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**RESEARCH ARTICLE**

# ENZYME SPECIFICITY AND CYTOTOXICITY OF HIGHLY PURIFIED ASPARAGINASE ENZYME OF FROZEN SEEDS OF *PISUM SATIVUM VAR JOF. AGAINST L20B TUMER CELL LINE*

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**ABSTRACT**

Purified asparaginase that's highly purified extracted from frozen plant seeds of *Pisam sativum var J of.* were used to determine the specificity of enzyme against different substrates. Enzyme specificity was 783.1, 28.1, 11.4, and 13.7 U/mg of L-aspargine, L-aspartic acid, L-glutamine, and L-glutamic acid respectively. The cytotoxicity effect of purified asparaginase of frozen seeds of *Pizum sativumvar J of.* against L20B tumor cell line measured at wave length of 450, 492 and 620nm respectively, it showed that's purified asparaginase has maximum inhibitory effect on tumor cell line reached to 33, 33, and 23% growth at 450, 492 and 620nm respectively. Cell survival and remaining activity of L20B tumor cell line after treatment with purified asparaginase reduced to 68, 65 and 77% respectively after incubation with purified asparaginase at concentration of 150 µg/ml, while the cell line remaining activity percentage reduced to 66.6, 67.7 and 76µg/ml respectively.



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## INTRODUCTION

The enzyme purified asparaginase (E.C.3.5.l.l) an aminohydrolase catalyses asparagine hydrolysis to yield L-aspartate and ammonia (Borek and Jaskolski, 2001). Purified asparaginase is a widely distributed enzyme and present in plant, animal tissue and microorganisms including bacteria, yeast and fungi (Siddalingeshwara and Lingappa, 2011). In general, plant enzymes are relatively more stable at wide range of pH and temperature than corresponding enzyme derived from microorganisms and animals. In recent years, purified asparaginase has attracted much attention in both pharmaceutical and food industrial applications (Taeymans *et al*., 2005). Purified asparaginase is a therapeutically important protein used in combination with other drugs in the treatment of acute lymphocytic leukemia (Tabandeh and Aminlari, 2009; Sunitha *et al*., 2010). On the other hand, one of the properties of enzymes that makes them so important as a diagnostic and research tools is the specificity they exhibit relative to the reactions they catalyze (Daniel *et al*., 2010). It showed that potassium dependant purified asparaginase is strictly specific for L-asparagine. While slight specificity has been showed for potassium-independent enzyme from the *A. theliana* plant (Bruneau *et al*., 2006). Modern clinical treatments of childhood acute lymphoblastic leukemia (ALL) employ enzyme-based methods for depletion of blood asparagine in combination with standard chemotherapeutic agents (Richards and Kilberg, 2006). In human, acute lymphoblastic leukemia cell lines have been markedly inhibited by purified asparaginase as the cell cycle arrest in G1 phases (Ueno *et al*., 1997). The aim of the study is the determination of the enzyme specificity and cytotoxicity of highly purified asparaginase from frozen source of plant seeds *(Pisum sativum)* instead of fresh sources.

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## MATERIALS AND METHODS

### Determination of Enzyme specificity

The specificity of purified asparaginase against different substrates was studied by incubating the purified enzyme with L-asparagine, L-aspartic acid, L-glutamine and L-glutamic acids at 200mM, pH 8.0 at optimum temperature for the activity for 30 minutes. Enzyme activity was then assayed after each treatment.

### Antitumor activity of purified asparaginase

An in vitro study was conducted to investigate the antitumor activity of purified asparaginase enzyme on tumor cell line (L20B), (Freshney, 1994) as follows:

### Subculture of L20B tumor cell line

Cell suspension of L20B tumor cell line was prepared by treating 25ml of cell culture with 2ml of trypsin-versine solution after the formation of monolayer surface of cell suspension, 20 ml of growth medium which was supplemented with 10% fetal bovine serum was added to inactivate trypsin activity. After that the viability of the cultured cells were counted by using trypan blue. The viability should be more than 95%, then cell suspension was mixed gently and transferred into a micro titer plate (200 µl/well). Each well must be containing 1x105 cell/well. Plates were then incubated at 37°C until 60-70% confluence of the internal surface area of the well for L20B cell lines (Toolan, 1954; Freshney, 1994).

