



Purification and characterization of L-asparaginase producing bacteria MNTG-7

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ABSTRACT

Several techniques have been described for recovery and purification of L-asparaginase from different sources. The present study was undertaken to recover and purify L-asparaginase from mutated MNTG-7 cell line using three-step procedure involving ammonium sulphate precipitation, ion exchange and gel filtration chromatography followed by the characterization of the purified enzyme. Protein molecular mass markers, DEAE cellulose and Sephadex G-200 matrices, Agarose, acrylamide, bis-acrylamide, sodium dodecyl sulfate (SDS), TEMED, ammonium persulphate and bovine serum albumin were used in the purification process.

Keywords: L-asparaginase, MNTG-7, anticancer agent.

INTRODUCTION

Microbial enzymes and co-enzymes are widely used in several industries, notably in detergent, food processing, brewing and pharmaceuticals. They are also used for diagnostic, scientific and analytical purposes. Since ancient times they have been used in the preparation of fermented foods, especially in oriental countries (Reed, 1975). At present economically the most important enzymes are proteases, glucoamylases, glucose isomerase, and pectinases.

L- asparaginase is enzyme acting on L- asparagine and is widely used as anticancer agent. The reason it is preferred for the purpose is it is biodegradable, non-toxic and can be administered at the local site quite easily. The antineoplastic activity is attributed to the depletion of L- asparagine by the action of L-

asparaginase from *Erwinia carotovora* is reported by Lee et al., (1989). Mushburn et al., (1961) purified *E. coli* L- asparaginase and demonstrated its tumour inhibitory activity. Cedar et al., (1968) reported that the L- asparaginase is synthesized at constant rates by *E. coli* under anaerobic conditions. The crystal structure of the enzyme from *E. coli* was studied by Swain et al., (1993). Asparaginase is preferred as it is biodegradable Stecher et al., (1999), Verma et al., (2007) and (Ferrare et al., 2004). Kidd in 1953 reported the action against tumour cells in mice and rats when Guinea pig serum was found to have this enzyme. Mushburn and Wriston detected similar activity from *E. coli* cells. But the enzyme L-asparaginase is responsible for this activity was suggested by Broome (1963). Later on some workers like Yellin and Wriston (1966) confirmed this

observation that L- asparaginase is the antitumour factor.

Most industrially important enzymes are extracellular i.e. secreted by the cells into the ambient medium, from where they have to be recovered by removal and separation from the cellular and other solid materials.

Several techniques have been described for recovery and purification of L-asparaginase from different sources (Youssef and Al-Omair, 2008; Prista, A 2001; Rozalska, M, 1989; Mesas et al., 1990; Lee et al., 1989; El-Bessoumy et al., 2003; Dunlop et al., 1978; Khushoo et al., 2004; Manna et al., 1995; Joseph Rpberts, 1976; Campbell et al., 1967; Barbara Rowley et al., 1967; John W et al., 1974; Kristiansen et al., 1970; Krasotkina et al., 2004; John A et al., 1976; Gaffar S. A. et al., 1977; Law and Wriston, 1971; Raymond et al., 1971).

The present study was undertaken to recover and purify L-asparaginase from mutated MNTG-7 cell line using three-step procedure involving ammonium sulphate precipitation, ion exchange and gel filtration chromatography followed by the characterization of the purified enzyme.

MATERIALS AND METHODS

Chemicals

Protein molecular mass markers were purchased from Genei, Bangalore, India. DEAE cellulose and Sephadex G-200 matrices were procured from Amersham Pharmacia Limited, Hong Kong. Agarose, acrylamide, bis-acrylamide, sodium dodecyl sulfate (SDS), TEMED, ammonium persulphate and bovine serum albumin were purchased from Sigma Aldrich Chemical Company, U.S.A. All other reagents were Qualigens and Loba grade products.

Preparation of cell suspension

Cell suspension (10ml) from stock culture of MNTG-7 was inoculated to the 45 ml of optimized production medium in 250 ml Erlenmeyer flask. The flasks were incubated at 37°C on rotary incubator shaker for 24 h (120 rpm). At the end of incubation, 5ml broth was collected and centrifuged at 4000 rpm for 10 min. and assayed for L-asparaginase activity.

