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Studies on the Recombinant Production in *E. coli* and Characterization of Pharmaceutically Important Thermostable L-Asparaginase from *Geobacillus thermodenitrificans*

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ABSTRACT

L-asparaginase is ubiquitously found in microbes, animals and plants. The asparaginase of bacterial origin has been extensively applied in the treatment of childhood lymphomas and leukaemia. However, the glutaminase activity of enzyme can cause serious side-effects triggering a search for highly specific and more stable enzyme. In the present study the gene consisting of 972bp coding for 323 amino acids was PCR amplified from *Geobacillus thermodenitrificans* DSM-465 was PCR amplified and recombinant plasmid pET22b-Asn was prepared. L-asparaginase was produced in BL21 (DE3) RIL codon plus strain of *E. coli* as heterologous protein under 0.3mM IPTG. The recombinant enzyme was purified by DEAE-Sephadex colum based anion-exchange chromatography. On SDS-PAGE, the enzyme exhibited a molecular weight of about 36 KDa. Its specific activity was 1650 U per mg of protein with about 5% glutaminase activity and no activity against D-asparagine. Optimal enzyme activity was found at 75°C and pH 9. The K_M value of 5.9mM was found for L-asparagine. *In silico* studies have shown that the enzyme exists as a homotetramer. Molecular docking studies have revealed that Gly199, Lys300, Arg198, Arg197, Tyr201, Tyr323 are putative active site residues interacting the substrate. Anti-proliferative effect of purified enzyme was determined quantitatively using non-cancerous human fibroblast cells (FB) and hepatocellular carcinoma cells (HepG2). A dose of 5U/ml of enzyme has shown an anti-proliferative effect up to 65 percent on HepG2 cells.

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Key words
L-asparaginase, Geobacillus, Cloning, 3D model, Anti-proliferative.

INTRODUCTION

L-asparaginase is an enzyme responsible to catalyze the breakdown of L-asparagine into ammonia and L-aspartate. The enzyme has therapeutic applications against all kind of lymphomas including non-Hodgkin's lymphoma and leukemia (Narta *et al*., 2007; Younes *et al*., 2008; El-Naggar *et al*., 2016). The anticancer use of enzyme is based on the nature of reaction it catalyzes. Being the essential amino acid for cancer cells, L-asparagine cannot be produced by lymphoblasts and their growth stops (Stams *et al*., 2003; Kotzia and Labrou, 2007). However, there is no effect on normal cells as they can produce L-asparagine. The enzyme also has importance in the maintenance of food quality and has been used to minimize the production of acryl amide like molecules in the fried or baked foods like the potato chips (Tareke *et al*., 2002; Rosen and Hellen, 2002). The acrylamide production is induced by interaction of reducing sugars with available free asparagine. The acrylamide As the free asparagine is reduced by the activity of asparaginase, the acrylamide formation is prevented (Onishi *et al*., 2015). L-asparaginase is ubiquitous enzyme found in animals, microorganisms, fungi and plants (Bhargavi and Jayamadhuri, 2016; Doriya and Kumar, 2016). In the recent past, the recombinant DNA technology has been used to produce the clinically and pharmaceutically important enzymes and proteins in different gene expression systems. Most preferably the industrial production of these proteins is carried out as recombinants in genetically modified bacteria such as *Escherichia coli* and *Erwinia chrysanthemi*. Bulk production of L-asparaginase is necessary to meet the ever increasing demand of enzyme as therapeutic and in food industries. The genes encoding asparaginases from different organisms including *Bacillus subtilis* (Jia *et al*., 2013), *Zymomonas mobilis* (Einsfeldt *et al*., 2016), *Thermococcus kodakarensis* (Hong *et al*., 2014), *Pseudomonas fluorescens* (Kishore *et al*., 2015), viruses (Zeshan *et al*., 2018) and *Homo sapiens* (Mohammadnejad *et al*., 2013) have been expressed in *E. coli* and the products have been characterized. Since the toxicity of therapeutic asparaginase mostly stems

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out from its glutaminase activity (Chan *et al*., 2013), there is continuous search for highly substrate specific enzyme from new species. The present study was aimed at the recombinant production and characterization of L-asparaginase from an unexplored strain of Geobacillus. The purified enzyme was characterized and evaluated for its anticancer activity using normal human fibroblast (FB) and hepatocellular carcinoma (HepG2) cell lines.

