Chapter 2

Purification and characterization of the enzyme
Chapter 2(A)  

Purification and characterization of the enzyme
4A.1. Introduction

In recent decades, advances in biotechnology have increased the potential usage of protein products. The emergence of new and promising research activities in molecular biology and immunology has expanded continuously seeking the number of proteins (enzymes) that need to be purified and characterized. Microbial L-asparaginase is one among them. Owing to its high efficacy in treatment of acute lymphatic leukaemia and application in food industries to reduce acrylamide levels, special emphasis in this direction is required. L-asparaginase from bacteria yielded less enzyme activity, and had showed difficulty in extraction. Also, there were allergic effects due to long-term administration resulting in hypersensitivity, anaphylaxis, allergic reactions, and other toxic reactions (Narta et al., 2007). L-asparaginase from fungal sources was found to be an alternative. L-asparaginase from *Aspergillus niger* and *Aspergillus oryzae* (Laan et al., 2008; Matsui et al., 2008) have been patented for industrial use. However, the application of the enzyme from fungal sources is at infancy. The present report comprises of detailed studies on the purification, characterization and kinetic properties of extracellularly produced L-asparaginase from *Cladosporium* sp.

4A.2. Materials

4A.2.1. Chemicals

L-asparagine was purchased by Sigma (St. Louis, USA). DEAE cellulose anion exchanger, sepharose 6B, acrylamide, bis-acrylamide, sodium dodecyl sulfate (SDS), TEMED, bovine serum albumin and standard proteins for SDS-gel electrophoresis were purchased from Pharmacia, Uppsala, Sweden. Coomassie brilliant blue G-250 was from Eastman Kodak Co., Rochester, NY, USA. Glycine and sodium acetate were from E. Merck (India) Ltd., Mumbai, India. Ammonium persulfate was from Hi Media Laboratories Ltd., Mumbai, India. β-mercaptoethanol was purchased from Fluka, Switzerland. SDS-PAGE markers were from Bangalore Genei Pvt. Ltd., Bangalore, India. All other chemicals were of the highest analytical grade and purchased from standard chemical companies.
4A.2.2. Reagents

4A.2.2.1. Sodium acetate buffer (pH: 4.0–6.0)

<table>
<thead>
<tr>
<th>Components</th>
<th>Grams/litre (15 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a). Sodium acetate</td>
<td>40.8</td>
</tr>
<tr>
<td>b). Acetic acid</td>
<td>18.0</td>
</tr>
</tbody>
</table>

Solution a and b were mixed together to obtain required pH range 4.0 to 6.0 and the volume was made up to 1 L with dist. H₂O.

4A.2.2.2. Sodium Phosphate buffer (pH: 6.0–8.0)

<table>
<thead>
<tr>
<th>Components</th>
<th>Grams/litre (15 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a). NaH₂PO₄.H₂O</td>
<td>2.07</td>
</tr>
<tr>
<td>b). Na₂HPO₄.H₂O</td>
<td>2.13</td>
</tr>
</tbody>
</table>

Solution a and b were mixed together to obtain required pH range 6.5-8.0 and the volume was made up to 1 L with dist. H₂O.

4A.2.2.3. Carbonate buffer (pH: 8.0-10.5)

<table>
<thead>
<tr>
<th>Components</th>
<th>Grams/litre (15 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a). Sodium carbonate</td>
<td>1.59</td>
</tr>
<tr>
<td>b). Sodium bi Carbonate</td>
<td>1.27</td>
</tr>
</tbody>
</table>

Solution a and b were mixed together to obtain required pH range 8.5-10.5 and the volume was made up to 1 L with dist. H₂O.

4A.2.2.4. 1 M Tris-HCl (pH 7.0)

TRIS base : 158 g

Tris base was dissolved in 500 ml distilled water and pH was adjusted to 7.0 with Conc. HCl. Then the volume was made up to 1 L with distilled water.

4A.2.2.5. Preparation of Sepharose 6B

Sepharose 6 B of particle size of 45-165 μm, which gives a bed volume of 15-20 ml per gram of dry gel was used. The exclusion limit of Sepharose 6B is 10 kDa - 400 kDa for proteins. Fifteen grams of Sepharose 6B dry powder was allowed to swell in 500 ml of 25 mM Tris-HCl pH 7.0 containing 1.2% NaCl (w/v) for 72 h. After three changes of the buffer, the slurry was packed into a glass column (100 x 0.9 cm) at a flow rate of 15 ml/h. The column was stored in buffer containing 0.05% sodium azide.
4A.2.2.6. Bradford reagent
Bradford reagents were prepared according 3A.3.8.

4A.2.2.7. Preparation of solutions for SDS-PAGE
4A.2.2.7.1. Monomer solution
Acrylamide : 116.8 g
N, N-Methylene-\textit{bis}-acrylamide : 3.2 g
dist.\(\text{H}_2\text{O}\) : 400 ml
Stored at 4°C in brown bottles.

4A.2.2.7.2. Running gel buffer
1.5 M Tris : 180.15 g/L
pH was adjusted to 8.8 with 2 N HCl and stored at 4°C.

4A.2.2.7.3. Stacking gel buffer
0.5 M Tris : 60.05 g/L
pH was adjusted to 6.8 with 2 N HCl and stored at 4°C.

4A.2.2.7.4. Tris-glycine running buffer (Tank buffer)
0.25 M Tris : 12 g
Glycine : 57.6 g
10% SDS solution : 1.5 g
dist.\(\text{H}_2\text{O}\) : 4 L.

4A.2.2.7.5. Ammonium persulfate (APS) (Prepared fresh): 10 g/100ml.

4A.2.2.7.6. 2X Loading Buffer
Stacking gel buffer : 5 ml
10% SDS solution : 8 ml
Glycerol : 4 ml
\(\beta\)-mercaptoethanol : 2 ml
dist.\(\text{H}_2\text{O}\) : 20 ml
Bromophenol blue : 0.02 g
Divided in aliquots and stored at -20°C.

4A.2.2.7.7. 1 % Coomassie Blue Solution (Stock solution)
Coomassie Blue R-250 : 2 g
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\[ \text{dist.} \text{H}_2\text{O} : 200 \text{ ml} \]
Filtered after preparation and stored in brown bottles.

4A.2.2.7.7.1. Coomassie Blue staining solution

Coomassie Blue 1% : 125 ml
Methanol : 500 ml
Acetic acid : 100 ml
\[ \text{dist.} \text{H}_2\text{O} : 1 \text{ L} \]

4A.2.2.7.8. Destaining Solution

Acetic acid : 7 %
Methanol : 10 %

4A.2.2.7.9. Separating gel (12.5%)

Monomer : 16.675 ml
Running buffer : 12.5 ml
10% SDS : 0.5 ml
dist.\text{H}_2\text{O} : 10.09 ml
10% APS : 208 \(\mu\)l
TEMED (Tetramethylethylenediamine) : 16.67 \(\mu\)l

4A.2.2.7.10. Stacking gel

Monomer : 1.33 ml
Stacking buffer : 2.5 ml
10% SDS : 0.1 ml
dist.\text{H}_2\text{O} : 6.1 ml
APS : 50 \(\mu\)l
TEMED : 5 \(\mu\)l

4A.2.2.8. Native page
4A.2.2.8.1. Separating gels (4A.2.2.7.9)
4A.2.2.8.2. Stacking gels (4A.2.2.7.10)

Separating gel (7.5%) was prepared as described above without the addition of SDS.

Stacking gels (5%) was also prepared similar to that for SDS-PAGE excluding SDS from the gel.
4A.2.2.8.3. Loading buffer

Loading buffer (4A.2.2.7.6) was prepared without SDS and β-mercaptoethanol.

4A.2.2.8.4. Tank buffer

Tank buffer (4A.2.2.7.4.) was also prepared just as in SDS-PAGE without SDS.

4A.2.2.9. Silver staining

4A.2.2.9.1. Fixation solution

\[
\begin{align*}
\text{Methanol} & : 12.5 \text{ ml} \\
\text{Glacial acetic acid} & : 2.5 \text{ ml} \\
\text{Formaldehyde} & : 125 \mu\text{l} \\
\text{dist.} \text{H}_2\text{O} & : 35 \text{ ml}
\end{align*}
\]

First mix water and methanol on a stirrer and then add acetic acid slowly followed by formaldehyde.

4A.2.2.9.2. Pretreatment solution

| 4A.2.2.9.2.1. Sodium thiosulphate | : 10 mg |
| dist.\text{H}_2\text{O} | : 50 ml |

| 4A.2.2.9.2.2. Silver nitrate | : 100 mg |
| Formaldehyde | : 12.5 µl |
| dist.\text{H}_2\text{O} | : 50 ml |

4A.2.2.9.3. Developer solution

| Sodium carbonate | : 1.5 g |
| Sodium thiosulphate | : 0.5 mg |
| Formaldehyde | : 37.5 µl |
| dist.\text{H}_2\text{O} | : 50 ml |

4A.3. Methods

4A.3.1. Purification of L-asparaginase

4A.3.1.1. Extraction of L-asparaginase by counter current extraction

L-asparaginase produced from Cladosporium sp. grown on wheat bran by solid state fermentation was air dried. 1 Kg air-dried mouldy bran was divided into 10 parts and one part was loaded onto a column and was soaked in distilled water at 1:10 ratio. After 1 hour of soaking, the enzyme was filtered
and added to fresh batch of mouldy bran. This process was repeated till all the mouldy bran was extracted.

**4A.3.1.2. Precipitation**

Crude enzyme filtrate was precipitated by adding different ice cold solvents like isopropanol, ethanol, acetone and methanol at 1:2 ratio with constant stirring for 2 h at 4 °C and the precipitate was separated by centrifugation at 10, 000g for 20 min at 4°C. The precipitate was dissolved in a minimal volume of phosphate buffer (50 mM, pH 7.0) and dialyzed against water for 24 h at 4°C and assayed for enzyme activity.

**4A.3.1.3. Ion exchange chromatography**

The DEAE cellulose matrix was first swelled by boiling 10 grams of the gel with 200 ml of dist.H2O. The gel was then equilibrated with 15 mM Tris-HCl buffer diluted from 1 M stock (4A.2.2.4) and packed into a column of 2 cm X 20 cm. The column was further equilibrated and used for purification of the enzyme. Methanol precipitated enzyme, which showed highest activity was passed through DEAE cellulose column using phosphate buffer (50 mM, pH 7.2) for elution. The packed bed volume was 20 ml. Crude enzyme was loaded on the column and the column was washed with equilibration buffer to remove the unbound fractions. Bound proteins were eluted by an increasing salt gradient. The unbound and bound fractions were checked for enzyme activity according to Drainas et al. (1977). L-asparaginase activity containing fractions obtained after the ion exchange chromatography were pooled, dialyzed extensively with distilled water and concentrated with bench top lyophilizer.

