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Research Article

IN VITRO ANTIOXIDANT AND ANTICANCER ACTIVITY OF L-ASPARAGINASE FROM *ASPERGILLUS FLAVUS* (KUFS20)

SONIYAMBY AMBI RANI [1], LALITHA SUNDARAM[1*], PRAVEESH BAHULEYAN VASANTHA[2]

[1]Department of Microbiology, Karpagam University, Coimbatore-641021, Tamil Nadu, India, [2]Department of Microbiology, Karpagam Arts and Science College, Coimbatore, Coimbatore-641021, Tamil Nadu, India , Email: lara9k@gmail.com

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ABSTRACT

The *in vitro* antioxidant and antitumor activity of partially purified L-asparaginase enzyme from *Aspergillus flavus* (KUFS20) was studied. The enzyme showed a good scavenging activity against DPPH with IC_{50} value of 263.63 µg/ml. It also showed total reducing activity. To determine anticancer activity, different concentration of partially purified L-asparaginase was tested on MCF-7 cancer cell line by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The enzyme showed a significant antiproliferative activity and a dose dependent effect was observed. Minimum inhibition of 4.78% was shown by enzyme at concentration 1.953 µg/ml and maximum inhibition (67.85%) was observed at 1000 µg/ml. From the result it concludes that this L-asparaginase can be used for the development of new preparations for the therapy of tumour.

INTRODUCTION

L-asparaginase enzyme (EC 3.5.11) specifically catalyzes L-asparagine to L-aspartate and ammonia[1] and play important roles both in metabolism of all living organisms as well as in pharmacology[2]. L-asparaginase is the first enzyme with antitumor activity to be intensively studied in human beings. It is an enzyme drug choice for acute lymphoblastic leukemia in children used in combination therapy[3]. The important application of the L-asparaginase enzyme is in the treatment of acute lymphoblastic leukemia, Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarbom and melanosarcoma[4]. L-asparaginase broadly distribute among the plants, animals and microorganisms. The microbes are a better source of L-asparaginase, because they can be cultured easily and the extraction and purification of enzyme from them is also convenient, facilitating the large-scale production[5].

The anti-leukemic effect of L-asparaginase is a result of rapid and complete depletion of the circulating pool of L-asparagine as in a great number of patients with lymphoblastic leukemia, the malignant cells depend on exogenous source of L-asparagine to be able to survive, mean while, the normal cells are able to synthesize L-asparagine[6-7].

Antioxidants are extensively studied for their capacity to protect organism and cell damage that are induced by oxidative stress. Oxidative damage plays a significantly pathological role in human disease. Cancer, emphysema, cirrhosis, atherosclerosis and arthritis have all been correlated with oxidative damage[8]. Breast cancer is the most common malignancy in women. Approximately one-third of the women with breast cancer developed metastases and ultimately died of the disease. Despite the fact that many tumors initially respond to chemotherapy, breast cancer cells can subsequently survive and gain resistance to the treatment[9]. Further, it has become increasingly important in the treatment of a number of major solid tumors, particularly metastatic and drug-resistant breast cancers[10].

This paper deals with the production of L-asparaginase from *Aspergillus flavus* (KUFS20) through solid state fermentation by using orange peel as a substrate, and to study its antioxidant and anticancer activity. There were no reports on production of L-asparaginase by using orange peel as substrate.

MATERIALS AND METHODS

Microorganism

Aspergillus flavus (KUFS20) isolated from garden soil collected from Coimbatore, India was used for the present study. The isolated strains was identified and maintained on modified Czapek Dox medium containing (g/l of distilled water): 2.0 glucose, 10.0 L-asparagine, 1.52 K_2HPO_4, 0.52 KCl, 0.03 $FeSO_4 \cdot 7H_2O$, 0.05 $ZnSO_4 \cdot 7H_2O$, 0.3 $NaNO_3$, and 15.0 Agar at pH 6.2.

Production of L-asparaginase by solid state fermentation

The production of L-asparaginase was carried out by using 20 g of orange peel as a substrate under solid state fermentation. The initial moisture content of substrate was adjusted to 40% with phosphate buffer, pH 6. The flask was inoculated with 3% (10^6 spores/ml) spore suspension under sterile conditions and incubated at 35°C for 4 days. The extracellular crude enzyme was prepared at the end of the fermentation period by the addition of 50 ml of a 0.01 M phosphate buffer pH 6.2 to the fermented medium. After shaking for 30 min, the suspension was centrifuged at 8,000 rpm for 20 min. The cell free supernatant was used as the crude enzyme.