Preparation of inoculum

Inoculum was prepared as described earlier with MNTG-7 cells.

Submerged fermentation

Five ml of inoculum (10% v/v) was inoculated to the optimized production medium (45ml) contained in 10 number of 250 ml EM flasks. The flasks were incubated at 37°C on rotary incubator shaker for 24 h (120 rpm). At the end of fermentation, the entire medium was pooled together and centrifuged using Remi C 24 cooling centrifuge at 10,000 rpm at 4°C for 15 min. The supernatant containing the crude enzyme was recovered and subjected to purification studies.

Analytical method

The L-asparaginase activity was determined as described earlier.

Preparation of reagents

1. Saline solution (0.9% w/v NaCl): 900 mg of NaCl was weighed accurately and dissolved in 100 ml of distilled water.
2. Acrylamide solution: 29.2 g of Acrylamide and 0.8 g of N, N'-methylene bis acrylamide were dissolved in distilled water and made up to 100 ml with distilled water. This solution was filtered through Whatmann No.1 filter paper and stored in an amber coloured bottle at 4°C.
3. Separating gel buffer (1.5 M Tris-HCl, pH 8.8): 18.15 g of Tris-HCl was dissolved in about 80 ml of distilled water and the pH was adjusted to 8.8 with 6 N HCl and the volume was made up to 100 ml with distilled water.
4. Stacking gel buffer (0.5 M Tris-HCl, pH 6.8): 6.05 g of Tris-HCl was dissolved in about 80 ml of distilled water and the pH was adjusted to 8.8 with 6 N HCl and made up to 100 ml with distilled water.
5. SDS (10% w/v): 1 g of SDS was weighed and dissolved in 10 ml of distilled water.
6. 10% APS (ammonium persulphate): 500 mg of APS was weighed accurately and dissolved in 5 ml of distilled water. This solution was freshly prepared just before use.
7. Running buffer for SDS-PAGE: Tris (3 g), glycine (14.4 g) and SDS (1 g) were weighed accurately

and dissolved in 1 L of distilled water. The pH of the solution was adjusted to 8.3 with 0.1 M HCl.

8. Sample buffer for SDS-PAGE: 6.25 ml of 0.5 M Tris-HCl (pH 6.8) was added to 2.5 ml of β -mercapto ethanol, 10% SDS (10 ml) and glycerol (5 ml). The pH was adjusted to 6.8 with 0.1 M HCl.

Purification of L-asparaginase

The purification protocol includes different steps sequentially like ammonium sulphate precipitation, ion exchange and gel filtration chromatographics. After each step, the L-asparaginase activity and total protein content were determined. SDS-PAGE was also carried after each step.

RESULTS AND DISCUSSION

Purification of L-asparaginase

Ammonium sulphate precipitation (step I)

Isolation of L-asparaginase was most effective with ammonium sulphate precipitation. 45% concentration of this salt gave a precipitate rich in L-asparaginase activity. After fractionation with ammonium sulphate (45% saturation) and dialysis, the specific activity of L-asparaginase was increased from 3.67 to 30.24 IU/mg. The dialyzate was subjected to lyophilization which resulted 1.02g of protein with 45.82% yield and 8.24 fold purification.

Ion exchange chromatography (step II)

Utilization of Ion exchange chromatography resulted in excellent purification index. The lyophilized protein was dissolved in 0.05 M Tris-HCL buffer of pH 8.6 and was applied on DEAE-cellulose column and was eluted with discontinuous gradient of NaCl (0.1 to 0.5M). The elution profile of ion exchange chromatography is shown in Fig. 6.2. The enzyme recovered from ammonium sulphate was fractionated into 5 major protein peaks and 2 minor peaks. It was observed that L-asparaginase was eluted from the column with 0.2 M NaCl concentration. The protein in each fraction was monitored by measuring the optical density at 280 nm. All fractions (27-33) which constituted a single peak and showed good L-asparaginase activity were pooled together dialyzed, lyophilized and stored for further purification. The specific activity of the L-asparaginase after ion exchange chromatography was found to be 75.26

IU/mg. A yield of 32.33% and 20.51 fold purification was achieved after ion exchange chromatography.

