4. Purification and characterization of pterin deaminase from *Aspergillus terreus* JQ436691

4.1 Introduction

With the advancement in the field of proteomics, new enzymes have been identified time and again. Thousands of enzymes are already known, it would be a critical mistake to assume that all enzymes have been recognized and explored for its properties. One such enzyme which has not been much investigated is pterin deaminase (E.C.No.3.5.4.11) which belongs to the class hydrolase.

Since 1982, detailed characterization and investigation of pterin deaminase for its antitumour property, other significant structural, biochemical and physiological properties were not observed and remained unexplored. For pterin deaminase to be ventured as cancer therapeutic agent, more information pertaining on the protein sequence, structure and other properties along with the detailed mechanism of action is essential. Therefore, there raise a need to resolve these questions to efficiently manipulate the enzyme.

Protein purification is a series of processes intended to isolate a single type of protein from a complex mixture. Protein purification is vital for the characterization of the function, structure and interactions of the protein of interest. The starting material is usually a biological tissue or a microbial culture. Protein purification is usually a multi-step process exploiting a wide range of biochemical and biophysical characteristics of the target protein, such as its source, relative concentration, solubility, charge and hydrophobicity. The ideal purification strives to obtain the maximum recovery of the desired protein, with minimum loss of activity, combined with the maximum removal of other contaminating proteins. Separation and isolation of proteins from a complex biological sample is very difficult task. More frequently, two, three, or more steps are needed for the eventual purification of the desired target protein. The main challenge in proteomics is to find the most efficient protocols for different sample types. Proteins are fragile molecules that denature readily at extremes of temperature and pH. Each protein offers its own unique set of physicochemical characteristics. The methods used for Purification and Characterization of Pterin Deaminase from *Aspergillus terreus* JQ436691
protein purification should be mild, to preserve the native conformation of the molecule and its bioactivity. In most cases, a reliable assay is essential to target a specific protein.

The purification step for pterin deaminase have reported by Takika et al. (1979) from bacterial extract of *Bacillus megaterium* precipitated using protamine treatment (0.1 volume of 2%), ammonium sulfate fractionation (30%) and dissolved in small volume of 0.01M potassium phosphate buffer, pH7. Then the ammonium sulphate precipitate was removed by gel filtration through a Sephadex G-25 column (4x16cm). Then eluate is purified on DEAE cellulose column (2 x 20 cm) equilibrated with 0.01M potassium phosphate buffer, pH 7.0 and then applied to a column chromatography on hydroxylapatite column (1.5 x 2.5 cm) equilibrated with potassium phosphate buffer, pH 7.0. The flow rate was 24 ml/h and 4 ml fractions were collected. The molecular weight of the enzyme was estimated by gel filtration through a sephadex G-150 column (1.5 x 87cm) equilibrated with 0.1M potassium phosphate buffer, pH 7.0. The molecular weight of the enzyme was found to be 110,000.

Further, purification of pterin deaminase was reported by Kusakabe et al., (1979) from fungal extract of *Aspergillus sp* Y8-5(ATCC20413) grown in wheat bran and precipitated using acetone dissolved in 2 liters of 0.02M Tris-HCl buffer (pH 7.0). The dialyzed solution (2.4 liters) was applied to a DEAE-cellulose column (10 x 100 cm) equilibrated with 0.02 M Tris-HCl buffer (pH7.0). After the column is washed with the buffer, the enzyme was eluted with a linear gradient of 0 to 0.6 M NaCl. The active fractions were combined, concentrated and passed through a sephadex G-75 column (6 x100 cm) equilibrated with 0.02M Tris- HCl buffer (pH7.0).

Protein identification and characterization are essential components of the discovery phase of any proteomics effort. In recent years mass spectrometry (MS) has emerged as a powerful tool to quickly and efficiently identify proteins in biological samples (Steen and Mann, 2004). MS became more compatible with the analysis of biopolymers, proteins, nucleic acids and carbohydrates with the introduction in the late 1980’s of two soft ionization methods; electrospray ionization (ESI; Fenn et al., 1989) and matrix-assisted laser desorption /ionization (MALDI; Karas and Hillenkamp, 1988). These methods are referred to as ‘soft’ because they do not (in the most part) degrade the
molecule during the ionization process. Thus the mass analysis of large (>10000 mass units (U) biologically derived polymers was realized.

Thus, the present study was aimed to purify extracellular pterin deaminase using gel filtration, ion exchange chromatography and identification by MALDI- MS. Further, the purified enzyme was characterized by substrate specificity against folic acid, effects of temperature, pH and inhibitors on enzyme activity and the interaction studies of zinc finger protein receptor with folic acid were studied by molecular docking.

4.2. Materials and Methods

4.2.1. Microorganism and crude enzyme preparation

*Aspergillus terreus* spore suspension (4%) was inoculated into wheat bran, moistened with distilled water and grown for 4 days at room temperature. The culture filtrate from the medium was obtained by adding 50 ml of distilled water filtered by Whatmann filter paper. The aqueous extract was centrifuged at 6000 rpm for 20 min at 4 ºC to remove the cellular debris and the supernatant was collected. This was treated as the crude preparation of the enzyme. It was estimated for enzyme activity (Kusakabe *et al*., 1976) and protein (Lowry *et al*., 1951), and was subjected for first purification step of acetone precipitation.

