			

Figure 1: Prime sequences of gene capA, ps1, ps2, ps3, ps4 (Moazamian, Bahador, Azarpira, and Rasouli, 2018).

3 RESULTS

3.1 Analyze the PCR Results

PCR is a rapid and highly sensitive method for detecting and identifying Bt genes (Carozzi, Kramer, Warren, Evola, and Koziel, 1991). The efficacy of PCR for cry genes and ps genes identification relies on the alternation of conserved and variable nucleotide regions. Through comparison and inquiry, the existence of parasporin-2 genes in the parasporal protein produced by Bacillus thuringiensis strain was confirmed (Figure 1).

3.2 SDS-PAGE and Western Blot Analysis

Protein gel electrophoresis provides an obvious display of complex protein collections from a biological sample. The gels can be compared with each other to evaluate the similarities and differences between samples. The two-dimensional gel provides separation and information on two important physical properties of protein components in the sample, namely, apparent molecular mass. Western blot was used to isolate and purify parasporin-2 in combination with anti-Parasporin-2 (which can be obtained by injecting antibodies into rabbits). Through SDS analysis, the paracrystal protein produced by Bacillus thuringiensis HepG2 was hydrolyzed after being hydrolyzed by protease K, and the reference strains of SDS-PAGE swimming lane 3-

6 were parasporin-1,2,3,4. (Figure 2) Primary and secondary of parasporin-2 antibodies were detected and imaged by Western Blot using immunofluorescence (Lenina, Naveenkumar, Sozhavendan, Balakrishnan, Balasubramani, and Udayasuriyan, 2014).

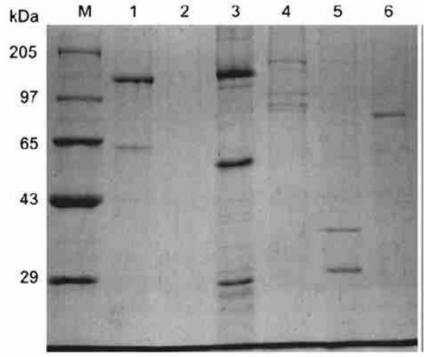

Figure 2. Lanes 3–6 reference strains of parasporin (PS4, PS3, PS2 and PS1) (Lenina, Naveenkumar, Sozhavendan, Balakrishnan, Balasubramani, and Udayasuriyan, 2014).

3.3 Cytotoxicity Analysis

Detect the proportion of HegG2 and L-O2 cells distraction and the degree of cytotoxicity. Each group for possible results corresponds to the groups in *Table 2*.

Table 5 shows all the possible outcomes, but since many of the outcomes were not possible, by making a separate table of the following possible outcomes.

Table 4: All outcomes of cytotoxicity assay and Hemolytic assay.

THE CPR/CT OF GROUP 3				THE CPR/CT OF GROUP 4				THE CPR/CT OF GROUP 5				THE CPR/CT OF GROUP 6				OUTCOMES
CPE	-	CT	++++	CPE	-	CT	++++	CPE	-	CT	++++	CPE	-	CT	++++	OUTCOME 1
												CPE	++++	CT	-	OUTCOME 2
								CPE	++++	CT	-	CPE	-	CT	++++	OUTCOME 3
												CPE	++++	CT	-	OUTCOME 4
				CPE	++++	CT	-	CPE	-	CT	++++	CPE	-	CT	++++	OUTCOME 5
												CPE	++++	CT	-	OUTCOME 6
								CPE	++++	CT	-	CPE	-	CT	++++	OUTCOME 7
												CPE	++++	CT	-	OUTCOME 8
CPE	++++	CT	-	CPE	-	CT	++++	CPE	-	CT	++++	CPE	-	CT	++++	OUTCOME 9
												CPE	++++	CT	-	OUTCOME 10
								CPE	++++	CT	-	CPE	-	CT	++++	OUTCOME 11
												CPE	++++	CT	-	OUTCOME 12
				CPE	++++	CT	-	CPE	-	CT	++++	CPE	-	CT	++++	OUTCOME 13
												CPE	++++	CT	-	OUTCOME 14
								CPE	++++	CT	-	CPE	-	CT	++++	OUTCOME 15
												CPE	++++	CT	-	OUTCOME 16

The possible results are as follows:(1) As the *Table 6* shows, it means group 3&4 have high proportion on cell damage and high degree of cytotoxicity. Group 1,2,5&6 have low proportion on cell damage and low degree of cytotoxicity. (2) As the *Table 7* shows, it means all groups have low proportion on cell damage and low degree of cytotoxicity. (3) As the *Table 8* shows, it means group 1&2 have high proportion on cell damage and high degree of cytotoxicity. Group 3,4,5&6 have low proportion on cell damage and low degree of cytotoxicity, etc.