Sephadex G-200 gel filtration chromatography (step III)

The lyophilized protein obtained after DEAE-cellulose column was then subjected to further purification using molecular sieving. Molecular filtration of partially purified L-asparaginase was performed in Sephadex G-200. The elution profile of gel filtration chromatography is shown in Fig. 6.2. From the elution profile, it was observed that the enzyme was eluted in four fractions (14-17) constituting the peak. These fractions were pooled, dialyzed and finally lyophilized. The specific activity of the purified L-asparaginase was found to be 228.52 IU/mg. After gel filtration chromatography, 25.97% yield and 62.27 fold purification was achieved.

Employing a three step purification protocol involving ammonium sulphate precipitation, DEAE ion exchange chromatography and Sephadex G-200 gel filtration chromatography, the L-asparaginase was purified to 62.27 fold over the activity present in the crude enzyme with 25.97% yield.

SDS-PAGE (step IV)

Protein fractions after each step of purification, viz., ammonium sulphate fractionation, DEAE-cellulose chromatography and Sephadex G-200 gel filtration chromatography along with crude extract were analyzed by SDS-PAGE. The electrophoretic pattern is shown in Fig. 6.3.

From the electrophoretic pattern it is evident that the crude and ammonium sulphate fractionations contained many protein bands and were not properly resolved. DEAE-cellulose ion exchange chromatography showed three distinct protein bands, where as Sephadex G-200 gel filtration chromatographic fraction showed a single protein band corresponding to single peak of enzyme activity observed in gel filtration elution profile. It indicated that protein was purified to apparent homogeneity and is composed of only a single polypeptide chain.

The molecular weight of the enzyme was determined by comparison of the migration distances of standard marker proteins (Andrews, 1965). Standard solutions of bovine serum albumin (67 kDa), ovalbumin (43 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa),

carbonic anhydrase (29 kDa) and lysozyme (14 kDa) were used to create a molecular weight ladder on the gel. The molecular weight of the purified protein was determined by interpolation from a semi-logarithmic plot of relative masses vs the R_f values (relative mobility) depending on the relative mobility. The molecular weight of the purified fraction was deduced from the graph to be 36 kDa which coincided with the band of glyceraldehyde-3-phosphate dehydrogenase which was used as a marker protein.

Characterization of purified enzyme

Effect of pH on enzyme activity and stability

The effect of pH on enzyme activity and stability was tested at various pH values as described earlier. The results are shown in Fig. 6.4. The highest enzyme activity was found to be at pH 9.0 and the stability was found to be more between pH 8.0-10.0.

Temperature optimum and thermal stability

The activity of the purified enzyme was determined at different temperatures ranging from 10-100°C. The maximum enzyme activity was found to be upto 40°C and the stability was found to be more between 50-60°C Fig. 6.4.

Effect of metal ions on activity of L-asparaginase

The effect of metal ions on L-asparaginase activity was examined and the results are presented in Table 6.1. The results suggested that the L-asparaginase activity was stimulated by Ca^{+2} , Na^+ and K . Enzyme activity was inhibited by Cu^{2+} , Hg^{2+} and Cd^{2+} ions.

Effect of inhibitors and chelators on L-asparaginase activity

Inhibition studies primarily give an insight of the nature of the enzyme, its cofactor requirements and nature of the active center (Sigma and Mooser, 1975). The effect of different inhibitors on enzyme activity of the purified fraction was studied and the results are presented in Table 6.2. Of the inhibitors tested, PMSF and pCMB completely inhibited enzyme activity while DFP exhibited 92% inhibition. In this regard, PMSF sulphonates are the essential serine residues in the active site of the enzyme and has been reported in the complete loss of enzyme activity. This indicated that it is a serine protease. In the case of others, the L-asparaginase activity was not inhibited by EDTA and β -ME, while a slight inhibition was observed with iodoacetate and benzamide.

