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Anticancer Properties of Highly Purified L-Asparaginase from *Withania somnifera* L. against Acute Lymphoblastic Leukemia

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Abstract *Withania somnifera* L. has been traditionally used as a sedative and hypnotic. The present study was carried out for the purification, characterization, and in vitro cytotoxicity of L-asparaginase from *W. somnifera* L. L-Asparaginase was purified from the fruits of *W. somnifera* L. up to 95% through chromatography. The purified L-asparaginase was characterized by size exclusion chromatography, polyacrylamide gel electrophoresis (PAGE), and 2D PAGE. The antitumor and growth inhibition effect of the L-asparaginase was assessed using [3-(4, 5-dimethyl-thiazol-2yl)-2, 5-diphenyl-tetrazolium bromide] (MTT) colorimetric dye reduction method. The purified enzyme is a homodimer, with a molecular mass of 72±0.5 kDa, and the p*I* value of the enzyme was around 5.1. This is the first report of the plant containing L-asparaginase with antitumor activity. Data obtained from the MTT assay showed a LD_{50} value of 1.45±0.05 IU/ml. *W. somnifera* L. proved to be an effective and a novel source of L-asparaginase. Furthermore, it shows a lot of similarity with bacterial L-asparaginases EC-2.

Keywords Antitumor activity · L-Asparaginase · MTT · Purification · *Withania somnifera* L.

Introduction

Withania somnifera (L.) Dunal was considered a rasayana herb, which works on a nonspecific basis to increase health and longevity. The species name *somnifera* means "sleep-making" in Latin, indicating its attributed sedating properties. Extracts from the fruits, leaves, and seeds of *W. somnifera* L. were traditionally used for the Ayurvedic system as aphrodisiacs, diuretics, and for treating memory loss. The Japanese patent applications are related to the use of the herb as a skin ointment and for promoting reproductive fertility.

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W. somnifera L. is reported to have anticarcinogenic effects in animal and cell cultures by decreasing the expression of nuclear factor-kappaB, suppressing intercellular tumor necrosis factor, and potentiating apoptotic signaling in cancerous cell lines [1].

The enzyme L-asparaginase (ASN) (EC.3.5.1.1; L-asparagine aminohydrolase) catalyzes the deamination of L-asparagine (Asn) to L-aspartate and ammonia. The enzyme has been isolated from a variety of sources: animals and plant cells, yeast, fungi, and bacteria. It is identified as an effective agent in the therapy of certain types of lymphoma and leukemia. ASN can induce complete remission in up to 80% of patients suffering from acute lymphoblastic leukemia. The principal sources of asparaginase used extensively are from the bacteria *Escherichia coli* or *Erwinia carotovora* [2]. Using amino acid sequences and biochemical properties as criteria, enzymes with asparaginase activity can be divided into several families [3]. The two largest and best-characterized families include bacterial and plant-type asparaginases. The bacterial-type enzymes have been studied for over 40 years [4]; their homologs are found in some mammals and in fungi [5]. In particular, enzymes such as glutamin-(asparagin)-ases (EC 3.5.1.38) [4], lysophospholipases (EC 3.1.1.5) [6], and the α-subunit of Glu-tRNA amidotransferase (EC.3.5.–) [7] can also be considered part of the bacterial asparaginase family. It has been shown on the basis of kinetic and structural studies that two conserved amino acid motifs are responsible for the activity of the abovementioned proteins [8].

L-Asparaginase has been isolated from a number of sources. These include *Proteus vulgaris* [9], *E. carotovora* [2], *Acinatobacter* [10], *Serratia marcescens* [11], *Mycobacterium bovis* [12], *Streptomyces griseus* [13], *Achromobacteraceae* [14], guinea pig liver [15], and *Pisum sativum* L. [16]. Though L-asparaginase has been reported in many higher plants, little work has been carried out on the characterization of L-asparaginase from higher plants. The presence of an amidase in barley roots capable of hydrolyzing L-asparagine had been reported earlier, and the distribution of L-asparaginase in *Lupinus luteus* and *Dolichos lablab* seedlings has also been reported [17].