4.2.2. Acetone precipitation

The crude extract was precipitated with four volume of acetone. It was left at 4ºC over night followed by centrifugation at 8000 rpm for 20 min. The pellet was collected and dissolved in 0.05 M Tris -HCl buffer, pH 8.6. The precipitated pellet was checked for enzyme activity and protein (Lowry *et al*., 1951).

4.2.3. Purification of pterin deaminase from *Aspergillus terreus* JQ436691

4.2.3.1. Gel filtration chromatography

The protein was purified by sephadex G-25 column (50 x 2.3 cm) and further purified by sephadex G- 75 column (50 x 2.3cm) with Tris- HCl (50 mM, pH 8.6) as eluent. First the column was packed with sephadex G-25 and stabilized. Acetone precipitated samples were loaded into the column and 30 fractions were collected in vials of flow rate 2 ml /min. The collected fractions were checked for enzyme activity and
protein, and the fractions showing the higher enzyme activity were pooled together. This active fraction was loaded onto sephadex G-75 column and eluted with the same buffer. Again flow rate of 2ml per min each of 30 fractions were collected and checked for enzyme activity and protein. The active fractions were pooled and dialyzed with 50 mM Tris- HCl, pH 8.6 (Kusakabe et al., 1974).

4.2.3.2. Ion exchange chromatography

DEAE-cellulose of 5 g was dissolved in Tris-HCl buffer and was left over night and was used to make the column. The column was pre-equilibrated with 50 mM Tris- HCl (pH 8.6) at a flow rate of 1ml /min. Finally, 2 ml of pooled peak fractions collected were applied to the ion exchange column. The column was washed with two column volume of the above buffer and the adsorbed protein was eluted using a linear gradient of NaCl (0-0.6 mM) in 50 mM Tris-HCl (pH 8.6). Two ml/min (flow rate) of 30 fractions collected were analyzed for enzyme activity and protein; fractions having better enzyme activity were pooled together and stored in deep freezer for later use. The determination of soluble protein was carried out by the Folins phenol reagent method (Lowry et al., 1951) with BSA as standard. The enzyme activity was calculated by estimating the released ammonia (Mashburn and Wriston, 1964) using the statistically optimized assay conditions. The active fractions were further used for the studies described below and for the biological characterization of enzyme.

4.2.3.3. Molecular weight determination

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE 12%) of purified enzyme was carried out in Tris- glycine buffer (pH 8.6) at 100 V for 3 h at room temperature and proteins in the gels were stained with coomassie blue R-250 (Laemmeli, 1970). The apparent molecular weight of pterin deaminase was determined using standard molecular weight markers in SDS-PAGE. The marker proteins were β-galactosidase (116.0 KDa), BSA (66.2 KDa), ovalbumin (45.0 KDa), lactate dehydrogenase (35.0 KDa, 125.0 KDa), lactoglobulin (18.4 KDa) and lysozyme (14.4 KDa).
4.2.3.4. Thinlayer chromatography

The product analysis was carried out using thin layer chromatography by checking the fluorescence property of the product. 10 µl of the sample was placed on TLC slides and run in the solvent chloroform: methanol (1:10 ml) and checked for the fluorescence under UV light (Levenberg and Hayaishi, 1959).

4.2.4. Characterization of pterin deaminase from Aspergillus terreus JQ436691

The characterization of purified enzyme included: determination of optimum pH, temperature, kinetic data and inhibitor.

4.2.4.1. Determination of optimum pH of purified pterin deaminase enzyme

To determine the optimum pH of purified enzyme, Tris -HCl buffer (0.05M) was used with pH variations of 4, 5, 6, 7, 8 and 9. The temperature and other parameters were kept constant and enzyme assay was done (Kusakabe et al., 1979).

4.2.4.2. Determination of optimum temperature of purified enzyme

To find the optimum temperature, the variations of temperature used were 30°C, 35°C, 40°C, 45°C and 50°C. The specific activity of each sample was then determined by the method of Mashburn and Wriston, (1964).

4.2.4.3. Determination of enzyme kinetics data of purified enzyme

This is one of the important parameters for evaluating the potential usefulness of the enzyme for anti-leukaemic therapy. The initial velocity of the sample was estimated in a range of folic acid concentrations (5 to 30 µM).

The Michaelis-menten (K_m) constant and maximum reaction rate (V_max) of the purified enzyme was determined based on equation of Lineweaver-Burk plot (Lineweaver and Burk, 1934) and Eadie- Hofstee plot (Eadie, 1942., Hofstee, 1959). The double reciprocal plot was obtained from the Line weaver-Burk equation, which states that,

\[ \frac{1}{V_o} = \frac{K_m}{V_{max}} \times \frac{1}{s} + \frac{1}{V_{max}} \]
When $1/V_o$ was plotted against $1/(s)$, a straight line was obtained. This line had a slope of $K_m / V_{max}$, an intercept of $1/V_{max}$ on the $1/V_o$ axis and an intercept of $1/K_m$ on the $1/(s)$ axis. Double reciprocal plot had the advantage of allowing a much more accurate determination of $V_{max}$.