The above characterization studies indicated that the enzyme has a molecular weight of 36 kDa. The enzyme has stability over a wide pH and temperature ranges and was stimulated by sodium and potassium ions and inhibited by serine protease inhibitors.

FIGURES AND TABLES

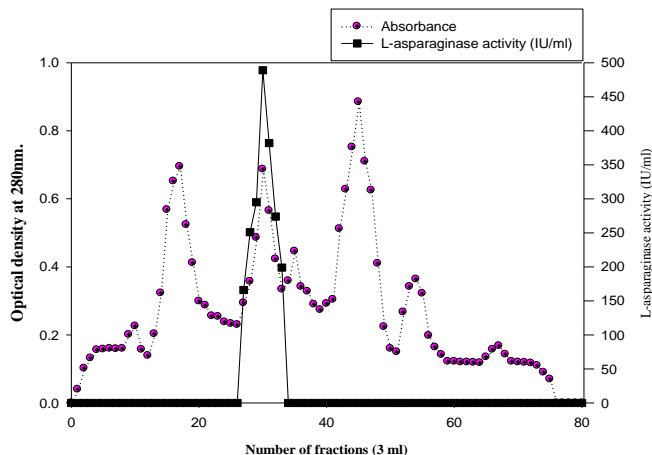


Fig. 6.1: Elution profile of DEAE-Cellulose column chromatography

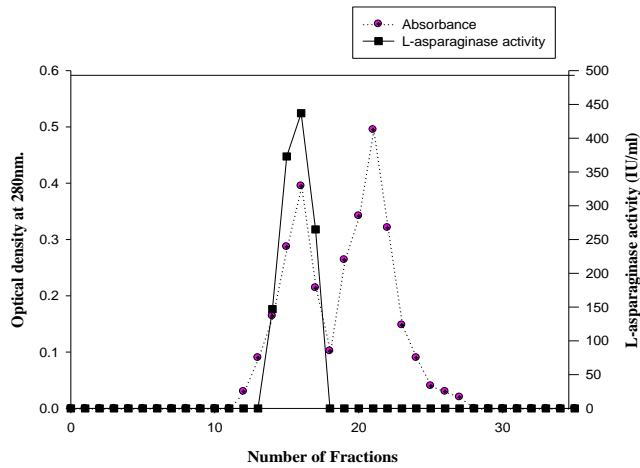


Fig. 6.2: Elution profile of Sephadex G 200 gel filtration chromatography

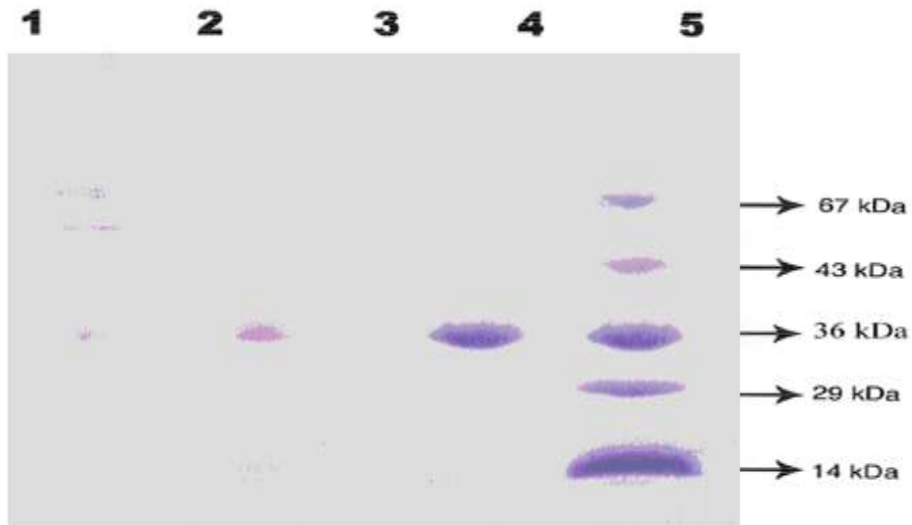


Fig. 6.3: SDS-PAGE of L-asparaginase from MNTG-7 cell line.

