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Partial purification and characterization of L-asparaginase from an endophytic *Talaromyces pinophilus* isolated from the rhizomes of *Curcuma amada*

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ABSTRACT

L-Asparaginase is a commercially significant enzyme. There exists a demand for a broad variety of microbial L-asparaginases with characteristics compatible with its different applications. Endophytic microorganisms, in particular are emerging as potential sources of L-asparaginases.

The current work involves partial purification and characterization of L-asparaginase from *Talaromyces pinophilus*, an endophytic fungus isolated from the rhizomes of *Curcuma amada*. Maximum enzyme activity could be achieved at pH 8.0 and with temperature 28 °C. The enzyme Exhibits 95 % and 98% of its total activity at physiological pH and temperature, respectively. The enzyme activity is largely unhindered in the presence of metal ions such as Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}. Increase in the enzyme activity in the presence of thiol groups and reduction in the same upon addition of thiol blockers indicates the involvement of cysteine in the enzyme's catalytic activity. The enzyme is a heterodimer of 62 kDa and 39 kDa. The enzyme has a K_m of 6.4 mM, its turnover number towards L-asparagine is 286.3 s^{-1}. The enzyme has 16% glutaminase activity; its K_m towards glutamine is 13.3 mM and turnover number is 54.6 s^{-1}.

Our results highlight that L-asparaginase from endophytic *Talaromyces pinophilus* could be considered as potential candidate for clinical and industrial trials, owing to its efficiency and biochemical properties. To the best of our knowledge, this is the first report on partial purification and characterization of L-asparaginase from an endophyte.

1. Introduction

L-Asparaginase is commercially a very significant enzyme. It is one of the primary drugs used in the treatment of acute lymphoblastic leukaemia (ALL), a prevalent form of paediatric cancer [1]. L-asparaginase is also used in food industry to reduce the formation of acrylamide in food products that are processed at temperatures above 100 °C [2]. Although L-asparaginases from *Escherichia coli* and *Erwinia chrysanthemi* are commercially available for the treatment of ALL, their therapeutic utilization is hindered by untoward complications involving hypersensitivity and several other toxic side effects [3,4]. For utilization in the food industry, just a couple of L-asparaginases are available with the brand names Acrylaway® and PreventAse®, both of which are derived from *Aspergillus* sp. These limitations advocate the exploration for more L-asparaginases from newer microbial sources. The applications of L-asparaginases, and the need and scope for new L-asparaginases have been reviewed in detail by Krishnapura et al. [5]. Probing for structurally and functionally diverse L-asparaginases from relatively unexplored sources like the endophytic diversity residing in the medicinal plants appears promising.

Several microorganisms are endowed with L-asparaginase producing ability [6]. However, with constant additions of newer zones of applications to the enzyme, L-asparaginases from newer microbial sources with different activities and properties are sought which facilitate their subjection to new processing conditions, and thereby expand their industrial use. Very few L-asparaginases are highly active at physiological pH and temperature. Therefore, there is a need for L-asparaginases displaying efficient enzyme activity at physiological conditions for its use as a therapeutic enzyme. Furthermore, for applications in food industry, the enzyme must possess sufficient activity over a large range of pH and temperature, and have high enzyme activity and substrate affinity for efficient and speedy conversion of L-asparagine [5].

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The current work deals with purification of L-asparaginase from *Talaromyces pinophilus*, an endophytic fungus isolated from the rhizomes of *Curcuma amada,* using strategies such as ammonium sulphate precipitation, dialysis, and Sephadex gel-filtration chromatography. The effect of pH, temperature, and various effector molecules on the enzyme activity, and the enzyme's molecular weight, were studied. Characterization of an enzyme plays a vital role in understanding the properties and behaviour of an enzyme as a catalyst. These data also help in improving the enzyme's catalytic efficiency and in tailoring it to suit its applications.

2. Materials and methods

2.1. Microorganism

Talaromyces pinophilus previously isolated in our laboratory from the rhizomes of *Curcuma amada* [7], was used for the production of L-asparaginase enzyme. The ITS/5.8 S rRNA gene sequencing of the organism was carried out at IMTECH, Chandigarh, India. The data was deposited in GenBank and was assigned the accession No. KJ372306.

2.2. L-Asparaginase assay

The enzyme activity of L-asparaginase was determined by measuring the amount of ammonia formed, using direct nesslerization method based on Imada et al. [6]. One Unit of L-asparaginase is the amount of enzyme which liberates 1 μmol of ammonia in 1 min at 37 °C.

2.3. Protein estimation

Total protein estimation was carried out using Bradford method [8]. A standard curve was established by measuring the absorbance at 595 nm of known concentrations of Bovine Serum Albumin (BSA).

