CHAPTER 9

Effect of the Purified L-Glutaminase on Growth of Carcinoma Cells

9.1. Introduction

Tumor cells are characterized as rapidly dividing cells and consequently they need a constant supply of both energy and nitrogen substrates. The consumption of glucose as energy substrate and amino acid as nitrogen substrate by malignant tumor cells is necessary to regulate their cellular metabolism (Jimenez et al., 1989; Medina et al., 1992). Glutamine donates its amide (γ nitrogen) group for synthesis of nucleotides and other non essential amino acids (NEAAs) (Voet and Voet, 1995; Ahluwalia et al., 1990; Cowan et al., 2012). Tumor cells such as hematopoietic tumors, hepatomas, Ehrlich carcinoma, and He-La are reported to have very low expression level of glutamine synthetase and hence uptake glutamine from exogenous source (Brown et al., 2008; Roberts et al., 2001). These tumor cells are being starved with the essential nutrient, glutamine in presence of L-glutaminase (Ahluwalia et al., 1990; Cowan et al., 2012; Griffiths et al., 1993; Sinsuwan et al., 2012).

Several microbial and mammalian glutaminase were purified and characterized. Mammalian glutaminase and some of the microbial glutaminase were found to be unsuitable for therapeutic use because of their high K_m values and requirement of phosphate esters or maleate for their activation (Medina et al., 1992; Bonthron, 1990; Hartman and McGrath, 1973, Hartman and Stochaj, 1973; Hartman, 1968). Glutaminase-asparaginase from *Achromobacter* sp. (Roberts et al., 1972; Spiers and Wade, 1979), *Pseudomonas* 7A (Roberts et al., 1970; Roberts, 1976; Roberts et al., 1979), and *Bacillus sp.* (Moharam et al., 2010) were reported to have low K_m, good activity in a physiological milieu and showed good inhibitory effect on growth of tumor cells (Verma et al., 2007). The discovery of new microorganism producing

glutaminase with good therapeutic effect is highly desired (Moharam et al., 2010). Hence an attempt has been made to produce glutaminase with desired therapeutic effect from novel bacterial source.

In this chapter, effect of different concentration of purified L-glutaminase from *Bacillus cereus* MTCC 1305 was studied on growth of Hep-G2 hepatocellular and HCT-116 colon carcinoma cell lines.

9.2. Materials and Methods

9.2.1. Growth medium and other chemicals

Growth medium RPMI 1640 was purchased from Sigma Aldrich (USA). The heat inactivated fetal bovine serum used in these growth medium to inactivate possible compliment immunological effects, was purchased from Sigma Aldrich (USA). The antibiotic solution "L-Glutamine–Penicillin–Streptomycin" containing 200mM L-glutamine, 10,000units penicillin, 10mg/ml streptomycin, used for culture growth, was purchased from Sigma-Aldrich (USA). MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was purchased from Sigma-Aldrich (USA) chemicals.

9.2.2. Cell culture preparation

Cell lines of hepatocellular Hep-G2 and colon carcinoma HCT-116 were obtained from ATCC, USA. Cells were cultured in RPMI-1640 media containing 10% fetal bovine serum at 37˚C in a CO_2 incubator with atmosphere of 5% CO_2. The medium of cell cultures was replaced in 3-4 days with fresh media. The cells were removed from the cell culture flasks by the following method:

i. Growth medium was decanted from the cell culture flask ($25cm^2$) and confluent cells in flasks were washed twice with Calcium and Magnesium free phosphate buffer saline (PBS).

ii. 0.5ml trypsin solution (0.25%) was added and dispersed evenly on horizontal surface of flask.

iii. The flasks were incubated at 36˚C until all cells were detached from the surface. The complete detachment of cells was checked by examining under an inverted microscope (Labkron Company, India).

iv. Cells were re-suspended in 4.5ml growth medium to halt the action of the trypsin.

v. Cell suspension was gently aspirated for few times using a fine Pasteur pipette to break up cell clumps.

vi. Cell suspension was poured into 15ml centrifuge tubes.

vii. The tubes were centrifuged at 500×g for 10 minutes and supernatant was discarded. Cell pellets were re-suspended in 1ml growth medium RPMI 1640.

viii. Accurate number of cells in suspension was counted using haemocytometer (improved Neubauer) by undergoing following steps.

- 0.2ml of trypan blue (0.1% w/v in PBS solution) was added to 0.2ml of the cell suspension. Viable cells were not stained by tryptan blue, while non vibale cells were blue stained.
- Mixture was mixed immediately with a fine Pasteur pipette and both side of haemocytometer chamber was filled with sufficient volume of this mixture.