**4A.3.1.4. Size exclusion chromatography**

The dialyzed and lyophilized sample from the previous step was loaded on 50 mM phosphate buffer (4A.2.2.2) pH 7.0 pre-equilibrated Sepharose 6B column (1.1 cm x 100 cm). The protein elution was done with the same buffer at a flow rate of 0.4 ml/min. Fractions of 5 ml each were collected up to 40 tubes. The absorbance at 280 nm was recorded and active fractions were pooled, concentrated and dialyzed against distilled water. This concentrated fraction was stored at -20°C.
4A.3.2. SDS-PAGE

About 15-20 μg of protein was dissolved in 20 μl of the sample buffer and loaded on the gel. The gel apparatus was connected to the power supply with the lower electrode connected to the positive power supply lead. After the electrophoretic run, proteins were visualized using Coomassie brilliant blue R-250 (4A.2.2.7.7.1).

4A.3.3. Silver staining Procedure

SDS PAGE gel after electrophoresis was kept in fixative solution (4A.2.2.9.1) for 1 hr. Sodium thiosulphate (4A.2.2.9.2.1) solution was added as a pre treatment step and kept for 10 min on rocker followed by addition of silver nitrate containing formaldehyde (4A.2.2.9.2.2) for 20 min. Developer solution (4A.2.2.9.3) was added till band appear and the same fixation solution (4A.2.2.9.1) was added to store the gel. The gel was washed thrice with dist.H₂O after every step.

All reagents were prepared freshly in salt free glass wares.
AgNO₃ was prepared freshly just before use.
All steps were carried out on a rocker with constant shaking.

4A.3.4. High Performance Liquid Chromatography

Purified enzyme was chromatographed in Shimadzu HPLC 10AT series with a UV–vis variable wavelength detector (SPD-M10AVP, Shimadzu) using C-18 reverse phase column (300 x 6 mm, 5 μm Hypersil), isocratic condition with 80% acetonitrile and 0.01% trifluoroacetic acid (TFA) as mobile phase at 1 ml/min flow rate and analyzed at 280 nm at 28 ºC according to Latha et al. (2012).

4A.3.5. Determination of molecular weight

The lyophilized L-asparaginase obtained from the sepharose 6B column was subjected to sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE) for ascertaining the homogeneity and to estimate the molecular weight of the protein. SDS–PAGE was performed on a slab gel containing 10 % polyacrylamide, which was employed in order to achieve separation of proteins (Laemmli, 1970). Alcohol dehydrogenase (150 kDa), Bovine serum albumin (66 kDa), Ovalbumin (43 kDa), Carbonic
anhydrase (29 kDa), Lactoglobulin (18 kDa), Cytochrome C (12.4 kDa) and Aprotinin (6 kDa) were used as reference standards. Proteins were stained with coomassie brilliant blue R-250 and also silver staining. Native PAGE of the purified L-asparaginase was performed on 7.5% polyacrylamide gel in glycine buffer at 5 ± 1°C as described by Gallagher, (1999).

4A.3.6. Molecular weight determination by size exclusion chromatography

The molecular weight of the purified L-asparaginase was estimated by gel filtration chromatography through a column (1.1 cm × 50 cm) of sepharose 6B. The column was equilibrated with 50 mM phosphate buffer, pH 6.3. The column was calibrated with blue dextran and standard molecular weight marker proteins. The elution volume (V_e) of each marker protein and void volume (V_v) of the column were estimated. A plot of V_e/V_v against log of MW (molecular weight) was used to determine the molecular weight of L-asparaginase.

4A.3.7. Localization of L-asparaginase on polyacrylamide gels

Purified L-asparaginase was electrophoresed in native gel. Agar (1.5%) containing 10 mM L-asparagine and 2.5 % phenol red was layered on electrophoresed acrylamide gel. This was incubated overnight with buffer to keep the gels wet. After 24 h the L-asparagine containing agar sheet was observed for clear zone formation.

4A.3.8. Effect of pH and temperature on enzyme activity and stability

The effect of pH on purified enzyme was investigated in the pH range 4.0–10.5 (using 15 mM sodium acetate buffer for pH 4.0–6.0(4A.2.2.1), sodium phosphate buffer for pH 6.5–8.0 (4A.2.2.2) and carbonate-bicarbonate buffer for pH 8.5–10.5 (4A.2.2.3)). The enzyme was incubated in these buffers for 1 h and the assay was carried out as given above. The stability of the L-asparaginase at optimum reaction pH was determined by incubating the enzyme at optimum pH of 6.3 for different time intervals and assaying the residual activity in 15 mM phosphate buffer (pH 6.3). The optimal temperature for L-asparaginase activity of the purified enzyme was measured by incubating the enzyme-substrate mixtures for 1 h at various temperatures (8
°C to 60 °C) at pH 6.3 and the liberated ammonia and β-aspartylhydroxamate were measured. Thermal stability of the purified L-asparaginase was measured in terms of residual activity after incubation of enzyme in 0.01 M L-asparagine with 15 mM sodium phosphate buffer (pH 6.3) at 30 °C upto 6 h.

4A.3.9. Temperature reversibility

Loss of enzyme activity as a function of temperature was carried out as follows: Enzyme was incubated (2 sets) at different temperature (8 °C to 50 °C) for 1 h. One set of samples were removed and cooled immediately in ice bath and residual activity was measured. Other set of tubes were removed at required time intervals and allowed to reach ambient temperature slowly followed by activity measurement as given above.

4A.3.10. Substrate specificity

Activity was determined with D-asparagine, DL-asparagine, L-glutamine, D-glutamine, D-aspartic acid, DL-aspartic acid, L-glutamic acid, thioacetic acid, thioacetamide, thiourea, L-histidine, L-aspartic acid, L-cysteine, glutathione, glycine, L-arginine and L-asparagine as substrates at a final concentration of 10 mM. The relative activity was expressed as the percentage ratio of the enzyme activity determined against different structure analogs of L-asparagine to enzyme activity with L-asparagine. The rate of the reaction was measured in terms of the ammonia released because of enzyme action.

4A.3.11. Effect of effectors, detergents and metal salts on L-asparaginase activity

PMSF (Phenyl-Methyl-Sulfonyl Fluoride), NBS (N-Bromo Succinimide), EDTA (Ethylene Diamine Tetra Acetic Acid), Iodoacetamide, Cysteine and Sodium azide at 1 mM, β -ME at 2 mM level and Urea at 2.5 mM level were evaluated for their effectiveness on L-asparaginase activity using L-asparagine (10 mM) as substrate at optimum pH and temperature for 1 h. SDS was studied at 2 % concentration. Triton X-100, Tween-80 and Tween-20 at 1.0 % level. Metals including NaCl, Na₂SO₄, Na₂CO₃, KCl, K₂SO₄, KI, KNO₃, CoCl₂, CuSO₄, MgCl₂, MgNO₃, MgSO₄, Pb(CH₃COO)₂·3H₂O, ZnSO₄·7H₂O, FeCl₃, FeSO₄·H₂O, NH₄Cl, NH₄NO₃, (NH₄)₂SO₄, and CaCl₂ at 1
mM were evaluated for their effect on L-asparaginase activity by incubating the enzyme at optimum pH and temperature for 1 h.

4A.3.12. Determination of kinetic constants

The kinetic constants, $K_m$ and $V_{max}$, of the purified L-asparaginase were determined by the method of Lineweaver and Burk, (1934) with different concentrations (0.5 mM – 2 M) of L-asparagine as a substrate. Reactions were performed in 15 mM sodium phosphate buffer (pH 6.3) at 30°C.

4A.3.13. Inactivation kinetics of L-asparaginase

The enzyme inactivation kinetics was determined in the presence of NAC, cysteine, β-ME and GSH at concentrations of 50-400 µM and SDS at 0.01 to 1 % using different L-asparagine concentrations in the range of 10 to 500 µM. The assay was carried out at different substrate concentrations, keeping the enzyme concentration constant. The type of inhibition was deduced by analyzing the Lineweaver-Burk plots of $1/V$ vs $1/S$ at different inhibitor concentrations. The inhibitory constant $K_i$ was determined from the Dixon plot (Dixon, 1953) where $1/V$ vs [I] were plotted at different substrate concentrations. In all cases, enzymatic activity was assayed under temperature and pH optima. Alternatively data were fitted to Lineweaver-Burk and Michaelis-Menten’s equation to obtain the kinetic constants by applying linear regression and non-linear regression respectively. L-asparagine hydrolysis was studied in terms of change in values of kinetic parameters ($K_m$ and $V_{max}$) in presence and absence of inhibitor compounds. Enzymatic activity was determined as mentioned above.


4A.3.14.1. UV- Spectral profile of purified L-asparaginase

The UV-visible spectrum of purified L-asparaginase at 0.8 mg/ml was determined using Shimadzu, UV-160A, spectrophotometer.

4A.3.14.2. Fluorescence spectroscopic profile of purified L-asparaginase

Fluorimetric measurements were carried out using Shimadzu model No.RF 5301PC fluorescence spectrophotometer in the ratio mode using slit widths of 5 mm for excitation and emission. The Fluorimetric assay of the enzyme was conducted by following increase in the intensity of emission
fluorescence 330 to 340 nm (excitation at 280 nm). The spectra were recorded with a 5nm band width for both excitation and emission monochromators. Blank solutions containing corresponding concentrations of the denaturant at 280 nm ($\lambda_{exc}$) had an absorbance value less than 0.1 and therefore the inner filter effect was considered insignificant.

4A.3.14.3. CD measurements

CD measurements were made using Jasco J-810 automatic spectropolarimeter fitted with a xenon lamp. The instrument was calibrated with d-(-) 10-camphor-sulphonic acid. Slits were programmed to yield 1 nm bandwidth at each wave length from 320 to 190 nm. The chart speed, wavelength expansion and time constant of the instrument were set to obtain the best signal-to-noise ratio. The lamp was purged continuously with nitrogen before and during experiments. The scans were recorded thrice. The far UV CD spectra were recorded between 190 and 260 nm using a quartz cell of 1 mm path length. The near UV CD spectra were recorded between 260 and 320 nm using a quartz cell of 1 cm path length. The enzyme protein concentration used was 0.9 mg/ml. The base line for the spectrum was obtained using buffer blank (15 mM phosphate buffer, pH 6.3) and buffer containing respective denaturant. The measurements were made at ambient temperature. Mean residue ellipticity values $[\theta]_{mrw}$ were calculated following the method of Adler et al. (1973) using II5 for mean residue weight. CD analyses were done using the programs CONTIN and K2D recommended for proteins with predominant $\beta$-structure.