### Detection of Cell Growth Inhibition

To detect the growth inhibition of tumor cell line L20B, culture of this cell line was incubated with different concentrations 150, 75, 37.5, 18.75, 9.37, 4.68, 2.34 and 1.17µg/ml of purified asparaginase in six times duplicate for each treatment. Negative control was achieved by incubating culture of cell line with maintenance medium and serum, then plates were incubated at 37°C in an incubator supplemented with 5% $CO_2$ for 48 hours. After elapsing the incubation period, 50 µl of neutral red was added to each treatment (50 µl/well) and re-incubated for 2 hours. After incubation, cell culture in each plate was washed three times with phosphate buffer saline then 100 µl of destaining buffer solution was added to each well to remove the excess dye from viable cells. Optical density of each well was measured by using ELISA reader at a transmitting wave length 450 and 492 nm (Freshney, 1994; Mahony *et al*., 1989). Remaining activity percentage, Cell survival percentage and Inhibition rate was also measured. The inhibitory rate was measured according to (Wang *et al*., 2003) as follows:

$$\text{Inhinition ratepercentage} = \frac{\text{O.D. of control - O.D. of test}}{\text{O.D. of control}} \times 100 \text{ [Wang } et\ al.\text{, 2003]}$$

Remaining activity percentage calculated with the following formula:

$$\text{Remaining activity percentage} = \frac{\text{O.D. of control well (cells only)}}{\text{O.D. of test well (treated cells)}} \times 100 \quad \text{[Henson } et\ al.\text{,1989]}$$

Cell survival percentage calculated with the following formula:

$$\text{Cell survival percentage} = \frac{\text{Average of O.D. control well (cells only)}}{\text{O.D. of test well (treated cells)}} \times 100 \text{ [Freshney,1994]}$$

## RESULTS AND DISCUSSION

### Enzyme specificity against different substrates

In order to investigate the specificity of purified asparaginase produced from frozen seeds of *Pisum sativum var J of.* toward different substrates. Different substrates (L-asparagine, L-glutamine, L-aspartic acid and L-glutamic acid) were added to the purified enzyme at a concentration of 200 mM and the reaction mixture was incubated at 37°C for 30 minutes, then the activity and remaining activity were determined. Results indicated in Table (1) showed that the highest activity 783.1U/ml was obtained when L-asparagine was used as a substrate while less enzyme activity was recovered when L-glutamine, L-glutamic acid and L-aspartic acid were used. Purified asparaginase activity using these substrates was 28.1, 11.4, and 13.7U/ml respectively. These results indicated that the purified asparaginase extracted from *Pisum sativum var Jof.* was more specific for asparagines as natural substrate. This property of the enzyme is very essential in treatment of patients when incomplete removal of asparagines is required (Manna *et al*.,1995; Campbel *et al*., 1967). In other study, It found that purified asparaginase II from *E.coli* gives highest specificity against asparagines keeps 100% remaining activity in comparison to L-glutamine, L-glutamic acid and D-L aspartic acid at a concentration of 0.01 mM that held remaining activity of 3.5,0.0 and 0.0% respectively (Mokrane, 2003).

**Table 1. Specificity of purified Asparaginase from frozen seeds *Pisum sativum var Jof.* Against different substrates**

| Substrate | Substrate Conc. (mM) | Activity (U/ml) |
|---|---|---|
| L-asparagine | 200 | 783.1 |
| L-aspartic acid | 200 | 28.1 |
| L-glutamine | 200 | 11.4 |
| L-glutamic acid | 200 | 13.7(2). |

The cytotoxicity effect of purified asparaginase extracted from frozen seeds of *Pisum sativum var Jof.* was studied on L20B cell line (passage 18), a transgenic mouse cell lines (L20B) expressing the human Poliovirus surface receptor (Pvr, CD155) which is regarded as a significant technological advancement for selectively isolating Poliovirus from clinical samples (David Featherstone, 2002; Sushma *et al*., 2003). L20B cell line, represent one of the effective cytotoxicity assays, it were also used to determine the effect of sliver particles and it showed that's there is no significance change in cell viability expect the higher concentrations of sliver particles 10 µg/ml which showed significant reduction of cell growth (20). Coupe transversal of the used L20B cell line for determination of cytotoxicity was showed in Figure (1).