- Viable cells were counted in each of the four corner squares bordered by triple lines.
- Mean count of the total viable cells per four corner squares was then calculated
- Viable cell concentration per ml was calculated using the following formula:

$$C_1 = t \times t_b \times {}^1/_4 \times 10^4 \text{.....(9.1)}$$

Where, t = Total viable cell count of four corner squares, t_b =Correction for the trypan blue dilution (counting dilution was $1/t_b$),1/4 = Correction to mean cells per corner square,10^4 = Conversion factor for counting chamber,C_1 = Initial cell concentration per ml

- The working cell concentration was obtained by mixing 1 volume of the original cell suspension with 11 volumes of the growth medium. Dilution factor (D) was calculated to determine the working cell concentration per ml (C_2) using following method:

$$\text{Dilution factor (D)} = \frac{C_2(\text{Working cell concentration})}{C_1(\text{Initial cell concentration})} \text{.....(9.2)}$$

ix. Cell suspension was diluted with growth medium to the desired concentration based upon counting the cells.

9.2.3. MTT cell viability assay

L-glutaminase from *Bacillus cereus* MTCC 1305 was purified with specific activity 7.44U/mg. Standard solution of 100µg/ml purified protein containing 0.744U of L-glutaminase was prepared. Cancer cells (5×10^4) were seeded in each well of 96 wells of microtitre plate containing 0.1ml of RPMI medium. The different concentration of

purified L-glutaminase (10, 20, 40, 60, 80, and100µg/ml) was added to each well after 24hrs. 10µl of MTT (5mgml^{-1}; stock solution, Sigma) was added to each well and cell viability was assessed after 4hrs. The medium from well was discarded and formazan blue crystals, which formed in the cells, were dissolved with 100µl DMSO (Di-methyl sulfoxide). The rate of color production was measured at 570nm in a spectrophotometer (Schimadzu). All experiments were conducted under the standard laboratory illumination. The relation between viability of cell and concentration of purified enzyme containing L-glutaminase was plotted to get the viability curve of each tumor cell line for the specified enzyme (Mosmann, 1983). The effective concentration of L-glutaminase required to inhibit growth for each cell line was determined in term of IC_{50}.

9.3. Results and Discussion

A wide variety of human cancer cell lines (pancreatic cancer, acute myelogenous leukemia and small cell lung cancer) were reported to be sensitive of glutamine starvation (Wu et al., 1978). Some glutamine analogues like Azaserine, azotomycin, 6diazo-5-oxo-L-norleucine and acivicin were used as possible chemotherapeutic agent to inhibit growth of human malignant tumor (Duvall, 1960; Roberts et al, 1979; Ovejera, 1979; Houchens et al., 1979; Medina et al., 1992). Acivicin from *Streptomyces sviceus* was used as an antitumor agent in a variety of mouse tumor models and human tumor xenografts (Poster et al., 1981). The inhibitory effect of L-glutamic acid gamma-mono-hydroxyamate against tumor cells like L1210 leukemia, B16 melanoma (Vila et al., 1990) was also studied. Use of these chemotherapeutic agents for treatment of malignant tumor caused cytotoxic effect on nearby normal

cells, and hence not preferred for cancer therapy (Medina et al., 1992). The deamination property of microbial glutaminase to hydrolyze glutamine could be also used to inhibit growth of glutamine dependent tumor cells. Some microbial glutaminase-asparaginase and asparaginase were used as a drug for the treatment of acute lymphoblastic leukaemia (Roberts et al., 1972; Roberts et al., 1979; Spiers and Wade, 1979; Gallagher et al., 1989; Roberts et al., 2001; Moharam et al., 2010). L-asparaginase from *Helicobacter pylori* CCUG 17874 also showed inhibitory effect on growth of gastric epithelial cell lines (Cappelletti et al., 2008) and used for treatment of acute lymphoblastic leukemia (Scotti et al., 2010). Glutaminase-asparaginase from *Pseudomonas* 7A was also reported to have considerable antineoplastic activity against a variety of rodent leukemia, ascites tumors and solid tumors like walker 256 carcinosarcoma, B16 melanoma (Holcenberg and Teller, 1976; Holcenberg et al., 1978; Roberts and McGregor, 1991).