4A.3.14.4. Tryptic digestion of purified protein

1nmol of purified enzyme was dissolved in 100μl of 50 mM NH$_4$HCO$_3$ pH 7.5 – 8.5. To this 40μM of freshly prepared DTT was added and incubated for 15 min at 50°C. Then added 1 μl of 100 mM 4-vinyl pyridine and incubated for 15 min at ambient temperature. Trypsin was added to the enzyme at enzyme to trypsin ratio of 50: 1. The reaction mixture was incubated at 37°C for 2 – 3 h. The reaction was stopped with the addition of 5 μl of 10 % TFA. The digested protein was centrifuged at 10,000 rpm for 20 min at 4°C and stored as aliquots at -20°C.
4A.3.14.4.1. MALDI-TOF/TOF of the digested protein

The dried residue (4A.3.14.4) was dissolved in a minimum volume of distilled water/TFA (100:0.1v/v) and applied to Bruker Daltonics Ultraflex MALDI-TOF/TOF system (Bruker- Daltonics, Bremen, Germany) in refractive positive ion mode. The spectra were recorded and each peak obtained was assigned with all possible combination of amino acids to the total molecular weight of the peak and blasted to get the matching peptide sequence.

4A.3.14.4.2. MALDI was performed in Ultraflex TOF/TOF, Bruker Daltonics German.


Laser: N2 Laser, 337nm, 50Hz.
No. of shots averaged: 300shots.

4A.4. Analytical

4A.4.1. Protein estimation by Bradford Reagent
Bradford assay was carried out according to 3A.5.1.

4A.4.2. Assay for L-asparaginase activity
L-asparaginase activity was quantitatively measured by estimating the formation of β-aspartyl hydroxamate (3A.5.3.2).

4A.5. Results and Discussion

4A.5.1. Purification of L-asparaginase produced by Cladosporium sp.
L-asparaginase was purified using solvent precipitation, DEAE cellulose column and Sepharose 6B gel filtration. The purification steps of L-asparaginase of Cladosporium sp. is given in Table 4A.1. The specific activity of the enzyme increased with every step of purification with a minimum loss in quantity. The purified enzyme after gel filtration step had a specific activity of 83.3U (Table 4A.1). After every purification procedure, the peak fractions with the enzyme activity were analyzed using SDS-PAGE. The enzyme precipitated out with methanol was observed to have better activity than other
precipitants. Removal of few contaminating proteins using DEAE cellulose column was found to be a suitable step. Peak fractions of DEAE cellulose column were pooled together and loaded onto equilibrated Sepharose 6B gel filtration column for purification and in the eluent two protein peaks and one enzyme activity peak were obtained (Figure 4A.1). Peak fractions showing enzyme activity were pooled, dialysed extensively against distilled water and lyophilized. L-asparaginase from Bacillus strain DKMBT10 was purified by 2 steps to 43 % recovery (Moorthy et al., 2010). L-asparaginase from Pseudomonas aureginosa 50071 was purified to 106 folds by 3 steps (ammonium sulphate precipitation, gel filtration on sephadex G-100 column followed by CM sephadexC50 column) (Bessoumy et al., 2004). The purity of the purified enzyme was analysed by SDS and native PAGE electrophoresis and also by HPLC analysis. SDS-PAGE (Figure 4A.2a) revealed the presence of two bands while native PAGE (Figure 4A.2c) showed only one band which indicated that L-asparaginase purified from Cladosporium sp. was homogeneous.

**Table 4A.1: Summary of steps employed in purification of L-asparaginase from Cladosporium sp.**

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total activity (IU)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>9067.27</td>
<td>32360</td>
<td>0.096</td>
<td>1</td>
</tr>
<tr>
<td>Methanol precipitation</td>
<td>29160</td>
<td>540</td>
<td>1.8</td>
<td>18.75</td>
</tr>
<tr>
<td>DEAE-cellulose column</td>
<td>98820</td>
<td>180</td>
<td>18.3</td>
<td>190.62</td>
</tr>
<tr>
<td>Sepharose 6B</td>
<td>99960</td>
<td>40</td>
<td>83.3</td>
<td>867.7</td>
</tr>
</tbody>
</table>

L-asparaginase activity was assayed according of Drainas et al. (1977). One unit (U) of L-asparaginase is equivalent to 1 µmol of β-aspartyhydroxamate formed in one minute/mg of protein under assay conditions. Protein concentration was determined by measuring absorbance at 280 nm using BSA as a standard.
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Figure 4A.1: Purification profile of L-asparaginase from Cladosporium sp. by gel filtration on Sepharose 6B column

The concentrated and dialysed enzyme was applied to a 1.1 cm x 100 cm column, equilibrated and eluted with with 50 mM phosphate buffer pH 7.0. Fractions (5 ml) collected from the column were assayed for proteins content at 280 nm and L-asparaginase activity. Flow rate was maintained at 24 ml/h.

- L-asparaginase activity
- Protein OD @ 280nm.

4A.5.2. Molecular weight determination

The L-asparaginase from Cladosporium sp. is active as hetero trimer with the approximate total molecular mass of 120 kDa. SDS-PAGE revealed only two distinctive bands that was indicated by the pure preparation of L-asparaginase (Figure 4A.2a). SDS-PAGE, showed a homodimer of 37.5 ± 0.5 kDa and another sub unit of 47 kDa. Gel filtration indicated the enzyme with a molecular weight of 117 kDa (Figure 4A.2b). The apparent molecular mass of bacterial L-asparaginase were 91-150 kDa, L-asparaginase enzyme of Aspergillus niger had a molecular mass of 39 kDa (Dharmsthiti and Luchai, 2009; Mukherjee et al., 1999; Warangkar and Khobragade, 2010). The purity of the enzyme was also confirmed by HPLC (Figure 4A.3), where it showed a single peak. Purified L-asparaginase was electrophoresed in native gel. 1.5 % agar layer containing 10 mM L-asparagine and 2.5 % phenol red showed clear zone after 24 h incubation indicating the presence of L-asparaginase.
Figure 4A.2a: Determination of the molecular weight of the purified L-asparaginase from *Cladosporium* sp. by SDS–PAGE

SDS–PAGE was carried out on 10% gel. Lane 1: crude preparation; lane 2: purified L-asparaginase after Methanol precipitation; lane 3: purified L-asparaginase after size exclusion chromatography; lane 4: standard protein markers of different molecular weights.

Figure 4A.2b: Gel filtration chromatography using Sepharose 6B column

Standard molecular weight proteins used included chymotrypsinogen (25 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), BSA (66 kDa), catalase (240 kDa) and ferritin (398 kDa).
Figure 4A.2c: Native-PAGE of the purified L-asparaginase from *Cladosporium* sp.

![Native-PAGE](image)

Figure 4A.3: HPLC profile of the purified L-asparaginase from *Cladosporium* sp.

![HPLC profile](image)
4A.5.3. Effect of pH, temperature and stability

L-asparaginase from *Cladosporium* sp. was active between pH values of 4.0 to 6.0 with an optimum at pH 6.3. At higher pH, the enzyme activity decreased (Figure 4A.4a). L-asparaginase of *Aspergillus niger* AK10 functioned maximally at pH 8.6 and was most stable when stored at pH 8.0-8.6 (Dharmsthiti and Luchai, 2009). L-asparaginase activity of *Serratia marcescens* and actinomycetes had a broad pH optimum from pH 5 to 9.0 (Khamna et al., 2009; Novak and Phillips, 1974). The pH optimum of purified *staphylococcal* L-asparaginase was found to be between 8.6 and 8.8 (Sobis & Mikucki, 1991).

The reaction rate of L-asparaginase was measured at various temperatures ranging from 4 to 60 °C; Maximum activity was obtained at 30 °C. At higher temperatures, the reaction rate declined sharply (Figure 4A.4b). Maximum activity of *Pseudomonas aeruginosa* 50071 L-asparaginase (Bessoumy et al., 2004) and *Erwinia carotovora* L-asparaginase (Kamble et al., 2006) have been observed at 37°C and 35°C respectively. Interestingly, the crude enzyme was clearly more stable than the purified one, which could be due to the presence of other substances that helped to stabilize the enzyme. This was similar to those reported for other *Aspergillus* sp. enzyme (Tari et al., 2008) and *Aspergillus niger* AK10 (Dharmsthiti and Luchai, 2009). Both L-asparaginase and L-glutaminase activities were stable at 52 and 62 °C for 30 min; and both activities were completely inactivated at 80 °C in 5 min. In our studies L-asparaginase enzyme was stable for 3 h at optimum pH (Figure 4A.5a) and temperature (Figure 4A.5b).

When the enzyme was incubated at different temperatures for 1 h and then assayed immediately for enzyme activity, there was progressive loss in enzyme activity with increase in temperature (Figure 4A.6). Gradual cooling of the enzyme after incubation at different temperatures did not show any progress in enzyme activity. Loss in enzyme activity was 10.5, 20.6, 22.68, 22.68 and 28.68 %, respectively when the activity was measured immediately after exposure to different temperatures (8-50 °C). Slow cooling of the enzyme after incubation respectively between 8-50 °C resulted still more loss.
in enzyme activity i.e., 22.68, 53.09, 61.34, 69.58 and 73.56 % activity was lost.

**Figure 4A.4a: Effects of pH on the activity of the L-asparaginase enzyme from *Cladosporium* sp.**

Effect of pH on the enzyme activity was assayed in a pH range of 3.0–9.0.

**Figure 4A.4b: Effects of temperature on the activity of the L-asparaginase enzyme from *Cladosporium* sp.**

Effect of temperature on the enzyme activity was assayed at various temperature range from 8-60 °C in 15 mM sodium phosphate buffer (pH 6.3).
Figure 4A.5a: pH stability of L-asparaginase enzyme from *Cladosporium* sp.

![Graph of pH stability](image1)

Stability of purified L-asparaginase at pH 6.3 after incubation for different time intervals.

Figure 4A.5b: Temperature stability of L-asparaginase enzyme from *Cladosporium* sp.

![Graph of temperature stability](image2)

Stability of purified L-asparaginase at 30°C after incubation for different time intervals.
Purification and characterization of the enzyme

Figure 4A.6: Influence of temperature reversibility of the purified L-asparaginase from *Cladosporium* sp.

The enzyme solution in sodium phosphate buffer (15 mM, pH 6.3) was incubated for 1 h at various temperatures, and then the residual enzyme activities were assayed immediately for one set and with the other set assay was carried out after the assay mixture attained ambient temperature slowly. The experiment was done in triplicate. Error bars show the percent error.

