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Purification and Biochemical Characterization of L-Asparaginase from *Aspergillus niger* and Evaluation of Its Antineoplastic Activity

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Abstract: ***L-asparaginase is a chemotherapeutic drug used in the treatment of lymphoblastic leukemia. In the present study, the extracellular L-asparaginase produced by strain Aspergillus niger was purified, characterized. Moreover, its antiproliferative activity was evaluated. The apparent molecular weight of the enzyme was found to be 136 kDa. The optimal pH and temperature for the enzyme were 9.0°C and 40°C, respectively. The enzyme retained 100% of the activity at 40°C for 120 min. L-asparaginase against human normal cells did not show cytotoxicity. However, in the human leukemia cell line A431 the antiproliferative effects of L-asparaginase was observed after 96 h of incubation. For the first time, an L-asparaginase from fungus was evaluated as an antitumor agent in human cells lines and further investigations should be conducted to improve the knowledge about this enzyme.***

Keywords: *Aspergillus niger*; L-Asparaginase; Antineoplasic Activity; Leukemia cell line viz. A431

1. Introduction

The enzyme L-asparaginase (L-asparagine amino hydrolase, E.C. 3.5.1.1) is an important component in the treatment of pediatric acute lymphoblastic leukemia (ALL) and catalyzes the hydrolysis of asparagine into aspartic acid and ammonia. This conversion provokes the asparagine starvation in the blood plasma and induces the death of malignant cells, since they are unable to synthesize asparagine and reduced levels of asparagine inhibits protein synthesis in leukemic cells [1]. Current studies of L-asparaginase therapy have also started in adults [2]. The effect and half-life of L-asparaginase depends on some factors such as antibody formation, plasmatic proteases, formation of asparagine via asparagine synthetase and microbial source [3]. Over the years, several bacterial L-asparaginases have been reported and only a few reports about L-asparaginase produced by filamentous fungi have been made. Among these reports are the L-asparaginase production by *Aspergillus tamari*, *A. terreus* [4], *A. niger* [5], *A. nidulans* [6], and in some yeast, but their antiproliferative activities were not analyzed. Currently, there are three asparaginases preparations available for therapy, two of them are native and produced by the bacteria *Escherichia coli* and *Erwinia chrysanthemi*. The other one, also from *E. coli* is conjugated and its elimination half life is approximately six days, five times longer than the native *E. coli* and nine times longer than the *Erwinia* preparations [2, 7]. The bacterial L-asparaginases are targets of antibodies and proteases, moreover side effects are observed during the treatment using this enzyme. Great efforts have been made to modify and immobilize these L-asparaginases in order to decrease their immunogenicity effects and increase their half-life. L-asparaginase from other sources, like eukaryotic microorganisms, should lead to an enzyme with less adverse effects. In this study, the extracellular L-asparaginase produced by *Aspergillus niger* was purified, biochemically characterized, and the antiproliferative activity of enzyme was evaluated against two leukemic cells lines.

2. Material and Methods

2.1 Microorganism

Aspergillus niger was isolated from soil. The fungus has been maintained by weekly transfers on slants of PDA medium.

2.2. L-Asparaginase Production

Enzyme production was optimized in Czapek Dox's modified liquid medium in two steps: the pre fermentation medium containing 0.2% (w/v) glucose, 2% (w/v) L-proline, 0.2% (w/v) NH_4NO_3, 0.15% (w/v) KH_2PO_4, 0.05% (w/v) KCl, 0.05% (w/v) $MgSO_4{\cdot}7H2O$ and 0.001% (w/v) $ZnSO_4{\cdot}7H_2O$, $FeSO_4{\cdot}7H_2O$ and $CuSO_4{\cdot}5H_2O$, the pH was adjusted to 8.5 with KOH, this medium was inoculated with 1×10^7 spores·mL^{-1} and incubated at 120 rpm for 17 h at 30°C. The culture was filtrated; the mycelium was collected and inoculated in the fermentative medium, which was similar to the medium used in previous step except for the absence of NH_4NO_3. The culture was reincubated for 96 hours at the same previous conditions.