MATERIALS AND METHODS

Materials

Expression plasmid vector pET-22b (+) was obtained from Novagen (Darmstadt, Germany). PCR primers were synthesized by Macrogen 9South Korea). E. coli strain, BL21(DE3) codon plus strain, LB-medium, tris-base, ampicillin, IPTG, acetic acid glacial, sodium chloride, ethanol absolute, trichloroacetic acid, Nessler's reagent, L-asparagine, L-Glutamine were provided by Millipore Sigma (St. Louis, MO, USA). DEAE-Superdex from GE Healthcare Bio-Sciences (Pittsburgh, PA, USA). The acrylamide gels were purchased from Bio-Rad (Hercules, CA, USA). All the chemicals and reagents used were of molecular biology grade.

PCR amplification and cloning of asparaginase gene

A complete ORF of gene coding for asparaginase was PCR amplified under optimized conditions. Cloning of the gene was carried out using DNA manipulation procedures as described in the literature (Sambrook and Russell, 2001). Primers were selected from the genomic DNA sequence of subject strain available in the gene bank. The restriction sites for *Nde*I and *BamH*I were added at the termini of primer sequences to obtain the sticky ends of amplified gene. The PCR product was purified and ligated into pTZ57R/T plasmid vector which was used for the transformation of *E. coli* cells. The cells transformed with pTZ57R/T plasmid were selected initially on the basis of blue and white colony colours. The confirmation of recombinant plasmid was made by the restriction analysis of isolated plasmids.

Production of recombinant enzyme

Plasmid pTZ57R/T isolated from recombinant cells were restricted by *Nde*I and *BamH*I. Same pair of enzymes was used for the restriction of pET22b (+). The purified restricted plasmid and gene were ligated and competent bacterial cells were transformed by the procedure given with the gene cloning kit (Thermo-Fischer Inc. #K1214). An individual colony of BL21 cells harbouring the target gene was grown on LB medium supplemented with 100 µg/mL ampicillin in an incubation shaker adjusted at 37°C and 150 rpm to attain an absorbance of 0.5 at 600 nm. Bacterial cells transformed by pET22b (+) minus gene were used as the negative control. Production of recombinant enzyme was induced under 0.3mM IPTG at 30°C for 6 to 7 h, culture was harvested and stored at 4°C till further processing.

Enzyme purification and characterization

Bacterial cells were sonicated, subjected to centrifugation at 12,000×*g* and enzyme activity of clear supernatant was measured using modified procedure adopted from literature (Chohan and Rashid, 2013). Briefly, the release of ammonia was measured per minute at 75°C in a reaction mixture prepared in Tris-HCl buffer pH 9. The standard curve of ammonia for absorbance at 480 nm was used to estimate the micromoles of ammonia released. Purification process involved selective precipitation and anion-exchange chromatography. The molecular weight and purity of enzyme and total protein content were determined. Molecular weight was determined by SDS-PAGE. Enzyme activity, specific activity, percentage yield, V_{max}, K_M, effect of pH and temperature on the activity and stability of purified enzyme was determined. The enzyme activity against L-glutaminase was determined according to the procedure described by our previous studies (Shah *et al*., 2019).

3D modelling of protein

The structure of ASP-GT protein (L-Asparaginase of Geobacillus thermodenitrificans DSM-465) was not reported at the protein database bank (PDB), so we generated its 3D model by Swiss-Model (Biasini *et al*., 2014) and I-Tasser (Yang and Zhang, 2015), by using the crystal structure *'2wltA'*. L-Asparaginase protein of Helicobacter pylori that shares 96.6% global structural similarity, as template. Subsequently, a protein structure validation server RAMPAGE, was applied for the validation of protein model. It described the structure in terms of phi, psi and cBeta deviations in the form of Ramachandran plot for the protein model. Quaternary structure of asparaginase was determined by QSQE. ChemSpider and PubChem servers were applied to determine the 3D structures of potential enzyme substrates. Hex docking server was used for the molecular docking of the substrate molecules against ASP-GT (Macindoe *et al*., 2010). The binding free energy change of each docking complex was recorded to understand the interaction of Asp with different potential substrates.