4A.5.4. Effect of various effectors on L-asparaginase activity

L-asparaginase activity was assayed in the presence of different inhibitors/activators (Table 4A.2). β-ME, SDS, Cysteine, PMSF, and Iodoacetamide showed 100 % inhibition. Loss in activity by thiol reagent (β-ME) indicates the presence of S-S bridges. Inhibition in the presence of thiol group blocking reagents, namely, cysteine and iodiacetatamide provided additional proof for the role of sulfhydryl groups in the catalytic activity of the enzyme. Inhibition by PMSF indicated involvement of serine in the active site. Thiol reactivity was also observed with the purified L-asparaginase from *Erwinia carotovora* (Warangkar and Khobragade, 2010). Sodium azide inhibited the enzyme activity by 77 %. Detergents, Tween- 80 and Triton- X 100 enhanced the enzyme activity and there was 12 % loss in activity in the presence of Tween- 20. The metal chelating agent EDTA did not affect the L-asparaginase activity, which revealed that the enzyme was not a metalloprotein. Increase in enzyme activity in presence of NBS (1.25 folds) indicates the modification of tryptophan residues. Urea also did not show any
inhibitory effects on enzyme activity. L-asparaginase from *P. Carotovorum* completely lost its activity in 2 M urea, and only 21% of activity was retained in the presence of 2 mM of SDS (Kumar et al., 2011).

**Table 4A.2: Effect of inhibitors and/or activators on L-asparaginase activity**

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Relative activity (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 20</td>
<td>88.68</td>
</tr>
<tr>
<td>Tween 80</td>
<td>158.53</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>141.52</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>0</td>
</tr>
<tr>
<td>Urea</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>100</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>77.37</td>
</tr>
<tr>
<td>SDS</td>
<td>0</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0</td>
</tr>
<tr>
<td>PMSF</td>
<td>0</td>
</tr>
<tr>
<td>NBS</td>
<td>125.46</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>0</td>
</tr>
<tr>
<td>Control (without inhibitor)</td>
<td>100</td>
</tr>
</tbody>
</table>

PMSF, NBS, DTT, EDTA, Iodoacetamide, cysteine and Sodium azide at 1 mM, β-mercaptoethanol at 2 mM level and Urea at 2.5 mM level were evaluated for their effectiveness on L-asparaginase activity using L-asparagine (10 mM) as substrate at optimum pH and temperature for 1 h. SDS was studied at 2 % concentration. Triton X-100, Tween-80 and Tween-20 at 1.0 % level.

<sup>a</sup>Relative activity was determined by measuring β-asparylhydroxamate at optimal assay conditions.

The influence of various metal ions on the purified L-asparaginase activity is presented in Table 4A.3. Ca<sup>2+</sup>, Co<sup>2+</sup>, Cu<sup>2+</sup>, Mg<sup>2+</sup>, Na<sup>+</sup>, K<sup>+</sup> and Zn<sup>2+</sup> were very detrimental to enzymatic activity, whereas Fe<sup>3+</sup>, Pb<sup>2+</sup> and KI acted as enhancers. Inhibition of enzyme activity in presence of Hg<sup>2+</sup>, Cd<sup>2+</sup>, Cu<sup>2+</sup>, Mg<sup>2+</sup> and Zn<sup>2+</sup> might be indicative of essential vicinal sulphydryl groups of the
Purification and characterization of the enzyme for productive catalysis. Hg$^{2+}$ and Cu$^{2+}$ cations strongly inhibited the Staphylococcal L-asparaginase while Na$^+$ and K$^+$ cations strongly stimulated activity (Sobis & Mikucki, 1991). Similar observation has been made by Kumar et al. (2011).

**Table 4A.3: Effect of metal ions on L-asparaginase activity**

<table>
<thead>
<tr>
<th>Metals</th>
<th>Relative activity (%)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH$_4$Cl</td>
<td>21.65</td>
</tr>
<tr>
<td>NH$_4$NO$_3$</td>
<td>23.54</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>41.52</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>20.73</td>
</tr>
<tr>
<td>CoCl$_2$</td>
<td>83.05</td>
</tr>
<tr>
<td>CuSO$_4$</td>
<td>89.6</td>
</tr>
<tr>
<td>FeCl$_3$</td>
<td>184.95</td>
</tr>
<tr>
<td>FeSO$_4$</td>
<td>428.5</td>
</tr>
<tr>
<td>Pb(CH$_3$COO)$_2$.3H$_2$O</td>
<td>320.85</td>
</tr>
<tr>
<td>KCl</td>
<td>83.05</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>35.84</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>22.62</td>
</tr>
<tr>
<td>K$_2$SO$_4$</td>
<td>74.55</td>
</tr>
<tr>
<td>KI</td>
<td>362.38</td>
</tr>
<tr>
<td>KNO$_3$</td>
<td>81.16</td>
</tr>
<tr>
<td>Na$_2$CO$_3$</td>
<td>251.98</td>
</tr>
<tr>
<td>ZnSO$_4$</td>
<td>52.84</td>
</tr>
<tr>
<td>Na$_2$SO$_4$</td>
<td>16.02</td>
</tr>
<tr>
<td>NaCl</td>
<td>42.44</td>
</tr>
<tr>
<td>Mg(NO$_3$)$_2$.6H$_2$O</td>
<td>39.64</td>
</tr>
<tr>
<td>Control (without metal ion)</td>
<td>100</td>
</tr>
</tbody>
</table>

$^a$Relative activity was determined by measuring β-aspartylhydroxamate under assay conditions. Assay was carried out in the presence of 1 mM concentrations of each metal ion after incubating L-asparaginase with metal at optimum pH and temperature for 1 h.
4A.5.5. Substrate specificity

Substrate specificity of purified enzyme on various substrates is summarized in Table 4A.4. The purified enzyme had both L-asparaginase and L-glutaminase activities. Enzyme was not reactive to any of the other substrates tested. This property makes L-asparaginase of Cladosporium sp. potentially very useful in food applications. Similar observations have been made by Kumar et al. (2011), except that the enzyme had very low activity against D-asparagine, DL-asparagine, D-glutamine, D-asparatic acid, succinamic acid, L-asparagine-t-butyl ester HCl and N-α-acetyl L-asparagine. Sarita and Wamik, (2012) found that the L-asparagine was most suitable substrate for the L-asparaginase of E. carotovora MTCC 1428. The substrate specificity for L-asparaginase by E. carotovora was determined by Howard and Carpenter, (1972) and they found that the relative specificity of L-asparaginase towards the L-asparagine was maximum among all the substrates used.

4A.5.6. Kinetic parameters of purified L-asparaginase

The enzyme showed typical Michaelis-Menten kinetics at lower substrate concentrations and the apparent $K_m$ and $V_{max}$ values for L-asparagine is 0.1 mM and 4.00 μM/mg/min respectively (Table 4A.5, Figure 4A.7a). Eadie-Hofstee plot showed $K_m$ and $V_{max}$ of 0.132 M and 4.5 μmol/ml/min respectively (Figure 4A.7b). The efficiency of substrate utilization was estimated by $V_{max}/K_m$ ratios (Table 4A.5) and the hydrolysis efficiency of L-asparagine was at least 8 fold higher than that of L-glutamine indicating the greater affinity of the enzyme towards L-asparagine. The $K_m$ and $V_{max}$ of purified L-asparaginase from P. Carotovorum MTCC 1428 were 0.657 mM and 4.45 U/µg respectively. The substrate affinity in terms of $K_m$ is very low (0.1 mM), which is 9-10 times lower than the reported cytosolic bacterial L-asparaginases (Kumar et al., 2010). Higher $K_m$ values for bacterial L-asparaginases have been reported by Kumar et al. (2010). On the other hand, a lower Km value (0.058 mM) was obtained for L-asparaginase from Erwinia chrysanthemi 3937 (Kotzia and Labrou, 2007).
Table 4A. 4: Effect of different substrates on L-asparaginase activity

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-asparagine</td>
<td>N.D.</td>
</tr>
<tr>
<td>DL-asparagine</td>
<td>N.D.</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>3.87</td>
</tr>
<tr>
<td>D-glutamine</td>
<td>N.D.</td>
</tr>
<tr>
<td>D-aspartic acid</td>
<td>N.D.</td>
</tr>
<tr>
<td>DL-aspartic acid</td>
<td>N.D.</td>
</tr>
<tr>
<td>L-glutamic acid</td>
<td>N.D.</td>
</tr>
<tr>
<td>thioacetic acid</td>
<td>N.D.</td>
</tr>
<tr>
<td>thioacetamide</td>
<td>N.D.</td>
</tr>
<tr>
<td>Thiourea</td>
<td>N.D.</td>
</tr>
<tr>
<td>L-histidine</td>
<td>N.D.</td>
</tr>
<tr>
<td>L-aspartic acid</td>
<td>N.D.</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>N.D.</td>
</tr>
<tr>
<td>glutathione</td>
<td>N.D.</td>
</tr>
<tr>
<td>Glycine</td>
<td>N.D.</td>
</tr>
<tr>
<td>L-arginine</td>
<td>N.D.</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>3.82</td>
</tr>
</tbody>
</table>

N.D. Not detected.
aSpecific activity was determined in the presence of 10 mM concentrations of each substrate at optimum pH and temperature.

Table 4A.5: Kinetic constants of purified L-asparaginase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Linear regression LB Plot</th>
<th>Non Linear regression (MM equation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_m (mM)</td>
<td>V_max (μmoles/mg/min)</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>0.100</td>
<td>4.0</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>0.108</td>
<td>0.61</td>
</tr>
</tbody>
</table>

The reaction was performed at pH 6.3 and 30°C at different concentrations ranges of L-Asparagine and L-Glutamine (0.5 mM-2 M). Data represent the mean of three replicates ± SE.
Figure 4A.7: Determination of $K_m$ and $V_{\text{max}}$ of purified L-asparaginase for L-asparagine by non-linear regression analysis of experimental steady-state data

**Figure 4A.7a:** Plot of the reaction velocities ($V$) vs substrate concentration ($S$: 0.5 mM–2.0 M) fitted to the Michaelis–Menten equation ($K_m = 0.1$ mM and $V_{\text{max}} = 4.44$ µmol/ml/min). **Figure 4A.7b:** The corresponding Eadie-Hofstee plot ($K_m = 0.132$ M and $V_{\text{max}} = 4.5$ µmol/ml/min) for L-asparaginase catalyzed reaction.