2.3. L-Asparaginase Assay

L-asparaginase activity was determined according Drainas and Pateman [6] and modified as follows: 0.6 mL 20 mmol·L^{-1} Tris-HCl buffer, pH 8.0; 0.2 mL 100 mmol·L^{-1} stock L-asparagine solution; 0.2 mL 1 mol·L^{-1} stock hydroxylamine solution; and 1mL culture broth were mixed and incubated at 37°C and 120 rpm. After 30 min 0.5 mL ferric chloride reagent [10% (w/v) $FeCl_3$ plus 5% (w/v) trichloroacetic acid in 0.66 mol·L−1 HCl] was added. One unit of L-asparaginase is the amount of enzyme that re-

leases 1 mmol NH3 or aspartic acid per minute at 37°C at the specific conditions just mentioned. The reaction mixture contained 0.5 mL of 0.04 M of L-glutamine; 0.5 mL of 0.5 M Tris-HCl buffer; pH 7.2; 0.5 -1.0 mL culture broth (concentrated or not) and distilled water a final volume of 2.0 mL. It was incubated at 37°C for 30 - 60 min. and the reaction was stopped with 0.5 mL of 1.5 M of trichloroacetic acid. In fact 0.1 mL of mixture just mentioned and 0.2 mL of Nessler's reagent were added to 3.7 mL of distilled water. After 20 min, the absorption was measured at 450 nm.

2.4. Determination of Protein Concentration

The concentration of protein was determined by the Bradford [9] method with bovine serum albumin as a standard.

2.5. Separation and Purification of L-Asparaginase

The extracellular L-asparaginase was purified in three chromatographic steps, and after each step, the fractions were analyzed for activity and absorbance at 280 nm.

Step 1: The culture fluid was separated from mycelium by filtration. Then 900 mL was dialyzed against 5 mmol·L−1 Tris-HCl buffer, pH 8.0 (buffer A) overnight at 4°C, and 20 mL were applied to a DEAE—Sepharose Fast Flow column (2.5 × 22.5 cm) pre-equilibrated with 20 mmol·L−1 Tris-HCl buffer, pH 8.0 (buffer B). The bound proteins were eluted by step-wise increases in NaCl (100 and 150 mmol·L−1) at flow rate of 120 mL·h−1. Fractions (5.0 mL) were collected.

Step 2: The fractions with L-asparaginase collected from DEAE—Sepharose Fast Flow were pooled, dialyzed against buffer A, and loaded on a Sephacryl S-200 HR column (1.0 × 58.0 cm). The protein elution was performed with the buffer B containing 150 mmol·L−1 NaCl at a flow rate of 9.6 mL·h−1. Fractions (2.0 mL) were collected.

Step 3: The fractions from step 2 that contained L-asparaginase were pooled, dialyzed against buffer A, and applied again on a Sephacryl S-200 HR column (1.0 × 5.8 cm) however the flow rate was reduced at 6.0 mL·h−1. The fractions (0.8 mL) were pooled and dialyzed against buffer A over- night at 4°C. The samples were assayed and used for further characterization.

2.6. Electrophoresis Analysis

Polyacrilamide gel electrophoresis (SDS-PAGE) as described by Laemmli [11] was performed using 4% (w/v) stacking gel and 7.5% (w/v) acrylamide slab gel at a constant current of 20 mA. Protein bands were stained with silver nitrate according to Blum *et al*. [12].

2.7. Characterization of Purified L-Asparaginase

The optimum values of pH and temperature of purified enzyme were determined over a pH range of 2.2 - 10.6 (citrate-phosphate buffer [pH 2.2 - 7.8], Tris-HCl buffer [pH 8.2 - 9.0], and carbonate-bicarbonate buffer [pH 9.5 - 10.6]) and temperatures from 25°C to 60°C. Km was determined from double reciprocal plots (Linewear-Burk) incubating the pure enzyme with different concentrations of substrate at temperature and pH optima. The molecular mass was estimated by chromatography on Sephacryl S-200 HR using different protein molecular weight markers: cytochrome C (12.4 kDa), α-lactalbumin (14.2 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa). The column was pre-equilibrated with buffer B, the proteins were eluted with the same buffer containing 150 mmol·L^{-1} NaCl at a flow rate of 6.0 mL·h^{-1}. The thermo stability of purified L-asparaginase was determined by pre-incubating the enzymes in 100 mmol·L^{-1} Tris-HCl buffer (pH 8.0) at different temperatures (40°C, 50°C, and 60°C) for 120 min. Samples were collected at 15, 30, 60, 90, and 120 min, and the residual activity was assayed. Proteolytic resistance was evaluated after digestion of 180 µg of pure asparaginase and modified with 60 µg of bovine trypsin in a total volume of 1.0 ml at 37°C. Samples were collected from each solution at 5, 10, 15, 30, and 60 min and assayed for the residual activity. All the stability studies were repeated in triplicate and values are shown as mean ± SD.