L-asparaginase assay

L-asparaginase activity was measured following the method of Imada *et al*[11]. This method utilizes the determination of ammonia liberated from L-asparagine in the enzyme reaction by the Nessler's reaction. Reaction was started by adding 0.5 ml supernatant in to 0.5 ml 0.04 M L-asparagine and 0.5 ml 0.05 M tris (hydroxymethyl) aminomethane (HCl) buffer, pH 7.2 and incubated at 37ºC for 30 min. The reaction was stopped by the addition of 0.5 ml of 1.5 M trichloroacetic acid (TCA). The ammonia released in the supernatant was determined calorimetrically by adding 0.2 ml Nessler's reagent into tubes containing 0.1 ml supernatant and 3.75 ml distilled water and incubated at room temperature for 10 min. Absorbance of the supernatant was read using a UV- spectrophotometer (UV-2450, SHIMADZU) at wavelength of 450 nm.

Partial purification of L-Asparaginase

The purification was carried out at 4ºC on the crude extract, according to the modified method of Distasio *et al*[12].

Finely powdered ammonium sulfate was added to 80% saturation. The mixture was left for 12 h at 4°C, followed by centrifugation at 8,000 rpm for 20 min at 4°C. The precipitate was dissolved in a 0.01 M phosphate buffer pH 8.5 and dialyzed overnight against the same buffer at 4°C. The enzyme preparation obtained from the above step was further purified by passing through a column of activated DEAE-cellulose previously equilibrated with 0.01M Phosphate buffer, pH 6.2. A total of 30 fractions were collected at the flow rate of 5 ml/30 min. Fractions showing high activity were pooled and used for further studies.

In vitro antioxidant scavenging activity by DPPH and total reducing power assay

The partially purified enzyme was lyophilized and used to study its *in vitro* antioxidant effect. The antioxidant activity of the L-asparaginase was checked by DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity. 0.3 ml of different concentration (100, 200, 300,400 and 500 µg/ml) of sample was taken and made up to 0.4 ml with distilled water. To this added 0.6 ml of 100 M DPPH reagent in methanol. The reaction mixture was

incubated for 20 min under dark and the reading was taken at 517 nm. The decrease in absorbance at 517 nm was taken as the antioxidant capacity of the sample. L-ascorbic acid was taken as standard[13].

The reducing power was determined according to the method of Lin *et al*[14]. Different concentration (100, 200, 300,400 and 500 µg/ml) of sample (0.25 ml) was mixed with 0.25 ml of 200 mM sodium phosphate buffer (pH 6.6) and 0.25 ml of 1% potassium ferricyanide. Then the mixture was incubated at 50°C for 20 min. After 0.25 ml of 10% trichloroacetic acid was added to the mixture to stop the reaction, the mixture was centrifuged at 3000 rpm for 10 min. The supernatant (0.5 ml) was mixed with 0.4 ml of deionized water and 0.1 ml of 0.1% ferric chloride solution, allowed to stand for 10 min, and the absorbance was measured at 700 nm. Higher absorbance indicated higher reducing power. L-a*scorbic acid* was taken as standard.

***In vitro* anticancer study**

Human breast cancer (MCF-7) cell lines were obtained from National centre for cell sciences, Pune (NCCS). The cells were maintained in RPMI-1640 supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37°C.

The cytotoxicity of samples on MCF-7 cells was determined by the MTT assay[15]. Cells (1×10^5/well) were plated in 100 µl of medium/well in 96-well plates (Costar Corning, Rochester, NY). After 48 hours of incubation, the cell reaches the confluence. Then the cells were incubated in the presence of various concentrations of the samples in 0.1% DMSO for 48h at 37°C. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 20 µl/well (5 mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide cells (MTT) phosphate- buffered saline solution was added. After 4 h incubation, 0.04M HCl/ isopropanol were added. Viable cells were determined by the absorbance at 570 nm with reference at 655 nm. Measurements were performed in 3 times, and the concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically. The absorbance at 570 nm was measured with a microplate reader (Bio-Rad, Richmond, CA), using wells without sample containing cells as blanks. All experiments were performed in triplicate.

RESULT AND DISCUSSION

Production and purification profile of L-asparaginase

L-asparaginase, an important therapeutic enzyme has been isolated from a number of sources. In the present study, the enzyme was purified from the *Aspergillus flavus* (KUFS20) using ammonium sulphate precipitation, dialysis and DEAE cellulose chromatography. The yield, activity, protein content and purification fold of the purified L-asparaginase from the various steps are summarized in table 1. The enzyme activity was determined by using L-asparagine as a substrate and found to be 339.16 U/g. The partial purification of the L-asparaginase crude extract that was affected by the ammonium sulphate (80%) precipitation showed the most of the enzyme activity was preserved in the precipitate. The total protein decreased from 6919.07 to 991.65 mg in the ammonium sulfate precipitation step. The specific activity increased to 518.45 from 12.01 U/mg (Table 1).