**4A.5.7. Kinetics of inhibitor Compounds on L-asparaginase**

The enzyme activity was determined in the presence of inhibitor compounds and were analysed by Lineweaver-Burk and Dixon plots. An increase in inhibitor concentration in the reaction medium revealed an increase in $K_m$ and a decrease in $V_{\text{max}}$ values, which corresponds to a non-competitive inhibition (Segal 1975). Further increase in the concentrations of inhibitor compound decreased the catalytic activity of L-asparaginase from *Cladosporium* sp.; Table 4A.6 shows the inhibition results with L-asparagine as the substrate. All five inhibitors used in this study inhibited L-asparaginase non-competitively. SDS was the most potent inhibitor with an apparent $K_i$ of 65.92 µM/L followed by GSH, L-cysteine, β-ME and NAC with 50.48, 44.61 40.28 and 20.83 µM/L respectively. These results are in agreement with results reported for L-asparaginase from *Erwinia carotovora* and
Chapter 2A

Purification and characterization of the enzyme

Streptomyces radiopugnans MS1 (Warangkar and Khobragade, 2010; and Kumar and Selvam, 2011). Thus biocatalyst studied in the presence of the inhibitors will be more useful to improve a potential biotechnological purpose and to increase the catalytic efficiency of L-asparaginase.

Table 4A.6: Inhibition constants of purified L-asparaginase

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$ (µM/ L)</th>
<th>$K_m$ (mM/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAC</td>
<td>20.83</td>
<td>0.166</td>
</tr>
<tr>
<td>GSH</td>
<td>50.48</td>
<td>0.041</td>
</tr>
<tr>
<td>β-ME</td>
<td>40.28</td>
<td>0.098</td>
</tr>
<tr>
<td>L- cysteine</td>
<td>44.61</td>
<td>0.111</td>
</tr>
<tr>
<td>SDS</td>
<td>65.92</td>
<td>0.112</td>
</tr>
</tbody>
</table>

Inhibition constants measured in the presence of inhibitors, NAC: N-acetyl cysteine, GSH: glutathione reduced, β-ME: β-Mercaptoethanol, L- cysteine and SDS: Sodium Dodecyl Sulfate, at concentrations of (50-400 µM) and SDS (0.01 to 1 %). The inhibition constants were measured after pre incubation of enzyme with each inhibitor at 30°C at different L-asparagine concentrations in the range of 10 to 500 µM. Data represent the mean of three replicates ± SE.

4A.5.8: Spectroscopic studies

The UV-Vis spectrum of the L-asparaginase in 50 mM phosphate buffer pH 6.3 is presented in Figure 4A.8. The spectrum showed absorption maxima at 220 and 280 nm are typical of proteins. The shoulder at 292 nm indicated the presence of tryptophan.
L-asparaginase enzyme had an excitation maximum of 280 nm and the emission maximum of 320 nm (Figure 4A.9). In general, the aromatic amino acids exhibit fluorescence in any protein and proteinaceous enzymes in the range 310 nm to 340 nm. The hydrophobicity of these aromatic amino acids determines the intensity of fluorescence. Moreover, an exposure of aromatic amino acids such as tyrosine, tryptophan and phenylalanine those are buried in the core of the protein to hydrophilic or aqueous environment results in the reduction of protein fluorescence.

Figure 4A.9: Fluorescence spectrum of L-asparaginase enzyme in 15 mM phosphate buffer, pH 6.3
The near-UV CD spectrum exhibited a minimum at 215 nm. The secondary structure analysis of the enzyme suggested a predominance of β-structure in the enzyme. The enzyme L-asparaginase had 42.2% β-structure, 24.6% random 31.7% α-helix and 1.5% turns (Figure 4A.10).

Figure 4A.10: CD spectrum of purified L-asparaginase enzyme

![CD spectrum of purified L-asparaginase enzyme](image)

The molecular weight obtained from MALDI-TOF data (370 amino acids) was 39.24 kDa with extinction co-efficient of 22460 cm/M. The final amino acid sequence of this L-asparaginase was Phe-Ala-Leu-Gly-Thr-Asp-Ala. All mass spectra obtained in reflector mode and were calibrated using several matrix ion peaks as internal standards. L-asparaginase from *Aspergillus niger* has been reported to be a glycoprotein with a primary sequence of 378 amino acids and a calculated molecular weight of 39.58 kDa (Yingling, 2006). The primary sequence of the enzyme band at 37 kDa was deduced by LC-MS/MALDI-TOF. The sequence obtained is given below.

1  MPTPHPITALASLAPSASPLLMTTNEFFVFTNANGLNFTQMNTIPKVTIIAYYYVLAGS
61  PSSSTALCGYTAGVGLSLIDGAPSTFDHANVAGADVANVGSEDITSSTALISSKLN
121  VVCEDWDAGAVITHTDTLETAFFDLATVNCGKPIVVGAMRPTAAWMQCNILEAVT
181  ASTSARDRGAMVVMNDRIASAYVVTKNANTMDFKAAMSCGLHEGEMISNTQFFFYPV
241  PTGKVFADITNVTEIPRVDCMHQEMMHNTMNCQSMGAQQIVGAGQAGGYTTSFNEAIED
301  VINRLEIPVVQSMRTVNGEVPSDVSSTATHIASGYNPQKRILLGLLSQGKNITEI
361  ADVFALGTDA.
4A.6. Summary and Conclusions

- A new fungal strain capable of producing L-asparaginase enzyme was purified.
- L-asparaginase from *Cladosporium* sp. was purified to 867 folds by three steps of purification.
- SDS-PAGE showed a homodimer of 37.5±0.5 kDa another subunit of 47 kDa.
- Gel filtration indicated a molecular weight of 117 kDa.
- HPLC and native page confirmed the purity of the enzyme.
- The purified L-asparaginase produced by *Cladosporium* sp. showed pH and temperature-stability for 3 hrs at pH 6.3 and 30°C.
- It hydrolysed both L-asparagine and L-glutamine.
- The enzyme was observed to have serine to be in the active site.
- The enzyme was found to posses S-S bridges.
- L-asparaginase had a $K_m$ of 0.1 mM and $V_{max}$ 4 µmoles/mg/min.
- The primary sequence of HPLC purified enzyme was deduced by LC-MS-MALDI-ESI.
- These properties render L-asparaginase a potential candidate for applications primarily in food industry to reduce acrylamide content in fried and baked foods.
4A.7. Bibliography


Chapter 2(B)

Modification of L-asparaginase
**4B.1. Introduction**

Although L-asparaginase is produced by various microorganisms, L-asparaginase from *Escherichia coli* and *Erwinia chrysanthemi* have been used in the treatment of leukemia for the last 40 years. Unfortunately, a therapeutic response by patients showed some evidence of toxicity effects including pancreatitis, diabetes and coagulation abnormalities (Verma et al., 2007). Thus, there is need for development of this potent anti-tumor enzyme with lower side effects to alleviate this problem. Modification of the native enzyme is required due to its short half-life and high immunogenicity. Chemical modification of the enzyme using N-bromosuccinimide (Mardanyan et al., 2001), 2,4,6-trinitrotoluene (Nyanhongo et al., 2006), Reductive alkylation (Hernandez et al., 2005), 2,3,4-trinitrobenzenesulfonate (Oveimar et al., 2012a), Immobilization of the enzyme using polyacrylamide (Emel et al., 2006), albumin polymers (Sarka et al., 2012), succinylation and acetylation (Xueqiong et al., 2011), methoxy-poly(ethylene) glycol (Ratzka et al., 2012), glutaraldehyde (Tardioli et al., 2011) and glyoxyl-agarose supports (Oveimar et al., 2012b) have been attempted. Immobilization onto solid supports facilitates recovery and re-use of the enzyme. In addition, increased enzyme stability can be achieved by both immobilization and chemical modification techniques (Jessica et al., 2010). In this section attempts were made to modify the enzyme by both chemical modification techniques, to examine the potential of L-asparaginase from *Cladosporium* sp. for the long term operational stability, improved activity and half-life.

**4B.2. Materials and Methods**

**4B.2.1. Materials**

Bovine Serum Albumin (BSA), Ovalbumin (OVA), Glutaraldehyde, 2,4,6-trinitrobenzenesulfonic acid (TNBS), Trypsin, Iodoacetamide, Glycine and N-Bromosuccinimide (NBS) were purchased from Sigma-Aldrich chemical company. Mono-methoxy Poly-Ethylene-Glycol (PEG) was obtained from Hi-Media laboratories, Mumbai. Blood serum samples were obtained from healthy Swiss mouse from the animal house maintained at CFTRI, Mysore. All the other chemicals used were of analytical grade and purchased from standard chemical companies.
4B.2.1.1. Reagents

4B.2.1.1.1. Phosphate buffer saline (0.15 M) (pH: 6.8)

<table>
<thead>
<tr>
<th>Components</th>
<th>Grams/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>20.4</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>26.1</td>
</tr>
<tr>
<td>NaCl</td>
<td>24.0</td>
</tr>
<tr>
<td>KCl</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Sodium chloride and KCl were added to this buffered solution and the volume was made up to 1 L with distilled water.

4B.2.1.1.2. Glycine (1 %)

1.0 g of glycine dissolved in 100 ml distilled H₂O.

4B.2.1.1.3. NaCl (1 %)

1.0 g of Sodium chloride dissolved in 100 ml distilled water.

4B.2.1.1.4. Phosphate buffer saline (50 mM) (pH: 7.4)

<table>
<thead>
<tr>
<th>Components</th>
<th>Grams/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>6.8</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>8.7</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.0</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Sodium chloride and KCl were added to this buffered solution and the volume was made up to 1 L with distilled water.

4B.2.1.1.5. Sodium borate buffer (0.6 M) (pH 9.5)

<table>
<thead>
<tr>
<th>Components</th>
<th>Grams/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium borate</td>
<td>228.82</td>
</tr>
<tr>
<td>Boric acid</td>
<td>37.09</td>
</tr>
</tbody>
</table>

4B.2.1.1.6. Sodium acetate buffer (50 mM) (pH 4.7)

<table>
<thead>
<tr>
<th>Components</th>
<th>Grams/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium acetate</td>
<td>136</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>60</td>
</tr>
</tbody>
</table>
4B.2.1.1.7. 2, 4, 6- trinitrobenzenesulfonic acid

To 140 µl of 5% TNBS prepared in distilled water, 860 µl of distilled water was added.

4B.2.2. Methods
4B.2.2.1. L-asparaginase enzyme

L-asparaginase was produced on large scale in tray fermenter by solid state fermentation (SSF) using Cladosporium sp. (3B.4.7) and purified to homogeneity (4A.5.1).