The plant-type enzymes have been studied less thoroughly. In plants, L-asparagine is the major nitrogen storage and transport compound, and it may also accumulate under stress conditions [18]. Asparaginases liberate from asparagine the ammonia that is necessary for protein synthesis. There are two groups of such proteins, called potassium-dependent and potassium-independent asparaginases [19, 20]. Both enzymes have significant levels of sequence similarity. The plant asparaginase amino acid sequences did not have any significant homology with microbial asparaginase but was 23% identical and 66% similar to a human glycosylasparaginase [17].

The L-asparaginases of *Erwinia* and *E. coli* have been reported for many years as effective drugs in the treatment of acute lymphoblastic leukemia. Their main side effects are anaphylaxis, pancreatitis, diabetes, leucopoenia, neurological seizures, and coagulation abnormalities. Hence, an attempt has been made to find out novel sources of this enzyme from plants. In this paper, we report the new potential sources of L-asparaginase, its purification, characterization of the enzyme, and its antitumor activity from *W. somnifera* (L.) Dunal.

Materials and Methods

Plant Material

W. somnifera (L.) Dunal was collected from the botanical garden of the Department of Biosciences, Sardar Patel University V V Nagar, Gujarat (India). A voucher specimen of the

plant was submitted to the department herbarium. Identification of the plant was confirmed through comparison with herbarium specimen number 10337.

Extraction of Protein

Two grams of the obtained fruits was washed thoroughly with tap water followed by sterile distilled water to remove surface dust and other extraneous material and homogenized in liquid nitrogen using a homogenizer. Extraction of proteins was carried out with three volumes of 0.15 M KCl solution, centrifuged, and the supernatant separated. This was designated as the crude extract. All the steps were carried out at 4 °C. The enzyme activity was measured with Nessler's method.

Purification of the L-Asparaginase

The crude enzyme was precipitated out between 40% and 60% of ammonium sulfate saturation. The pellet was collected by centrifugation at 9000×*g* at 4 °C and dissolved in water. The dissolved pellet was loaded on to a Sephadex G-25 (Pharmacia) column (1.5×15.0 cm) using 0.1 M sodium borate buffer, pH 8.6 as an eluant in order to remove the salts from the previous step. Further purification was done by gel filtration using Sephadex G-75 (Pharmacia) with column dimensions of 1.5×20.0 cm using 0.1 M sodium borate buffer as an eluant. The peak active fractions of G-75 were loaded into CM-Sephadex C-50 with 0.01 M sodium borate buffer (Pharmacia) and ion exchange chromatographic resin. The enzyme was eluted using a gradient of 0.1–0.5 N NaCl. The above procedure was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), protein estimation, and activity assay.

Electrophoresis

The subunit molecular mass of the L-asparaginase from *W. somnifera* L. was estimated by SDS-PAGE in a discontinuous buffer system [21]. In addition, the molar size of native L-asparaginase was estimated by chromatography on Sephacryl S-400 HR (1.6×25.5 cm; Pharmacia). The column was equilibrated with phosphate buffer of 1.0 mM, pH 7.0. The proteins were eluted with the equilibration buffer at a flow rate of 1.5 ml/h. Different protein molecular weight markers were used for SDS-PAGE and NSephacryl S-400 HR.

2D Gel Electrophoresis

Purified protein sample was loaded onto Readystrip IPG strips (Bio Rad) with a pH range of 3–10 NL (nonlinear) and layered with 0.8 ml of cover oil to prevent the gel from drying and urea crystallization. The gel was run on the PROTEAN IEF cell (Bio Rad Laboratories) at 30 V to rehydrate the gel strip for 12 h, followed by the running programs: 30 V for 12 h with 360 V h, 500 V for 1 h with 500 V h, 1,000 V for 1 h with 1,000 V h, and 8,000 V for 2 h with 1,600 V h. The voltage ramped automatically based on the increasing resistance from the strip as excess ions moved out of the strip. After first-dimension IEF, the strip was washed to remove the cover oil and equilibrated in 5 ml of equilibration buffer containing 50 mM Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, and 1% dithiothreitol (DTT) for 12–15 min. The strip was then subjected to a second equilibration with 5 ml of equilibration buffer with 1.5% iodoacetamide substituted for the DTT for an additional 12–15 min, followed by SDS electrophoresis at 100 V for 4 h. All chemicals were brought from Bio Rad Laboratories. At the end of electrophoresis, the gel was stained with silver staining method [22].

