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Biochemical characterization and antitumor activity of three phase partitioned L-asparaginase from *Capsicum annuum* L.

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ABSTRACT

In this study, three phase partitioning (TPP) as an efficient bioseparation method, was explored for the first time to purify stable uncontaminated L-asparaginase from *Capsicum annuum* L., and its antitumor activity was evaluated. With the optimized system parameters, the enzyme was purified to homogeneity and having 6.83-fold with 567.4% recovery of its activity. The optimum pH and temperature of the TPP purified enzyme were determined as 8.5 and 40 °C, respectively. The stability studies of the enzyme activity envisaged that the enzyme is stable up to 45 °C and retained its activity over a wide range of pH (5.0–9.0). The purified enzyme showed good scavenging activity, and its total antioxidant capacity was found to be 88.58 μM ascorbic acid equivalent. The antiproliferative activity of the purified L-asparaginase was also investigated against the three human cancerous cell lines.

1. Introduction

The manufacture of an enzyme for use as a drug is an important facet of today's pharmaceutical industry. L-asparaginase has received increased attention in current years for its anticarcinogenic potential [1]. Asparaginase (L-asparagine amidohydrolase E.C 3.5.1.1) catalyzes the hydrolysis of L-asparagine to produce L-aspartic acid and ammonia. L-asparaginase is widely used in chemotherapy, and it displays a range of interesting properties, like being important agents in the therapy of malignancies of the lymphoid system, acute lymphoblastic leukemia and non-Hodgkin's lymphoma [2,3]. These significant properties of L-asparaginase require further attention, to purify better quality asparaginase from easily available natural resources, for their indispensable biological and medicinal applications. Using amino acid sequences and biochemical properties, enzymes with asparaginase activity can be divided into several families [4]. Among these, the two largest and best-characterized families were recognized, which include bacterial and plant-type asparaginases. Normally microbial asparaginase is used as an antitumor and anti-leukemia agent. Nevertheless, the utilization of asparaginase from these sources is initially limited due to the potential toxicity and several side effects [5]. Another use of asparaginase is its frequent requirement for a cancerous patient, because of its short half life, and the instability of the drug in the treatment process, which results in side-effects in patients [6]. Furthermore, asparaginase contaminated with glutaminase activity causes the depletion of glutamine in the blood [6]. Low glutamine in the blood may lead to serious effects on the biochemical functions in the body of patients [6,7].

The production of asparaginase, on a large scale being produced from different species of microorganisms and plants has been reported. Unfortunately, most of them have a low recovery of enzyme activities and poor biochemical properties [8,9]; while some of them are highly stable, but have low purity and recovery of enzyme activities [10–12]. This prevents asparaginase from medicinal use on a large scale in some application fields. Therefore, the extraction of pure asparaginase from easily available natural resources is important, and to treat cancer patient devoid side effects. The problems encountered during the isolation, extraction and purification of plant enzymes were extensively studied [13]. Some of the problems in plant extract are due to the presence of a rigid cell wall, phycocolloids, phenolic compounds, and the high

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viscosity of the extract. However, traditional purification methods such as column chromatography, and salt or solvent precipitation techniques are limited by their complicated operation, long separation time required, and little recovery of activity [12,14]. In addition, those methods share a common problem in scale-up, which makes them uneconomical and impractical.

Since the applications of L-asparaginase in medical fields are broadening, it is important to discover new sources and novel methods to produce and purify asparaginase, and understand the nature and properties of these enzymes for their efficient and effective usage. A survey of several plants revealed that green chillies (*Capsicum annuum* L.) contained an appreciable amount of L-asparaginase [15,16]. Three phase partitioning (TPP) is an innovative method for the successful extraction and purification of various enzymes from both plant and bacterial sources [17,18]. It uses ammonium sulfate with a certain saturation to precipitate the protein, and t-butanol was added to make a three-phase layer, and to remove some small molecular weight compounds such as lipids, phenolics and some detergents [17]. This method was scalable, and could be used directly with crude suspensions. However, TPP has not been reported to be used in green chilli enzyme extraction.

In the present research, a practical approach, which exploits the known effect of system parameters, such as salt and solvent concentration, temperature and pH of the crude extract upon enzyme purity and activity recovery, was used. This approach was followed to evaluate the feasibility of using TPP for the recovery of asparaginase from green chillies as a first step in the development of a prototype bio-process. Meanwhile, electrophoresis experiments were carried out to ascertain the purity of the separated asparaginase. The obtained L-asparaginase enzyme was further biochemically characterized and its antioxidant potential and antitumor effects against various cancerous cell lines, were studied.