Homogeneity of the enzyme was obtained by three steps of purification. The purified enzyme in SDS-PAGE, showed a homodimer of 37.5 ± 0.5 kDa and another sub unit of 47 kDa and a single band in native PAGE (4A.5.2). The results revealed no detectable electrophoretic contamination. One unit of enzyme activity is referred to that of enzyme which produces one μmol of β-aspartylhydroxamate per min under given assay conditions. Specific activity is expressed as U/mg of protein. The L-asparaginase from Cladosporium sp. has a molecular weight of 120 kDa and specific activity of 83.3 U/mg. The purified enzyme has a half-life of 12.26 h at ambient temperature. This purified enzyme was used for studies on the enzyme modification to improve some important properties.

4B.2.2.2. Modifications of L-asparaginase
4B.2.2.2.1. Preparation of Ovalbumin (OVA)-L-asparaginase bioconjugates

L-asparaginase Ovalbumin conjugate was produced according to Poznansky et al. (1982) by incubating 2mg of L-asparaginase and 20 mg of ovalbumin with 20 mg of L-asparagine and volume was made up to 3 ml by Phosphate buffer saline PBS (150 mM, pH 6.8) (4B.2.1.1.1). The mixture was vortexed, chilled to 4°C for 10 min. 50 µl of 25 % glutaraldehyde was added followed by gentle mixing for 4 to 5 h. Cross linking reaction was stopped by addition of 20 mg glycine followed by 24 h dialysis against mixture of 1% glycine (4B.2.1.1.2) and 1%NaCl (4B.2.1.1.3) solution at 4°C. Finally, filtration was done to separate the modified enzyme with unreacted molecules. The modified enzyme was purified by preparative poly acrylamide gel
electrophoresis and the purified modified enzyme was lyophilized and stored at 4 °C.

4B.2.2.2.2. Preparation of BSA-L-asparaginase bio-conjugates

BSA (50 mg) was dissolved in 50 mM phosphate buffer saline (pH 7.4), (4B.2.1.1.4) onto which 0.5 ml L-asparaginase (10 mg/ml) was added along with 0.5 ml of L-asparagine (5 mg/ml) as an active centre protector. Addition of glutaraldehyde followed by mild homogenization at 4°C. The volume was made up to 5 ml with 50 mM phosphate buffer saline. The reaction mixtures were kept in an orbital shaker for 24 h at 4°C. After incubation the reaction was stopped by adding glycine (100 mg) and then dialysed with excess of water for 24 h to remove unreacted molecules. The modified enzyme thus obtained was purified by preparative poly acrylamide gel electrophoresis, concentrated by lyophilization and stored at 4°C (Zhang et al., 2005).

4B.2.2.2.3. Modification of tryptophan groups with NBS

Tryptophan groups of L-asparaginase were modified according to Williams, (1975), using NBS. The modification was carried out by adding NBS (1mM) aliquots to 5 ml of L-asparaginase enzyme in phosphate buffer (15 mM, pH 6.3) (4A.2.2.2). Additions were made slowly as the solution was being stirred on a magnetic stirrer. This procedure minimized formation of turbidity in the solution. After 1 h stirring at 28±2°C, the modified enzyme was dialysed against distilled H₂O for 24 h. The modified enzyme was purified by preparative poly acrylamide gel electrophoresis and stored at 4°C.

4B.2.2.2.4. Modification of L-asparaginase with PEG

Modification of L-asparaginase with PEG was carried out as per Soares et al. (2002). The following were taken in a reaction vessel: 1.5 ml of phosphate buffer (15 mM, pH 6.3) and water (to a final volume of 50 ml). To this 1 ml of L-asparaginase (0.268 mg protein/ml) and 100 mg mono-methoxy PEG were added. The solution was maintained on magnetic stirrer at 28°C for 2 h. The mixture was allowed to stand for 16 h at 8°C. The PEG asparaginase was purified by passing through Sephadex G-150 column, equilibrated with 15 mM phosphate buffer (pH 6.3) and eluted with the same buffer at a flow rate of 0.2 ml/min.
4B.2.2.3. Preparative Poly acrylamide gel electrophoresis

Native PAGE (4A.2.2.8) of the modified L-asparaginase was performed on 7.5% polyacrylamide gel in glycine buffer at 5±1°C as described by Gallagher. (1999). One part of the gel was stained by silver staining technique (4A.2.2.9). The other half of the gel was approximated with the stained portion. The region of the gel corresponding to the modified enzyme band was excised and suspended in phosphate buffer (pH-6.3, 15 mM). The gel was macerated in the buffer and the supernatant obtained after centrifugation was used as modified enzyme source.

4B.2.3. Analytical methods

4B.2.3.1. Determination of enzyme modification degree

TNBS method was used to determine the degree of enzyme modification due to ε-amino groups (Habeeb, 1966). The TNBS reaction was carried out as follows. 200 µl of 0.6 M sodium borate buffer, pH 9.5 (4B.2.1.1.5) was mixed with 200 µl of a solution of conjugate in 0.05 M sodium acetate buffer, pH 4.7 (4B.2.1.1.6) and 50 µl of 0.2 M NaOH. The reaction was initiated by the addition of 50 µl of a freshly prepared solution of TNBS (7.2 mg/ml of water). The reaction mixtures were kept at 25°C in dark for 60 min and then the spectrophotometric readings were taken at 367 nm. BSA, OVA, PEG and NBS without conjugation were also run to calculate the difference in the number of free lysine groups. % conjugation was calculated by calculating the difference in O. D. of conjugated and non-conjugated molecules.

4B.2.3.2. Estimation of L-asparaginase activity

Assay for native and modified L-asparaginases was performed for the formation of β-aspartylhydroxamate (3A.5.3.2). Authentic β-aspartylhydroxamate was employed as standard. One unit (U) of L-asparaginase activity was defined as the amount of enzyme capable of producing 1µmol of β-aspartylhydroxamate per minute under assay conditions. The product formed was estimated at 500 nm. Total protein content was measured according to Bradford, (1976), using bovine serum albumin (BSA) as a standard (3A.5.1).
4B.2.3.3. Thermal stability of free and modified L-asparaginase

Enzyme solutions (0.1 ml) were added to 3.7 ml phosphate buffer (15 mM, pH 6.3). The mixtures were incubated at 28 °C and 37 °C for 0 to 24 h. After incubation for different time intervals, substrate L-asparagine was added to each reaction tube at 10 mM level. The assay was done as under (3A.5.3.2). Relative activities were calculated by taking the highest enzyme absorbance value as 100 %.

4B.2.3.4. Thermal inactivation kinetics and inactivation energy estimation

Kinetics of thermal inactivation of L-asparaginase, were studied by incubating the native and modified enzymes at two different temperatures (28 and 37°C) in the absence of substrate. Aliquots were withdrawn at intermittent intervals and assay was performed as mentioned in 3A.5.1. The residual activity was expressed as percentage of the initial activity. Slopes of a semi-logarithmic plot of residual activity vs time were used to calculate the inactivation rate constants ($k_d$) and apparent half-lives ($t_{1/2}$), i.e. the time after which the enzyme retains 50 % of its initial activity under defined conditions, was calculated by Equation (4B.1).

$$t_{1/2} = \frac{\ln 2}{k_d} \quad \text{(Eq. 4B.1)}$$

The temperature dependence of $k_d$ was analyzed using the Arrhenius plot (Shuler and Kargi, 2002). The inactivation energy was calculated from the Arrhenius equation as

$$\ln(K_d) = \ln(K_o) - \left(\frac{E_d}{R} \right) \frac{1}{T} \quad \text{(Eq. 4B.2)}$$

The values of $E_d$ and $k_d$ were estimated from the slope and intercept of the plot of $\ln (k_d)$ vs $1/T$, respectively (Canan et al., 2008).

4B.2.3.5. Estimation of thermodynamic parameters

Inactivation rate constant ($k_d$) of L-asparaginase as a function of temperature at 28 & 37°C was determined both in the presence and absence of selected additives. Activation enthalpy ($\Delta H^*$) was calculated according to the equation

$$\Delta H^* = E_d - RT \quad \text{(Eq. 4B.3)}$$
Where, R is the universal gas constant, and T is the absolute temperature. The values for free energy of inactivation ($\Delta G^*$) at different temperatures were obtained from the equation

$$\Delta G^* = -RT \ln (k_d h k T) \quad \text{(Eq. 4B.4)}$$

Where, h is the Planck’s constant and k is the Boltzmann constant. Activation entropy ($\Delta S^*$) was calculated from equation

$$\Delta S^* = (\Delta H^* - \Delta G^*) / T \quad \text{(Eq. 4B.5)}$$

### 4B.2.3.6. Effect of inhibitors

Effect of few inhibitors which inhibited native enzyme (such as MgCl$_2$ and iodoacetamide) was studied for L-asparaginase activity. The enzymes (native and modified) were incubated with 1 mM concentration of MgCl$_2$ and iodoacetamide separately for 1 h and then L-asparaginase activity was assayed by estimating the product $\beta$-aspartyhydroxamate formed at 500 nm. Highest enzyme absorbance value was taken as 100 % to calculate relative activity.

### 4B.2.3.7. Resistance to trypsin digestion

1 ml of enzyme solution was dissolved in 0.5 mg/ml of trypsin and the mixture was incubated at 37 °C for different time intervals (1 to 25 min). 0.1 ml samples were taken after incubation to determine the residual enzyme activity. Relative activities were calculated by taking the enzyme absorbance value at 0 min as 100 % (Qian et al., 1997).

### 4B.2.3.8. Studies on in-vitro half-life of L-asparaginase in blood serum

Aliquots of native and modified L-asparaginase were added to blood serum at 1:9 ratio. The reaction mixture was incubated at 37°C for 24 h. Samples were removed at regular intervals to determine residual L-asparaginase activity.

### 4B.2.3.9. Intrinsic fluorescence measurement

Fluorimetric measurements were carried out using Shimadzu model no. RF-51PC fluorescence spectrophotometer in the ratio mode using split widths of 5 mm for excitation and emission. Protein solution of 0.05-0.1 mg/ml concentrations was taken in a cuvette to record the spectra at emission...
between 330 to 340 nm after exciting at 280 nm. The spectra were recorded with a 5 mm band width for both excitation and emission monochromators.

4B.2.3.10. Determination of secondary structure

Circular dichroism (CD) measurements were made with a JASCO-J20C automatic recording spectro polarimeter. The instrument was calibrated with dextro-10-camphor-sulphonic acid. Slits were programmed to yield 1 nm bandwidth at each wave length from 350 to 200 nm. The chart speed, wavelength expansion and time constant of the instrument were set to obtain the best signal- to- noise ratio. The lamp was purged continuously with nitrogen before and during experiments. The scans were recorded twice. Mean residue ellipticities \([\theta]_{\text{mrw}}\) were calculated by standard procedures (Adler et al., 1973). A value of 110 for mean residue weight was used. The CD spectra were analyzed to estimate the secondary structure (Kelly and Price, 2000). The measurements were made at ambient temperature and each spectrum represented the average of three accumulations. CD analyses were done using the programs CONTIN and K2D recommended for proteins with predominant \(\beta\)-structure.