The Eadie-Hofstee plot which was plotted with $V_o$ against $V_o/(s)$ confirmed the $V_{max}$ and $K_m$ values in a very simple way and also manipulated departures from linearity which was not apparent in double reciprocal plot (Eadie, 1942 and Hofstee, 1959).

**4.2.4.4. Enzyme inhibitors**

The effect of inhibitors on relative pterin deaminase activity were determined by incubating enzyme for 30 min with each of the various inhibitors *viz.*, sodium cyanide NaCN, sodium fluoride , NaF, EDTA, mercaptoethanol, SDS and lumazine at concentrations 0.1mM and 10 mM before addition of substrate. The residual enzyme activity was determined using pterin deaminase assay (Takikawa, *et al.*, 1979). All the experiments were carried out in three sets and the average obtained values were taken for analysis.

**4.2.5. Protein identification approaches - MALDI TOF- MS**

Mass spectrometry has been a significant driving technology in the formation of the field of proteomics. Computer algorithms have been developed which use mass spectrometric data (either peptide or fragment ion masses) in correlative based approaches to identify proteins in sequence databases. Protein bands were excised, from SDS PAGE gel destained with 50 % acetonitrile in 0.1M ammonium bicarbonate and dried in a speed vacum evaporator. The dried gel pieces were reswollen with 3 µl of 3mM Tris- HCl, pH 8.8 and containing 50 ng trypsin. After 15 min, 3 µl water was added and left at room temperature for 12 h. Two microliters of 30% acetonitrile, containing 0.1% trifluoroacetic acid was added and the content was vortexed for 3 min and sonicated for 5 min at 4ºC. About 1µl was applied onto the dried matrix spot. The matrix consisted of 15 mg nitrocellulose and 20 mg cyano-4 hydroxycinnamic acid in 1.0 ml of acetone: isopropanol (1:1 v/v mixture). About 0.5 µl portion of matrix solution was applied on the sample target. Specimen was analyzed in a time of flight (TOF) Bruker Daltonics mass
spectrometer equipped with a reflectron. An accelerating voltage of 20 KV was used. Calibration was again total spots and background (Wilm, et al., 1996; Perkins, et al., 1999). Spectra was interpreted with mascot software (Matrix science Ltd London, UK). The probability score calculated by the software was used as criterion for correct identification (http://www.matrix science.com /help /scoring-help.html).

4.2.6. Sequence Retrieval

The amino acid sequence of zinc finger protein (Aspergillus terreus) was retrieved from Swiss-Prot Database (http://www.uniprot.org/uniprot/Q0CQV1).

>tr|Q0CQV1| Q0CQV1_ASPTN Zinc finger protein 160 OS=Aspergillus terreus (strain NIH 2624 / FGSC A1156) GN=ATEG_03933 PE=4 SV=1

MAPGSGRDFNCSWEDCGKSFNRKSDLCRHYRIHTNERPYHCNFKDCNKSFIQRSALTVHS
RTHTGEKPHCDHEGCHKAFAQDSLARHRRIHTGRRPYICQEPICRKTTLTKHQRNSHF
PPGAMHRTSEDLPDRSYQPVTAQHDQYLLAQQPYYPPPTSTPTTTEFFPPQPIQITQ
VPAVQEAAPPPIVTHSPVAPVDVQAQQQYMQLMQQYRYESARGYVSPEFQPA
FQAGVPPIEGHALMVTPQNFAYKQTRLLNQPEGTDWGFGLVG

On UNIPROT database, the only protein sequence is available for zinc finger protein from Aspergillus terreus strain NIH 2624 (Birren et al., 2005). That was the reason the zinc finger protein sequence from Aspergillus terreus strain NIH 2624 was selected as a template for interaction studies using bioinformatics tool in order to model the structure of this putative protein.

4.2.6.1. Homology modeling

The homology modeling for zinc finger protein of Aspergillus terreus was carried out in following steps; such as Template selection from the Protein data Bank (ID:2I13), Sequence-template alignment, Model building and validation. The sequence alignment of the Protein - BLAST program against the Protein data bank was used to identify the sequence similarity. The target sequence of template 3D structure of protein was extracted from the PDB database. To identify the high resolution crystal structure was used for homology modeling. Molecular modeling was performed by Modeller 9.11
software (Eswar et al., 2006). The modeled structure of protein was visualized by PyMol Visualization Software (DeLano, 2002). The energy minimization of modeled structure was carried out by Swiss-pdb Viewer program in Gromos 96 force field (Schwee et al., 2003). Structure validation was done with Ramachandran plot analysis (Laskowski et al., 2005) to determine the stability of the modeled structure at SAVS (Structural analysis verification server) server.

