5. PURIFICATION AND CHARACTERIZATION OF L-ASPARAGINASE

5.1. INTRODUCTION

Once an enzyme geared up for therapeutic application, the enzyme should be purified and characterized, before its final diligence. Enzymes are being emerged as therapeutic agents currently have been focused by pharmaceutical companies worldwide. Several features of the enzyme based therapy contribute to its growth by enlightening the risk–benefit ratio (Fekte et al., 2013). These features include tolerance, good efficacy, high specificity and reduced side effects. Before the release of an enzyme, the identity, heterogeneity, impurity content and activity has to thoroughly be investigated (Schneider et al., 2008).

The quality of the product (enzyme) may be achieved by wide range of analytical methods that combines with Ion–exchange chromatography (IEX), Reversed–phase liquid chromatography (RPLC), Hydrophobic interaction chromatography (HIC), Size exclusion chromatography (SEC), Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), Capillary isoelectric focusing (CIEF) and Capillary zone electrophoresis (CZE) (Beck et al., 2013). Lucy et al. (2003) reviewed the history and continuous evolution of ion–exchange chromatography and cation exchange chromatography (CEX) is the most widely used method for protein purification and characterization among different IEX methods. CEX is considered as the gold standard for protein purification, meanwhile parameters such as column type, mobile phase pH and salt concentration gradient are often need to be optimized for each and individual protein. IEX is an ancient and non–denaturing technique broadly utilized for the study of charge variants of proteins and accepted as a reference technique for the qualitative and quantitative evaluation of therapeutic proteins (Fekte et al., 2013).

103
L-asparaginase from bacterial origin can cause hyper sensitivity in the long-term usage leading to allergic reactions and anaphylaxis. The toxicity is partially attributable to the glutaminase activity of these enzymes (Oza et al., 2011). L-asparaginase with high asparaginase activity and negligible glutaminase activity are reported to be less troublesome during the course of antitumor therapy (Hawkins et al., 2004). The search for other asparaginase sources, with new immunological characteristics can lead to enzyme with less adverse effects. Furthermore, new studies have revealed potential application of this enzyme in prevention of leukemia. Therefore, introduction of new fermentation and purification protocols of L-asparaginase will be mandatory to satisfy these demands (Aghaeepoor et al., 2011).

So, in this chapter the properties of the crude enzyme were studied before it was purified by ion–exchange column chromatography. The enzyme L–asparaginase was purified to its homogeneity and the molecular weight of the enzyme was resolved with Sodium dodecyl sulphate – poly acrylamide gel electrophoresis (SDS – PAGE). The stability of the purified enzyme in the presence of substrate and various environmental factors were also studied elaborately.
5.2. MATERIALS AND METHODS

5.2.1. Purification of L-asparaginase

The crude enzyme prepared from strain *B. subtilis* RM4 was used for further purification of L-asparaginase at 4°C to 8°C.

The sequential steps followed in purification included ammonium sulfate precipitation, ion exchange and gel filtration chromatography. The molecular weight of the L-asparaginase was determined by SDS-PAGE. The molecular mass was determined by using matrix-assisted laser desorption/ionization - time-of-flight mass spectrometry (MALDI-TOF) and the purity of enzyme was checked using reverse phase high performance liquid chromatography (RP-HPLC). After each step, the L-asparaginase activity and total protein content were determined.

5.2.1.1. Ammonium sulfate precipitation

Ammonium sulfate removes water from the surrounding of the protein revealing hydrophobic patches, which come together and causes the protein to precipitate. The more a protein is hydrophilic, the more will be the ammonium sulfate needed. The fractionation range of ammonium sulfate needed to precipitate out the target protein was determined by performing analytical ammonium sulfate cut. Precipitation of L-asparaginase with ammonium sulfate was performed according to the method described by Zhang *et al.* (1995) with slight modification.

Ammonium sulfate was added to the supernatant in different concentrations ranging from 10 to 90%w/v saturation, with constant stirring in ice bath. The precipitate was removed by centrifuging at 10000 rpm for 10 min in cooling centrifuge (4°C). The supernatant was used for estimation of enzyme activity and protein content. The fractionation range of ammonium sulfate needed was determined and found to be 40 to 70% w/v. Initially the cell free supernatant was brought to 40% saturation with ammonium sulfate and kept at 4°C to 8°C overnight. After overnight equilibration, the
precipitate was removed by centrifuging at 10000 rpm for 10 min in cooling centrifuge at 4°C. The supernatant was further brought up to 70% saturation with ammonium sulfate and left overnight at 4°C to 8°C. The precipitate was collected by centrifuging at 10000 rpm at 4°C for 15 min. The precipitate was re-suspended in 10 ml of cold 50 mM Tris-HCl with pH 8.6 and desalted using sephadex G-25 column with the same buffer. An aliquot from this was used to determine the enzyme activity and protein content. The desalted protein solution was collected, stored at 4°C to 8°C and used in further steps of purification.