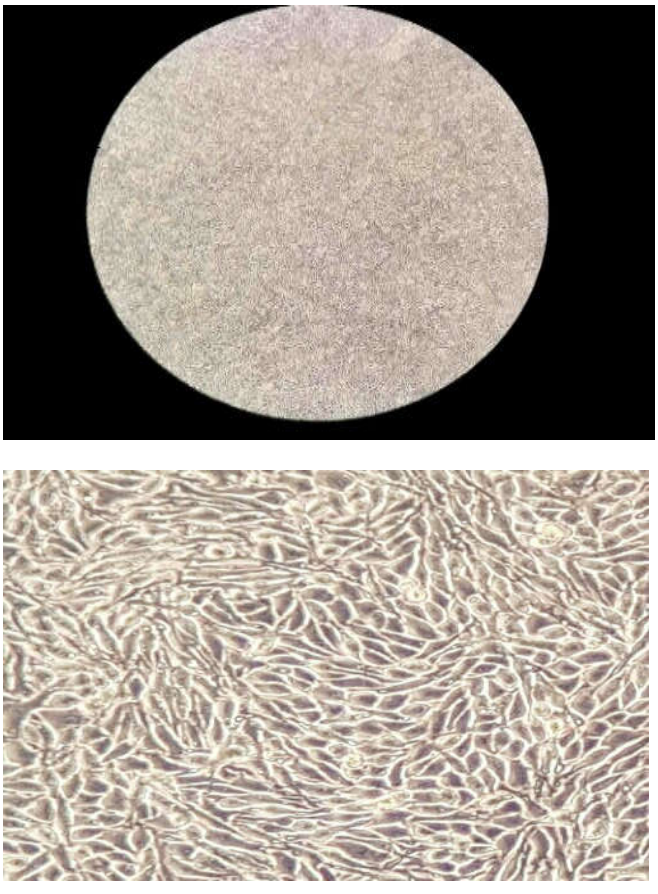

**Figure 1. Coupe transversal of the L20B cell line for determination of cytotoxicity of purified asparaginase**

L20B cell line evaluating purified asparaginase effect on L20B cell line on exposure time of 48 hours at various concentrations of purified asparaginase 150.0, 75.0, 37.5, 18.75, 9.37, 4.68, 2.34 and 1.17µg/ml using Neutral Red assay. The optical density measured at transmitting wave length 450, 492 and 620 nm. The Neutral Red assay is a cell survival/viability assay that was selected for determination of purified asparaginase cytotoxicity as the Neutral Red assay provides one of the most used cytotoxicity tests with many biomedical and environmental applications. It based on the ability of viable cells to incorporate and bind Neutral Red dye (Winckler, 1974; Guillermo *et al*., 2008). Tumor cells are incubated in the presence of Neutral Red dye following exposure to enzyme. Neutral Red was used for staining living cells as it is a vital stain. The dye readily penetrates cell membranes and accumulates intracellularly in lysosomes (Chazotte, 2011). The effect of purified asparaginase causes a decreased in the uptake and binding of Neutral Red. Tumor Cells are washed with phosphate buffered saline and treating with destaining solution to release any excess of dye taken up, then the damage level of tumor cells was evaluated by measuring the optical density of treated cell solution and comparing it to untreated negative control samples. Microtiter plate reader equipped with 450, 492 and 620 nm filter. Results indicated in Figure (2) showed that purified asparaginase showed a gradual decrease in the viability of the cells in comparison with negative controls.

