Purification and Characterization of Therapeutic Enzyme L-Asparaginase from a Tropical Soil Fungal Isolate

Fusarium Culmorum ASP-87

Abstract
L-asparaginase from fungal source finds extensive applications in pharmaceutical and food industries. L-asparaginase is an enzyme that catalyzes the hydrolytic cleavage of the amino acid L-asparagine to L-aspartic acid and ammonia. In the present study, L-asparaginase was purified and characterized from the fungus, Fusarium culmorum ASP-87, isolated from tropical soil. The enzyme was purified to homogeneity by ammonium sulfate precipitation, ion exchange followed by gel filtration chromatography, to 14.03 fold with a final specific activity of 16.66 U/mg of protein with 26.7% yield recovery. The molecular mass of the enzyme was 95 kDa. The purified L-asparaginase had a pH optimum of 8.0, and a temperature optimum of 40°C. The enzyme was stable at pH 8.0 and retained 100% activity up to 24 hrs, and at temperature 60°C retained 50% activity for 60 min. The purified enzyme was highly specific to the substrate L-asparagine and the Km and Vmax were found to be 3.57 mM and 0.5μmol/ml/min, respectively. The enzyme was activated by Mn2+ and Tween 80 and inhibited by Cu2+ and EDTA.

Keywords: Fusarium Culmorum ASP-87; L-Asparaginase; Soil Fungi; Ion Exchange Chromatography; Gel Filtration Chromatography

Introduction
Microbial L-asparaginase is one of the most important industrial enzymes of interest accounting for about 40% of the total worldwide enzyme sales [1]. The enzyme L-asparaginase amidase EC:3.5.1.1 belongs to an amidase group that catalyzes the hydrolysis of the amino acid L-asparagine to L-aspartic acid and ammonia. This enzyme has got much significance in medical field for the treatment of leukemia especially acute lymphoblastic leukemia (ALL) [2] and also widely used in baking and food industries to reduce the formation of carcinogenic acrylamides in biscuits and in deep fried potato [3,4]. L-asparaginase is widely distributed in plants, animals and microorganisms. However, L-asparaginase from microbial sources has gained much attention because of its high productivity. It is extracellular and therefore secreted in to the fermentation medium. Among microbes, this enzyme is produced by bacteria, fungi and actinomycetes. Microbial strains like Escherichia coli [5], Erwinia caratovora [6], Pseudomonas aeruginosa [7], Streptomyces grallargensis [8], Aspergillus terreus [9], Aspergillus niger [10], Penicillium breviciptomatum [1], Cladosporium sp [11] are the main source of L-asparaginase. Bacterial L-asparaginase has been reported to cause hypersensitivity leading to allergic reactions and anaphylaxis [12]. Hence, L-asparaginase from eukaryotic microorganisms is gaining much importance as it is known to have less adverse effects [13]. The main objective of the present work was to purify and characterize L-asparaginase from Fusarium culmorum ASP-87 isolated from tropical soil and to demonstrate its physio-chemical properties which are indispensable in both fundamental and applied research.

Materials and Methods

Strain and chemicals
The fungus F. culmorum strain ASP-87 used in this experiment was isolated from tropical soil. Media components used in the experiment were obtained from Hi-media (Mumbai, India). The substrate L-asparagine, DEAE-cellulose and Sephadex G-100 was procured from Sigma (Sigma-Aldrich, USA). All the chemicals were of analytical reagent grade.

Production of L-asparaginase by Fusarium culmorum ASP-87
The fungal culture was maintained on potato dextrose agar (PDA) slant at 4°C and sub cultured on PDA plates, incubated at 30°C for 6 days and used as inoculum. The culture medium used for the study was modified Czapek-dox medium containing g/l of, Glucose,2.0; L-asparagine,10.0; KH2PO4,1.52; KCl,0.52; MgSO4.7H2O,0.052; Ca(NO3)2,3H2O,trace; ZnSO4.7H2O,trace; FeSO4.7H2O,trace; pH 7.5 [14]. Modified Czapek-dox broth (200 ml) was prepared, sterilized and inoculated with F. culmorum ASP-87 spore suspension (10⁶/ml). The cultures were incubated at 30°C under shaken condition (120 rpm) for 4 days.

