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# Purification, characterization and antiproliferative activity of L-asparaginase from *Aspergillus oryzae* CCT 3940 with no glutaminase activity

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ABSTRACT

**Objective:** To explore the anti-proliferative activity of purified L-asparaginase from *Aspergillus oryzae* CCT 3940 (*A. oryzae*).
**Methods:** L-asparaginase was produced by submerged fermentation and purified to electrophoresis homogeneity by ionic exchanged chromatography in a fast protein liquid chromatographic system. The purified enzyme was characterized and used for the anti-proliferative assay against nine tumor cell lines and one non-tumor cell line.
**Results:** The free glutaminase L-asparaginase was purified 28.6 fold. L-asparaginase showed high stability under physiological condition, remaining stable in the pH range 7.0–8.0 after 1 h incubation at temperature range 30–45 °C. The Km and Vmax values of purified L-asparaginase were estimated as 0.66 mmol/L and 313 IU/mL, respectively. The purified enzyme could inhibit the growth of a broad range of human tumor cell lines at the concentrations studied. Also, the enzyme from *A. oryzae* CCT 3940 could inhibit tumor growth of leukemia cell line (K562) with a total growth inhibition value of (3.2 ± 2.5) IU/mL and did not inhibit the non-carcinogenic human cell line growth at the concentrations studied.
**Conclusions:** The sensitivity of the cells lines to purified L-asparaginase from *A. oryzae* CCT 3940 appeared to be concentration dependent affording a more significant decrease in cell growth than that observed for the commercial L-asparaginase from *Escherichia coli*. The L-asparaginase from *A. oryzae* CCT 3940 has a high potential for pharmaceutical exploitation in the treatment of leukemia.

## 1. Introduction

L-asparaginase is an important chemotherapeutic agent used to treat a variety of diseases of the lymphatic system, and lymphomas such as acute lymphoblastic leukemia [1]. The enzyme has also been applied in the treatment of Hodgkin's disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphoblastic leukemia, lymphosarcoma, reticulosarcoma and melanosarcoma [2]. In the normal animal cell, L-asparagine is not an essential amino acid for the maintenance of cell viability since cell supplies the absence of amino acid through the action of asparagine synthetase [3]. Asparagine synthetase is an intracellular enzyme responsible for the *de novo* synthesis of asparagine. However, the neoplastic cells are not able to induce the synthesis of asparagine synthetase, and they are dependent on the extracellular level of L-asparagine to protein synthesis. As L-asparagine is withdrawn from plasma by L-asparaginase, the resulting small concentration of this amino acid leads to disrupting protein synthesis and consequently cell growth inhibition in neoplastic cells [4]. Thus, L-asparaginase is used as an antitumor agent injecting the enzyme intravenously for lowering the concentration of L-asparagine, selectively affecting neoplastic cells dependent on this amino acid [5].

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There are three formulations of asparaginase widely used against acute lymphoblastic leukemia, the native L-asparaginase from *Escherichia coli* (*E. coli*) (Elspar®), its pegylated form (Oncaspar®) and L-asparaginase from *Erwinia chrysanthemi* (Erwinase®). The ability to substantially reduce the plasma levels of L-asparagine and keep it low for an extended period is the main feature responsible for the anti-neoplastic activity of L-asparaginase [6]. Despite significant progress in developing formulations of L-asparaginase, they are not free of adverse effects. There are some limiting factors for the use of L-asparaginase in chemotherapy, such as low catalytic activity that requires the use of high concentrations of each application [1], toxic effects, such as hyperglycemia, decreased serum albumin, lipoproteins, and fibrinogen, increased fat in the liver and some mild brain dysfunction [7]. The primary limiting factor is the development of hypersensitivity to the processing, which occurs in 50% of treated patients during the therapy with L-asparaginase from *E. coli* [8]. Even the pegylated form of L-asparaginase from *E. coli* also shows hypersensitivity reactions, thus requiring a shift to another form of L-asparaginase [9]. The hypersensitivity is associated with the production of antibodies, which may reduce the activity of L-asparaginase, causing the increase of the amino acid asparagine and possible development of drug resistance [10]. L-asparaginase also contributes to the activity of glutaminase, rapidly reducing the circulating glutamine concentrations in the plasma of patients, since this is converted into glutamic acid and ammonia [11]. The toxicity of asparaginase is partly attributable to the glutaminase activity of these proteins, and the cytotoxicity of L-asparaginase is determined primarily by its glutaminase activity [12]. Therefore, the activity of glutaminase in therapeutic preparations of L-asparaginases has been implicated in the cause of some side effects [1].