Table 1: Purification profile of L-asparaginase from *Aspergillus flavus* (KUFS20)

Steps	Total L-asparaginase activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme	83096	6919.07	12.01	100	1
Ammonium sulphate (80%)	65287.2	991.65	65.84	78.57	5.48
Dialysis	40732.2	114	357.3	49.02	29.75
DEAE cellulose chromatography	11406	22	518.45	13.73	43.17

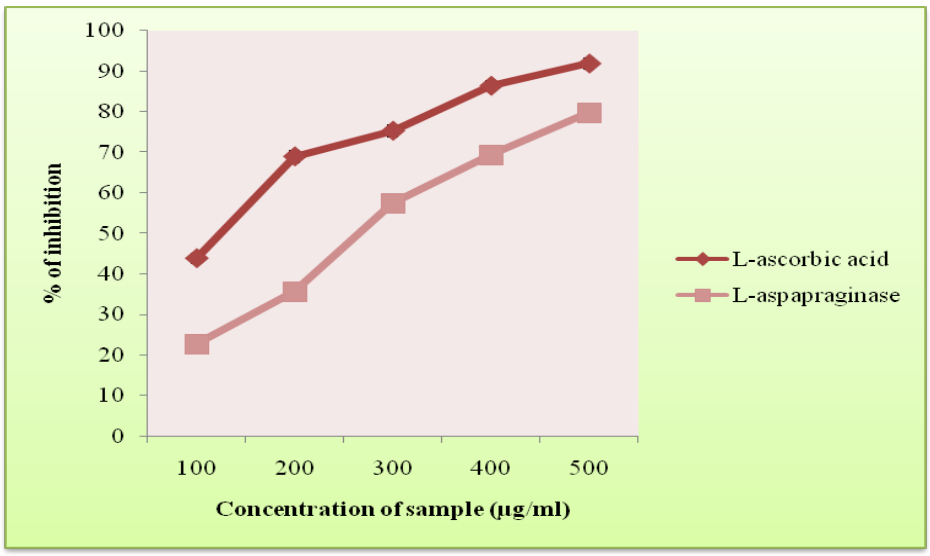

Fig.1: Scavenging activity of L-asparaginase by DPPH assay

Antioxidant activity of L-asparaginase by DPPH scavenging assay

Antioxidant properties, especially radical scavenging activities, are very important due to the deleterious role of free radicals in food and biological systems. The reduction of DPPH absorption is indicative of the capacity of the sample to scavenge free radicals, independently of any enzymatic activity. The method widely used to predict free radical, the ability of sample to transfer H atoms to radicals is based on 1,1,-diphenyl-2-picrylhydrazyl in the DPPH assay. The antioxidants were able to reduce the stable radical DPPH to the yellow colored diphenyl-picrylhydrazine. Figure 1, illustrates the DPPH radical scavenging ability of L-asparaginase and standards. L-ascorbic acid was used as reference radical scavengers. L-asparaginase showed excellent DPPH radical scavenging activity that was enhanced with increasing concentration. The IC_{50} value for L-ascorbic acid and L-asparaginase were 123.28 and 263.63 µg/ml respectively.

Maysa *et al*[16] who studied the antioxidant activity of L-asparaginase from *Bacillus sp* R36 by DPPH scavenging assay. Their result showed that *Bacillus sp* R36 possessed low scavenging activity with high SC50 values of 325.4 µg.ml.

Reducing power of L-asparaginase

The antioxidant activities of natural components might have a reciprocal correlation with their reducing powers. L-asparaginase showed a good reducing power activity. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of each antioxidant sample. Reducing power of a compound served as a significant indicator of its potential antioxidant activity. Reducing power of enzyme and L-ascorbic acid were enhanced by increasing concentration of sample (Figure 2).

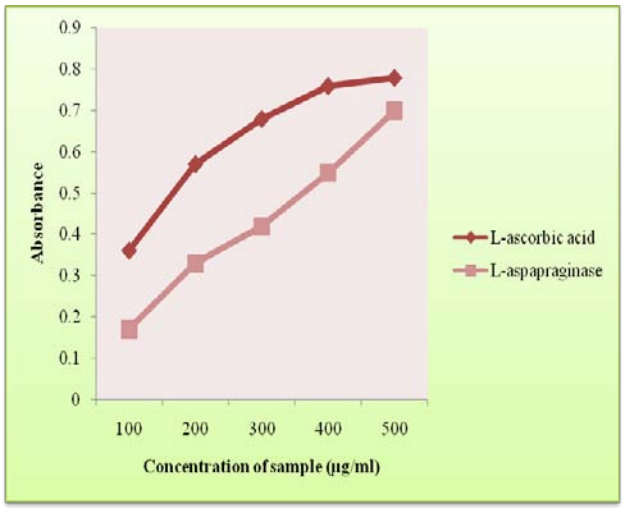

Fig.2: Total reducing power of L-asparaginase

Free radicals are chemical entities that can exist separately with one or more unpaired electrons. The generation of free radicals can bring about thousands of reactions and thus cause extensive tissue damage; lipids, proteins and DNA are all susceptible to attack by free radicals. Antioxidants may offer resistance against oxidative stress by scavenging the free radicals[17].