5.2.1.2. Dialysis

Dialysis membrane is transparent in nature and contains the small pores which allow the smaller molecules to pass through the membrane and retains the large molecules inside the bag thus purifies the solution (enzymes) from the excess impurities when placed in an exchange buffer which are changed continuously to obtain the purified enzyme at 4°C.

12 cm of the dialysis membrane-50 (avg. flat width 22.5 mm, avg. diameter 14.3 mm having capacity 1.61ml/cm HI – MEDIA, Mumbai) were cut and placed in 100 ml of 2% sodium bicarbonate to which 1 mM EDTA was added with pH 8.0 to chelate any metal ions. The tubes were boiled for 10 min in 1 mM EDTA and tubes were washed with distilled water thoroughly for 10 min and allowed to cool. The activated dialysis tubes were washed with distilled water and tied tightly at the one end with sterilized yarn. The solution containing precipitated enzymes of strain \textit{B. subtilis} RM4 was placed inside the dialysis tube separately and was tied on another end. Then tubes placed in 1500 ml of 5 mM Tris-HCl pH 8.0 containing 1 mM MgCl$_2$ over night at 40°C with stirring conditions by using magnetic stirrer. After completion of dialysis, the dialysates were centrifuged at 8000 rpm for 20 min at 40°C. Then the supernatant were subjected for an ion exchange column chromatography for further purification and elution of enzyme.
5.2.1.3. Ion exchange chromatography (IEC)

Ion exchange chromatography was performed using Q-Sepharose column in Fast Protein Liquid Chromatography (FPLC) (AKTA prime plus, Amesham Biosciences, USA). The sample obtained after desalting was diluted to 50 ml of 50 mM Tris-HCl buffer and used in IEC. DEAE-sepharose anion exchange column was equilibrated with 50 mM Tris-HCl buffer (pH 8.6). Then the sample was added to the equilibrated column, and the column was washed with two bed volumes of the same buffer to remove any unbound protein. The sample was eluted as 6 ml/fraction using NaCl gradient (0.1 to 0.5M) at flow rate of 30mlh⁻¹. The protein content and enzyme activity were determined. The fractions showing peak L-asparaginase activity were pooled together and concentrated by dialysis against 50 mM Tris –HCl buffer at 4°C (El-Bessoumy et al., 2004).

5.2.1.4. Gel filtration chromatography (GFC)

The sample obtained after dialysis was chromatographed on a column of Sephacryl S 200, high resolution column (Pharmacia, 16/60) which was pre-equilibrated with 0.05M Tris-HCl buffer of pH 8.6. The sample was eluted with the same buffer at 24 mlh⁻¹ flow rate as 4 ml fractions. The enzyme activity and the protein content of the fractions were determined. Fractions with peak enzyme activity were pooled together and concentrated by dialysis against 50 mM Tris-HCl buffer and stored at 4 to 8°C. The homogeneity of the protein was checked by SDS and native PAGE.

5.2.2. Partial characterization of the enzyme

5.2.2.1. Effect of pH on enzyme activity and stability

The effect of pH on enzyme activity was studied by estimating the activity in different pH ranges from 4.0 to 10.0 with different buffers viz., phosphate buffer (pH 4.0 to 7.0), Tris-HCl (pH 8.0) and glycine-NaOH (pH 9.0 to 10.0). The enzyme was incubated in contact with different relevant buffers at 37°C for 48 h to determine its stability in different pH. The relative activities were measured.
5.2.2.2. Effect of temperature on enzyme activity and stability

The optimum temperature range for enzyme activity was determined by incubating the assay mixture at different temperatures. Temperature ranges 20°C, 25°C, 30°C, 35°C, 40°C and 45°C at pH 8.0 for 5 h and the residual activity was determined by Nessler’s method.

5.2.2.3. Effect of inhibitors and chelators on enzyme activity

The effect of different inhibitors were used to optimize the enzyme activity. Inhibitors viz., dithiothritol (DTT, 5mM), β-mercaptoethanol (MCE, 5mM) and chelator like ethylene diamine tetra acetic acid (EDTA, 5mM) and iodio acetic acid (IAA, 5mM) were used. The enzyme was pre-incubated with respective inhibitor or chelator at 37°C for 1 h and then the enzyme assay was performed under standard conditions.

