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Research Article

IN VITRO CYTOTOXICITY OF L-GLUTAMINASE AGAINST MCF-7 CELL LINES

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ABSTRACT

Breast cancer prevention research has made notable steps forward in the past decade. Accumulating evidences suggest the beneficial effects of amino acid-depleting enzymes in lowering the risk of various cancers. Herein, we investigated the *in vitro* antioxidant (DPPH and ABTS assays) and antitumor effects of L-glutaminase produced by *Aspergillus flavus* KUGF009 against MCF-7 cell lines by MTT assay. Collectively, these findings indicate a crucial role of L-glutaminase in breast cancer.

Keywords: Antioxidant, L-glutaminase, MCF-7 cell line, MTT assay.

INTRODUCTION

Among women, breast cancer is the most devastating cause of cancer mortality, both in the developed and the developing world[1]. Although several noteworthy progresses in the past decades highlights the understanding of molecular and cellular mechanisms and the development of therapeutic strategies, the survival rate for breast cancer has not changed significantly[2]. There is an increasing need for search of new antitumor compounds, as the available anticancer drugs in the treatment of cancer is often unsatisfactory to the normal cells.

In recent years, biomedical sciences accentuate the involvement of glutaminase and other amino acid-depleting enzymes as agents for treating tumors[3]. The tumor cells display tremendous dependence on the exogenous supply of L-glutamine as a growth substrate, since they do not demonstrate the L-glutamine synthetase[4]. The ongoing challenge of the tumor cells on its survival can be blocked by the action of L-glutaminase (EC 3.5.1.2), an amidohydrolase enzyme which generates L-glutamic acid and ammonia from L-glutamine[5].A variety of microorganisms, including bacteria, yeast, moulds and filamentous fungi, have been reported to produce L-glutaminase, of which the most potent producers are fungi[6]. Microbial glutaminases are supposed to be more stable than plant and animal counterparts[7]. It also acts as proteolytic endopeptidase, which hydrolyses the peptide bonds present in the interior of the protein molecules[8].

As a practical application of the excellent L-glutaminase producing ability of *Aspergillus flavus* KUGF009, the present study was carried out to gain further insight into the antioxidant effects using free radical-scavenging activity determination (DPPH and ABTS assay) and also its *in vitro* anticancer activity against human breast carcinoma cell line (MCF-7) using MTT assay.

MATERIALS AND METHODS

Preparation of enzyme extract

Our previous paper reported a potent L-glutaminase producer, *Aspergillus flavus* KUGF009 isolated from fungal infected coconut (wet kernel)[9] during our preliminary screening study of L-glutaminase producing microorganisms.

Purification of L-glutaminase

The purification was carried out using crude enzyme extract[10]. The enzyme was purified by the following steps at 4°C, unless otherwise mentioned. Finely powdered ammonium sulfate was added to the crude extract. The L-glutaminase activity was associated with the fraction precipitated at 80% saturation. The precipitate was collected by centrifugation at 9,000 g for 15 min, dissolved in 0.01 M phosphate buffer, pH 8.5 and dialyzed against the same buffer. The dialysed fractions were collected and freeze-dried (Alpha 1-4, Christ, Germany), and the lyophilised enzyme was used for the following assays.

DPPH antioxidant activity

Free radical scavenging activity of L-glutaminase was determined by using rapid, simple and inexpensive method involving the use of the free radical, 2, 2-diphenyl-1-picrylhydrazyl (DPPH)[11]. DPPH is a free radical of violet colour. The antioxidants present in the sample scavenge the free radicals and turn it into yellow colour. The colour change from violet to yellow is proportional to the radical scavenging activity. Briefly, sample stock solutions (1.0 mg/ml) were diluted to final concentrations of 50, 40, 30, 20, 10 µg/ml, in ethanol. One ml of a 0.3 mM DPPH ethanolic solution was added to 2.5 ml of sample solutions of different concentrations, and allowed to react at room temperature. After 30 minutes, the degree of reduction of absorbance was recorded in UV–Vis spectrophotometer at 518 nm (Shimadzu UV–Vis 2450).

The percentage of scavenging activity was calculated as

$$\mathbf{AA\ \% = 100 - \{[(ABS_{SAMPLE} - ABS_{BLANK}) \times 100] / ABS_{CONTROL}\}}$$

Percentage of radical scavenging activity was plotted against the corresponding concentration of the extract to obtain IC50 value.