***In vitro* anticancer activity of L-asparaginase**

In the present study, the cytotoxicity effect of L-asparaginase on MCF-7 (human breast cancer) cells was characterized by MTT cell viability assay. Cultures of MCF-7 cells were treated with the L-asparaginase at different sample concentration and the results were shown in Table 2. It can be found that the incubation of tumour cells with the sample significantly reduced the viability of these cells and the dead cells were significantly increased with high extract concentration. At concentration of 1.953 µg/ml the sample reduced the cell viability to 95.22 % (4.78 % death). The IC_{50} value of L-asparaginase was found to be 120.875 µg/ml. The dead cells reached to 67.85 % at 1000 µg/ml. From this result it was clear that the sample has an inhibitory effect on MCF-7 (Figure 3a and 3b). There was a significant association between the concentration of sample and the inhibitory effect as shown in Table 2.

Table 2: Cytotoxicity of L-asparaginase on human breast cancer cell.

Concentration (µg/mL)	Cytotoxicity activity	IC_{50}
Cell control	100	
1.953	4.78±1.35	
3.906	14.88±1.87	
7.812	24.45±1.55	120.875 µg/mL
15.625	34.88±1.22	
31.25	39.77±0.60	
62.5	44.77±1.21	
125	50.85±0.45	
250	57.85±0.98	
500	61.44±0.55	
1000	67.85±1.23	

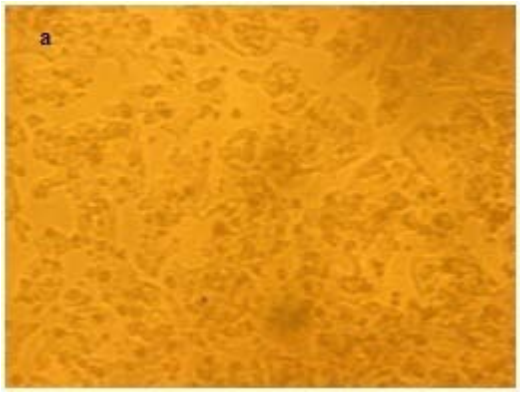

Fig.3a: MCF-7 normal cell line

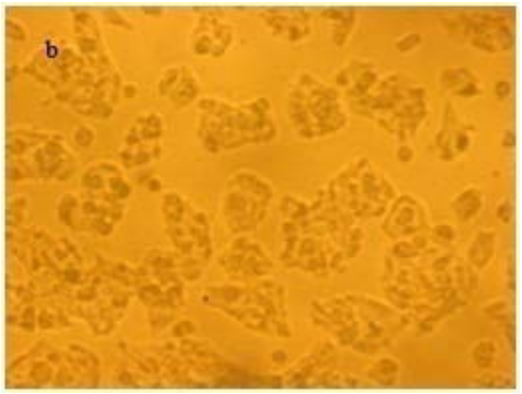

Fig.3b: Cell inhibition by L-asparaginase

Abakumova *et al*[18] studied the cytotoxicity activity of L-asparaginase from *Yersinia pseudotuberculosis* and from *Erwinia carotovora* using several tumour cell lines such as MCF-7 (human breast adenocarcinoma), LnCap (human prostate carcinoma), NGUK1 (rat gasser node neurinoma), Jurkat and Molt-4 (human T-lymphoblastic leukemia). The cell growth inhibition data indicate that *Y. pseudotuberculosis* L-asparaginase significantly inhibits growth of

leukemia and solid tumour cells. Pritsa *et al*[19] also studied the anticancer activity of L-asparginase from *Thermus thermophilus* against different tumour cell line include K-562 (chronic myelogenous leukemia), Raji (Burkitts's lymphoma), SK-N-MC (primitive neuroectodermal tumour), HeLa (cervical cancer), BT20 and MCF-7 (breast cancers), HT-29 (human colon cancer), and OAW-42 (ovarian cancer).

CONCLUSION

Present study indicates that the local soil isolates *Aspergillus flavus* (KUFS20) can be used as potential source of L-asparaginase. Furthermore, the purified L-asparaginase showed antioxidant activity against DPPH and potential anticancer activity against MCF-7 cell line and should be considered for further pharmaceutical use as anticancer agents.

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