Cytopathic effects of activated parasporal inclusion proteins produced from Iraqi isolates of Bacillus thuringiensis

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Abstract

Six isolates of Bacillus thuringiensis were isolated from Iraqi soil characterized as non- insecticidal and non- hemolytic parasporal inclusion proteins. Bacterial isolates were propagated on nutrient broth. Then, the parasporal inclusion proteins were extracted and processed with proteinase K and trypsin. The major protein segments produced of 64KDa were characterized and tested for cytocidal activity against human leukemic T- cells (CLL) (Chronic lymphoid leukemia). Results indicated that the treated parasporal proteins of four isolates (Bt2, Bt3, Bt4 and Bt6) showed strong cytotoxicity with no significant differences between normal lymphocytes and leukemic lymphocytes. Two isolates BtA1 and BtA5 show discriminative cytotoxicity between normal and leukemic lymphocytes at low extent causing cell ballooning, nucleus fragmentation and finally cell rapture. This may be referring to the ratio of unsaturated fatty acids included in cell membrane.

Key words: B. thuringiensis, hemolytic, activated parasporal protein, cytocidal, leukemic cells.


Introduction

Bacillus thuringiensis is a sporogenic soil bacterium which form a characteristic crystalline inclusion composed of insecticidal crystal protein (Cry), this protein is highly specific for larvae of several insects (Beegle and Yamamoto 1992, Common 1996). Insecticidal Cry protein is highly toxic for number of pest insects belonging to orders of lipedoptera, Hymenoptera, Diptera, Homoptera, Orthopter and Coleoptera. Feitelson et. al. 1992 found strain of B.thuringiensis active against nematods, mites and protozoa. Most strains isolated from natural environment are not known to have biological activity (Ohba and Aizawa 1986, Mizuki, 1999). This may lead to the fact that non-insecticidal strain have biological activities that are not discovered yet. Mizuki et al. (1999) reported unusual property of parasporal inclusions protein of non-insecticidal B. thuringiensis isolated from soil, on human leukemic cell recognizing activity. This protein was named "parasporin". Also Mizuki et al. 2000, discovered new isolates of B.thuringiensis which can specifically kill leukemic cell. Unusual activity associated with non-insecticidal and non-hemolytic parasporal protein, against cancerous cell was produced by B.thuringiensis isolated from soil samples (Yamashita et al. 2000). Parasporin-2, a new crystal protein isolated from B.thuringiesis and characterized as non-insecticidal and non-hemolytic. This protein can recognize and kill human liver and colon cancer cells as well as some cases of human culture cells (Kitata et al. 2006).
The protein (parasporin) is classified into four families; PS1, PS2, PS3 and PS4 according to cytotoxicity spectra and activity level (Ohba et al.2009), however, Brasseur et al. 2015 found that treatment of parasporin-2Aal, isolated from a novel B.thuringiensis strain, with proteinase-K lead to produce molecules specifically cytotoxic to endometrial, liver, breast, colon, cervix, and prostate cancer and do not show toxicity against normal cell. Paraspolire-4 inclusion bodies produced by recombinant Escherichia coli were solubilized and activated with new method and purified by anion-exchange chromatography (Okumura et. al.2008). A Ph.D. thesis by Domanska (2016) investigated the effect of recombinant PS-3 on the human hepatic cancer cell line HePG2 and elucidate its mode of action. 

Parasporins are classified into six groups by the committee of parasporin classification and nomenclature as parasporine 1(PS1).PS2.PS3.PS4.PS5 and PS6 taking the amino acid homology into account (Yilmaz et al.2017). Parasporin -3 structurally related to toxin synthesized by commercially produced tranecgenic insect-resistant plant, with the notable exception of an additional C-terminal β-trefoil ricin domain (Krishnan et al. 2017)

This research conducted to study the cytopathic activity of activated parasporal inclusion protein, produced by Iraqi isolates of B.thuringiensis, on chronic lymphocytic leukemia cells (CLL) in vitro.