Culture of Leukemic Cell

Primary leukemia cells from patients were obtained from peripheral blood, leukapheresis, or bone marrow specimens under an IRB-approved protocol. All samples were obtained from patients newly diagnosed with leukemia prior to the administration of chemotherapy. Lymphoblasts were isolated following the method of Moriji Miura [23] and cultured in RPMI-1640 supplemented with 20% fetal calf serum in a humidified atmosphere containing 5% CO_2.

Antitumor Activity

Initially, the leukocyte culture was prepared in triplicate for further experiments. To the first leukocyte culture, a tube consisted of 0.1 ml phosphate buffer; to the second, the tube contained 2.0 IU of L-asparaginase from *W. somnifera* in 0.1 ml phosphate buffer; and to the third, 1% polyhydroxyalkanoate (PHA) and 2.0 IU of L-asparaginase from *W. somnifera* L. After 48 and 72 h of incubation, cellular viability was determined by direct cell counts using a hemocytometer.

Cytotoxicity Assay

The growth inhibition effect of L-asparaginase was assessed using the [3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl-tetrazolium bromide] (MTT) colorimetric dye reduction method [24, 25]. One hundred thirty-five microliters of exponentially growing lymphoblasts at 48 h was plated at a density of 0.5×10^4 to 1.0×10^4 cells/well in a 96-well microtiter plate with L-asparaginase added to each well at concentrations specified below. After 24 h of continuous drug exposure, 15 µl of MTT (final concentration 0.5 mg/ml) was added to each well, the plates were incubated for 4 h at 37 °C, and the absorbance was measured at 550 nm using a spectrophotometer. 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. For single drug assays, replicates of three wells were used for each drug concentration. Combination effects were analyzed using nonconstant drug combination ratios at single drug 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 3, and 5 IU drug concentrations. After subtraction of blank values, the leukemic cell survival (LCS) was calculated by the equation:

$$\text{LCS} = (\text{OD treated well}/\text{mean OD control wells}) \times 100\%$$

Drug sensitivity was assessed by the LC_{50}, the drug concentration lethal to 50% of the cells.

Table 1 Purification of L-asparaginase from *Withinia somnifera* L.

Sample	Volume (ml)	Protein (mg)	Activity (IU)[a]	Specific activity (IU/mg)	Fold purification	Recovery (%)
Crude	10.0	102.4	11,960.00	117.25	–	100
40–60% $(NH_4)_2SO_4$ precipitation	05.0	51.4	9,850.12	191.63	1.64	82
Gel filtration (G-75)	8.0	16.3	7,950.00	487.13	4.16	66.47
Ion exchange	3.0	3.7	5,698.00	1540	13.14	47.64

[a] IU, one international unit (IU) of L-asparaginase is that amount of enzyme which liberates 1 µmol of ammonia 1 min at 37°C