ABTS radical scavenging capacity

The $ABTS^{+}$ assay was based on the procedure described by Re et al. (1999)[12]. $ABTS^{+}$ radical [2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] was freshly prepared by adding 5 ml of 4.9 mM ammonium persulfate solution to 5 ml of 14 mM ABTS solution and kept for 16 h in dark. This solution was diluted with ethanol (99.5%) to yield an absorbance of 0.70±0.02 at 734 nm and the same was used for the assay. To 950 µl of ABTS radical solution, added 50 µl of extract solutions (25-500 µg/ml) and the reaction mixture was vortexed for 10 sec. After 6 minutes the absorbance was recorded at 734 nm and compared with the control ABTS solution. Percentage inhibition was calculated from the formula

Percentage inhibition= [1– (absorbance of test/absorbance of control)] ×100

Determination of cell viability by MTT Assay

MCF-7 human breast carcinoma cell line was purchased from National Center for Cell Sciences (NCCS), Pune. The cell lines were grown at 37 °C in a 5% CO_2, 95% air humidified atmosphere, in DMEM supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) to which penicillin (100 U/mL) and streptomycin (100 µg /mL) and amphotericin B (5 µg/ml) had been added.

The growth-inhibitory effect of L-glutaminase (freeze dried dialysed fractions) on the cancer cell lines was determined using a colorimetric test, MTT assay. Cells were seeded in 96-well plates at a density of $5x10^3$ cells per well in 100 µl of medium with FBS then cultured in a CO_2 incubator at 37 °C for 24 h. After which, 20 µl of L-

glutaminase fraction (or 20 µl of DMEM medium with FBS for the control) was added to each well and the plate was incubated for 96 h. After incubation, 20 µl MTT stock solution (5 mg/ml in phosphate-buffered saline or PBS, pH 7.5, filtered through 0.22-µm cellulose acetate filter; Sigma, St. Louis) was added to each well, incubated for 4 h at 37 °C then the solution was decanted. To stop succinate-tetrazolium reductase activity and solubilise formazan crystals, 100 µL of propanol was then added to each well. Absorbance was read on a plate reader (VERSAmax, Molecular Devices, Saint Gregoire, France) at 540 nm[13].

The percentage growth inhibition was calculated using the following formula and the concentration of enzyme extract needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

Growth inhibition (%) =100 – (AE×100/AC)

where AE and AC are the mean absorbencies measured for cell viability in culture medium containing only enzyme extract and PBS respectively.

Statistical analysis

Data were expressed as means ± SD. The mean values were calculated based on the data taken from at least three independent experiments (n = 3). Statistical analysis was performed by using the Student's t-test. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The partial purification of the L-glutaminase crude extract that was affected by the ammonium sulfate (80%) precipitation showed that most of the enzyme activity was preserved in the precipitate. This could be observed by total protein decreased from 4,150 to 674 mg in the ammonium sulfate precipitation step. The specific activity increased to 497 and 1,900 IU/mg after dialysis.

L-glutaminase can scavenge radicals generated *in vitro* by DPPH and ABTS scavenging assays by donating their hydrogen atom indicating L-glutaminase, a potent antioxidant[14,15,16].