2.8. Cell Culture and Cell Preparation

The human leukemia cell line A431, Stong *et al*. [13] were purchased from the American Type Culture Collection and were maintained in RPMI (GIBCO, USA) supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin. Human peripheral blood mononuclear cells (PBMC) were purified from heparinized venous blood drawn from healthy donors. PBMC were isolated by centrifugation on Ficoll-Paque (Pharmacia-LKB, Uppsala, Sweden) density gradients (1.077 g/mL) at 1000 rpm for 15 min at room temperature and subsequently resuspended in RPMI. All cell cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere. The counting and cell viability tests were determined using the test of Trypan blue exclusion.

2.9. Proliferation Assay

Cells were seeded in 96-well plates at 1 × 104 cells per well. After 24 h, L-asparaginase was added at concentrations of 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL and 200 µg/mL. At different time points (48, 72, and 96 h) of continuous drug exposure, 10 µl of MTT dye (3 mg/mL was added in each well. The plates were incubated for 2 h at 37°C and the formazan product was measured at 450 nm by using a microplate reader (Bio-Rad Laboratories). The experients were performed in triplicate in three independent sets. Values are shown as mean ± SD. Cell survival was calculated by subtracting the background absorbance of media alone and then dividing the absorbance of test wells by the absorbance of the control (untreated) wells.

3. Results

3.1. Biochemical Characterization of L-Asparaginase

The purification of L-asparaginase was carried out by three steps with a final yield of 7.28% and a purification fold of 10.67 (**Table 1**, **Figure 1**). The molecular weight of the native enzyme determined by its mobility on the gel filtration column and SDS-PAGE was estimated to be 136

kDa (**Figure 2**). The Km value of the native enzyme using asparagine as a substrate and determined from Lineweaver-Burk plot was found to be 2.42 mM (**Figure 3(a)**). The optimum pH for enzyme was found to be 9.0 and at physiological pH the enzyme retained 70% of maximum activity (**Figure 3(b)**). L-asparaginase exhibited the highest levels of activity at 37°C (**Figure 3(c)**) and was stable at this temperature for until 120 min. (**Figure 3(d)**).

Table 1: Purification steps of L-asparaginase produced by *A.niger*

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold purification	Yield %
Crude extract	23.10	139.70	6.04	1.00	100
Step 1	2.19	48.69	22.23	3.68	34.85
Step 2	0.37	17.18	46.70	7.72	12.30
Step 3	0.16	10.16	64.55	10.67	7.28

Step 1: DEAE-Sepharose; Step 2: Sephadex G 150; Step 3: Sephadex G 25.