**Materials and Methods**

Six isolates of Bacillus thuringiensis (Bt1, Bt2, Bt3, Bt4, Bt5 and Bt6) were isolated from soil samples and characterized biologically. Bioactivity against erythrocytes and insect larvae were tested (Almaadhidi 2010).

**Isolation of parasporal bodies (spore +crystal)**

1- **Zonal rate centrifugation using NaBr:**

The lysed culture was centrifuged at 9000 rpm for 15 minutes at 40°. From the three layers formed, the upper two layers were collected, the final pellet resulted was wished with ddH2O and then resuspended in 15ml ddH2O and sonicated for 2 minute. Different concentration of NaBr (26%-38%) was prepared and used for separation; step gradient was prepared using a Pasteur pipette with syringe. Five ml. of the emulsion was gently added on a top of the gradient and centrifuged at 18000 rpm for 2hrs at 4C°. Bands of crystal and spores were harvested with Pasteur pipette after removing the above layer.

2- **Discontinues sucrose gradient:**

Spores and crystals were recovered by centrifugation at 9000 rpm, and washed once with NaCl (1M) and 2-3 times in ddH2O, free crystal band was syringed, while spores pellet was discarded. After washing by water, crystals were lyophilized for long storage period.

**Toxin activation**

Harvested crystals were solubilized in SME buffer at 37c° for one hour and adjusted to pH 8 by 1M HCl. Soluble crystals were divided into two sets , each contain 1 mg of protein. First set treated with proteinase K, the second set was treated with Trypsin. After incubation at 37C° for 1.5hrs, 0.1 mg/ml PMSF solution was added to stop the proteolytic process. Activated protein become ready for further examination, including different fragments which used for cytopathic experiments.
Hemolytic assay (Koni and Ellar, 1994):
Ten ml of heparinized erythrocytes were washed twice in TBS buffer and resuspended in equal volume of the same buffer, sample of 0.5 ml erythrocytes suspension was added to 1.0 ml of serially diluted alkali solubilized toxin or purified activated parasporal protein fragment, then incubated at 37°C for 45 min, and centrifuged at 2500 rpm for 2 min. Hemoglobin released was measure at 540 nm absorbance. The 50% hemolytic dose (HD50) was defined as the amount of protein needed to release half of hemoglobin from erythrocytes (Pfannenstiel et al. 1984).

Isolation of lymphocytes:
One volume of heparinized (10 IU/ml) blood sample was diluted either with equal volume of TBS or equal volume of RPMI 1640 and then placed slowly and carefully to avoid disturbances of blood layer on top of two volume of lymphofloat 4000 solution in new blood collection tube (silicon coated tube), and centrifuged for 25 min. at 450 g. Viable lymphocytes accumulated as a white turbid layer on top of the lymphofloat layer, erythrocytes and dead cells sediment to the bottom of the tube. Lymphocytes was transferred to fresh tube using positive pipette, then diluted in half volume of TBS or RPMI 1640, centrifuged at 250 g, supernatant was discharged, while the pellet was resuspended in equal volume of TBS or RPMI 1640 in fresh tube and used immediately for dose-respond or cytotoxicity tests.

Dose-response:
Cell suspension (90µl) containing $2 \times 10^4$ cells was delivered to each well micro test plate and pre-incubation at 37°C for 10 hours. The well then received 10 µl of the sample solution. The proteinase-K (30 µg/ml) activated crystal protein or trypsin (30µg/ml) activated crystal protein were prepared in 10-fold serial dilution with the same solubilizing buffer (Na2Co3 containing β-mercaptoethanol and EDTA). After incubation with activated crystal protein, cells were examined at adequate intervals for 24 hours, for the ability to uptake trypan blue stain. Stained cells were counted using hemocytometers chambers. (All cell dilutions were prepared in PBS or TBS). Five wells were used for each dilution, the test was repeated three times.

Results and Discussion
Crystalline protein (δ-endotoxin) produced by B. thuringiæsis are inert (not active) in solid phase (Delecluse et al. 1991). The two methods used for isolation of parasporal crystal showed the sodium bromide gradient (NaBr) proved to be very suitable method for isolation parasporal bodies from spores and cellular debris (Fig.1).