5.2.2.4. Effect of metal ions for the enzyme activity

The effect of different metal ions on the enzyme was studied by pre-incubating enzyme with 10 mM magnesium, cobalt, copper, manganese, ferrous and zinc for 1 h at 37°C. The relative enzyme activity was estimated.

5.2.2.5. Substrate specificity of purified L-asparaginase

Different substrates namely L-asparagine, D-asparagine, DL- asparagine, D-aspartic acid, L-aspartic acid amide, L-glutamine, D-glutamine, L-glutamic acid and succinamic acid were used to investigate the specificity of L-asparaginase from B. subtilis RM4. The substrates were prepared in potassium phosphate buffer of pH 8.5 (0.25 M) at 10 mM concentration. The reaction mixture was incubated for 15 min and relative activity was expressed as the percentage ratio of enzyme activity.
5.2.3. Electrophoresis Analysis

5.2.3.1. Determination of molecular weight of enzyme by SDS-PAGE

Native PAGE of the purified L-asparaginase was performed by using 12% polyacrylamide gel in glycine buffer at 5±1°C as per standard protocol described by Gallagher et al. (1999). SDS-PAGE was performed following the modified method of Laemmli et al. (1970) with a 12.5% separating acrylamide gel (pH 8.8) and 5% stacking gel (pH 6.8) containing 0.1% SDS. Standard-L-asparaginase and standard markers sample [included phosphorylase (97.4kDa), bovine serum albumin (66.2kDa), ovalbumin (43kDa), carbonic anhydrase (31kDa), trypsin inhibitor (20.1kDa), lysozyme (14.3kDa) and purified asparaginase] was applied to individual well. After completion of electrophoresis in a Tris-glycine buffer (pH 8.3) at 120 V for 3 h at room temperature, the gel was retained under 100 ml of fixing solution (which includes 50% methanol, 12% glacial acetic acid and 56 µl of 36% of formaldehyde) for 10 min. Then the gel was washed with 100 ml of 20 % of methanol for 10 min and then the gel was retained under 100 ml of 0.02% of sodium thio sulphate as a sensitizing solution for 2 to 3 min. Finally, the gel was maintained under 50 ml of 0.2% of silver nitrate solution for 15 min under dark condition and the gel was rinsed with water for two times to observe the clear band of markers and L-asparaginase sample on the wells. The subunit molecular weight and intact molecular weight of L-asparaginase was determined by using SDS – PAGE and native PAGE respectively.

5.2.4. Peptide mapping

Peptide or protein digestion, at a ratio of 1:100 to 1:200 (w/w) of enzyme to the substrate is recommended. The protein substrate in 100 mM ammonium bicarbonate or 100 mM Tris-HCl was dissolved at pH 8.5. The Tris-HCl buffer is incompatible with MALDI analysis and the ammonium bicarbonate buffer should be used when MALDI analysis will follow. The trypsin in 1 mM HCl to a concentration of 1 mg / ml is dissolved and added to the substrate protein solution. The recommended incubation
time is between 2 and 18 h at 37°C depending on the enzyme to substrate ratio. After trypsin digested, the protein sample was used for MALDI-TOF (Klammer Maccoss et al., 2006).

5.2.5. Molecular mass determination

The native molecular weight of purified enzyme was determined by gel filtration chromatography through a column (1 cm × 50 cm) of sephacryl S-200. The column was calibrated with blue dextran and standard molecular marker proteins. The elution volume ($V_e$) of each marker protein and void volume ($V_o$) of the column were estimated. A plot of ($V_e/V_o$) against log $M_r$ was used to find the native weight ($M_r$) of L-asparaginase. The intact molecular mass was also determined by matrix assisted laser desorption ionisation time-of-flight (MALDI-TOF), using Bruker Ultra flex MALDI-TOF/TOF mass spectrometer equipped with a 337 nm nitrogen laser (IISC, Bangalore). The protein sample was mixed with equal volume of saturated matrix solution [sinapinic acid (10 mg /ml) in 50% acetonitrile/ H$_2$O with 0.1% trifluoroacetic acid (TFA)]. The L-asparaginase was mixed with matrix (1:1) 1 μl of the sample was spotted on the probe plate and dried by steam and the spectra were recorded in the reflectron positive ion mode using Bruker Daltonics (Andrew et al., 1964).