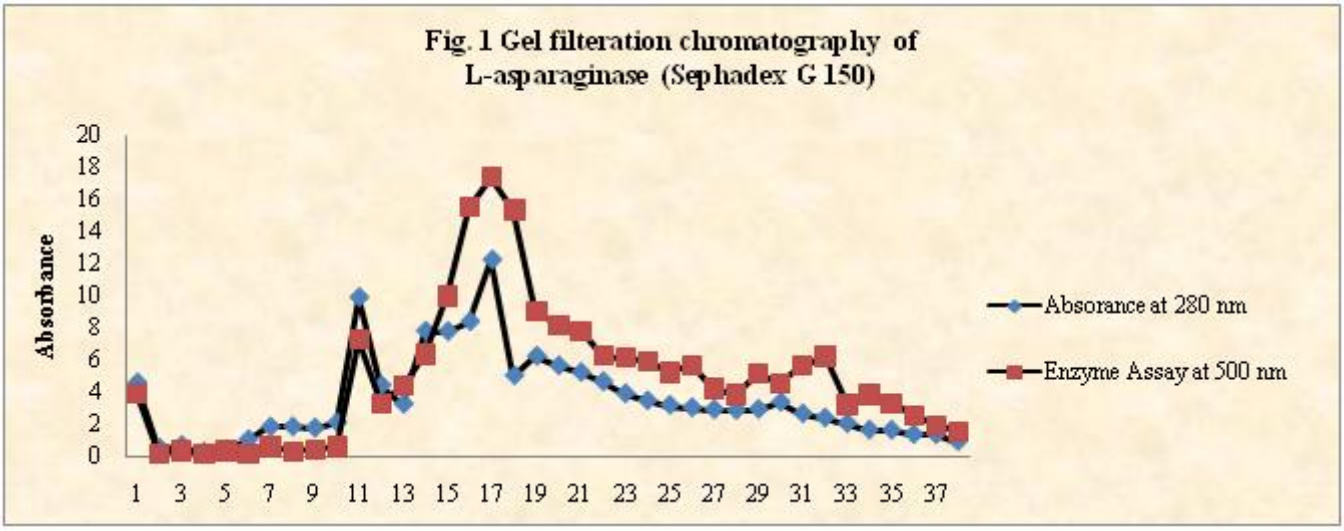
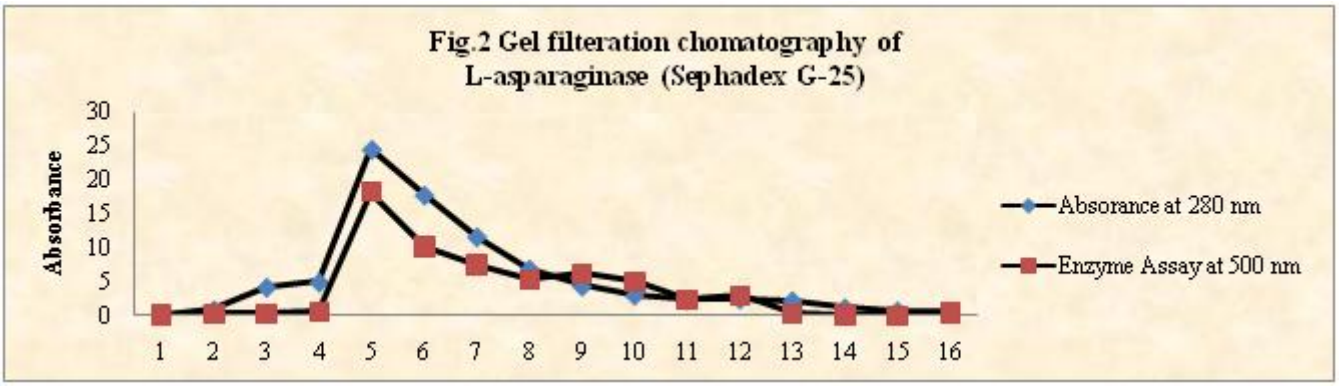


Figure 1: Chromatographic separation of L-asparaginase produced by *A. niger*. (a) The crude extract was dialyzed, concentrated and applied to DEAE-Sepharose which was eluted in 20 mmol·L^{-1} Tris-HCl pH 8.0 under a flow of 120 mL·h^{-1}



(b) The fractions containing enzyme activity were pooled and applied to gel filtration column of Sephadex G-25 with the same buffer under a flow of 9.6 mL·h^{-1}

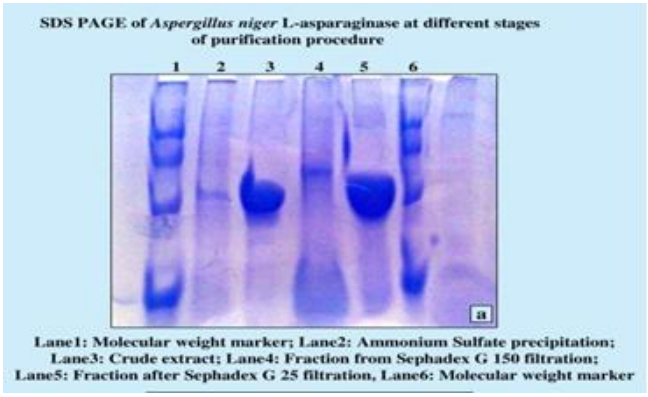


Figure 2: Polyacrylamide gel electrophoresis of purified L-asparaginase from *A. niger*