Figure 1: Isolation of Parasporal crystals using sodium bromide (A) and sucrose gradients (B). Red arrow represents parasporal crystals, Blue arrow represents spores layer, and Black arrow represents cell debris. Thus, preparations of parasporal crystals by this method contained virtually no spores and were relatively free of cell wall. Also, NaBr has the ability to inhibit the proteolytic protease activity which
giving another important criterion makes NaBr method efficient in protection of crystals against
digestion protease released during cell lyses (Ang and Nickerson, 1978). In comparison, sucrose
gradient seems to be less efficient in segregation of spores away from crystals (Fig. 2).

Figure 2: SDS-PAGE analysis of total crystal protein. S, standard proteins. Molecular weights of
standard proteins were: A-globulin (150 KD), Ova-transferrin (80 KD), Pepsin (67 KD), albumin (50
KD), Trypsin (20 KD), lysozyme (14 KD) 1, BtA2; 2, BtA3; 3, BtA1; 4, BtA5; 5, BtA4; 6, BtA6.

Determination of dose response (EC50) is important for evaluation of toxins fragments effects
produced by BtA isolates after activation of parasporal protein with proteinase-K. Intact protein
produced by six isolates used in this study was tested for lysing erythrocytes and insect larvicide.
Result showed that only BTA2 and BTA3 had hemolytic activity and larvicideal, while the other had no
effects (table 1).

**Table 1: effect of intact parasporal protein produced by B. thuringinesis isolate on lysing of
erythrocyte and killing insect larvae.**

<table>
<thead>
<tr>
<th>B. thuringinesis isolates</th>
<th>Hemolytic Activity</th>
<th>Activity against insect larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sheep erythrocyte</td>
<td>Human erythrocyte</td>
</tr>
<tr>
<td>BtA1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>BtA2</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>BtA3</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>BtA4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BtA5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BtA6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Quantitative measurement of effective dose and potency of protein for hemolyzing erythrocytes was
measured after solubilizing in four different buffers (table 2). Also, results showed that, Bt2 and Bt3
were effective at low level, and the other four strain showed negative results. The intact parasporal
proteins have mild or no bio-activity unless be activated (Clairmont et al. 1998). Activation occurs
normally in insect midgut, mediate by protease (deMaagd et. al. 2001), or induced by treatment with
insect gut extract (Crickmore et al. 1995) or by protease treatment (Ohba et al. 2009). Parasporal
proteins from all six BTA isolates were treated with proteinase K at different concentration to
investigate the ability in lying erythrocytes and determine the optimal protease concentration to be used in further experiments.

Table 2: Potency and effective dose of intact parasporal protein produced by six isolates of *B.thuringensis* on lysing erythrocytes after Solubilizing in four buffers

<table>
<thead>
<tr>
<th>Solubilization buffer</th>
<th>Hemolytic activity (HD₅₀)</th>
<th>BtA1 protein</th>
<th>BtA2 protein</th>
<th>BtA3 protein</th>
<th>BtA4 protein</th>
<th>BtA5 protein</th>
<th>BtA6 protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM NaOH, 1mM EDTA (pH10.5)</td>
<td>ND</td>
<td>2.5±0.2</td>
<td>2.4±0.4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>50 mM Na₂CO₃, 1mM EDTA, β-mercaptoethanol (pH10.5)</td>
<td>ND</td>
<td>1.5±0.1</td>
<td>1.3±0.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>50 mM Na₃CO₃ (pH10.5)</td>
<td>ND</td>
<td>2.4±0.2</td>
<td>3.3±0.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>50 mM Na₂HPO₄ 50 mMKH₂PO₃ (pH10.5)</td>
<td>ND</td>
<td>2.3±0.5</td>
<td>3.1±0.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Results shows that two isolates BTA4 and BTA6 have the ability to lyses human red cells and the isolates BTA2, BTA3 showed weak hemolytic activity even before activation and became more active after activation with proteinase K, while BTA1 and BTA5 have no hemolytic activity against red cells and the optimal concentration of proteinase -K for activation was 0.03mg/ml. (table3).