The extensive research is, therefore, going on worldwide with eukaryotic L-asparaginases looking for less adverse effects than those observed for bacterial enzymes [2,13]. However, few reports are available for the extracellular secretion of L-asparaginases by fungi, their characterization, and purification. The search for an L-asparaginase producing organism with no glutaminase activity, relevant biochemical characteristics, and high purification yields is a continuous exercise.

This paper deals with the purification and biochemical characterization of a glutaminase-free L-asparaginase from *Aspergillus oryzae* CCT 3940 (*A. oryzae*) and its anti-proliferative activity against human cancer cell lines in comparison with L-asparaginase from *E. coli* and the cytostatic antibiotic doxorubicin.

## 2. Materials and methods

### 2.1. Chemicals

Commercial L-asparaginase from *E. coli* was purchased from Megazyme (Wicklow, Ireland). Nessler's reagent was purchased from Merck® (Darmstadt, Germany); L-asparagine and L-glutamine were purchased from Sigma–Aldrich® (Missouri, USA). Other reagents were obtained from Synth® (São Paulo, Brazil).

### 2.2. Microorganisms

The filamentous fungi *A. oryzae* CCT 3940 was previously selected as an L-asparaginase producer strain from the Culture Collection of André Tosello Foundation, Campinas, SP, Brazil. The strain was periodically subcultured and maintained on potato dextrose agar slants.

### 2.3. L-asparaginase production

Conidial suspension was prepared from freshly raised seven-day-old culture on potato dextrose agar medium with a 5 mL of sterile 0.3% Tween 80 solution. The suspension was aseptically transferred to 250 mL Erlenmeyer flask containing 50 mL of modified and previously optimized Czapek Dox medium [14] composed of 5.0 g glucose, 10.0 g proline, 2.0 g L-asparagine, 5 g yeast extract, 1.52 g $KH_2PO_4$, 0.52 g KCl, 0.52 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CuNO_3 \cdot 3H_2O$, 0.01 g $ZnSO_4 \cdot 7H_2O$ and 0.01 g of $FeSO_4 \cdot 7H_2O$ per liter and initial pH adjusted to 7.0. The flasks were incubated at 30 °C and 150 r/min for 72 h. The fungi cultures were filtered through a paper filter, and the filtrate was called crude extract.

### 2.4. Purification of L-asparaginase

Crude extract filtrate was precipitated by adding ammonium sulfate with constant stirring until 80% saturation and incubated overnight at 5 °C to assure that the precipitation had come to completion. The precipitate was separated by centrifugation at 7 722 r/min for 30 min at 5 °C. The protein precipitate was resuspended in 0.01 mol/L Tris–HCl buffer (pH 8.0) and dialyzed against distilled water. The chromatographic purification steps were performed in a fast protein liquid chromatography (GE Healthcare, Uppsala, Sweden) with a flow rate of 1 mL/min, temperature of 20 °C and volume fraction of 0.5 mL. The elution of protein was estimated by measuring the absorbance at 280 nm (Beckman DU-640, CA, USA). The dialyzed enzyme was filtered through a 0.45 μm membrane filter (Millipore, Billerica, MA, USA) and loaded onto a Q Sepharose™ Fast Flow 1 mL column (GE Healthcare, Uppsala, Sweden) that was pre-equilibrated with 10 mmol/L Tris–HCl (pH 7.0). The bound enzyme was eluted with the NaCl gradient: 0–1.0 mol/L, in the same buffer. Fractions containing L-asparaginase activity were pooled, dialyzed against distilled water and concentrated by lyophilization. The lyophilized fraction was resuspended in Tris–HCl buffer (pH 7.0) and then it was applied to an SP Sepharose™ Fast Flow 1 mL column (GE Healthcare, Uppsala, Sweden) that was pre-equilibrated with 10 mmol/L Tris–HCl (pH 7.0). The protein elution was done with the same buffer, and the bound enzyme was eluted with a linear NaCl gradient: 0–1.0 mol/L. Eluted proteins were pooled, collected, dialyzed against distilled water and lyophilized. After resuspension in Tris–HCl buffer (pH 7.0), the enzyme was applied to the CM Sepharose™ Fast Flow 1 mL column (GE healthcare, Uppsala, Sweden) previously equilibrated with 10 mmol/L Tris–HCl (pH 7.0). The proteins were eluted with a linear NaCl gradient from 0.0 to 1.0 mol/L and collected in 0.5 mL fractions. The active fractions were pooled, dialyzed against distilled water concentrated by lyophilization and used for the characterization studies.

### 2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for purified L-asparaginase

The lyophilized L-asparaginase obtained from the CM Sepharose™ Fast Flow column was subjected to SDS-PAGE to