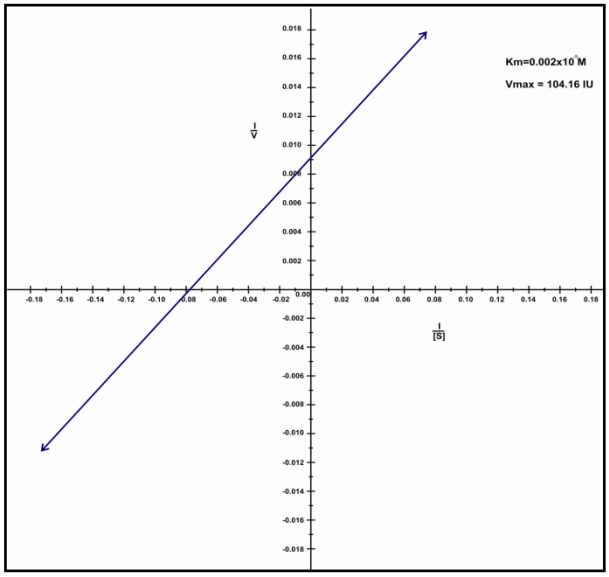
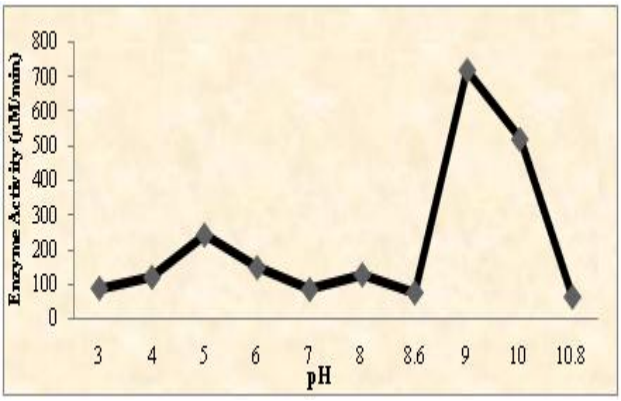
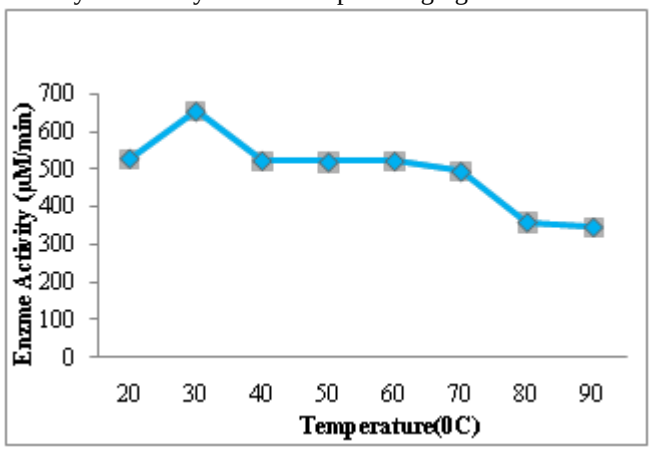


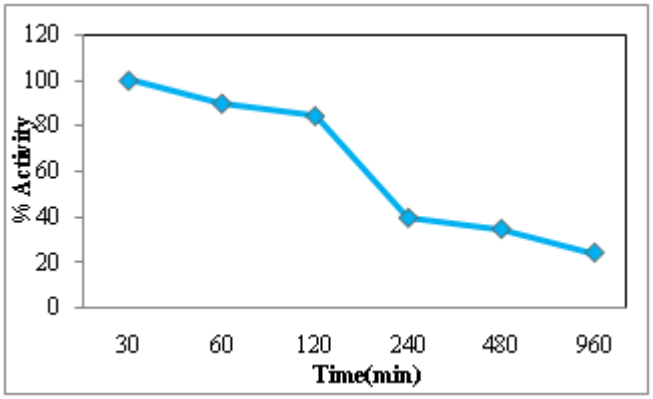
Figure 3: Characterization of L-asparaginase obtained from *A. niger*. (a) The Km value was determined by incubate the enzyme at different concentrations of asparagines



(b) The optimal pH of activity was assessed by measuring the enzyme activity at different pHs ranging from 3 to 10.8



(c) The optimal temperature for activity was assessed by measuring the enzyme activity at different temperatures between 20°C and 90°C



(d) For thermostability test the native enzyme was incubated at optimum temperature at 37°C for different time of up to 960 min.