Assay of L-asparaginase activity
The activity of L-asparaginase in the culture filtrate was assayed using the method of Imada [15]. The rate of hydrolysis of L-asparagine was determined by measuring the ammonia released using Nessler’s reagent. A mixture of 0.5ml of enzyme extract, 0.5ml of 0.04M L-asparagine, 0.5ml of 0.05M Tris-HCl
buffer (pH 7.2) and 0.5ml of distilled water was incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.5ml of 1.5M trichloroacetic acid (TCA). The ammonia released in the supernatant was determined colorimetrically by adding 0.2ml of Nessler’s reagent into tubes containing 0.1ml of supernatant and 3.7ml of distilled water and incubated at room temperature for 20 min. The absorbance was read at 450 nm. One international unit (IU) of L-asparaginase activity is defined as the amount of enzyme required to produce 1μmol of ammonia per min under the conditions of the assay.

**Purification of L-asparaginase**

Ammonium sulfate precipitation: A four day old culture filtrate (200ml) of *F. culmorum* ASP-87 grown in modified Czapek-dox broth was collected after centrifugation at 8000 rpm for 10 min at 4°C. All subsequent purification steps were carried out at 4°C. The crude enzyme was subjected to ammonium sulfate precipitation and the protein precipitate at 80% salt saturation was allowed to stand overnight. The precipitate was collected by centrifugation at 10,000 rpm for 15 min and resuspended in 0.01M Tris-HCl buffer (pH-7.2) and dialyzed overnight against the same buffer.

Ion exchange chromatography: The dialyzed ammonium sulfate fraction was applied to an anion exchange chromatography on DEAE cellulose (1.5×15cm) column pre-equilibrated with the Tris-HCl buffer (0.01M; pH 7.2). The unbound proteins were washed repeatedly for five times and then eluted with the same buffer containing NaCl (1M) by a stepwise gradient at a flow rate of 1 ml/min. Fractions containing L-asparaginase activity were pooled and lyophilized.

**Estimation of protein:** The concentration of protein was estimated by the method of Bradford [16] using bovine serum albumin as the standard.

**Determination of molecular weight of purified protein by SDS-PAGE:** The molecular weight of purified L-asparaginase was determined by (SDS-PAGE) the method of Laemmli [17]. SDS-PAGE was performed using a 12% polyacrylamide gel. The proteins were stained with coomassie brilliant blue R-250. The molecular weight of purified enzyme was determined using standard molecular weight markers (Bio-Rad).

**Enzyme characterization**

**Effect of pH on enzyme activity and stability:** In order to determine the effect of pH on purified L-asparaginase, the purified enzyme was pre-incubated in 0.1 M buffer in the range between pH 3 and pH 11 without the addition of substrate, the enzyme activity was determined under standard assay conditions. Buffers used were sodium phosphate-citrate (pH 3.0-6.0), tris-HCl (pH 7.0-9.0) and glycine-NaOH (pH 10.0-11.0). In order to determine the pH stability, the purified enzyme was pre-incubated in pH buffer range between pH 3 and pH 11 at room temperature for 24hrs. The residual activity was determined for every 12 hrs intervals under standard assay conditions. The activity of the enzyme at zero minute of the reaction was considered as 100% and served as control.

**Effect of temperature on enzyme activity and stability:** In order to determine the effect of temperature on purified L-asparaginase, the enzyme activity was studied at different temperatures ranging from 20°C to 80°C at 5°C increments, the enzyme activity was determined under standard assay conditions. Thermal stability of the purified enzyme was evaluated by incubating the enzyme for 10, 20, 30, 40, 50, 60 minutes at different temperatures ranging from 30°C to 80°C. The residual activity was determined under standard assay conditions. The activity of the enzyme at zero minute of the reaction was considered as 100% and served as control.