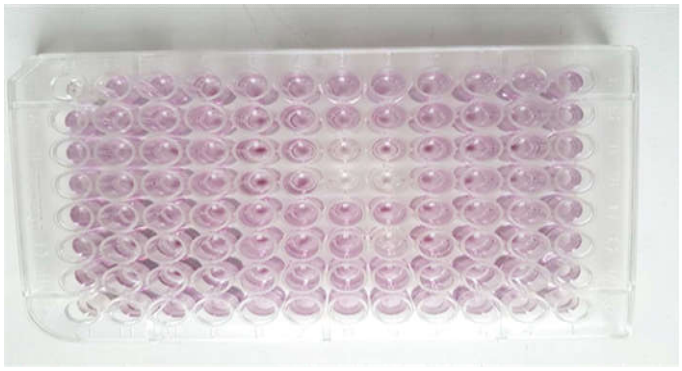

**Figure 2. Cytotoxicity of purified asparaginase from *Pisum sativum var Jof.* seeds against L20B cell line using Neutral Red assay**

**A:** Test sample: the L20B cell line after treatment with purified asparaginase (six times duplicate at each concentration)
**B:** Blank: the L20B cell line after treatment with only phosphate buffered saline
**NC:** Negative control: the L20B cell line without any treatment

The 96-microtiter plate was used as a standard tool for cytotoxicity analysis, as indicated in Appendix (1), which viewed the example map (Plate configuration) of micro titer plate. The percentage of inhibition growth rate represents the cytotoxicity effect of purified asparaginase on L20B cell line. Growth inhibition percentage, Remaining activity percentage and cell survival percentage were estimated. Results indicated in Figure (3) showed that purified asparaginase has significant cytotoxicity effect on L20B cell line in concentration range between 150 to 1.17 µg/ml in comparison with the negative control (the same cell line without any treatment) and blank (the same cell line treated only with phosphate buffered saline). Results showed that growth inhibition of L20B cell line, which measured at 450,492 and 620 nm, was increased gradually with the increase of asparaginase concentration treated with. After determination of cytotoxicity at wave length 450, 492 and 620 nm respectively, Maximum Growth inhibition ratewas reached33, 33and 23% respectively when the cell culture of L20B treated with 150 µg/ml of purified asparaginase, then the inhibitory effect was decreased to 30, 32, and 14% respectively after treatment with 75 µg/ml of purified asparaginase. It showed that's Maximum Growth inhibition rate was reached 39 and 34% respectively after treatment with 75 µg/ml of purified asparaginase from fresh *Pisum sativum var J of.* after determination at wavelength 450 and 492 nm respectively, using Neutral red assay (Khalaf, 2012).

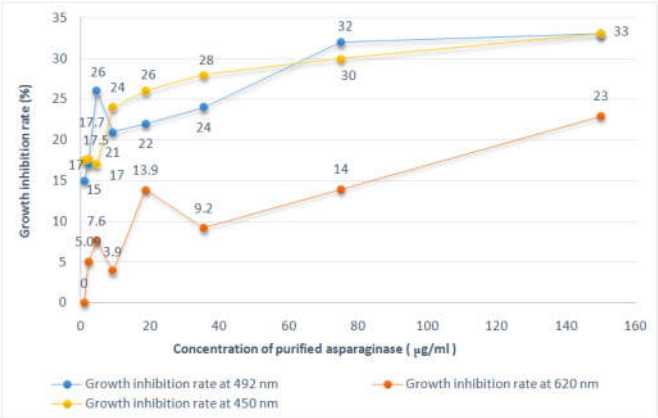


**Figure 3. Cytotoxicity effect of purified asparaginase extracted from frozen seeds of *Pisum sativum var Jof.* on L20B cell line after incubation period for 48 hours at 450, 492, and 620 nm**

On the other hand, Cell survivals percentage and remaining activity percentage of L20B tumor cell line after treatment with purified asparaginase (for 48 hours) was depends on the concentration of purified asparaginase at both wave lengths 450, 492 and 620 nm. Results indicated in Figure (4) showed that cell survival of L20B cell line at 450 nm was highly reduced after incubation with purified asparaginase at a concentration of 68 µg/ml while the cell line remaining activity percentage was reduced to 69 and 66.6% after incubation with purified asparaginase concentration of 150 and 75 µg/ml respectively.