5.2.6. Purity analysis on Reverse phase - High performance liquid chromatography (RP-HPLC)

The purified native L-asparaginase purity was analysed on C8 (46 mM x 150 mM, ZORBAX-C8) reverse phase high performance liquid chromatography (RP-HPLC) column. The sample of 100 μl (20μg) was loaded onto column using a solvent-A (100% Water: 0.05% TFA V/V) and solvent- B (100% Acetonitrile (ACN): 0.05% TFA V/V). The experiment was carried on linear gradient of 0 to100% achieved in 70 mins. The protein was monitored at 280 nm (Thakur et al., 2002).
5.3. RESULTS

5.3.1. Purification of L-asparaginase

The results of purification of L-asparaginase from *B. subtilis* RM4 are summarized in Table 14. Activity guided analytical ammonium sulfate cut method was used to determine the fractionation range for precipitation of the target protein. The fractionation range was found to be 75% w/v saturation of ammonium sulfate. With preparative ammonium sulfate precipitation, the yield was 2.74 % and purification fold was 69.93. The desalting was performed using sephadex G25 and the elute was diluted to 50 ml with 50 mM Tris-HCl buffer (pH 8.6) and further purified by IEC. While analysing previous procedures, dialysis was used as second step immediately after the ammonium sulfate precipitation which gave sufficient yield of enzyme. The purification of glutaminase-free L-asparaginase was involved in ammonium sulphate precipitation followed by IEC using DEAE sepharose column (Figure 37b). Different protein peaks were observed and the target protein was eluted with 0.2 M NaCl. The purification fold and yield after IEC was found to be 4.48 and 56.23 respectively (Table 14). Elute from IEC was subjected to dialysis and concentrated. Analysing the above studies, DEAE column was found to be important in distinguishing L-asparaginase and free L-glutaminase in crude extract. Free glutaminase was not eluted from DEAE Sepharose. Hence, DEAE column was intentionally used as a first column in the purification of L-asparaginase from *B. subtilis* RM4 to eliminate the possibility of separation of L-asparaginase and L-glutaminase. DEAE column was found to be effective column in the initial step of purification of L-asparaginase. Further the enzyme preparation obtained was found to be stable after purifying in DEAE column (Figure 37 b). Further dialysis in later stage of purification would be advantageous which yields relatively pure material for subsequent GFC.

Final purification was achieved by gel filtration chromatography (GFC) using Sephacryl S-200. The pooled high active fractions of DEAE column were applied on
The target protein was eluted in three fractions. The yield and purification fold of the pooled fractions was found to be 19.11 and 27.31 respectively (Table 14). The specific elution profile of L-asparaginase on Sephacryl S 200 column clearly indicates only one sharp distinctive peak and homogenous form of L-asparaginase (Figure 37 c). The specific activity and purity of enzyme increased with every step of purification with minimum loss in quantity giving a final recovery of almost 27%. This was found to be a good recovery of enzyme reported so far. The commercially available asparaginase (Colpase) was used as a standard and the distinct peak was recorded and given in Figure 37 a. Further the GFC removed major contaminants there by making it vital for further chromatographic separation and it was found to be an ideal column. This suggest that GFC should be final column to achieve homogeneity of the enzyme L-asparaginase. The pooled high active fractions of Sephacryl S 200 were lyophilized and used for further analysis of the enzyme. In general, antitumor study of L-asparaginase requires milligram level of protein contained considerable amount of enzyme and their specific activity.

5.3.2. Assessment of homogeneity and molecular weight of L-asparaginase producing B. subtilis RM4

The enzyme was purified with SDS- PAGE and the molecular weight of the denatured enzyme was calculated from the Rf values and it was found to be 35.26 kDa (approx. 35 kDa) (Figure 38 a). Native PAGE separation indicated a band representing a molecular weight of 144 kDa (Figure 38 b). It was also evident from Figure 38 c that no other band appeared. From the bands observed, it can be claimed that the enzyme was purified to near homogeneity.

5.3.3. Partial characterization of L-asparaginase

The enzyme activity was determined in the presence of different modifiers (pH, temperature, metal ions, inhibitors, chelators and substrate specificity) and the results are presented in Figures 39 to 42 and Table 15. The enzyme was found to be stable at
wide pH range from 4.0 to 10.0. The partially purified L-asparaginase was found to be stable at pH 8.0. There was 20% decrease in activity when stored at pH 10.0 for 48 h. Hence, in this study alkaline Tris HCl buffer system was used (50 mM, pH 8.6) in all the purification steps (Figure 39). The enzyme was stable when stored at various temperatures ranging from 20°C to 45°C. The optimum temperature for enzyme activity was found to be 30°C (Figure 40). Storage temperature above 35°C affected the enzyme activity.