Table 3: Hemolytic activity of proteinase-K activated parasporal protein produced by *B.thuringensis* isolates.

<table>
<thead>
<tr>
<th>Proteinase K mg/ dl</th>
<th>Hemolytic activity (HD₅₀)</th>
<th>BtA1 toxin</th>
<th>BtA2 toxin</th>
<th>BtA3 toxin</th>
<th>BtA4 toxin</th>
<th>BtA5 toxin</th>
<th>BtA6 toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.003</td>
<td>ND</td>
<td>1.1±0.4</td>
<td>1.9±0.2</td>
<td>2.9±0.2</td>
<td>ND</td>
<td>1.2±0.2</td>
<td></td>
</tr>
<tr>
<td>0.03</td>
<td>ND</td>
<td>0.4±0.5</td>
<td>0.7±0.1</td>
<td>1.5±0.1</td>
<td>ND</td>
<td>0.5±0.1</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>ND</td>
<td>0.7±0.3</td>
<td>1.4±0.3</td>
<td>1.8±0.1</td>
<td>ND</td>
<td>0.8±0.1</td>
<td></td>
</tr>
</tbody>
</table>

Brasseur *et al.* (2015) found that proteinase-K activated parasporin-2Aal protein isolated from a novel *B.thuringensis* 4R2, strain was particularly cytotoxic to breast, endometrial, cervix, liver, colon and prostate cancer, and doesn’t show toxicity against normal cells. When the parasporal protein activated with trypsin, the same as in proteinase-k activation was made using the same optimal concentration (0.03 mg/ml). Parasporal protein structure for most strains made of different polypeptide (Waalwijk, *et al.*1985). SDS-page analysis of total crystal proteins for six Bt. strains showed different patterns and distance of migration in the gel (Fig.2). Protein of isolates BtA2, BtA3, BtA4 and BtA6 have a minor
band of about =-68KD, Bt1 and Bt5 contains major band with molecular weight of about =-180KD, which is very high in compare with major bands of other isolates (table 4).

Table 4: SDS-PAGE molecular weights analysis of total digested parasporal protein fragments of B.thuringiesis isolates.

<table>
<thead>
<tr>
<th>Fragments M. Wt.</th>
<th>Digested parasporal protein of B.thuringiesis isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
</tr>
<tr>
<td>150.000</td>
<td>169.091</td>
</tr>
<tr>
<td>80.000</td>
<td>142.275</td>
</tr>
<tr>
<td>67.000</td>
<td>93.917</td>
</tr>
<tr>
<td>50.000</td>
<td>77.480</td>
</tr>
<tr>
<td>20.000</td>
<td>71.102</td>
</tr>
<tr>
<td>14.000</td>
<td>63.200</td>
</tr>
</tbody>
</table>

Molecular weights calculated in KD, using Bioprofile program. S- Standard proteins, 1-BtA2, 2-BtA3, 3-BtA1, 4-BtA5, 5-BtA4, 6-BtA6.

The strains which produced toxin active against lepidoptera all contained a major band migration at an apparent molecular weight of 135KD (Tyell et al.1981), while the non-insecticidal activity of protein have a molecular weight of about 180Kd (Yamashita et. al. 2000, Lee et. al. 2001). Parasporin exhibit cytocidal activity at low molecular weight when degraded by proteinases into smaller molecules of 40 to 60 KD. Trypsin and proteinease- K activated parasporin express strong cytocidal effect against human leukemic T cells and uterus cervix cancers cells (Mizuki et al. 2000, Almas et al. 2015).

BtA1 and BtA5 showed the same pattern of effects on Hep2 and CLL cancerous cells. The effective concentration (EC50) values of BtA1 protein were 100, 40 and 10 µg/ml for normal lymphocytes, Hep2 and CLL cells respectively, which means that normal lymphocytes were more resistant than HeP2 and CLL cells (table 5).