2. Materials and methods

2.1. Chemical and reagents

All the chemicals and reagents used were of an analytical grade and were purchased from Hi-media, India. All the reagents were prepared using milli-Q water (Millipore, USA). Three different cancerous cell lines, Hela (Human, Cervix), A549 (Human Lung Carcinoma) and KB (Human Oral Squamous Carcinoma) were procured from the cell repository-NCCS Pune, India. Hela and KB cell lines were cultured in Eagle's minimal essential medium with 2.0 mM L-glutamine,1.5 g/L $NaHCO_3$, 0.1 mM non essential amino acids, and 1.0 mM sodium pyruvate and supplemented to contain 10% (v/v) fetal calf serum. Likewise A549 cell was cultured in DMEM F-12 Ham with 2.0 mM L-glutamine adjusted to contain1.5 g/L $NaHCO_3$ and 10% fetal calf serum. Cells were grown at 37 °C, and 5% CO_2 in humidified air.

2.2. Extraction of L-asparaginase

Green chillies (100 g) were purchased from the local market and washed with tap water, followed by distilled water prior to use. Extraction was carried out by homogenized chillies using 0.15 M KCl by mortar and pestle, and made up to 300 mL total volume. The homogenized sample was centrifuged at 8000*g* for 15 min. The supernatant was filtered and used for extraction. All the steps were carried out at 4 °C.

2.3. Purification of L-asparaginase by three-phase partitioning

TPP was carried out as described by Roy and Gupta [9]. The crude extract was subjected to TPP for the purification of L-asparaginase, by addition of the ammonium sulfate to attain a particular saturation, followed by the addition of a t-butanol at particular pH in centrifuged tubes, and allowed to stand for 1 h at a specific temperature. After incubation for 1 h, the mixture was centrifuged at 5000*g* for 10 min, when the extract separated into three phases. The interfacial layer and lower aqueous layer were collected, and the former was re-dissolved in 0.15 M KCl, and these samples were dialyzed overnight against 0.015 M KCl, to remove excess salt, and assayed for enzyme activity and total protein concentration. Parameters, such as ammonium sulfate (w/v), crude: t-butanol ratio (v/v), temperature (°C) and pH were optimized as follows.

2.4. Optimization of TPP purification parameters

In order to find out the optimum ammonium sulfate saturation, salt was added to the crude extract so as to attain different saturations (10–70%) (w/v), as the ratio of the crude: t-butanol was fixed as 1.0:1.0 and the temperature and pH of the system were maintained as 28 ± 2 °C and 7.0, respectively. With the optimized ammonium sulfate saturation, the ratio of the crude and t-butanol was varied from 1.0:0.25 to 1.0:2.0 at a fixed temperature (28 °C) and pH 7.0. The effect of temperature was optimized by carrying out TPP at a standing time (1 h) at different temperatures ranging from 30 °C to 60 °C at a constant pH 7.0. The optimum pH of the TPP was determined by adjusting the pH of the crude extract (5.0–9.5) after saturating it with ammonium sulfate followed by the addition of t-butanol of an optimized quantity.

2.5. Determination of enzyme activity and protein concentration

The enzymatic assay procedure consists of 0.1 mL of enzyme extract mixed with 0.1 mL of L-asparagine as substrate; 1 mL of 50 mM Tris buffer (pH 8.6) and 0.9 mL of milli-Q water comprised the assay system. The mixture was incubated for 30 min at 37 °C. After incubation, the reaction was stopped by adding 0.1 mL 1.5 M trichloroacetic acid (TCA). 0.2 mL of this mixture was added to 4.3 mL water, and the released ammonia due to L-asparagine catalysis was estimated by adding 0.5 mL of Nessler's reagent. The intensity of the color was measured at 436 nm, using the UV-Spectrophotometer (SL-159, Elico Ltd., Hyderabad, India). The absorbance was compared with the ammonium sulfate standard. One international unit (IU) of L-asparaginase is that amount of enzyme which liberates 1 μM of ammonia in 1 min at 37 °C. The total protein was determined by Lowry's method [20], with bovine serum albumin as the standard (BSA). Assays were carried out in duplicates and the averages were used for the calculations. Specific activity was calculated and expressed as unit per milligram (U/mg) of protein.

2.6. Biochemical properties of TPP Purified L-asparaginase

2.6.1. Effect of temperature on the activity and stability of L-asparaginase

In order to determine the effect of temperature on the activity of L-asparaginase, the assay was carried out over the temperature range of 20–55 °C [21]. The relative activities were expressed in percentage as the ratio of the purified L-asparaginase activity obtained at a certain temperature to the maximum activity obtained at the given temperature range. To check the thermal stability, the enzyme was incubated at different temperatures (30–45 °C) for different time intervals. After the incubation period, the enzyme aliquots were withdrawn, brought to room temperature and then assayed at optimal assay conditions and the residual activity was determined.