- Lane 1: Crude extract
- Lane 2: Ammonium sulphate precipitation
- Lane 3: DEAE-Cellulose ion exchange chromatography
- Lane 4: Sephadex G-200 gel filtration chromatography
- Lane 5: Protein molecular weight markers

Fig.6.4: Effect of pH on L-asparaginase activity and stability

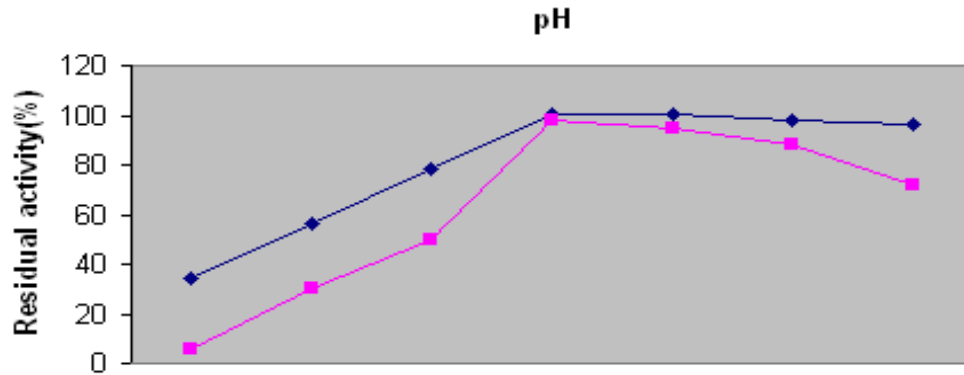


Fig.6.5: Effect of temperature on L-asparaginase activity and stability

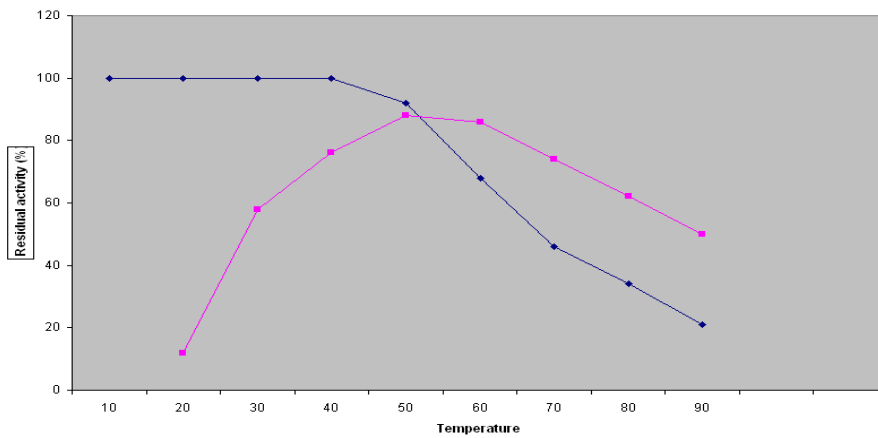


Table 6.1: Effect of metal ions on L-asparaginase activity

Metal ions (10 mM)	Residual L-asparaginase activity (%)
Control	100
Ca ²⁺ (CaCl ₂)	116
Cu ²⁺ (CuSO ₄)	00
Cd ²⁺ (CdCl ₂)	00
Na ⁺ (NaCl)	118
Hg ²⁺ (HgCl ₂)	00
K ⁺ (KCl)	140
Zn ²⁺ (ZnCl ₂)	10
Ni ²⁺ (NiCl ₂)	60
Fe ³⁺ (FeCl ₃)	52
Mg ²⁺ (MgSO ₄)	83
Mn ²⁺ (MnCl ₂)	32

Table 6.2: Effect of inhibitors and chelators on L-asparaginase activity

Inhibitor/chelator (5 mM)	% Residual L-asparaginase activity
Control	100
DFP	08
PMSF	00
pCMB	00
β -ME	100
EDTA	100

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