Cell culture growth and treatment

To determine the anticancer activity of enzyme, human HepG2 cells (hepatocellular carcinoma cells)

were used as cancerous and human FB (fibroblast) cells were used as non-cancerous cell lines. Fibroblasts were grown on Fibroblast Growth Medium (116-500, Sigma Aldrich) in culture plates. The medium was kept to surface area ratio at 1ml per 5 cm^2. Plates were coated with 10 % gelatine before seeding cells. Cells were allowed to grow at 37°C, 5% CO_2 humidified incubator for 24-36 h. Media was changed every other day until the cells reach 60% confluency. Cells were subcultured when the culture reached 80% confluency. HepG2 were grown on DMEM medium in culture plates. Cells were allowed to grow at 37°C, 5% CO_2 humidified incubator for 24-36 h. Procedures modified from literature (El-Naggar *et al*., 2016) were adopted to determine the cytotoxicity and anticancer activity of enzyme on FB and HepG2 cells. Media containing 6×10^4 cell per millilitre were treated by serially diluted enzyme concentrations to establish the IC_{50} using neutral red assay method (Weyermann *et al*., 2005). The activity time of enzyme was also determined in the cell line media.

RESULTS

Cloning and expression of asparaginase gene

The complete open reading frame of asparaginase gene consisting of 972bp was PCR amplified, ligated, T/A cloned into pTZ57R/T and sub-cloned in pET22a (+) expression plasmid, respectively. *E. coli* cells successfully transformed with recombinant pET22a-Asp plasmid were grown in the LB medium containing 0.3 mM IPTG to induce the gene expression. The sonicated cellular extract gave a protein band at about 36kDa on SDS-PAGE representing L-asparaginase (Fig. 1).

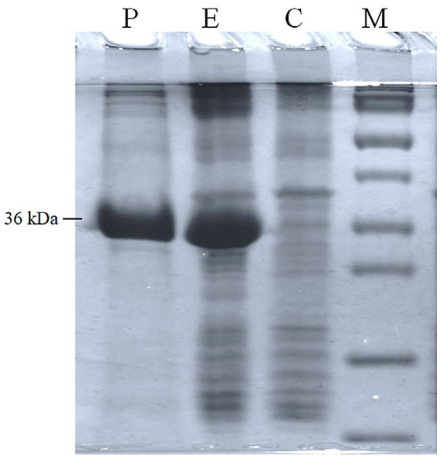

Fig. 1. SDS-PAGE image showing partially purified enzyme (Lane P), gene expressed in *E. coli* (Lane E), negative control (Lane C) and protein marker (Lane M).