Inhibition of enzyme activity with different stimulation in metal chelators, viz., EDTA inferred that the enzyme was not a metalloprotein. Furthermore, stimulation of the enzyme activity with the reducing agents, and inhibition in the presence of thiol group blocking reagents such as Iodo acetic acid (IAA), β-mercaptoethanol and DTT (cysteine protease inhibitor) provided additional proof for the role of sulphhydril groups in the catalytic activity of the enzyme (Figure 41).

Among the metal ions tested, enzyme activity for divalent metal ions was observed to be very detrimental for enzymatic activity by Cu²⁺, Co²⁺, Mn²⁺ and Zn²⁺. Whereas Mg²⁺ and Fe³⁺ acted as a moderate enhancer (Figure 42).

The substrate specificity of the purified enzyme obtained from *B. subtilis* RM4 was determined. No positive hydrolysis was observed when D-asparagine, L-glutamine, D-glutamine, D-aspartic acid, L-glutamic acid, sinnamic acid and L-aspartic acid amide used separately as substrates. It was observed that the enzyme was very specific for its natural substrate, L-asparagine and no glutaminase activity was observed with L-glutamine as substrate. Therefore, the novel purified glutaminase-free L-asparaginase reported in this study will be an advantageous and value-added product (Table 15).
5.3.4. Peptide Mapping

Peptide mapping is considered to be one of the most specific methods for identifying changes in protein structure. The peptide fragments of native L-asparaginase are similar to *Bacillus subtilis subsp. spizizenii* TU-B10. The score of each peptide fragments are identical with *Bacillus subtilis subsp. spizizenii TU-B10* (Figure 43).


*Bacillus subtilis subsp. spizizenii*


5.3.5. MALDI TOF

Native L-asparaginase molecular mass was analysed by MALDI-TOF and the L-asparaginase molecular weight of 36498.052 kDa was identified (Figure 44). The data obtained from MALDI-TOF MS and gel exclusion chromatography were supported by the SDS and Native PAGE analysis.

5.3.6. Reverse Phase – High Performance Liquid Chromatography (RP-HPLC)

RP-HPLC for protein separation prior to intact protein characterization and peptide mass mapping by MS analyses. It takes advantage of fast mass transfer kinetics to provide efficient separation of peptides and proteins. Protein separation by RP-
HPLC was compared to SDS-PAGE electrophoresis, in terms of resolution and the sensitivity of their associated detection technique (UV detection at 214 nm Coomassie stain respectively). Intact protein masses were determined for RP separated proteins by MALDI-TOF MS. Peptide recovery and sensitivity of analysis by MS after in-well digestion of the separated proteins was measured and compared with results obtained from in-gel digestion. The purity of the L-asparaginase was confirmed in RP-HPLC. While lyophilized enzyme was analysed in RP-HPLC, a clear peak of L-asparaginase (retention time 10) was observed in the profile while for the standard L-asparaginase the retention time found to be 12 [Figure 45 (a) and (b)]. It revealed that the purified L-asparaginase was found to be 100% pure and ultimately in evidence of the homogenous form of the strain RM4 B. subtilis L-asparaginase. The procedure adopted for purification of L-asparaginase was proved to be very effective and vital for attaining a homogenous form of enzyme. It highly refined the enzyme for further evaluation of antitumor activity. The purified L-asparaginase was stored at 4°C for subsequent characterization.
Table 14: Purification steps of the L-asparaginase from *B. subtilis* RM4

<table>
<thead>
<tr>
<th>S.NO</th>
<th>Purification steps</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Total activity (IU)</th>
<th>Recovery (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cell free supernatant</td>
<td>2500</td>
<td>19,975</td>
<td>490</td>
<td>40.765</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Ammonium sulphate precipitation</td>
<td>200</td>
<td>13970</td>
<td>125</td>
<td>111.76</td>
<td>2.74</td>
<td>69.937</td>
</tr>
<tr>
<td>3</td>
<td>DEAE Sepharose fraction</td>
<td>50</td>
<td>7856</td>
<td>43</td>
<td>182.69</td>
<td>4.48</td>
<td>56.23</td>
</tr>
<tr>
<td>4</td>
<td>Sephacryl S 200</td>
<td>25</td>
<td>5456</td>
<td>7</td>
<td>779.42</td>
<td>19.11</td>
<td>27.314</td>
</tr>
</tbody>
</table>

Table 15: Substrate specificities of purified L-asparaginase from *B. subtilis* RM4