4.2.6.2. Selection of ligand

The selection of folic acid (ID: CID 6037) compound was retrieved from PubChem database. The folic acid compound was downloaded in sdf format and this file were converted into pdb file using OpenBapel 2.3.2 software.

4.2.6.3. Autodock4.0

The study of docking was performed by the program Auto dock 4.0. Pique EM 2010 The modeled Zinc finger protein and folic acid compound were prepared using MGL Tool 1.5.4. The grid box of dimension 56 x 58 x 56 was created surrounding the active sites with grid points having coordinates value X=2.194, Y= -10.028 and Z=1.806. The input parameters were analyzed using a genetic algorithm and set of 100 runs for docking procedure. The complex protein having lowest Binding energy and ligand efficiency, with more number of hydrogen bonds was chosen for better result.

4.3. Results and Discussion

Enzyme production has gained a recent take off the world over and there is still a lot of potential boost in this area of recent industrial production. It is likely that a number of traditional mechanical industrial processes will be shifted to bioprocesses. Furthermore, with the advancement of knowledge, enzyme technology will explore new areas of employment in human life but the production potential of enzymes is not uniform globally and it favors more to the developed world. Although genomic studies can reveal valuable information about molecular mechanisms in organisms, the proteome analysis is often essential for better understanding of the biological processes.
4.3.1. Purification of acetone extract of *Aspergillus terreus* JQ436691

The purification of the protein is an essential first step in the study of its physical and biological properties. As enzymes are unstable molecules with physiochemical organizations, even slight change in this organization reduces the activity of the enzymes. Fungi can produce both intracellular as well as extracellular enzymes. Separation of one protein from all others is typically the most laborious aspect of protein purification. Earlier reports available are only for the fungus *Aspergillus* Y85 regarding the partial purification of unexplored enzyme. In the present investigation for the first time an attempt has been made to purify the enzyme pterin deaminase from *Aspergillus terreus* JQ436691 and the crude extract contained 826 units and 125 mg of protein concentration which was lower compared to earlier report by Kusakabe *et al.* (1979) who obtained crude extract of *Aspergillus* Y8-5 1140 units of enzyme and 0.004 mg/ml of protein in 190 litres of enzyme.

Pterin deaminase from *Aspergillus terreus* was purified by fractionation with acetone precipitation followed by dialysis to remove undesirable molecules. The total protein decreased from 125 to 105.5 mg and the specific activity increased from 6.608 to 7.582 IU/mg respectively in the acetone precipitation step (Table 4-1). Levenberg and Hayaishi (1959) used the solvent acetone for partial purification from *Aspergillus* Y8-5.

According to Levenberg and Hayaishi (1959), the specific activity of pterin deaminase in *Alcaligenes metalcaligenes* after protamine sulphate precipitation was increased from 1.0 to 1.3 U/mg. Successive treatments with heat and activated charcoal resulted in the specific activity of 3.0 and 5.1 U/mg, respectively. The crude extract supernatant of L-asparaginase from *Aspergillus terreus* precipitated by ammonium sulphate having a specific activity of 240 (U/mg protein / ml) and yield of 57 % as reported by Azad Chandrasekhar (2012).

4.3.2. Desalting column chromatography

The precipitated sample was loaded on to equilibrated SephadexG-25 column. The elution pattern of sephadex G-25 showed that the fractions 17 and 18 had maximum enzyme activity and protein concentration (Fig.4-1). The specific activity increased from...
6.608 to 7.582 IU/mg (Table 4-1). The specific activity of protease increased by about 2.3 times by Sephadex G-25 column separation compared to crude enzyme preparation in the earlier report (Su et al., 1967).

**4.3.3. Gel filtration column chromatography**

Further peak fractions were collected from G-25 and loaded onto equilibrated sephadex G-75 column chromatography. The elution profile showed activity peaks of enzyme and protein concentration in fractions 18 and 19 (Fig.4-2). The specific activity increased from 9.435 IU/mg to 11.17 IU/mg (Table 4-1). Rembold and Simmersbach (1969) have purified a pterin deaminase from rat liver by ammonium sulphate precipitate and sephadex G-100 column chromatography and the purification fold was increased up to 23%.

**4.3.4. Ion exchange chromatography**

The pooled active fractions from sephadex G-75 was loaded onto DEAE cellulose column equilibrated with 50mM Tris-HCl buffer, pH 8.6. The elution profile showed active peaks in fractions 14 and15 with higher enzyme activity and protein concentration (Fig4-3). In the final purification step, about 39.97 fold purity with specific activity of 14.35 IU/mg of pterin deaminase was obtained (Table 4-1). Sahu et al. (2007) have reported purity of L-asparaginase of 18.01 fold and specific activity 13.57 of IU/mg using sephadex G 200 column. In *Bacillus megaterium*, purification of pterin deaminase was performed by treatment with protamine and ammonium sulphate and subjected to DEAE cellulose and hydroxyl apatite columns. The specific activity was increased by 90% (Takikawa et al., 1979).