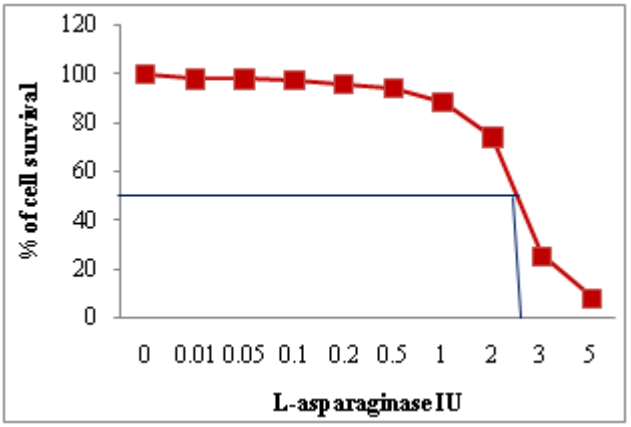


Figure 4: Cell viability of leukemic cells (A431) after treatment with purified L-asparaginase from *Aspergillus niger*

3.2 Cytotoxic Activity

The leukemic cells (A431) incubated with L-asparaginase had their cell viability by 50% in a concentration of 250 $\mu g \cdot mL^{-1}$ in an incubation time of 72 h, but after this time, the cell viability increased again (**Figure 4**).

4. Discussion

Since the first observations that some L-asparaginases demonstrate anti-leukemic activity, great progress has been made in the therapeutic protocols that combine L-asparaginases with other chemotherapeutic drugs. However, the favorable effects achieved with L-asparaginase in leukemia treatment are accompanied of the undesired side effects as thrombosis, pancreatitis, renal complications, liver damage among others. For this reason, several reports has showed different enzyme formulations and new sources of L-asparaginase included those from eukaryotic organisms, to find less toxic enzymes that also show suitable antineoplastic effects. Our studies of L-asparaginase produced by *A. niger* started determining of the good conditions for its production and the its highest levels were detected if L-proline 2% was the nitrogen source [14] and it is in accordance with the results reported by Sarquis *et al.* [4]. The molecular weight found here for the enzyme was 136 kDa, similar value has been described to *E. coli* L-asparagi- nase, 134 kDa. However, the Km value of native

L-asparaginase was found to be 2.42 mM, while that from *E. coli* is 0.0125 mM [15]. It shows that the L-asparaginase from *A. niger* has less affinity for asparagine than L-asparaginase from *E. coli* but the L-asparaginase from *A. niger* was capable to inhibit the prolifiration of leukemia cells. According to Panosyan *et al*. [16] the effective deamination of glutamine by L-asparaginase appears to contribute to the decrease of asparagine depletion by depriving the asparagine synthetase of glutamine, the precursor asparagine biosynthesis. Bacterial L-asparaginase, used in therapeutic protocols, has low glutaminase activity but toxicity reactions are attributed to this activity [17, 18]. Herein L-glutaminase activity was not detected in crude enzyme (concentrated or not) produced by *A. niger* even after 60 min of the reaction. This should contribute significantly to diminution of side effects and it may be helpful in clinical practice.

The antiproliferative effects of L-asparaginase produced by *A. niger* was evaluated after 24, 48, 72 and 96 h of incubation of A431 leukemia cell line. This L-asparaginase caused 50% reduction in cell viability after 72 h on the cell line HL-60 and after 96 h on the cell line RS4;11 (**Figure 4**). Interestingly, cell proliferation of HL-60 cell increased after 72 h and this can be associated with multiple adaptive cellular mechanisms. Studies have demonstrated increased asparagine synthetase (AS) expression in cells treated with L-asparaginase. It has been hypothesized that this elevated activity allows these leukemia cells to become resistant to the treatment. Moreover, other adaptive processes may provide a substrate to asparagine synthetase such as aspartate or glutamine, which derive from intracellular and extracellular sources [19,20].

The results of the present study clearly indicate that the L-asparaginase produced by *A. niger* has a molecular weight similar to the *E. coli*, does not present glutaminase activity. Moreover, this L-asparaginase caused antiproliferative effects on A431 leukemia cell line. Altogether, these data prompted further investigations into the L-asparaginase produced by *A. niger*.

5. Conclusion

Phe characterization of the enzyme revealed an optimum at pH 9.0. This property of enzyme makes clear that enzyme produced by *Aspergillus niger* under the present study has effective carcinostatic property, because the physiological pH is one of the perquisites for anti tumor activity. The optimum temperature for L-asparaginase activity was found to be 37^0C which is the physiological temperature. This property of enzyme is most suitable for complete elimination of asparagines from the body when tumor patient is treated with L-asparaginase *in-vivo*. Even though the enzyme showed maximum activity at body temperature and physiological pH and its considerable stability over a wide range of *p*H and temperature makes it highly favorable to be exploited as a potent anticancer agent.

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