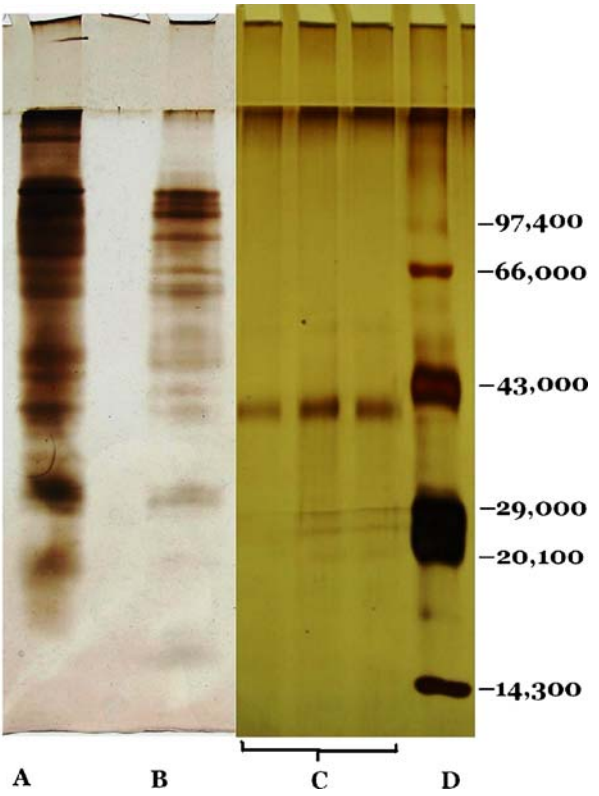

Fig. 1 Purification of L-asparaginase from *Withania somnifera*: *A* crude extract, *B* salt precipitation, and *C* pure form of L-asparaginase after all purification steps. *D* Protein molecular weight markers

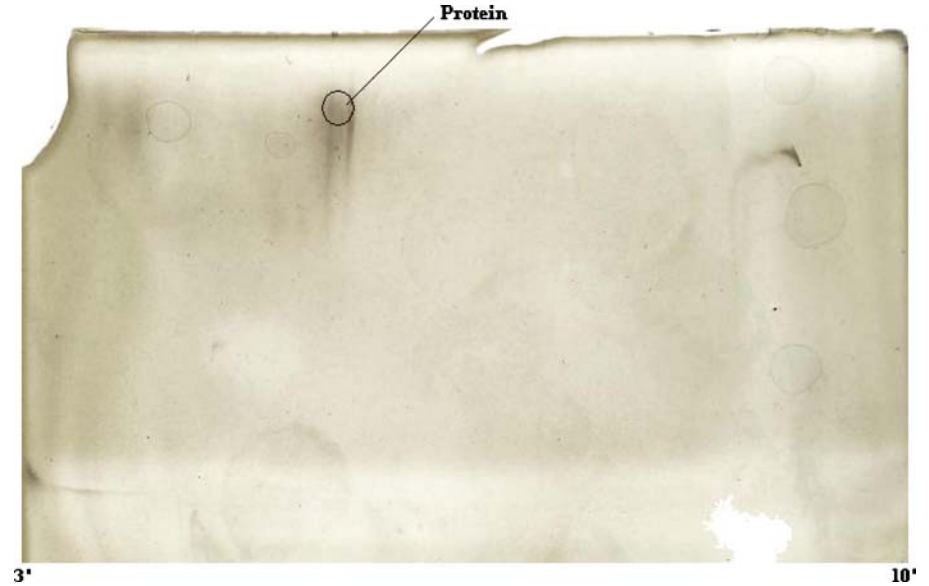

Fig. 2 Determination of p*I* value of L-asparaginase from *Withania somnifera* using 2D gel electrophoresis

Table 2 Influence of L-asparaginase inhibition of PHA-induced lymphocyte cell culture.

Nos.	Tube	Cell counting after 48 h	Cell counting after 72 h
1	Normal blood	24×10^3	47×10^3
2	Normal blood + L-asparaginase	10×10^2	12×10^2
3	Normal blood + 1% PHA + L-asparaginase	16×10^2	19×10^2
4	Patient blood	48×10^5	05×10^6
5	Patient blood + L-asparaginase	75×10^3	88×10^3
6	Patient blood + 1% PHA + L-asparaginase	84×10^3	91×10^3
7	Patient blood + 1% PHA + L-asparaginase (*E. coli*)	64×10^3	69×10^3

Statistical Analysis

Statistical calculations were carried out with the SPSS 10.0 for Windows software package (Statistica). Statistical significances were considered at P values less than 0.05. The percentages of cell viability were presented graphically in the form of histograms, using Microsoft Excel computer program.