**4.3.5. Homogenity test of purified enzyme by electrophoresis SDS-gel polyacrylamide electrophoresis**

The molecular weight of pterin deaminase was determined by 12% SDS PAGE. The Figure 4- 4 showed that only a single band was seen in SDS PAGE and the mobility of the band corresponded to apparent molecular weight of 35 kDa of protein molecular weight. Pterin deaminase in *Bacillus megaterium* was reported to have a molecular weight of 110 kDa (Takikawa et al.,1979). In *Tetrahymena pyriformis*, the molecular
weight was 33 kDa which have been studied by Prista and Kyriakidis (2001). A single major band corresponding to a molecular weight of approximately 35 kDa was found from *Helicobacter pylori* type II L-asparaginase by SDS-polyacrylamide gel electrophoresis (Dhavala *et al.*, 2008).

4.3.6. Thin layer chromatography

The product analysis was done with purified enzyme using thin layer chromatography. Lane 1 shows the fluorescence of standard lumazine. With the Rf value of 0.55 Lane 2, the enzyme incubated with substrate was found to be same as that of lumazine with the Rf value of 0.65. This indicated that the enzyme is capable of deaminating folic acid to yield product lumazine (Levenberg and Hayaishi, 1959).

4.3.7. Characterization of pterin deaminase from *Aspergillus terreus* JQ436691

4.3.7.1. Effect of pH on pterin deaminase activity

The pH of the medium plays a vital role in most of microbial process. Purified sample was assayed at various pH and the kinetic properties of the purified enzyme was shown by the shape of the curves representing activity as function of pH. The activity of purified enzyme from *Aspergillus terreus* can be seen in Fig. 4.5 A. The figure 4.5A showed that the optimum pH of purified pterin deaminase was 7. These results revealed that the purified enzyme was more stable towards pH in neutral compared to that basic range in *Aspergillus terreus*. Levenberg and Hayaishi (1959) have reported optimum pH for the enzyme from *Alcaligenes metalcaligenes* was between 6.3 and 6.7. Kusakabe *et al.* (1979) stated that pH 7 was optimum for purified pterin deaminase enzyme isolated from *Aspergillus* sp Y8-5.

4.3.7.2 Effect of temperature on pterin deaminase activity

The activity (%) of purified enzyme from *Aspergillus terreus* at various temperatures is presented in Figure 4-5 B. The optimum temperature of purified enzyme based on Figure 4-5B was 35°C. The L-asparaginase activity from *Bacillus megaterium* was found to be optimum at a temperature of 37°C (Mohapatra *et al.*, 1995).
4.3.8. Determination of kinetic data of purified enzyme from *Aspergillus terreus* JQ436691

In the present study based on the Lineweaver-Burk equation, the $V_{\text{max}}$ value obtained for pterin deaminase was 4.16 IU/ml, whereas $K_{\text{m}}$ value of pterin deaminase was 40 $\mu$M folic acid as a substrate (Fig. 4-6 A, B). The small $K_{\text{m}}$ value showed that the purified enzyme has better affinity towards folic acid substrate. Yandri *et al.* (2010) reported that the purified extracellular $\alpha$ amilase enzyme from *Bacillus subtilis* ITBCCB148 has small $K_{\text{m}}$ values for soluble starch substrate ($K_{\text{m}}$ value 2.5 mg ml$^{-1}$ and $V_{\text{max}}$ value 192.3 $\mu$mol ml$^{-1}$ min$^{-1}$) and has better affinity starch as substrate. Pattanaik *et al.* (2000) have reported the apparent $K_{\text{m}}$ and $V_{\text{max}}$ as 4.9 X 10-6 mol/l and 9.803 IU/ml respectively. In *Bacillus megaterium* and *Pseudomonas sp*, the $K_{\text{m}}$ was found to be 1.3 mM against 6- carboxypterin and 0.05 mM against pterin 6 carboxylic acid respectively for pterin deaminase (Rappold and Bacher, 1974; Takikawa *et al.*, 1979; Tsusue *et al.*, 1978).

The enzyme was almost fully inhibited (0% residual activity) by various inhibitors EDTA, Lumazine, Mercaptoethanol, SDS and at 10 mM concentrations compared to other inhibitors used for the study (Fig. 4-7). Mohapatra *et al.* (1995) reported that enzyme activity was strongly inhibited by EDTA. The decrease detected in the presence of EDTA could be due to its influence on the interfacial area between the substrate and enzyme (Silva Lopes *et al.*, 2002; Jinwal *et al.*, 2003). Deaminase activity could be effectively inactivated by lumazine. This determines that lumazine inhibits enzyme in the feed back inhibition pattern. This correlates to the report of Wurster and Butz (1980) who postulated that the end product of deamination of folic acid inhibits the activity of deaminase catalysing the reaction. In the present study sodium fluoride and sodium cyanide enhanced the enzyme activity whose mechanism of action has yet to be perceived.