4B.3. Results and Discussion

4B.3.1. Modification of L-asparaginase

Native L-asparaginase was modified as described under methods (4B.2.2.2) and the degree of modification of \(\varepsilon\)-amino groups in the molecule was determined. In all the modifications studied, 100 % activity was retained i.e. there was no loss in enzyme activity as compared with the activity of native enzyme. By conjugating BSA to L-asparaginase enzyme 3.6 fold of increase in activity was observed (Table 4B.1). With OVA, the substitution was observed to be still better with 10 fold increase in activity. NBS and methoxy-PEG modification showed 1.34 and 2.27 fold increase in activity respectively (Table 4B.1).
Table 4B.1: Specific activities of Native and Modified L-asparaginase enzymes

<table>
<thead>
<tr>
<th></th>
<th>Native enzyme</th>
<th>BSA modified</th>
<th>Albumin modified</th>
<th>NBS modified</th>
<th>PEG modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity@ 0 h</td>
<td>0.043</td>
<td>0.155</td>
<td>0.446</td>
<td>0.058</td>
<td>0.093</td>
</tr>
</tbody>
</table>

*Specific activity was measured without any incubation and the units were expressed in U/mg.

In our studies, among the modifications used, BSA and OVA modifications performed better in terms of increased activity followed by PEG and NBS. Increase in activity of the enzyme by BSA conjugation using glutaraldehyde is advantageous as it is considered to have more limited allergenic potential compared to OVA which is a significant human allergen (Dearman et al., 2000). Quiocho and Richards, (1964) first recognized the use of glutaraldehyde as enzyme crosslinking agent for stabilizing carboxypeptidase. The cross-linked enzyme retained 5% residual activity (Quiocho and Richards, 1964).

4B.3.2. Degree of enzyme modification

In general, the changes in the free amino groups of the enzyme elucidate the degree of modification of an enzyme either modified or bio-conjugated with another molecule. In our studies TNBS (Habeeb, 1966) was used to find the degrees of modification of ε-amino groups in L-asparaginase and modified L-asparaginase. The results showed that the amount of ε-amino groups in the L-asparaginase bio-conjugates after modification decreased to 64 %, 64.42 %, 28.58 % and 16.08 % of the original level in BSA, OVA, PEG and NBS modified L-asparaginase respectively.

4B.3.3. Thermal stability of native and modified L-asparaginase

The activity and stability of both native and modified enzymes were studied with respect to temperature. Studies were done at two temperatures 28 and 37 °C and the stability studies were carried out at these temperatures up to 24 h. Results are summarized in Figure 4B.1a & b. It was observed that there was 72 % loss of activity with native enzyme by 24 h of incubation at 28
°C (Figure 4B.1a). The loss in activity was reduced to 60 % with NBS modification and to 24 % by PEG modification for the same period of incubation and at the same temperature. Both BSA and OVA modified enzymes showed increase in activities. With BSA modification there was progressive increase in activity. By the end of 24 h, there was 8.5 fold of increase in activity. However with OVA, there was an increase in activity (2.73 fold) by 3 h which remained almost constant thereafter.

At 37 °C incubation temperature, there was 65 % loss of activity with native enzyme by 24 h of incubation (Figure 4B.1b). The loss in activity was reduced to 32 % with NBS modification and 29 % by PEG modification for the same period of incubation. The loss in activity was observed only after 6 h of incubation. In the first 3 h, there was enhancement in the enzyme activity by 2.8, 3.5, 1.43, 1.32 and 1.58 folds with native, BSA, OVA, NBS and PEG modified enzyme respectively. With further incubation for 3 h, native enzyme showed loss of activity that was 0.8 times compared to initial activity. With other modifications, there was 1.6, 1.39 1.8 and 1.1 fold increase in activity respectively with BSA, OVA, NBS and PEG modified enzymes as compared to 3 h activity. With BSA modification there was progressive increase in activity. By the end of 24 h, there was 7.62 fold of increase in activity. However with OVA, there was 2.6 folds increase in activity.

Figure 4B.1a: Thermal stability of native and modified L-asparaginase at 28 °C
Figure 4B.1b: Thermal stability of native and modified L-asparaginase at 37°C

Specific activity was plotted against time for a period of 24 h at: 28 °C & 37 °C. The experiment was done in triplicate. Error bars show the percent error.

4B.3.4. Thermal inactivation kinetics

Inactivation rate constant ($K_d$) of modified and native L-asparaginase, given in Table 4B.2 were calculated from the slope of semi-log plot of residual activity vs time and the constants were used for the estimation of half-life using Equation (4B.1). At 28 °C, the half-life of native enzyme was 12.26 h. Modified enzymes showed increase in half-life which was 17.46 and 30.66 h respectively for NBS and PEG modified L-asparaginase. At 37 °C, half-life of native enzyme was 15.56 h which was 27 % more compared to that shown at 28 °C. With modified enzymes, the trend followed was same. The half-lives were 22.5 and 31.79 h respectively for NBS and PEG modified L-asparaginase. The enzymatic degradation of L-asparagine is a very important reaction for the presumed clinical treatment of acute leukemia. When cross-linked with conjugates, the substrate of enzyme was observed to be a good protector of the enzyme active centre. The addition of protector during cross-linking, the substrate L-asparagine, protects the L-asparaginase active centre from denaturation and increases its half-life.
Inactivation energy of native L-asparaginase was 10498.49 kJ/mol. With enzyme modification there was an increase in inactivation energy except for PEG modified L-asparaginase (Table 4B.3). The $E_d$ of modified L-asparaginase was 11057.63 and 1570 kJ/mol respectively for NBS modified and PEG modified L-asparaginase. However, L-asparaginase modified with BSA and OVA did not show any deactivation till assay time i.e., 24 h.

**Table 4B.2: Half-life and Inactivation rate constant of native and modified L-asparaginase at 28 °C and 37 °C**

<table>
<thead>
<tr>
<th>Type of Enzyme</th>
<th>Temp. (°C)</th>
<th>Inactivation rate const ($k_d$)</th>
<th>Half life</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native Enzyme</td>
<td>28</td>
<td>0.056</td>
<td>12.26</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>0.044</td>
<td>15.60</td>
</tr>
<tr>
<td>NBS modified</td>
<td>28</td>
<td>0.039</td>
<td>17.45</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>0.030</td>
<td>22.50</td>
</tr>
<tr>
<td>PEG modified</td>
<td>28</td>
<td>0.022</td>
<td>30.66</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>0.021</td>
<td>31.78</td>
</tr>
</tbody>
</table>

*Units of half-life were expressed in hour (h) and Inactivation rate constant $k_d$ were expressed in per hour (1/h).
Table 4B.3: Thermal deactivation kinetics of native and modified L-asparaginase

<table>
<thead>
<tr>
<th>Type of Enzyme</th>
<th>Inactivation energy $E_d$ (kJ/mol)</th>
<th>Temp (°C)</th>
<th>$\Delta G^*$ (kJ/mol)</th>
<th>$\Delta H^*$ (kJ/mol)</th>
<th>$\Delta S^*$ (kJ/K mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native Enzyme</td>
<td>10498.49</td>
<td>28</td>
<td>5069.46</td>
<td>32.97</td>
<td>0.109</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>5070.49</td>
<td>-279.94</td>
<td>-0.90</td>
</tr>
<tr>
<td>NBS modified</td>
<td>11057.63</td>
<td>28</td>
<td>5081.73</td>
<td>592.11</td>
<td>1.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>5082.76</td>
<td>279.19</td>
<td>0.90</td>
</tr>
<tr>
<td>PEG modified</td>
<td>1570</td>
<td>28</td>
<td>5101.32</td>
<td>-8895.49</td>
<td>-29.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>5102.34</td>
<td>-9208.43</td>
<td>-29.70</td>
</tr>
</tbody>
</table>

*All L-asparaginase (native and modified) were studied for thermal deactivation kinetics. Inactivation energy ($E_d$), Free energy of inactivation ($\Delta G^*$), Inactivation enthalpy ($\Delta H^*$) and Inactivation entropy ($\Delta S^*$) were calculated for both the temperatures (28 °C and 37 °C).

4B.3.5. Evaluation of thermodynamic parameters

The changes in enthalpy ($\Delta H^*$) and entropy ($\Delta S^*$) are calculated using transition state theory according to Equation (4B.3) and Equation (4B.5) for the thermal inactivation. $\Delta H^*$ and $\Delta S^*$ were estimated in two temperatures studied. With increase in temperature to 37 °C, both $\Delta H^*$ and $\Delta S^*$ values decreased (Table 4B.3) and a slight increase in $\Delta G^*$ values was observed. All the modifications used followed the same trend. Thermal stability of the immobilized enzyme was improved due to attachment of multiple activated carbon to the enzyme molecule resulting in greater enzyme rigidity of enzyme conformation and increasing the activation energy for unfolding the enzyme. Similar to our studies, Tabandeh & Aminlari (2009) obtained noticeable improvement in thermal stability of modified enzyme. In contrast, Zhang et al. (2004) reported that the thermo stability of the immobilized L-asparaginase is very similar to that of the native enzyme and there were no apparent changes in the activities. $\Delta S^*$ decreased slightly with increase in temperature, and significant decrease in $\Delta H^*$ were observed. The decrease in $\Delta S^*$ indicated
that number of protein molecules in transition activated stage decreased, resulting in higher values of \( \Delta G^* \). Also, positive entropy values suggested that enzyme unfolding could be one of the rate determining step for the irreversible thermo-inactivation of L-asparaginase. Moreover, two major factors influencing the numerical values of \( \Delta H^* \) and \( \Delta S^* \) were solvent and structural effects and the information regarding the degree of solvation and the degree of compactness of protein molecule were given by \( \Delta S^* \) values (Dogan et al., 2008).

4B.3.6. Storage stability of native and modified L-asparaginase enzyme

Storage stabilities of both native and conjugated L-asparaginase were investigated in phosphate buffer at refrigerated and ambient temperatures. Native enzyme was found to lose its activity easily during storage at refrigerated and ambient temperatures. The loss in activity was faster at ambient temperature compared to storage under refrigeration conditions. When the enzyme was maintained at ambient temperature, there was progressive reduction in activity. Under refrigeration conditions, the stability of enzyme was more than storage at ambient temperature. Although there was reduction of activity, the reduction was more during storage at ambient temperature. With modified or bio-conjugated enzymes, BSA modified enzyme retained complete activity even at 60 days of storage. This was followed by PEG, OVA and NBS modified enzymes where 96, 87 and 79 % activities were retained. However native enzyme lost complete activity within 2 days at ambient temperature. At refrigerated conditions the enzyme activity was 20 % less by the end of 60 days.