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-asparagine</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Succinamic acid</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>D-aspartic acid</td>
<td>10</td>
<td>ND</td>
</tr>
<tr>
<td>D-aspargine</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>10</td>
<td>ND</td>
</tr>
<tr>
<td>L-glutamic acid</td>
<td>10</td>
<td>ND</td>
</tr>
<tr>
<td>DL- asparagine</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>L-aspartic acid amide</td>
<td>10</td>
<td>ND</td>
</tr>
<tr>
<td>D-glutamine</td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

ND = Not detected
Figure 37 (a): L-asparaginase Standard

AKTA profile - X axis – Time; Y axis - Absorbance at 280 nm

Figure 37 (b): Ion exchange chromatography - DEAE Sepharose for L-asparaginase from B. subtilis RM4

AKTA profile - X axis – Time; Y axis - Absorbance at 280 nm
Figure 37 (c): Gel filtration chromatography – Sephacryl S 200 for L-asparaginase from *B. subtilis* RM4

AKTA profile - X axis – Time; Y axis- Absorbance at 280 nm
Figure 38: Molecular weight analysis of purified L-asparaginase from *Bacillus subtilis* RM4

(a) SDS PAGE on 12.5% gel of stained with Coomassie brilliant blue (CBB) R-250

SDS PAGE showing different purification steps for L-asparaginase

Lane 1- Standard Markers
Lane 2- Crude sample
Lane 3- Ammonium sulphate (Supernatant)
Lane 4- Ammonium sulphate (Pellet)
Lane 5- Elution from DEAE Column
Lane 6- L-asparaginase from SEC Column

(b): Non-reducing PAGE (without SDS and β-mercaptoethanol) on 8% gel of stained with CBB R-250

Lane 1- Standard Marker
Lane 2- Purified L-asparaginase

The purified L-asparaginase molecular weight is approximately 144 kDa.
Figure 38  (c): Silver staining containing purified L-asparaginase (12 % of SDS- PAGE)

<table>
<thead>
<tr>
<th>KDa</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>97</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>68</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Lane 1- Standard L-asparaginase
Lane 2- Standard Markers
Lane 3- Purified L-asparaginase.

Figure 39: Effect of physical conditions on L-asparaginase activity - stability of purified L-asparaginase at different pH levels

[Graph showing relative activity (%) vs. pH]
Figure 40: Effect of physical conditions on L-asparaginase activity - stability of purified L-asparaginase at different temperature levels

![Temperature Stability Graph](image)

Figure 41: Influence of different inhibitors and effectors on L-asparaginase activity of purified L-asparaginase from *B. subtilis* RM4

![Bar Chart](image)
Figure 42: Inhibition activities of different metal ions on L-asparaginase activity of purified L-asparaginase from *B. subtilis* RM4

*100% of activity correspondent to 0.5U of enzyme; ND - not detected

Figure 43: Peptide Mapping
Figure 44: MALDI-TOF Mass Spectrum of Purified L-asparaginase from *B. subtilis* RM4

- Peak obtained due to single subunit of L-asparaginase (36498.052 Da)
- Molecular mass of the purified L-asparaginase *B. subtilis* RM4 is written at top of the peak

Figure 45 (a): RP-HPLC analysis of L-asparaginase from *B. subtilis* RM4

Figure 45 (b): RP-HPLC analysis of L-asparaginase standard
5.4. DISCUSSION

Purification of L-asparaginase was carried out using appropriate chromatographical techniques. The specific activity before purification was 490 U/mg protein. Upon precipitation with ammonium sulphate the specific activity was found to be increased by 11-fold and the total protein content was found to be decreased by 19 fold. This indicates that the fractionation range of ammonium sulfate used for precipitation was effective enough in removing proteins which are contaminants, with subsequent loss of total activity (approximately 42%). This is in accordance with Narayana et al. (2008) who stated that proteins containing hydrophobic amino acids will precipitate at lower salt concentration than the proteins containing hydrophilic amino acids.

With IEC, only 1.5% of the total protein from crude extract was eluted (73-fold decrease), but the specific activity was found to be increased by 27-fold. The specific activity after IEC was 56.23U/mg proteins. Different protein peaks observed in the elution profile indicates an efficient removal of contaminants. In most of the cases, ion exchange chromatography was used for purification of L-asparaginase, since these enzymes have pI value of 8.7 for Erwinia spp. and pI value of 5.0 for E. coli (Muller et al., 1998). The retention of L-asparaginase on DEAE sepharose column is dependent on pH of the buffer used. The adsorbed L-asparaginase on DEAE sepharose column was eluted with gradient of 0-200 mM NaCl. Similar studies proved to be evident for L-asparaginase from Pseudomonas aeruginosa 50071 purified in CM-Sephadex C-50 column up to 106-fold with 43% yield (El-Bessoumy et al., 2004). L-asparaginase from Streptomyces albido flavus was purified 99.3-fold with 40% recovery in the final CM-Sephadex C-50 purification step (Narayana et al., 2008). Similar type of variation in the activity of purified L-asparaginase from Erwinia carotovora was observed in the presence of thiol protecting and thiol blocking reagents (Warangkar et al., 2010). The
absence of glutaminase activity would minimize the risk factor for successful clinical studies (Gallagher et al., 1989 and Manna et al., 1995).