4.3.9. MALDI-TOF MS Analysis

Proteomics is defined as the large-scale study of proteins in particular for their structures and functions (Anderson and Anderson, 1998), and investigations of proteins
have become very important since they are the main components of the physiological metabolic pathways in eukaryotic cells. Proteomics increasingly plays an important role in areas like protein interaction studies, biomarker discovery, cancer prevention, drug treatment and disease screening medical diagnostics (Capelo et al., 2009). In traditional protein chemistry, proteins were identified by de novo sequencing using automated edman degradation. Today, this technique tends to be replaced by mass spectrometry, which is becoming one of the most powerful techniques in protein chemistry. The reason for this is a 100 fold increase in sensitivity and 10 fold increase in speed for protein identification.

Peptide masses were acquired with a range of 700 to 1400 m/z (Fig.4-8). Each spectrum was produced by accumulating data from 500 consecutive laser shots. Singly charged monoisotopic peptide masses were searched against Swiss-Prot and NCBI databases by utilizing the MASCOT Server 2.2 search engine (www.matrixscience.com; Matrix Science Ltd., London, United Kingdom). Up to one missed tryptic cleavage was considered, and the mass tolerance for monoisotopic peptide masses was set to 100 ppm. Spectra were interpreted with the Mascot software.

The protein was identified as zinc finger protein and showed only 30% match (Fig.4-8). It is one of the hyphothetical protein characterized by low identity to known annotated proteins (Galperin, 2001; Galperin and Koonin, 2004). The zinc act as a critical electrophile in many hydrolases. The role of zinc finger domain in cytidine deaminase enzyme has deamination and nucleic acid binding property (Hache et al., 2005). Similar putative zinc binding domain was identified in the Arabidopsis thaliana cytidine deaminase based upon primary sequence similarities to Escherchia coli (Kafer and Thornburg 2000). The earlier reports supports the present investigation and thus the identified protein from MALDI MS have similar function to the enzyme pterin deaminase and belongs to the class hydrolase.

Structure-based methods have been used to predict enzyme activities from experimentally determined structures; however, for the vast majority of proteins, no structures are available. Homology model-based virtual screening, especially with
modelling of protein backbone flexibility, may be broadly useful for enzyme function annotation and discovering new pathways and drug targets (Marti Renom et al., 2000).

4.3.10. Sequence retrieval

Zinc finger protein (Aspergillus terreus) sequence was retrieved from Swiss-Prot (accession number Q0CQV1). The sequence length is 284 AA and the molecular weight is 32,286 Da (Birren et al., 2005).

4.3.11. Homology modeling

The template crystal structure of zinc finger protein (PDB ID: 2I13/A, a six finger Zinc Finger Designed to recognize Ann Triplets-Mus musculus) was identified with the sequence similarity of 51% in the best hit. The lowest e-value of protein was 1e-22 and higher resolution 1.96 Å. The alignment of the target sequence, template protein structure was selected for homology modeling by Modeller 9.11. It has generated five models and DOPE score negative value of high was selected. The model no.4 (PDB.B99990004) was selected as the best model with DOPE score value of -15553.56 (Table 4-2) for further analysis. The Ramachandran plot is widely used in assessing the quality of experimental structures built using homology modeling (Ramachandran et al., 1963). The horizontal axis on the plot shows phi value, while the vertical shows psi values. Each dot on the ramachandran plot provides the phi and psi values for an aminoacid in a protein. Notice that counting in the left hand starts from -180 and extends to +180 for both vertical and horizontal axes. This is a convenient presentation and allows clear distinction of the characteristic regions of α helices and β sheets. The PROCHECK analysis of the modeled protein was performed and Ramachandran plot showed 79.1% of the residues were found in most favored regions (Fig. 4.9). Among which 36 residues were found in additional allowed region, 5 residues in generously allowed regions and 8 residues in disallowed regions. The above results show that the protein model is reliable (Fig. 4.10). The selection of folic acid (ID: CID 6037) compound was retrieved from PubChem database (Fig.4.11).

The interaction studies of zinc finger protein receptor with the folic acid compound was analyzed for the lowest binding energy, more number of hydrogen bonds formed and lowest ligand efficiency for the best interaction (Table 4-3). The bond Purification and Characterization of Pterin Deaminase from Aspergillus terreus IQ436691
distance between receptor and ligand below 3Å is the best. In our results, the folic acid compound docked with zinc finger protein receptor showed proper interaction. The receptor interactions with ligand are represented by Cyan, Green, Pink and Orange colors respectively (Fig.4-12A). The pink color represents ligand and green color protein residues, with hydrogen bond represented by orange color (Fig. 4-12B). The atomic interaction between HH12, HH22 atoms of the Arg 88, HZ1 atom of the Lys 78, Hydrogen atom of Glu 131(green color) and two oxygens, two OE2 atoms of folic acid (pink color) compound (Fig.4.12 A, B).