Table 5: effective concentration (EC50) of activated parasporal protein produced by B. thuringiesis on normal and cancer cells.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>EC50µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal cells</td>
</tr>
<tr>
<td>BtA1</td>
<td>100</td>
</tr>
<tr>
<td>BtA2</td>
<td>0.28</td>
</tr>
<tr>
<td>BtA3</td>
<td>1.3</td>
</tr>
<tr>
<td>BtA4</td>
<td>1.4</td>
</tr>
</tbody>
</table>
This finding indicates that this protein can discriminate (to a certain extent) between normal lymphocytes and HeP2 and CLL cells. The other four isolates (BtA2, BtA3, BtA4 and BtA6) proteins showed cytocidal activity and hemolytic activity and not discriminates between normal and cancerous cells (table 5). No remarks was appears on normal lymphocytes as a results of treatment with activated parasporal proteins of BtA1 or BtA5 (Fig.3A). However, activated parasporal protein of BtA2 and BtA3 showed extensive lytic effect on normal lymphocytes (Fig. 3 B). While, treatment of cancerous cell (CLL) with either BtA1 or BtA5 activated parasporal proteins showed remarkable cytopathic effect representing by granulation of cytoplasm, nucleus regeneration and obvious cell ballooning (Fig. 3C).

Figure 3: Normal human lymphocyte and CLL cancerous cell treated with activated parasporal protein produced by isolates of B. thuringiessis. A- Normal human lymphocytes treated for 12 hours with activated parasporal protein of BtA1 and BtA5. B- Normal human lymphocytes treated for one hours either activated parasporal protein of BtA2, 3, 4 or 6. C- CLL cancerous cells treated for 12 hours with either activated parasporal protein of BtA1 or BtA5. D- Cell lyses and ruptured of CLL cancerous cells treated for 12 hours with activated parasporal protein of BtA2, 3, 4 and 6.

The appearance of cytopathic effect on CLL cells depending on type of isolates used, it takes 12 hour when BtA1 and BtA5 used, while it take half to one hour period for BtA2 and BtA3. No discrimination in cytopathic effect was found between normal and cancerous cell when treated with parasporal protein of isolates BtA2, BtA3, BtA4 and BtA6. These proteins cause drastic or partial lyses and rupturing of cells and no recognition of cytoplasmic granulation or cell ballooning as shown when treated with BtA1 and BtA5 (Fig3D). The appearance symptom on normal and cancerous cells as a results of treatment with parasporal protein of BtA1 and BtA5 resembling what had found by many researchers, Okumura et al. (2011) studied the mode of anticancer action of parasporal protein, cell swelling and nuclear shrinkage were induced and the ballooned cells burst within 24 hrs. This appearance was found in this research within 12hours (Fig. 3C). Cell necrosis - like cytotoxicity against MOLT- 4 cells and the cytopathic effect was characterized by cell swelling (Lee et al. 2001). Microscopic observation indicate that the cell death was accompanied by coloid - osmotic swelling and cell lysis and other
mechanism (Namba et al. 2003). Also, Krishnan et al. (2017) found that the toxin induce a rapid and significant decrease in metabolic activity, depletion of adenosine triphosphate (ATP) and cell swelling with membrane damage. Mizuki et al. (2001) found as a results of screening 1744 B. Thuringiesis strains, parasporal protein has highly cytocidal effect against leukemia T-cells and other human cancer cells and can able to differentiate between leukemic and normal cells, particularly killing the former cells. These findings may lead to the use of B. thuringiesis inclusion proteins for medical purposes. Also, another result showed the toxin induce pore formation in artificial and biological membranes, cell swelling, rapid decrease in ATP level, irreparable membrane damage, and drop in metabolic activity (Domanska 2016). Parasporin- 2Aa1 is a selection cytotoxic protein that induces apoptosis in various human cell lines from diverse tissue (Brasseur et. al.2015), also, PS1Aa1 kills cancer cells through apoptosis and rapid increase of the intracellular Ca+2 concentration as a results of pore formation in plasma membrane (Ohba et al. 2009). Finally, commercial production can utilized through genetic engineering by recombination Escherichia coli with gene responsible for parasporin - 4 production (Okumura et al.2008).

References

- Kitata S, Abe Y, Shimada H, Kusaka Y, Matsuo Y, Katayama H, Okumura S, Akao T,