2.4. Partial purification of L-asparaginase

L-Asparaginase was produced by culturing *Talaromyces pinophilus* in the previously optimized liquid medium (Table S1) for 120 h at 28 °C. The fermentation broth was filtered using a Whatman No. 2 filter paper to remove the cell biomass. The cell-free filtrate was considered as the crude enzyme and was subjected to various purification strategies. L-Asparaginase activity and the specific activity of the enzyme were measured at each step of purification.

2.4.1. Ammonium sulphate precipitation

The crude enzyme was subjected to 40–95% ammonium sulphate saturation in increments of 5%. The supernatant of each of the fractionation step was centrifuged at 8000 × *g* for 15 mins at 4 °C, and the cut-off pellets were collected, suspended in 50 mM tris–HCl buffer (pH 7.4), and assayed for L-asparaginase activity and total protein content. The percentage of ammonium sulphate saturation required to precipitate L-asparaginase was determined.

2.4.2. Dialysis

Dialysis membrane was pretreated by boiling in 2% sodium bicarbonate and 1 mM EDTA solution. Ammonium sulphate cut-off pellet with maximum L-asparaginase activity was collected and dissolved in a small amount of 50 mM tris–HCl buffer of pH 7.4. This was dialysed for the removal of salts using dialysis membrane-150 (from HiMedia, India) with a molecular weight cut-off of 12–14 kDa. 50 mM tris–HCl buffer of pH 7.4 was used as the dialysis buffer. After dialysis, the enzyme solution was concentrated by reverse dialysis. The dialysis bags were immersed in poly ethylene glycol (PEG 6000) until the desired reduction in the volume was achieved. Thereafter, the enzyme samples were pooled together and subjected to gel-filtration chromatography.

2.4.3. Gel-filtration chromatography

Sephadex G-100 (medium-fine, Sigma–Aldrich) with bead size of 40–120 μm, swelling volume of 15–20 mL/g, and fractionation range of 4–150 kDa was used. A constant flow rate of 1 mL/min at 18 °C was maintained with 50 mM tris–HCl of pH 7.4 as the elution buffer. A total of 25 fractions of 1 mL each were collected and analysed for L-asparaginase activity and protein concentration. A chromatogram was plotted.

2.5. Characterization of the partially purified L-asparaginase

2.5.1. Effect of pH on the enzyme activity and stability

The effect of pH on the activity of the partially purified L-asparaginase was determined by assaying the enzyme activity at different pH values ranging from 4 to 10 using 50 mM of the following buffer systems: acetate buffer (pH 4.0–5.5), citrate buffer (pH 4.0–6.0), phosphate buffer (pH 6.0–8.0), Tris buffer (pH 7.4–9.0), borate buffer (pH 8.0–9.0), and carbonate buffer (pH 9.0–10.0). The use of two buffers with overlapping pH range compensates for any buffer-associated effects on the enzyme activity. The relative enzyme activities at different pH were calculated as a percentage of the highest enzyme activity.

To study the effect of pH on the stability of the enzyme, the enzyme was incubated in buffers with different pH (in the range 4.0–10.0) for different time durations up to a maximum of 8 h at 37 °C. The L-asparaginase activity was measured after different incubation periods, and the residual enzyme activity for each incubation period was estimated as a percentage of the highest enzyme activity at each pH.

2.5.2. Effect of temperature on the enzyme activity and stability

The effect of temperature (in the range of 15–50 °C) on the activity of L-asparaginase was determined by performing the enzyme assay at different temperatures. The relative enzyme activities at different temperatures were calculated as a percentage of the highest enzyme activity.

To study the effect of temperature on the stability of the enzyme, the enzyme was incubated at different temperatures (in the range 20–50 °C) for varying time intervals up to 8 h in 50 mM tris buffer of pH 7.4. The L-asparaginase activity was measured at different incubation periods and the residual enzyme activity for each incubation period was estimated as a percentage of the highest enzyme activity at each temperature.

Studies on reversibility of temperature-induced inhibition of the enzyme activity were also carried out. The enzyme was incubated at 15, 20, 40 and 50 °C for 8 h, following which it was incubated at 37 °C (in all the above four cases) and at room temperature (in the first two of the above cases) for 1 h and the enzyme activity was measured.

2.5.3. Effect of sodium and potassium salts

The effect of sodium chloride and potassium chloride were studied in the range 0.4–1% (w/v) concentration. The salts were incorporated in the reaction mixture of the enzyme assay.

2.5.4. Effect of metal ions and other effector molecules

Metal salts, ethylene diamine tetra acetate (EDTA) and sodium dodecyl sulphate (SDS) in the concentration range of 1–10 mM were incorporated into the enzyme assay reaction mixture. The relative enzyme activity was calculated as a percentage of the enzyme activity at zero concentration of the effector molecules. In the studies with respect to the effect of metal ions on the L-asparaginase