Thus the identified zinc finger protein (ID No.Q0CQV1) from *Aspergillus terreus* JQ436691 was proved to interact with folic acid. Earlier reports supported the identified zinc finger domain belongs to the class hydrolase and possess deamination action (Hache *et al.*, 2005). Based on these, it may be suggested that the selected zinc finger protein may have pterin deaminase activity. Depending on the presence or absence of the different pterin deaminases, the catabolism of pteridines results in either simple forms of pterin or lumazine. Exceptions are the pterin deaminases from rat liver (Rembold and Simmersbach, 1969) and *Drosophila melanogaster* (Gyure, 1970) which can catalyse the deamination of both aromatic and reduced pterins. Pterin deaminase is also denoted by researchers as folate deaminase as the enzyme replaces the amino group of folate at the 2-position of the pterin ring with a hydroxyl group (Bernstein and Vandriel, 1980). In the present investigation of folic acid interaction with zinc finger protein by molecular docking also revealed that the protein may now be assigned as pterin deaminase. This approach may be useful in the discovery of *in vitro* enzymatic and *in vivo* metabolical functions of unknown enzymes discovered in genome projects, especially for those targets with marginal sequence identities to template structures of known function.

### 4.4. Conclusion

Protein purification is vital in the characterization of protein of interest. It allows one to study the function of the protein, and its enzymatic activity. Purity will vary greatly depending on whether the novel protein is to be used for kinetic, sequence or crystallographic studies, or is to be injected into humans. Studies on the antitumour enzyme pterin deaminase relating to purification and characterization would open new
avenues in the application of the enzyme in the health care industry. In the present study fungi *Aspergillus terreus* JQ436691 extracellular pterin deaminase was purified from the culture filtrate using Gel filtration and ion exchange chromatography. The protein profile of DEAE eluent was studied using 12% SDS page gel electrophoresis. The molecular weight of the protein was apparently found to be approximately 35 k Da. The activity staining procedure on 12% native page showed a single fluorescence band similar to SDS-PAGE which confirmed the presence of enzyme. The mass spectra were interpreted with the mascot software. The protein was identified as zinc finger protein by MALDI TOF MS analysis belongs to the class hydrolase and possesses the property of deamination action to that of the enzyme pterin deaminase which is the first report for the organism *Aspergillus terreus* JQ436691. The interaction studies of zinc finger protein receptor with the folic acid compound showed the bond distance between receptor and ligand below 3Å is the best. In the present investigation of folic acid interaction with zinc finger protein by molecular docking also reveals that the protein may now be assigned as pterin deaminase.

The optimum conditions for enzyme was determined to be pH 7, temperature 35°C, Km 40 μM with folic acid as substrate and $V_{\text{max}}$ 4.16 (IU / ml). The enzyme was completely inhibited by EDTA, lumazine, SDS and mercaptoethanol which may be attributed to the fact that the enzyme may be a metallo enzyme. Further in depth confirmation studies on this unexplored enzyme with modern spectroscopic techniques, sequencing and X-ray crystallography would throw more light on the structure-functional relationships of pterin deaminase and its function.
Table 4-1. Purification of pterin deaminase enzyme from *Aspergillus terreus* JQ436691

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total enzyme activity (IU/ml)</th>
<th>Total protein content (mg/ml)</th>
<th>Specific activity (IU/mg)</th>
<th>Purification fold</th>
<th>Recovery yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>826.0</td>
<td>125.0</td>
<td>6.608</td>
<td>1.000</td>
<td>100.00</td>
</tr>
<tr>
<td>Acetone precipitate</td>
<td>800.0</td>
<td>105.5</td>
<td>7.582</td>
<td>1.136</td>
<td>96.80</td>
</tr>
<tr>
<td>Sephadex G-25</td>
<td>690.7</td>
<td>73.2</td>
<td>9.435</td>
<td>1.427</td>
<td>86.33</td>
</tr>
<tr>
<td>Sephadex G-75</td>
<td>592.1</td>
<td>53.0</td>
<td>11.170</td>
<td>1.690</td>
<td>71.68</td>
</tr>
<tr>
<td>DEAE Cellulose</td>
<td>330.2</td>
<td>23.0</td>
<td>14.350</td>
<td>2.171</td>
<td>39.97</td>
</tr>
</tbody>
</table>

1 unit: µM of ammonia released per ml per minute

Table 4-2. DOPE score of Modeled protein

<table>
<thead>
<tr>
<th>S.No</th>
<th>Modeled proteins</th>
<th>Dope score KJmol⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>seq.B99990001.pdb</td>
<td>-14443.45</td>
</tr>
<tr>
<td>2</td>
<td>seq.B99990002.pdb</td>
<td>-15231.18</td>
</tr>
<tr>
<td>3</td>
<td>seq.B99990003.pdb</td>
<td>-15017.15</td>
</tr>
<tr>
<td>4</td>
<td>seq.B99990004.pdb</td>
<td>-15553.56</td>
</tr>
<tr>
<td>5</td>
<td>seq.B99990005.pdb</td>
<td>-15214.47</td>
</tr>
</tbody>
</table>