4B.3.7. Resistance of native and bio-conjugated L-asparaginase against trypsin

The resistance of both native and bio-conjugated L-asparaginase against trypsin digestion is given in Figure 4B.2. It was observed that the resistance of modified L-asparaginase greatly improved when compared with that of native enzyme. By 25 min of hydrolysis, the native enzyme was mostly denatured while the modified enzyme lost only 20, 30, 35 and 38 % activity with BSA, OVA, PEG and NBS modifications respectively. Among the
modifications used, BSA was found to be more resistant to tryptic digestion. This may be attributed to the steric effects of the covalently cross linked L-asparaginase that protected the reactive site.

4B.3.8. Effect of inhibitors on the activity of native and modified enzyme

The enzymes (native and modified) were incubated separately with 1 mM concentration of MgCl₂ and iodoacetamide. The loss in activity in presence of these inhibitors was less compared to the native enzyme. When treated with 1 mM concentration of MgCl₂ for 1 h, the native enzyme lost 77% activity. The conjugated or modified enzymes retained complete activity as observed at 0 h of incubation. The same was true with iodoacetamide. The native enzyme lost most of the activity within 1 h of incubation with 1 mM of iodoacetamide. However, there was no loss in enzyme activity with all the other modified enzymes.

Figure 4B.2: Stability of the native and modified L-asparaginase against trypsin at 37°C

Enzyme activities were observed for assay time of 25 min. The experiment was done in triplicate. Error bars show the percent error.

4B.3.9. In-vitro half-life of L-asparaginase

In-vitro half-life of the native and modified enzyme in blood serum was investigated. The in-vitro clearance of native enzyme from blood was faster
with a half-life of 93.65 h (Figure 4B.3). *In-vitro* half-life of L-asparaginase increased with NBS modified L-asparaginase which had a half-life of 144.37 h. The modification resulted in a compound with longer half-life in blood plasma and improved therapeutic properties (Harms et al., 1991; Wehner et al., 1992; Derst et al., 1992). Comparatively, *E. coli* L-asparaginase modified with Polyethyleneglycol grafted Vinyl pyrrolidone-Maleic Anhydride Copolymer has shown a half-life of 53 h (Qian et al., 1997). With modification the half-life in serum got reduced except in NBS modified L-asparaginase which showed increase in half-life (144.37 h). The half-lives were 36.1, 42.26 and 72.95 h respectively with BSA, OVA and PEG modifications. The half-life of NBS modified L-asparaginase was 54 % higher than native enzyme, while all other modifications showed faster clearance in *in-vitro* conditions. This fact can be explained by the presence of non-specific globulins or proteases in serum. In other words, these results demonstrate that the human serum already contained natural antibodies and/or proteases capable of making L-asparaginase of *Cladosporium* sp. inactive. The modification with NBS protects L-asparaginase by inhibiting the binding of non-specific globulins or by proteolysis in serum. The half-life stability of PEG modified L-asparaginase was better at both 28°C and 37°C. However, data for deactivation for BSA and OVA modified L-asparaginase was not available till assay time of 24 h. Values for half-lives are given as the mean of at least three independent experiments.
Figure 4B.3: *In vitro* half life of native and modified L-asparaginase. Enzymes were incubated for 24 h in serum at 37 °C

![Graph showing serum half life of native and modified L-asparaginase](image)

The experiment was done in triplicate. Error bars are the mean of three replicates ± Standard deviation.

4B.3.10. **Spectral studies of native and modified L-asparaginase**

The control (untreated L-asparaginase) had an excitation maximum of 280 nm and the emission maximum of 330 nm (Figure 4B.4). The emission spectrum of BSA modified enzyme did not show drastic shift, but there was a reduction (36 RFU) in the intensity of emission fluorescence, compared native enzyme. The emission maxima of BSA modified enzyme was 337 nm and excitation maxima was 280 nm. No other changes were observed on the spectral pattern of BSA modified L-asparaginase. On the other hand, OVA modified enzyme showed an increase in the intensity of emission fluorescence (267 RFU), as compared to that of native enzyme. With OVA modified enzyme also, there was marginal shift and the emission maxima was 335 nm and excitation maximum was 280 nm. PEG modified enzyme showed maximum reduction of 242 RFU compared to the native enzyme. There was marginal shift and the emission maxima of PEG modified enzyme was 339 nm and excitation maxima was 280 nm. NBS modified enzyme showed maximum reduction of 153 RFU compared to the native enzyme. There was marginal shift and the emission maxima of NBS modified enzyme was 336 nm and
excitation maximum was 280 nm. In general, the aromatic amino acids exhibit fluorescence in any protein and enzyme in the range 310 nm to 340 nm. The hydrophobicity of these aromatic amino acids determines the intensity of fluorescence. Moreover, an exposure of aromatic amino acids such as tyrosine, tryptophan and phenylalanine those are buried in the core of the protein to hydrophilic or aqueous environment results in the reduction of protein fluorescence. The reduction in the fluorescence intensity of modified L-asparaginase enzyme in comparison with native enzyme was an indication of change of 3D conformation. Also, reduction in the intensity of emission fluorescence may be related to the reduced hydrophobicity of modified enzyme protein in comparison to the native enzyme. As the primary structure of L-asparaginase enzyme was intact enough after modification, the protein has shown its fluorescence at 330 nm. The emission maximum of native α-galactosidase from *Bacillus stearothermophilus* (NCIM 5146) did not change with modification at 336 nm, however decrease in the relative fluorescence intensity was observed with progress of modification (Gote et al., 2007).

**Figure 4B.4: Fluorescence spectrum of native and modified L-asparaginase**

Fluorescence spectrum was done with 0.05-0.1 mg/ml concentration of protein and spectra was recorded with 5 nm band width. The experiment was done in triplicate.
However, the secondary structure analysis showed some changes due to modification. CD spectral analysis of native enzyme showed only 0.8 % α-helix and 82.3 % β-sheets (Table 4B.4). Change in the secondary structure was observed in all L-asparaginase modifications. There was reduction in α-helix only in PEG modified L-asparaginase. There was an increase in α-helix in BSA, OVA and NBS modified enzymes. The increase in α-helix was very much in BSA modified enzyme. There was 31.7 % α-helix in BSA modified enzyme and the β-sheets were reduced from 82.3 % to 42.2 %. In OVA modified enzyme, α-helix increased to 15.3 % and β-sheets were decreased to 67.1 %. In PEG modified enzyme β-sheets increased to 87.2 % while α-helix was reduced to 0.6 %. In NBS modified enzyme, there was a slight increase in α-helix. It was 1.7 % while β-sheets were reduced slightly to 81.8 % (Table 4B.4). Random coils remained almost same in all modification except NBS modified enzyme where there was an increase from 16.9 % of native enzyme to 24.6 %. The far-UV CD spectrum exhibited a minimum at 217 nm. It is pertinent to note that the minimum at 217 nm is indicative of parallel β-structure. Band observed at 220–240 nm of the CD spectra of laccase from Trametes versicolor and glucose oxidase from Aspergillus niger with and without poly(ethyleneimine) (PEI) is attributed to the β-sheets arrangements in the enzymes secondary structure. The 30 % decrease in band intensity was due to disruption of the β-sheets induced by the polymer (PEI) for the Trametes versicolor enzyme (Zhang and Rochefort, 2011). Thus a rapid and inexpensive method for stabilization of enzymes by crosslinking or by introduction of monomeric or polymeric moieties can be achieved by chemical modification. Also, the introduction of functional groups and specificity-determining groups that are inaccessible by conventional mutagenesis techniques can be accomplished by chemical modification.
Table 4B.4: CD spectra showing the modification in $\alpha$ helix and $\beta$ sheets in native and modified L-asparaginase from *Cladosporium* sp.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\alpha$ helix (%)</th>
<th>$\beta$ sheets (%)</th>
<th>Random coils (%)</th>
<th>Turn (%)</th>
<th>RMS</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.8</td>
<td>82.3</td>
<td>16.9</td>
<td>0.0</td>
<td>518.51</td>
<td>100</td>
</tr>
<tr>
<td>L-asparaginase BSA</td>
<td>31.7</td>
<td>42.2</td>
<td>24.6</td>
<td>1.5</td>
<td>8.75</td>
<td>100</td>
</tr>
<tr>
<td>L-asparaginase NBS</td>
<td>1.7</td>
<td>81.8</td>
<td>16.5</td>
<td>0.0</td>
<td>352.78</td>
<td>100</td>
</tr>
<tr>
<td>L-asparaginase PEG</td>
<td>0.6</td>
<td>82.2</td>
<td>17.2</td>
<td>0.0</td>
<td>316.41</td>
<td>100</td>
</tr>
<tr>
<td>L-asparaginase OVA</td>
<td>15.3</td>
<td>67.1</td>
<td>16.6</td>
<td>1.0</td>
<td>44.04</td>
<td>100</td>
</tr>
</tbody>
</table>
4B.4. Summary and Conclusions

- Modification of L-asparaginase from *Cladosporium* sp. was tried to obtain improved stability and improved functionality.
- Modification of the enzyme was tried with Bovine Serum Albumin, Ovalbumin by crosslinking using Glutaraldehyde, N-Bromosuccinimide and Mono-methoxy Polyethyleneeglycol.
- Modified enzymes were studied for activity, temperature stability, rate constants \( (k_d) \) and protection to proteolytic digestion.
- Modification with Ovalbumin resulted in improved enzyme activity that was 10 folds higher compared to native enzyme, while modification with Bovine Serum Albumin through glutaraldehyde cross linking resulted in high stability of L-asparaginase that was 8.5 and 7.62 folds more compared to native enzyme at 28°C and 37°C by the end of 24 h.
- Modification also markedly prolonged L-asparaginase half-life and serum stability.
- N-Bromosuccinimide modified L-asparaginase presented greater stability with prolonged *in-vitro* half-life of 144 h to proteolytic digestion relative to unmodified enzyme (93 h).
- Fluorescence and CD spectral properties of the modified enzyme, point out conformational changes of L-asparaginase that perturbs the microenvironment of the aromatic amino acid residues such as tryptophan.
- The present work could be seen as a modified L-asparaginase produced with improved activity and stability and can be a potential source for developing therapeutic agents for cancer treatment.
4B.5. Bibliography


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