GFC was performed as final step of purification using Sephacryl G-200 chromatography. Glutaminase-free L-asparaginase was purified to homogeneity with 19.11% yield the specific activity after GFC was found to be increased by 73-fold, which is so far, the new report on L-asparaginase purification from B. subtilis RM4. The enzyme was purified approximately 27 fold with specific activity of 7.0 U/mg and found to be homogeneous, as evident from SDS-PAGE. Similarly, Kotzia et al. (2007) found the higher specific activity in Erwinia chrysanthami producing L-asparaginase which showed 118.7 IU/mg of protein and recombinant E. coli W3110 the L-asparaginase showed 174.49 IU/mg of protein (Youssef et al., 2008).

Three bands were observed in SDS-PAGE after IEC. And after GFC, only one band was observed. From the bands observed, it can be claimed that the enzyme was purified to near homogeneity. Therefore, sequential purification of L-asparaginase from isolate B. subtilis RM4 resulted in 27.314-fold pure enzyme. The molecular weight of the purified denatured enzyme was found to be 35.26 kDa. Native PAGE separation indicated a band representing a molecular weight of 144 kDa (Figure 38 b). This data is in accordance with Maita et al. (1980) and Hymavathi et al. (2009) who reported that L-asparaginase molecular weight ranges from 133 to 141 kDa. It is also evident from this study that (Figure 38 c) no other band appeared, this may be due to the fact that all low molecular weight proteins produced during fermentation and proteins contributing from the medium are dialyzed out. This enzyme being of high molecular weight, retained during dialysis and had 100 kDa cut-off PVDF membrane. These findings are in accordance with the previous reports of who stated that bacterial L-asparaginase is a homo tetramer (Aghaipyour et al., 2001, Aung et al., 2000 and Prakasham et al., 2010). Abakumova et al. (2009) reported that the pI of L-asparaginase isolated from Yersinia pseudotuberculosis was 5.4. The findings of the current study substantiate with the
available reports, which showed that the functional form of bacterial L-asparaginase exists as a tetramer of identical subunits, with molecular mass in the range of 140 – 160 kDa (Aghaiypour et al., 2001, Kozak et al., 2000 and Prakasham et al., 2010). Thus, the L-asparaginase tetramer have considered as dimers of dimers. Despite this fact, the active enzyme is always a tetramer (Khushoo et al., 2004). The molecular weight of 94 kDa was reported for L-asparaginase of Penicillium brevicompactum NRC 829 (Elshafei et al., 2012) and Aspergillus terreus (Balasubramanian et al., 2012). Another study showed 66 kDa for L-asparaginase from Penicillium spp. (Patro et al., 2012). The L-asparaginase from Cladosporium spp. had a molecular weight of 121 kDa (Kumar et al., 2013). Goodsell et al. (2005) has reported the tetrameric nature of L-asparaginase and the present enzyme could be a homo tetramer as a single band was observed not only in SDS-PAGE but also in native PAGE. The L-asparaginase molecular weight variability may be attributable to the genetic differences. While, Devi et al. (2012) represented L-asparaginase dimeric with two distinct bands of molecular weights 40.2 and 39.8 kDa were purified from Erwinia carotovora MTCC 1428 by total activity 0.34 and total protein 0.91 mg. The molecular weight of the enzyme determined in the present study has contrasted with that of Wakayama et al. (2005), who reported a 36 kDa glutaminase from Stenotrophomonas maltophilia. Similar studies were also reported by Kushoo et al. (2004) who stated that L-asparaginase II functional form exist as tetramer in E. coli with the molecular mass range from 140-160 KDa. Nevertheless, Avarmis et al. (2005) stated that there is an immense demand for homogenous form of L-asparaginase from new bacterial source which lacks glutaminase and other toxins for treatment of leukemia.