**Effect of metal ions, inhibitors and surfactants on L-asparaginase activity:** In order to determine the effect of metal ions, inhibitors and surfactants like Mn²⁺, Zn²⁺, Fe²⁺, Cu²⁺, Mg²⁺, Ca²⁺, Hg²⁺, Co²⁺, Fe³⁺, EDTA, β-mercaptoethanol, tween 80 and SDS on L-asparaginase activity, the purified enzyme was preincubated with different metal solutions at 5mM concentration and inhibitors and surfactants at 1mM concentration for 30 min and the residual activity of the enzyme was assessed. The activity of the enzyme without the addition of metal ions or inhibitors or surfactants was considered as 100% and served as control.

**Substrate specificity**

To assess the activity of L-asparaginase, different substrates such as L-asparagine, L-aspartic acid, L-glutamine and L-glutamic acid were used. The substrates were prepared in 0.05M tris-HCl buffer pH-7.5 at 10mM concentration. The enzyme was mixed with substrate and the reaction mixture was incubated for 30 min at 40°C and enzyme activity was determined. Results were expressed as relative percentage.

**Determination of kinetic constants**

The kinetic parameters $K_m$ and $V_{max}$ of purified L-asparaginase were determined by the method of Lineweaver and Burk [18] with different concentrations of L-asparagine (1mM-10mM) dissolved in 0.05M of tris-HCl buffer pH 8.0. The enzyme activity was determined by measuring the rate of hydrolysis of L-asparagine under standard assay conditions.

**Results and Discussion**

**Purification of L-asparaginase**

The enzyme L-asparaginase was purified from the culture filtrate of *F. culmorum* ASP-87 using ammonium sulfate precipitation, ion exchange chromatography followed by gel filtration. The purification procedure is summarized in (Table 1). The first step of purification by ammonium sulfate precipitation achieved 2.63-fold purification with 26% enzyme recovery. The second purification step was done by ion exchange chromatography using DEAE cellulose. This step showed 8.42-fold increase in enzyme activity with 6.57% enzyme recovery.
Table 1: Purification of L-asparaginase from *F. culmorum* ASP-87.

| Sample                          | Total Enzyme Activity (U) | Total Protein (Mg) | Specific Activity (U/Mg) | Purification Fold | Yield (%) |
|---------------------------------|---------------------------|--------------------|--------------------------|-------------------|-----------|
| Crude                           | 95                        | 80                 | 1.1875                   | 0                 | 100       |
| Ammonium sulfate precipitation  | 25                        | 8                  | 3.125                    | 2.6315            | 26        |
| Ion exchange                    | 6.25                      | 0.625              | 10                       | 8.421             | 6.578     |
| Gel filtration                  | 2.5                       | 0.15               | 16.666                   | 14.034            | 2.631     |

The final step of purification was performed by gel filtration using Sephadex G-100 column. The fractions showing L-asparaginase activity in this step were collected and pooled. The final step of purification resulted in 14.03-fold increase in enzyme activity with overall specific activity of 16.66 U/mg of protein and with a net yield of 2.6% enzyme recovery. L-asparaginase from various fungal species have been purified and characterized and reported earlier. L-asparaginase from *Penicillium brevicompactum NRC 829* was purified to 151.12 fold with a specific activity of 574.24 U/mg and yield of 39.90% [1] and L-asparaginase purified from *Mucor hiemalis* exhibited a specific activity 69 U/mg with 18.46% recovery and 4.59 purification fold [19]. Interestingly, L-asparaginase from *Cladosporium sp* was purified to 867.7 fold with a specific activity of 83.8 U/mg [11]. Although, the purification steps followed by various researchers are almost similar for different fungal species, the purification fold and yield varies. This could be due to the interference of different proteins present in the culture filtrate.

**Physio-chemical characterization of the enzyme L-asparaginase**

**Determination of Molecular weight:** The purified L-asparaginase from *F. culmorum* ASP-87 showed homogeneity and the molecular mass was estimated as 90 kDa, by SDS-PAGE analysis (Figure 1). The molecular weight of L-asparaginase from *F. culmorum* ASP-87 was almost similar to that of *Penicillium brevicompactum NRC 829* (94 kDa) and *Trichoderma viride* (99 kDa) [1,20]. Whereas, L-asparaginase from *Cladosporium sp* has a molecular weight of 117 kDa and *Aspergillus niger* with 48 kDa [13,21]. The variability in the molecular weight of L-asparaginase in different organisms may be inferable to its genetic diversities.