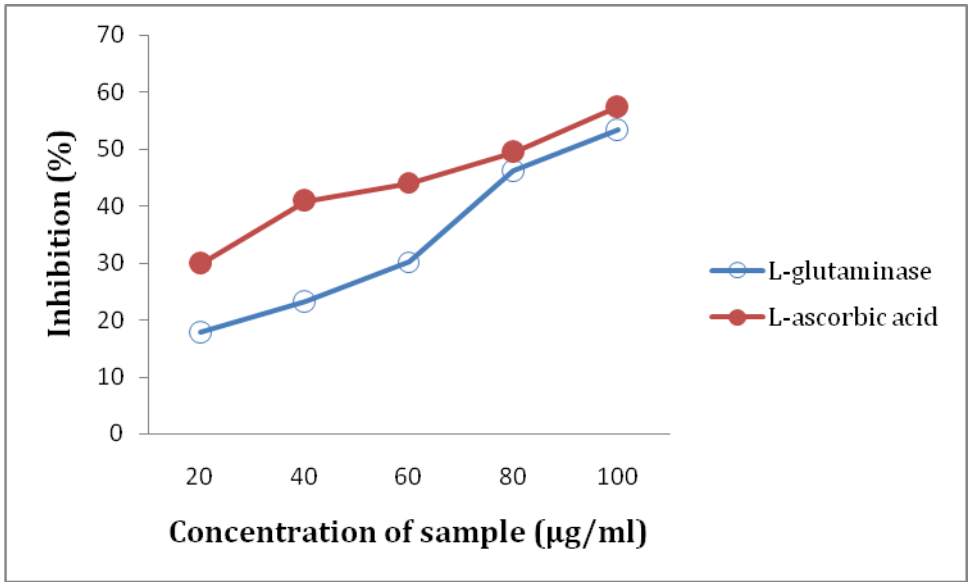

Fig.1: DPPH scavenging activity of L-glutaminase

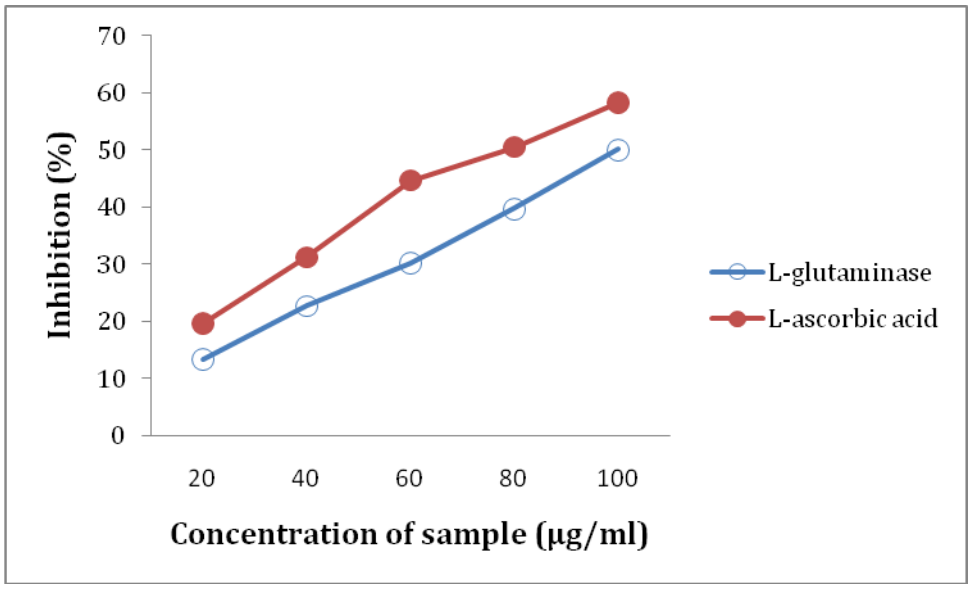

Fig. 2: ABTS radical scavenging activity of L-glutaminase

The ABTS assay is based on the generation of a blue/green $ABTS^{+}$, which is applicable to both hydrophilic and lipophilic antioxidant systems[17, 18]. The potential of L-glutaminase extracts to scavenge free radicals was also assessed by their ability to quench $ABTS^{+}$, which has a characteristic long wavelength absorption spectrum[19]. The experimental data depicts the concentration-dependent

decolourization of $ABTS^{+}$, expressed as IC50 values, by ethanolic extracts in comparison with ascorbic acid. The IC50 value of L-glutaminase is 98.53 µg/ml, whereas the IC50 of L-ascorbic acid is 80.52 µg/ml (Fig. 2).

Confrontation to chemotherapy is a commonly-encountered obstruction in the treatment of breast cancer [20, 21]. Such resistance can prove to be chief constraints to the effectiveness of the treatment, necessitating the use of increased doses which can lead to incapacitating cost that substantially reduce the patient's life. The cytotoxicity of the enzyme was examined in the MCF-7 cell lines by the MTT assay. *In vitro* screening models provide preliminary data in selecting the drugs for clinical trials. The cytoxicity studies indicated that the L-glutaminase is toxic (CTC_{50} 250 µg/ml) to the cell (Table 1).

Table 1: Percentage of growth inhibition by MTT assay in MCF-7 cell lines

	L-glutaminase (µg/ml)						CTC_{50} (µg/ml)
	1000	500	250	125	62.5	31.25	
Growth inhibition (%)	64.9	57.8	50.91	36.9	28.6	18.8	250

CONCLUSION

This profound protective effect of L-glutaminase may explain its extensive use in possible health benefits. Further studies are under progress to find out the exact mechanism of its anticancer activity.

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