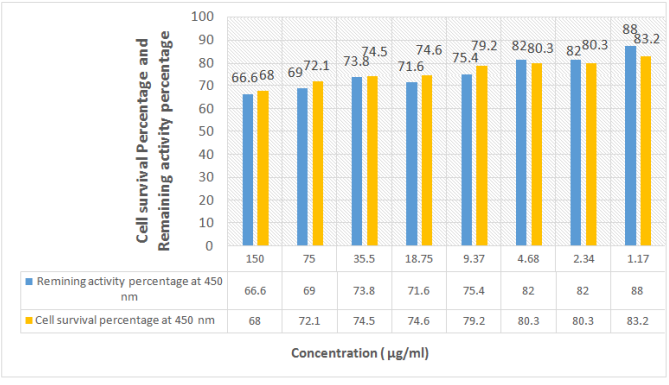


**Figure 4. Cytotoxicity effect of different concentrations of purified asparaginase from frozen seeds of *Pisum sativum var Jof.* on L20B tumor cell line after incubation for 48 hours measured at 450nm**

On the other hand, results indicated in Figure (5) showed that cell survivals percentage of L20B tumor cell line measured at 492 nm after 48 hour reaches the maximum reduction 65.0% after treatment with purified asparaginase at a concentration of 150 µg/ml, while the remaining activity of tumor cells was 66.5and 67.7%at concentration 150 and 75 µg/ml.

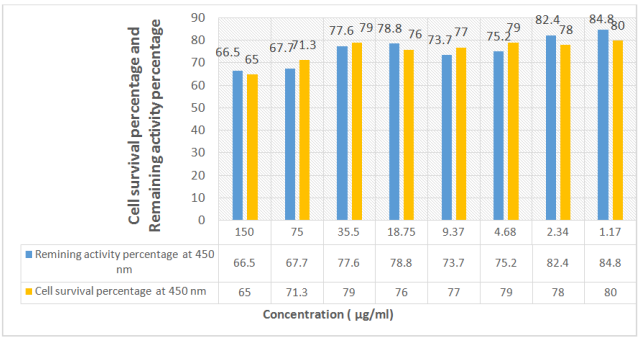


**Figure 5. Cytotoxicity effect of different concentrations of purified purified asparaginase from *Pisum sativum var Jof.* seeds on L20B tumor cell line after incubation for 48 hours measured at 492nm**

Figure (6) mentioned that cell survival and remaining activity of L20B cell line which measured at 620 nm showed that maximum reduction of cells and remaining activity after 48 hours of treatment with purified asparaginase at a concentration of 150 µg/ml. Cell survival and remaining activity of tumor cell line reaches 77 and 76% respectively.

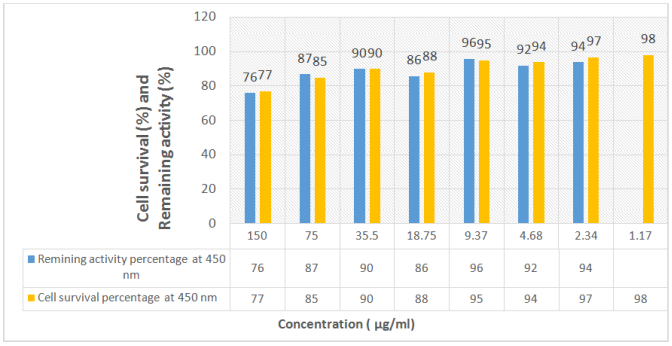


**Figure 6. Cytotoxicity effect of different concentrations of purified asparaginase from frozen seeds of *Pisum sativum var Jof.* on L20B tumor cell line after incubation for 48 hours measured at 620nm**