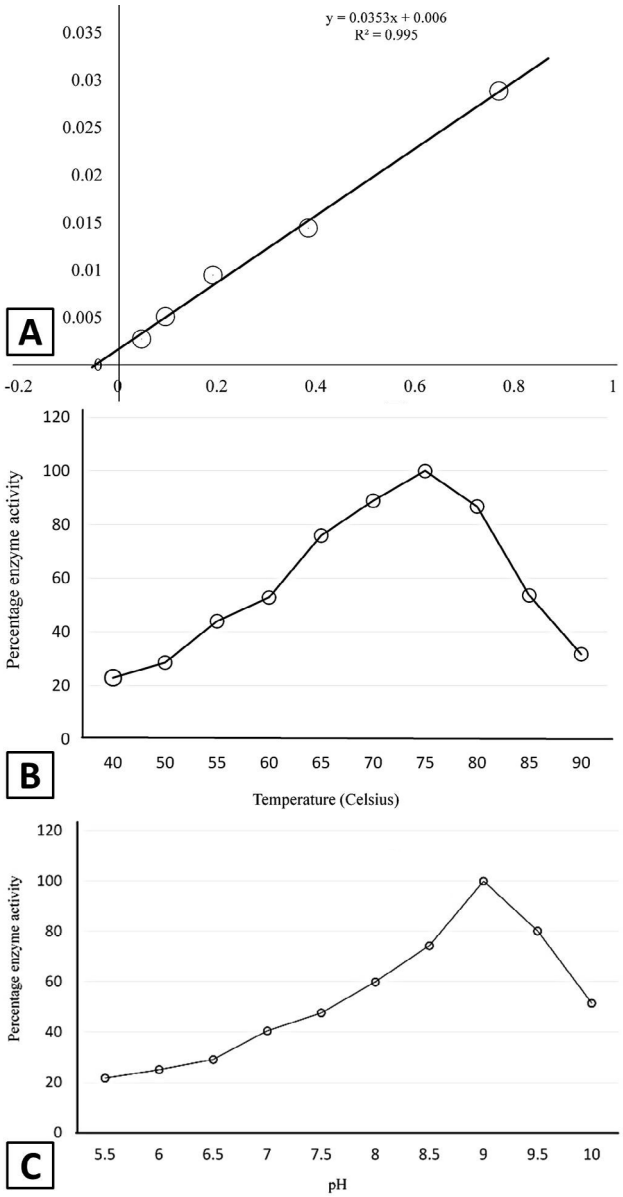

Fig. 2. Kinetic properties of recombinant asparginase. A, effect of substrate concentration on the enzyme activity indicating KM value of 5.9mM for L-Asparagine; B, effect of temperature on the enzyme activity; C, effect of pH, on the activity of enzyme.

Purification and properties of recombinant enzyme

The enzyme was found in soluble active form. Which was purified by anio-exchange chromatography based on DEAE-cellulose colum. The mobile phase consisted of 20mM phosphate buffer adjusted at pH 7.5, sample was applied on to the equilibrated column at a flow rate of 2 mL per minute, unbound protein was removed by the mobile phase and the bound proteins were eluted by a

linear gradient (0 to 0.5M) of sodium chloride prepared in the mobile phase. Eluted protein solution was collected in 3mL fractions, enzyme activity was measured and analysed on SDS-PAGE. The fractions with purified enzyme were pooled together. Specific activity of purified enzyme was 1650 units per mg of enzyme. The enzyme also exhibited glutaminase activity. However, it was less than 5% of the asparaginase activity in each batch. The enzyme has shown optimal activity at 75°C, pH 9, K_M value for L-asparagine was 5.9 mM (Fig. 2).

3D modelling of protein

A homotetramer model of Asp-GT was built using Swiss-Model and the QSQE server. It was visualized by PyMOL Molecular Graphics System, Version 1.2r3pre was applied to validate the quaternary structure of ASP-GT as a homo-tetramer depicting the structural clustering tree of ASP-GT homologs with other known structures (Fig. 3).

Docking and affinity calculations

Binding free energy changes of recombinant asparaginase was calculated as -ΔG 145.24 KJ/mole for L-asparagine and -ΔG 105.58 KJ/mole for L-glutamine. Our molecular docking study results have shown that Gly199, Lys300, Arg198, Arg197, Tyr201, Tyr323 are putative active site residues interacting the substrate (Fig. 4).

Anticancer activity of enzyme

The IC50 was found 3U/ml to 6U/ml for FB and HepG2 cells. The growth inhibitory effect of recombinant L-asparaginase on HepG2 cells was determined quantitatively. A selected dose of 5U/ml of enzyme was optimized for the treatment of cancer cells. The recommended dose has shown an anti-proliferative effect up to 65% on HepG2 cells. The enzyme has shown a half-life of 60h in the cell line growth media incubated at 37°C.