Table 4-3. Interaction of folic acid compound with zinc finger protein

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Binding energy (Kcal/mol)</th>
<th>Ligand_efficiency (Kcal/mol)</th>
<th>Hydrogen bond formed</th>
<th>Hydrogen bond residues</th>
<th>Distance between residues (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folic Acid</td>
<td>-4.65</td>
<td>-0.15</td>
<td>4</td>
<td>ARG 88</td>
<td>ARG 88 (1.791)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ARG 88</td>
<td>ARG 88 (2.079)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LYS 78</td>
<td>LYS 78 (2.042)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GLU 131</td>
<td>GLU 131 (2.178)</td>
</tr>
</tbody>
</table>
Fig. 4-1. Desalting column chromatography of pterin deaminase from *Aspergillus terreus* JQ436691 (Sephadex G-25)

Fig. 4-2. Gel filtration column chromatography of pterin deaminase from *Aspergillus terreus* JQ436691 Sephadex G-75
Fig. 4-3. Ion exchange - DEAE cellulose Column chromatography of pterin deaminase from *Aspergillus terreus* JQ436691

Elution profile of pterin deaminase from *Aspergillus terreus* JQ436691 from Fig.4-1 Sephadex G-25; Fig.4-2 Sephadex G-75 and Fig.4-3 DEAE cellulose. Details of chromatogram is given in materials and methods. Active fractions from DEAE cellulose was taken for further analysis.

Fig. 4.4 SDS PAGE profile of pterin deaminase

Gel profile of pterin deaminase: Fig. 4-4. SDS of pterin deaminase. SDS PAGE was performed in 12% gels under denaturing conditions and stained with coomasie blue. Lane 1. Fractions from Sephadex G-25; Lane 2 Fractions from Sephadex G-75 column; Lane 4, 5, 6 Fractions from DEAE cellulose column chromatography; Lane 7 Molecular weight markers. Details description of the analysis is given in materials and methods.
Fig. 4-5A Optimum pH of purified pterin deaminase enzyme

Fig. 4-5B. Optimum temperature of Pterin deaminase enzyme

Effect of various (A) pH and (B) temperature on the activity of partially purified enzyme was studied by incubation the enzyme in various buffer with different pH(4-9) and temperature (30°C-50°C), respectively. Details descriptions of the analysis are given in materials and methods. The enzyme showed maximum enzymatic activity at pH 6 and 35°C.
Fig. 4-6 A - Kinetics analysis of pterin deaminase for the substrate folic acid – Line weaver pot

![Line weaver pot](image)

Fig 4-6 B. Kinetics analysis of pterin deaminase for the substrate folic acid – Eadie Hofstee plot

![Eadie Hofstee plot](image)

Fig. 4-6 A. Line weaver pot showing the $V_{\text{max}}$ and $K_m$ value for the substrate folic acid of pterin deaminase.

Fig. 4-6 B. Eadie Hofstee plot showing $V_{\text{max}}$ of the pterin deaminase using folic acid as substrate.
Effect of various inhibitors on the activity of partially purified enzyme was studied. Detail descriptions of the analysis are given in materials and methods. The enzyme was almost fully inhibited (0% residual activity) by various inhibitors EDTA, lumazine, mercaptoethanol and SDS at 10 mM concentrations and the inhibitor sodium flouride and sodium cyanide enhance the activity at 0.1 mM and 10 mM.
**Fig. 4-8. MALDI-TOF MS Analysis**

Database: SwissProt 2012_06 (536489 sequences; 190389898 residues)

Taxonomy: Fungi (30734 sequences)

Timestamp: 6 Jul 2012 at 10:01:48 GMT

Top Score: 30 for RSV1_SCHPO, Zinc finger protein rsv1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=rsv1 PE=2 SV=1

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.

Protein scores greater than 57 are significant (p<0.05).

1. RSV1_SCHPO  Mass: 46788  Score: 30  Expect: 28  Matches: 1

2. Zinc finger protein rsv1 OS=Schizosaccharomyces pombe (strain 972 / ATCC 24843) GN=rsv1 PE=2 SV=1

Please purchase PDF Split-Merge on www.verypdf.com to remove this watermark.
Search Parameters

Type of search: Peptide Mass Fingerprint
Enzyme: Trypsin
Mass values: Monoisotopic
Protein Mass: Unrestricted
Peptide Mass Tolerance: ± 1.2 Da
Peptide Charge State: 1+
Max Missed Cleavages: 1
Number of queries: 10

Fig. 4.9. Screenshot of the Ramachandran plot for modeled zinc finger protein
(Aspergillus terreus)
Fig 4.10. 3D structure of modeled zinc finger protein (*Aspergillus terreus* JQ436691)

Fig 4.11. 3D structure of folic acid
Fig. 4.12 A, B. Modeled zinc finger protein docked with folic acid

A. The receptor interactions with ligand is represented by Cyan, Green, Pink and Orange colors respectively.

B. Pink color represents ligand and green color protein residues, with hydrogen bond represented by orange color. The atomic interaction between HH12, HH22 atoms of the Arg 88, HZ1 atom of the Lys 78, Hydrogen atom of Glu 131 (green color) and two oxygens, two OE2 atoms of folic acid (pink color) compound.
4.5. Reference