It showed that's cell survival percentage of L20B tumor cell line measured at 450 and 492 nm after 48 hours reaches the maximum reduction 70.8 % after treatment with purified asparaginase at a concentration of 4.68 µg/ml, while the remaining activity of tumor cells was 66.7 and 65.9% at concentration 75 and 37.5 µg/ml respectively (Khalaf, 2012). It showed that purified asparaginase has no significant effect on normal cell line after 48 hours of incubation (Teerayat *et al*., 2009), while It found that purified asparaginase from *W. sominifera* has slight antileukemia effect on leukemic cell line. In general, purified asparaginase can exert immunosuppressive effects (Oza *et al*., 2009). It showed that purified L-asparaginase had cytotoxic activity against various cancerous cell lines viz. Jurkat clone E6-1, MCF-7 and K-562 with IC50 of 0.22 IU, 0.78 IU and 0.153 IU respectively. However the enzyme had no toxic effect on human erythrocytes and CHO cell lines hence should be considered potential candidate for further pharmaceutical use as an anticancer drug (Mahajan *et al*., 2014).

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**Appendix 1. Example map of microliter plate that describe the treatment**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | TS (150 µg/ml) | TS (150 µg/ml) | TS (150 µg/ml) | TS (150 µg/ml) | TS (150 µg/ml) | TS (150 µg/ml) | B (5 µg/ml) | B (5 µg/ml) | B (5 µg/ml) | NC | NC | NC |
| B | TS (75µg/ml) | TS (75µg/ml) | TS (75µg/ml) | TS (75µg/ml) | TS (75µg/ml) | TS (75µg/ml) | B (2.5 µg/ml) | B (2.5 µg/ml) | B (2.5 µg/ml) | NC | NC | NC |
| C | TS (37.5 µg/ml) | TS (37.5 µg/ml) | TS (37.5 µg/ml) | TS (37.5 µg/ml) | TS (37.5 µg/ml) | TS (37.5 µg/ml) | B (1.25 µg/ml) | B (1.25 µg/ml) | B (1.25 µg/ml) | NC | NC | NC |
| D | TS (18.75 µg/ml) | TS (18.75 µg/ml) | TS (18.75 µg/ml) | TS (18.75 µg/ml) | TS (18.75 µg/ml) | TS (18.75 µg/ml) | B (0.62 µg/ml) | B (0.62 µg/ml) | B (0.62 µg/ml) | NC | NC | NC |
| E | TS (9.37 µg/ml) | TS (9.37 µg/ml) | TS (9.37 µg/ml) | TS (9.37 µg/ml) | TS (9.37 µg/ml) | TS (9.37 µg/ml) | B (0.31 µg/ml) | B (0.31 µg/ml) | B (0.31 µg/ml) | NC | NC | NC |
| F | TS (4.68µg/ml) | TS (4.68µg/ml) | TS (4.68µg/ml) | TS (4.68µg/ml) | TS (4.68µg/ml) | TS (4.68µg/ml) | B (0.15 µg/ml) | B (0.15 µg/ml) | B (0.15 µg/ml) | NC | NC | NC |
| G | TS (2.34µg/ml) | TS (2.34µg/ml) | TS (2.34µg/ml) | TS (2.34µg/ml) | TS (2.34µg/ml) | TS (2.34µg/ml) | B (0.07 µg/ml) | B (0.07 µg/ml) | B (0.07 µg/ml) | NC | NC | NC |
| H | TS (1.17 µg/ml) | TS (1.17 µg/ml) | TS (1.17 µg/ml) | TS (1.17 µg/ml) | TS (1.17 µg/ml) | TS (1.17 µg/ml) | B (0.03 µg/ml) | B (0.03 µg/ml) | B (0.03 µg/ml) | NC | NC | NC |

**TS**: Test Sample(L20B cell line treated with purified asparaginase)
**B**: Blank (L20B cell line treated with Phosphate buffered saline)
**NC**: Negative control (L20B cell line without any treatment)

*******