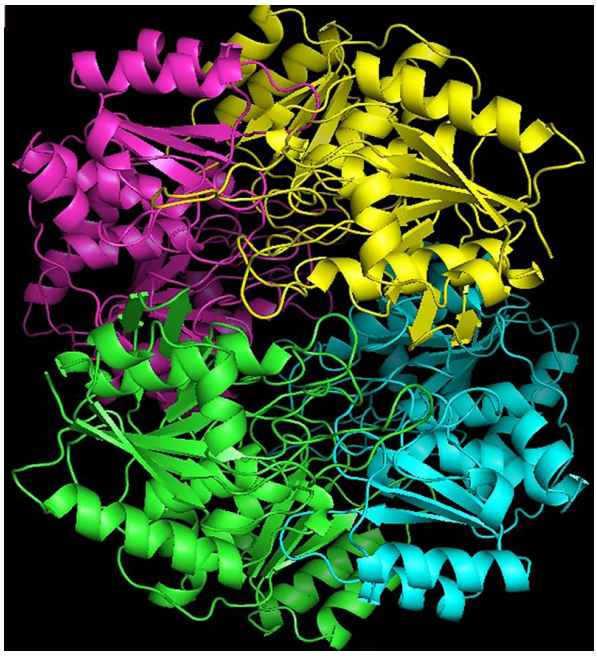

Fig. 3. ASP-GT monomer built by SWISS server, depicted in rainbow cartoons highlighting the secondary structure with different colours.

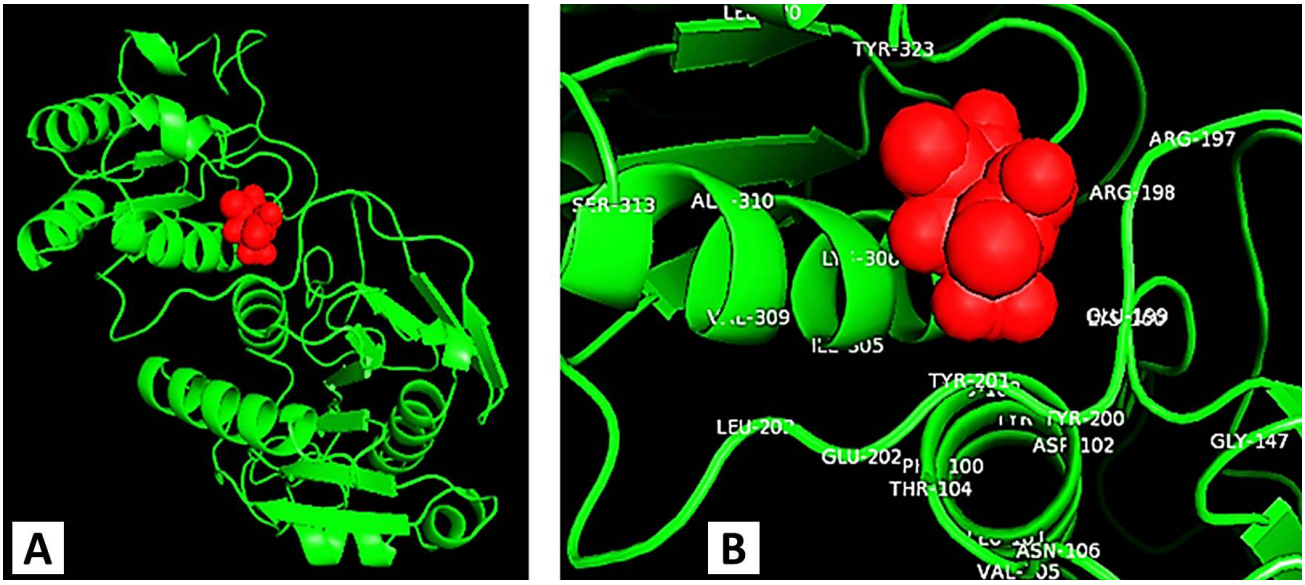

Fig. 4. Molecular docking photograph indicating the binding site residues of L-asparaginase with its substrate (L-asparagine).