**Effect of pH on enzyme activity and stability:** pH is a critical factor for stability and activity of purified enzyme, as it impacts on the ionic form of the enzyme active site residues. The effect of pH on the activity of purified L-asparaginase was done over a wide range of pH from 3.0 to 11.0 at 30°C. The results revealed that L-asparaginase from *F. culmorum* ASP-87 was active over a broad range of pH, optimum being pH 8.0 (Figure 2). Comparable results were reported by earlier workers in *Penicillium brevicompactum NRC 829* [1] and *Streptomyces sp.* [22]. But, on the contrary Lincoln and Monica reported that pH 7.0 was the optimum pH for the activity of L-asparaginase in *Trichoderma viride* and *Mucor hiemalis* [20,19]. The purified L-asparaginase retained 100% activity at pH 8.0 up to 24 hrs of incubation (Figure 3). However, L-asparaginase from *Trichoderma viride* retained 82% of its activity after 24 hrs of incubation [20] and *Mucor hiemalis* maintained its stability only for 4 hrs there after started declining [19]. The stability of L-asparaginase from *F. culmorum* ASP-87 at alkaline pH is a promising factor to be used as a therapeutic agent.
however, inactivated at temperature above 40°C while retaining 50% activity at 60°C for 1 hour. L-asparaginase from *Penicillium brevicompactum* NRC 829 was reported to be stable up to 1 hour at 37°C [1]. Considering the thermal stability of other organisms reported earlier, L-asparaginase from *F. culmorum* ASP-87 shows moderate thermostability, a factor of significant importance in pharmaceutical industry.

**Figure 5:** Effect of temperature on stability of purified L-asparaginase from *F. culmorum* ASP-87.

**Effect of metal ions, inhibitors and surfactants on L-asparaginase activity:** The influence of various metal ions on purified L-asparaginase activity was studied. Among the different metal ions tested, Mn\(^{2+}\) enhanced the activity of enzyme by 18% (Table 2) as reported by earlier worker in *Mucor hiemalis* [19]. Whereas, Cu\(^{2+}\) and Hg\(^{2+}\) inhibited the activity of enzyme by 84 and 80%, as observed by Archana in *Aspergillus nidulans* [23] and Kumar in *Pectobacterium carotovorum* [26]. However, other metal ions like Ca\(^{2+}\) and Mg\(^{2+}\) did not have much effect on enzyme activity. Among the two inhibitors tested, EDTA inhibited the activity by 88% while β-mercaptoethanol did not have any effect on enzyme activity. Among the two inhibitors tested, EDTA inhibited the activity by 88% while β-mercaptoethanol did not have any effect on enzyme activity. Among the two inhibitors tested, EDTA inhibited the activity by 88% while β-mercaptoethanol did not have any effect on enzyme activity. Among the two inhibitors tested, EDTA inhibited the activity by 88% while β-mercaptoethanol did not have any effect on enzyme activity. Among the two inhibitors tested, EDTA inhibited the activity by 88% while β-mercaptoethanol did not have any effect on enzyme activity. Among the two inhibitors tested, EDTA inhibited the activity by 88% while β-mercaptoethanol did not have any effect on enzyme activity. Among the two inhibitors tested, EDTA inhibited the activity by 88% while β-mercaptoethanol did not have any effect on enzyme activity. Among the two inhibitors tested, EDTA inhibited the activity by 88% while β-mercaptoethanol did not have any effect on enzyme activity. Among the two inhibitors tested, EDTA inhibited the activity by 88% while β-mercaptoethanol did not have any effect on enzyme activity. Among the two inhibitors tested, EDTA inhibited the activity by 88% while β-mercaptoethanol did not have any effect on enzyme activity. Among the two inhibitors tested, EDTA inhibited the activity by 88% while β-mercaptoethanol did not have any effect on enzyme activity. Among the two inhibitors tested, EDTA inhibited the activity by 88% while β-mercaptoethanol did not have any effect on enzyme activity. Among the two inhibitors tested, EDTA inhibited the activity by 88% while β-mercaptoethanol did not have any effect on enzyme activity. Among the two inhibitors tested, EDTA inhibited the activity by 88% while β-mercaptoethanol did not have any effect on enzyme activity. Among the two inhibitors tested, EDTA inhibited the activity by 88% while β-mercaptoethanol did not have any effect on enzyme activity.