The effect of different concentration of purified L-glutaminase from *Bacillus cereus* MTCC 1305 was also studied against Hep-G2 and HCT-116 carcinoma cell lines. The gradual inhibition in growth of Hep-G2 carcinoma cells was obtained in presence of different concentration of purified L-glutaminase as shown in Fig9.1. The cell growth inhibition gradually increased upto 60% with increased concentration of purified L-glutaminase upto 40µg/ml and beyond this concentration, it showed 50% inhibition. The effective concentration of purified L-glutaminase required to inhibit 50% growth of Hep-G2 carcinoma cells was determined in term of IC_{50} as 82.27µg/ml.

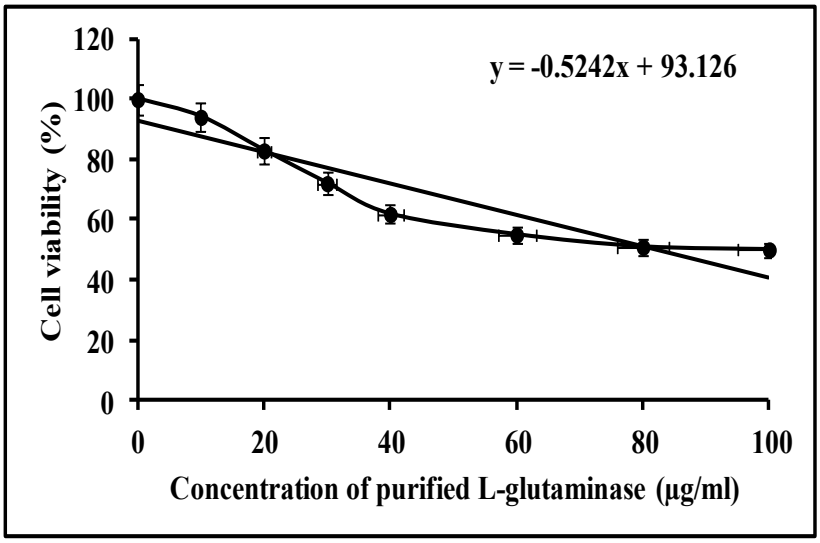

Fig.9.1. Anticancer activity of purified L-glutaminase from *Bacillus cereus* MTCC 1305 against Hep-G2 hepatocellular carcinoma cell line

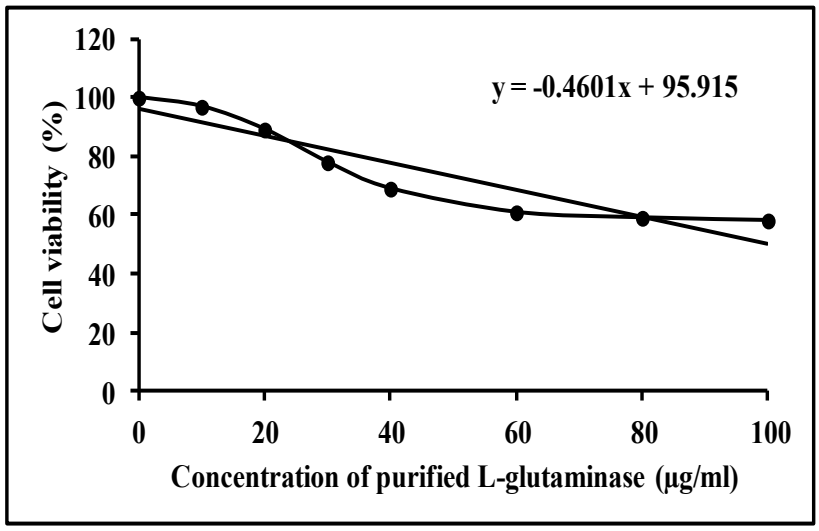

Fig.9.2. Anticancer activity of purified L-glutaminase from *Bacillus cereus* MTCC 1305 against HCT-116 colon carcinoma cell line

The effect of different concentration of purified L-glutaminase on growth of HCT-116 carcinoma cell line was also studied and its inhibition effect on cell viability is shown in Fig9.2. The effective concentration of purified L-glutaminase required to inhibit 50% growth of HCT-116 carcinoma cells (IC_{50}) was determined as 99.79µg/ml. The higher value of IC_{50} was obtained for HCT-116 colon carcinoma cell line in comparison to Hep-G2 hepatocellular carcinoma cells, which might indicate that higher concentration of purified L-glutaminase was required for 50% inhibition of growth of HCT-116 carcinoma cell line.

9.4. Conclusion

The purified L-glutaminase from *Bacillus cereus* MTCC 1305 showed good inhibitory effect against hepatocellular carcinoma (Hep-G2) and colon carcinoma (HCT-116) cell lines. This enzyme can be used as enzyme supplementation therapy for cancer in future.