DISCUSSION

Recombinant DNA technology has been exploited as a tool for the bulk production of therapeutic and industrially important proteins and enzymes. L-asparaginase is one of the therapeutic enzymes over produced sources from different in bacterial host strains (Oza *et al*., 2011; Einsfeldt *et al*., 2016). In the present study we have produced a recombinant of L-asparaginase from *Geobacillus thermodenitrificans* DSM-465 in *E. coli*. Genomic DNA of bacterial strain was purchased from DSMZ (Inhoffenstraße 7B 38124, Braunschweig, Germany). The PCR amplified gene coding for asparaginase was T/A cloned in pTZ57R/T and sub cloned in pET22b (+) expression vector. BL21 (DE3) codon plus cells of *E. coli* strain were transformed by the expression vector harbouring asparaginase gene. The enzyme expression and molecular weight was determined on SDS-PAGE (Fig. 1). It exhibited a molecular weight of about 36 kDa. Variable molecular weight of asparaginase has been reported from different species in literature ranging from 30 kDa to 45 kDa (Moorthy *et al*., 2010; Hong *et al*., 2014; Shakambari *et al*., 2018). *In silico* model of enzyme built in the present study (Fig. 3) indicated a homotetramer structure with an estimated molecular weight of 144 kDa. This is in accordance with the recent literature (Kumar *et al*., 2011; Sindhu and Manonmani, 2018). L- Asparaginase investigated in the present study has shown optimum activity at 75°C and pH 9. The enzyme has shown activity at broad range of pH and temperature (Fig. 2). Asparaginases with pH optima of 7 to 9 and temperature optima between 35°C to 90°C have been described in the literature. As for example enzyme from *Pseudomonas fluorescens* exhibited maximum activity at 34°C and pH 6 (Kishore *et al*., 2015), whereas, the enzyme from *Thermococcus kodakaraensis* has optimal activity at 40 to 90°C and pH 8 to 10 (Kumar *et al*., 2011; Chohan and Rashid, 2013). Though the *in silico* studies have shown a higher affinity of enzyme for L-glutamine, yet in the lab the enzyme has shown no activity with D-asparagine and very low activity with L-glutamine (5% of the activity against L-asparagine). There is a variety of asparaginases reported in the literature exhibiting different glutaminase activity. Zero to 10% glutaminase activity has been reported for asparaginases characterized from different species (El-Naggar *et al*., 2018; Nguyen *et al*., 2018). In the present study, a K_M value of 5.9 mM has been calculated for L-asparagine (Fig. 2) which correlates with the K_M value of asparaginase from *Thermococcus kodakaraensis* with K_M 5.5 mM (Chohan and Rashid, 2013), it differentiates with the K_M values reported for the enzyme isolated from *Streptomyces fradiae* (10mM) (El-Naggar *et al*., 2016), *Aspergillus niger* (0.81mM) (Vala *et al*., 2018), and *Penicillium digitatum* ($1x10^{-5}$M) (Shrivastava *et al*., 2012). The free energy change (ΔG) values calculated by molecular docking studies have shown a relatively high affinity of enzyme with the L-glutamine (-ΔG 105.58 KJ/mole). However, the enzyme indicated no activity against D-Asparagine and very low activity against L-glutamine. It questions the reliability of computer based molecular docking studies. The calculations made on the basis of docking studies have different indications in literature for their reliability. There are reports with 30 to 37% probability of correct measurement by computer based molecular docking (Tuccinardi *et al*., 2010; Ramírez *et al*., 2016). Our docking studies have predicted the active site amino acids (Fig. 4) that need further confirmation in the wet lab through x-ray or NMR studies. The purified enzyme was also evaluated for its applications as therapeutic agent in cancer treatment. The IC_{50} for both cell types was found between 3U/ml to 6U/ml. Our studies have found that 5U/ml of enzyme can be used a safe and effective dose for the treatment of hepatocellular cancer cells with an anti-proliferative efficiency up to 65%. The activity of enzyme was retained for 60 h in the cell lines under our growth conditions. Asparaginases from various species have been reported to for efficient anti-proliferative activity. A dose dependent effect of asparaginase on cancer cells has been reported (Einsfeldt *et al*., 2016). Enzymes with 40 h to 72 h half-life have been recently reported from *Pseudomonas fluorescens* (Sindhu and Manonmani, 2018) and *Saccharomyces cerevisiae* (Costa *et al*., 2016).

In conclusion, L-Asparaginase from the unexplored microbial species has been produced as a recombinant in *E. coli*. The enzyme has been characterized by biochemical and *in silico* approaches to describe for kinetic and putative structural properties. Enzyme activity at moderately high temperature and a broad range of pH and temperatures, low glutaminase activity and effective *in vitro* anti-proliferative effect advocates for its potential applications in the pharmaceutical and food industries.

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Statement of conflict of interests

The authors have no conflict of interests.

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