Results

L-Asparaginase was purified from *W. somnifera* using salt precipitation, gel filtration, and ion exchange chromatography. The recovery of the enzyme is shown in Table 1. Sodium borate buffer was used as the extraction buffer as the enzyme was most stable at this particular pH during purification. A single band was obtained after all the purification steps of chromatography. The specific activity of the enzyme increased with every step of purification with a minimum loss in quantity, giving a final recovery of 47.64% (Table 1). After every purification procedure, the fractions with the enzyme activity were analyzed using SDS-PAGE (Fig. 1).

The purified enzyme showed a band near 5.0 to 5.5 p*I* points of 3.0 to 10.0 non-linear gradients and 2D gel also showed that the protein sample was purified (Fig. 2). The molecular mass of L-asparaginase from *W. somnifera* is 72±0.5 kDa as determined by

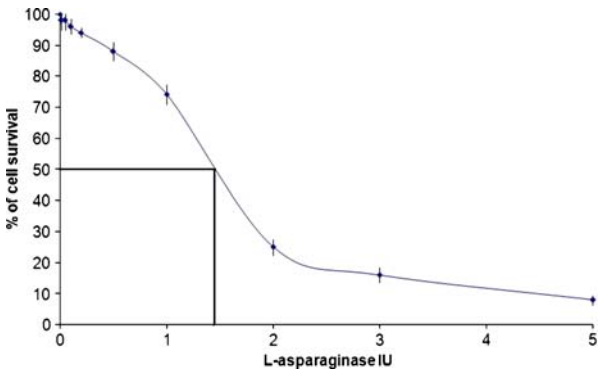

Fig. 3 Cytotoxic effect of L-asparaginase on cell survival in leukemia cells. The cells were treated in triplicate with each concentration for 24 h and the cell viability was determined by the MTT assay

molecular size chromatography on Sephacryl S-400 HR and polyacrylamide gel electrophoresis, respectively. The molecular mass of the subunit is 36±0.5 kDa according to SDS-PAGE, consistent with a homodimeric enzyme.

Table 2 shows the effect of L-asparaginase on lymphocyte cell culture when incubated with different combinations of normal blood with or without the enzyme or the enzyme with similar PHA for patient blood and cell counting at 48 and 72 h. A remarkable suppression of lymphocyte cell culture was observed on concentrations of 2 IU/tube of L-asparaginase from *W. somnifera.*

The results of the cytotoxicity of L-asparaginase to human leukemia cells are presented in Fig. 3. Data obtained from this assay indicated a strong dose–response relationship with regard to the cytotoxic property of L-asparaginase from *W. somnifera*. As indicated in this figure, there was a gradual decrease in the viability of leukemia cells, with increasing doses of L-asparaginase. Upon 24 h of exposure, the mean percentages of cell viability were 100±07, 98±03, 98±3.2, 96±03, 94±1.5, 88±2.8, 74±3.2, 25±2.6, 16±2.5, and 08±0.2 in 0, 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 3, and 5 IU of L-asparaginase, respectively. The drug dose required to cause 50% reduction in cell viability was computed to be 1.45±0.05 IU. These results showed that the enzyme induced a slight decrease in cell viability between 0 IU (control) and 0.5 IU, followed by a gradual decrease between 1 and 5 IU.

Discussion

W. somnifera popularly known as "Ashavgandha" is widely used in Ayurveda. However, this is the first report on the presence of L-asparaginase in this medicinally important plant species. It is assumed that the observed anticancerous property of the drug is due to the presence of L-asparaginase. The molecular weight of L-asparaginase is approximately half to that of prokaryotic asparaginase [26] and similar to plant L-asparaginase [27]. Findings from our study clearly demonstrated that L-asparaginase is highly effective against leukemia cells, showing a 24-h LD_{50} of 1.45±0.05 IU.

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