Table 5

	CPE	CT
Group 1	-	++++
Group 2	-	++++
Group 3	++++	-
Group 4	++++	-
Group 5	-	++++
Grouo 6	-	++++

Table 6

	CEP	CT
Group 1	-	++++
Group 2	-	++++
Group 3	-	++++
Group 4	-	++++
Group 5	-	++++
Grouo 6	-	++++

Table 7

	CEP	CT
Group 1	-	++++
Group 2	-	++++
Group 3	++++	-
Group 4	++++	-
Group 5	++++	-
Grouo 6	++++	-

Table 8

	CEP	CT
Group 1	-	++++
Group 2	-	++++
Group 3	++++	-
Group 4	-	++++
Group 5	-	++++
Grouo 6	-	++++

Table 9

	CEP	CT
Group 1	-	++++
Group 2	-	++++
Group 3	-	++++
Group 4	-	++++
Group 5	++++	-
Grouo 6	-	++++

Table 10

	CEP	CT
Group 1	-	++++
Group 2	-	++++
Group 3	++++	-
Group 4	-	++++
Group 5	++++	-
Grouo 6	-	++++

Table 11

	CEP	CT
Group 1	-	++++
Group 2	-	++++
Group 3	-	++++
Group 4	++++	-
Group 5	-	++++
Grouo 6	++++	-

Table 12

	CEP	CT
Group 1	-	++++
Group 2	-	++++
Group 3	-	++++
Group 4	++++	-
Group 5	++++	-
Grouo 6	++++	-

Table 5-12. The possible results 5-12. The above table reflects the results of Cytotoxicity assay and Hemolytic assay. An increase in the number of + indicates an increase in the value. For example, ++++ indicates that the value is too large, and + indicates that the value is small. And-means that the value is close to 0. CEP stands for proportion of damaged cells, and CT stands for the relative value of absorbance. The step of Cytotoxicity assay and Hemolytic assay should be repeated at least 3 times.

4 CONCLUSION

The morphology of Bacillus thuringiensis spores (cubic, spherical, rhomboid and irregular) was observed by isolation. It indicates the parasporal crystal protein. Through SDS-PAGE analysis and Western Blot, which can determine the existence of parasporin-2. Through the observation of brightfield confocal microscopy and MTT results, the absorbance of solution (which contain parasporin and protease K) were analyzed.

The cells observed under the microscope were compared with *Figure 3 and 4*, and the damage degree of the cells was calculated. The higher the degree of cell destruction, the higher the transmittance measured by MTT, indicating the stronger the cytotoxicity of the solution. The next step is to analyze possible results (*Table 5-12*). For possible result 1, it means parasporin-2 after action with protease K is toxic, and it don't have specific identification of cancer cells. Possible result 2 reflects neither parasporin-2 with protease K nor solutions containing only parasporin-2 are not toxic to cancer cells. In possible result 3, it means parasporin-2 is toxic to liver cells. As to possible result 4, which is closest to my prediction, reflects only after reaction with protease K, parasporin-2 can identify the cancer cells and kill them, but it is no toxic to normal liver cells. Besides, the solution contains only parasporin-2 are not toxic to HepG2 and L-O2. In possible result 5, contrarily with the possible result 4, the group with the solution contains only parasporin-2 has high damaged cells and high cytotoxicity, which means parasporin-2 is toxic and has identification of cancer cells. Possible result 6 reflects that parasporin-2 is toxic to cancer liver cells, whether it reacts with protease K. In possible result 7, which is contrary to the possible result 6. It means parasporin-2 is toxic to normal liver cells, whether it reacts with protease K. As for the possible result 8, parasporin-2 reacts with protease K isn't toxic to HepG2 but is toxic to L-O2, and parasporin-2 is toxic to not only live cancer cells and normal liver cells.

Protease K acts on the C terminal and activates parasporin-2 to identify and produce cytotoxicity on liver cancer cells. Parasporin-2 can specifically bound to the plasma membrane of liver cancer cells. It rapidly increases the membrane permeability, and that it dramatically alters the cytoskeleton and organelle morphologies. Thus, parasporin-2 is a cell discriminating, membrane-targeting, and pore-inducing toxin that subsequently causes irreversible intracellular decay in liver cancer cells. It can be found from other studies that the insecticidal spectra of many strains of Bacillus thuringiensis currently studied are very narrow. For example, cry crystals produced by a hemolytic bacillus thuringiensis studied in Japan produce toxins in only a few genera of the beetle family (Kaur, 2006). Therefore, Bacillus thuringiensis may have tremendous potential for non-insecticidal applications, such as the treatment of human cancers.

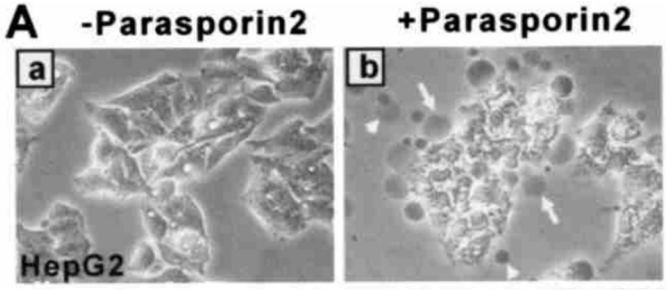

Figure 3: HepG2 cells with parasporin-2 under the brightfield confocal microscopy (S. Kitadam 2006).

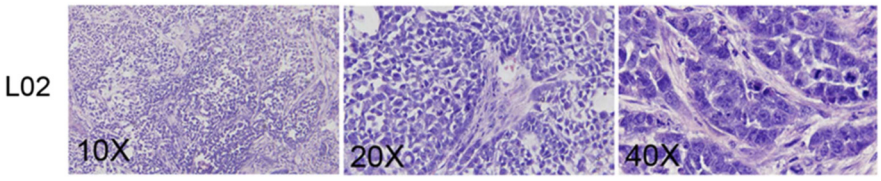

Figure 4: L-O2 cells under the brightfield confocal microscopy (X. Liang, G. Xu, Q. Gao, and X. Tao, 2016).

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