The enzyme was considerably stable at pH range of between 7.0 and 9.0 and temperature ranged from 30°C and 35°C. The peak activity was at pH 8.0 and 30°C. The enzyme showed stability at alkaline pH range (pH 8.0) as it retained 90% of its original activity after incubation for 24 h (Figure 39). Majority of the L-asparaginase
from *Erwinia* species showed alkaline pH optima (8.0 - 9.0) except L-asparaginase from *E. coli*, *Streptomyces* sp. strain PDK2 and *Streptomyces* sp. strains S3, S4, and K8 which exhibited acidic pH optimum of 5.0 - 6.0 (Basha *et al.*, 2009 and Dhevagi *et al.*, 2006). However, majority of previous reports suggests pH optima of L-asparaginase lie in alkaline pH conditions (Borkotaky *et al.*, 2002 and Kumar *et al.*, 2011). Prema *et al.* (2013) also supported the present findings and had reported pH 8.0 for the optimum activity of L-asparaginase from *Pseudomonas fluorescens*.

The temperature profile showed the highest L-asparaginase activity at temperature 30°C. The purified enzyme exhibited maximum activity at a temperature of 40°C. The activity decreased sharply above the optimum temperature range (30°C to 45°C) with almost 75% loss of its original activity at 45°C (Figure 40). No significant enzyme activity was lost when the purified enzyme was pre-incubated at 40°C for 60 min beyond this temperature the enzyme became increasingly unstable. Siddalingeshwara *et al.* (2010) reported the optimum temperature to be 30° - 37 °C for L-asparaginase of various fungal species. In contrast to the present study, Durban *et al.* (2006) who achieved high-level of recombinant Phospholipase C in *B. subtilis* WB800 at 37°C. Mahajan *et al.* (2012) and Prakasham *et al.* (2010) also reported similar results that 37°C was an optimum temperature for the production of L-asparaginase from *Bacillus licheniformis* (32.26 IU/ml) and *Bacillus circulans* (85 IU/ml). Similar results were also reported for L-asparaginase from *Pseudomonas aeruginosa 50071* (El-Bessoumy *et al.*, 2004).

The inhibition studies provide an understanding of the active site amino acid residues responsible for L-asparaginase activity. The enzyme activity was severely affected by β-mercaptoethanol. DTT, a cysteine protease inhibitor, IAA and EDTA slightly inhibited the enzyme. In this current study, the results which was plotted in Figure 41 clearly suggested that the purified enzyme *B. subtilis* RM4 is not an
metalloenzyme. A similar inhibition was reported by Kumar et al. (2013) who observed that L-asparaginase produced by *Erwinia carotovora* and *Streptomyces radiopugnans* MS1 were non metallo enzymes. The current result is in accordance with earlier reports where the fungal L-asparaginase exhibited higher affinity for L-asparagine than other amino acids (Gupta et al., 2012). On the other hand, in contrast to the current study, Elshafei et al. (2012) reported L-asparaginase as metallo enzymes in *Pseudomonas stutzeri* MB-405.

In the present study, Mg\(^{2+}\) acted as an inhibitor on purified L-asparaginase of *B. subtilis* RM4. Whereas, Cu\(^{2+}\), Mn\(^{2+}\), Fe\(^{3+}\) and Zn\(^{2+}\) did not alter the enzyme (Figure 42). Similarly, Mg\(^{2+}\) acted as an inhibitor on L-asparaginase of *Cladosporium* spp. (Kumar et al., 2013). Cu\(^{2+}\), Mg\(^{2+}\) also inhibited L-asparaginase activity of *Aspergillus aculeatus*, however, Mn\(^{2+}\), Ca\(^{2+}\), Fe\(^{2+}\) and Cu\(^{2+}\) did not alter the enzyme (Dange et al., 2011). L-asparaginase of *Cylindrocarpon obtusisporum* was also inhibited by Fe\(^{2+}\), Ni\(^{2+}\), Cu\(^{2+}\) and Hg\(^{2+}\)(Dange et al., 2011). Thus, the cations have different effects on L-asparaginase activity.

The stability of enzyme at varied pH and temperature reduces the cost involved in storage and transportation. The results of chromatography indicated that no effect on L-glutamine activity. Overall, the enzyme has high L-asparaginase activity and no L-glutaminase activity. It may thus be used as an antileukemic and anti-lymphoma agent since it will not have the side effects related to L-glutamine specificity by L-asparaginase. Therefore, this chapter embodies that IEC and GFC column were found to be important and suitable step for separation of L-asparaginase and very effective step for purification process. The modified procedure developed for the purification of L-asparaginase simplified purification difficulties and yielded sufficient quantity of enzyme to study the antitumor activity of the enzyme.