**Figure 4:** Effect of temperature on the activity of purified L-asparaginase from *F. culmorum* ASP-87.

**Substrate specificity**

The property of enzymes that makes them important as diagnostic tools is the specificity they exhibit towards the substrate. Among the different substrates investigated, the enzyme showed high specificity towards its natural substrate L-asparaginase, very low specificity towards L-aspartic acid, while no activity towards L-glutamine and L-glutamic acid (Figure 6). Our findings were in concordance with that of Prabhu, Sahira and Lincoln in *Vibrio costicola*, *Acinetobacter baumannii* and *Trichoderma viride* respectively [28, 29, 20]. This property of purified enzyme increases its potential to be used in therapeutics and food industries.

**Determination of kinetic constants**

The *K_m* and *V_max* of purified L-asparaginase from *F. culmorum* ASP-87 were determined using various concentrations of L-asparagine. The values of *K_m* and *V_max* were 3.1 mM and 0.77μmol/ml/min respectively (Figure 7), which is lower than the *K_m* value of L-asparaginase purified from *E. coli*, *Penicillium* sp and *Mucor hiemalis* [30, 31, 19]. However, a slightly higher *K_m* value of 12.5 mM and lower *K_m* value of 1.05 mM were reported.

**Figure 3:** Effect of pH on stability of purified L-asparaginase from *F. culmorum* ASP-87.

**Figure 6:** Effect of pH on the activity of purified L-asparaginase from *F. culmorum* ASP-87.
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The purified L-asparaginase from Fusarium culmorum ASP-87 has stronger affinity towards its natural substrate L-asparagine, a positive property to be useful towards the treatment of tumors.

**Conclusion**

L-asparaginase from microbial source is of great interest owing to its significance in pharmaceutical and food industries. Therefore, in the present study, an attempt is made to purify L-asparaginase from Fusarium culmorum ASP-87 and its physical and chemical characteristics were studied. The stability of L-asparaginase at alkaline pH and temperature is an advantage for pharmaceutical and food applications. The specificity of L-asparaginase to its natural substrate L-asparagine is of greater importance for making diagnostic biosensors. However, further in depth studies are required to optimize cost effective substrate for bulk production of enzymes. Although, a strain of Fusarium culmorum IFO5902 is been reported to possess L-asparaginase activity by Nakahama way back in 1973 [33], no attempt was made so far to purify and characterize the physio-chemical nature of the enzyme. This is the first report which gives the complete physical and chemical nature of the purified L-asparaginase from Fusarium culmorum, isolated and identified as one of the best producers of L-asparaginase by dye based rapid screening method among the 364 soil fungal isolates screened [34].

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**Table 2:** Effect of metal ions, inhibitors and surfactants on L-asparaginase activity.

| Metal Ions (5mm) | Relative Activity (%) |
|-----------------|-----------------------|
| Control         | 100                   |
| Zn^{2+}         | 77                    |
| Fe^{2+}         | 68                    |
| Cu^{2+}         | 16                    |
| Mg^{2+}         | 93                    |
| Ca^{2+}         | 96                    |
| Mn^{2+}         | 118                   |
| Hg^{2+}         | 20                    |
| Co^{2+}         | 72                    |
| Fe^{3+}         | 59                    |
| EDTA            | 12                    |
| β-mercaptoethanol| 76                   |
| Tween 80       | 116                   |
| SDS             | 0                     |
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