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Cloning, Purification, Characterization and Immobilization of L-asparaginase II from *E. coli* W3110

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Abstract: In the present study, we report the cloning of L-asparaginase II (*asn*II) gene from *E. coli* W3110 into pGEX-2T DNA vector. The L-asparaginase II enzyme (E.C.3.5.1.1) was overexpressed in *E. coli* BL21(DE3) and purified to homogeneity 238.4 fold by utilizing chromatography technique on DEAE-Sepharose fast flow, Glutathione S sepharose 4B columns and thrombin. SDS-PAGE of the purified enzyme revealed that has *Mr* of 40 kDa. In addition, we found that the enzyme can be efficiently immobilized in calcium alginate gelatin composites. The free enzyme has an optimum pH at 7.5 but this optimum pH is shifted to 8.5 for the immobilized enzyme. The optimum temperature, for free and immobilized enzyme were 37 and 50°C, respectively. The immobilized enzyme retained most of its activity at 60°C with high stability compared with the native enzyme when incubated at 60°C for 30 min.

Key words: Cloning, expression, purification, immobilization, characterization, glutathione s-transferase

INTRODUCTION

L-asparaginase (L-asparagine amidohydrolase, E.C 3.5.1.1.) is an enzyme which, catalyze the hydrolysis of L-asparagine to L-asparatate and ammonia according to the following equation:

$$\text{L-asparagine} + H_2O \xrightarrow{\text{L-asparaginase}} \text{L-aspartate} + \text{ammonia}$$

There are two forms of asparaginase: L-asparaginase I, an internal constitutive enzyme, and L-asparaginase II, an external enzyme which is secreted in response to nitrogen starvation. The two enzymes are biochemically and genetically distinct.

L-asparaginase II is widely distributed in both prokaryotic and eukaryotic cells and has been intensively studied over the past five decades. This enzyme is existing in many animal tissues, plants and bacteria. L-asparaginase II produced by a large number of microorganisms such as *E. coli* (Khushoo *et al*., 2004; Derst *et al*., 1994), *Erwinia cartovora* (Aghaiypour *et al*., 2001; Borisova *et al*., 2003), *Erwinia chrysanthemi* (Kotzia and Labrou, 2007), *Enterobacter aerogenes* (Mukherjee *et al*., 2000), *Pseudomonas aeruginosa* (El-Bessoumy *et al*., 2003), *Candida utilis* (Kil *et al*., 1995), *Thermus thermophilus* (Prista and Kyridio, 2001) and *Staphylococcus aureus* (Muley *et al*., 1998). Moreover, L-asparaginase II was demonstrated and characterized in higher plants on account of the vital role of this enzyme in the nitrogen nutrition (Sodek *et al*., 1980; El-Shora *et al*., 2005; Cho *et al*., 2007).

L-asparaginase II is composed of four similar monomers each monomer have 326 amino acid residue and the whole efficient enzyme exists as a tetramer of identical subunits, with molecular mass

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in the range between 140 and 160 kDa (Aung *et al*., 2000; Kozak *et al*., 2002). Each one of the four active sites is located between the N and C-terminal domains of two adjacent monomers. Thus, the L-aspraginase II tetramer can be treated as a dimer of dimers. Despite this fact, the active enzyme is always a tetramer (Khushoo *et al*., 2004).

L-asparaginase II is an important enzyme as therapeutic agent used in treatment of acute lymphocytic leukemia (mainly in children), Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticlesarcoma and melanosacroma (Stecher *et al*., 1999; Verma *et al*., 2007). The role of L-asparaginase II in lymphocytic leukemia cells treatment is based on the fact that these cells are not capable of synthesis L-asparagine and are rely on the exogenous sources to get hold of L-asparagine (Lee *et al*., 1989). On the contrary, normal cells are protected from L-asparagin starvation due to their ability to generate this essential amino acid (Duval *et al*., 2002). The antineoplastic activity attributed to the depletion of L-asparagine by the action of L-asparaginase II (Lee *et al*., 1989). Through many species producing L-asparaginase II as mentioned above only *E. coli* and *Erwinia cartovora* asparaginases are currently in medical use as efficient as drugs in the lymphocytic leukemia. because of high substrate affinity (Verma *et al*., 2007; Schwartz *et al*., 1966) and factors affecting the clearance of the enzyme from the media of the reaction (Stecher *et al*., 1999; Broome, 1965).

Unhappily, although the extensive use of L-aspraginase II , the therapeutic response of patients rarely occurs without some evidence of toxicity. The main side effects of L-asparaginase II are: pancreatitis, diabetes, liver dysfunction, neurological seizures, leucopoenia and coagulation abnormalities that may guide to intracranial thrombosis or haemorrhage (Duval *et al*., 2002). Another limiting factor of L-asparaginase II treatment is the development of allergic reaction, which ranges from mild allergic reactions to anaphylactic upset (Moola *et al*., 1994). Because the L-asparaginase II from *E. coli* is immunologically dissimilar, they provide an important alternative therapy to patients who become hyper-sensitive to one of the enzymes (Moola *et al*., 1994).

In this study we describe the cloning and overexpression the L-asparaginase II enzyme from *E. coli* W3110 by using the molecular biology tools. The overexpressed protein is purified, immobilized and optimal pH and temperature are studied for both free and immobilized enzymes.

MATERIALS AND METHODS

Chemicals

All the chemicals, restriction enzymes, DNA polymerase and DNA ligase used were AR or molecular biology grade and obtained from sigma, BDH chemicals LTD, Bio-Rad and stored as directed by supplier.

Media and Growth Conditions

LB medium was made by dissolving 10 g bacto-tryptone, 5 g yeast extract and 10 g NaCl in 1 L deionised water and sterilised by autoclaving. LB agar plates were prepared by adding 20 g agar to one liter of LB medium. The LB media was supplemented with 100 μg mL^{-1} ampicillin (LBA).

Bacterial Strains and Plasmid DNA

Escherichia coli W3110 strain (F-IN (*rrn*D-*rrn*E)1) (Bachmann 1972), DH5 strain (*sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi-1 rel*A1) (Hanahan, 1983), BL21(DE3) strain (*hsd S* gal(λc *its*857 *ind*1 *Sma*7 *nin*5 *lac Uv*5 *T7gene*1) (Studier and Moffatt, 1986) and pGEX-2T DNA plasmid (GST gene fusion plasmid IPTG inducible ApR) were kindly provided by Dr. Picksley, S.M. (Biomedical Science Department, Bradford University, UK).

Chromosomal and Plasmid DNA

Extraction and purification of both chromosomal and plasmid DNA were carried out as described by Sambrook *et al*. (1989).

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmili (1970).

Agarose Gels

DNA was analysed by using horizontal agarose gel electrophoresis. The DNA mixed with 1/6 volume of loading dye (10% w/v ficol 400, 0.06% w/v bromophenol blue and 0.5% w/v SDS) and loaded onto the 0.8% agarose gel in TAE buffer (0.04 M Tris-HCl pH 7.9, 5 mM sodium acetate, 1 mM EDTA). The electrophoresis was performed in TAE buffer and stained with ethidium bromide (0.5 μg mL^{-1}).

Restriction Enzyme Digestion

The digestion of DNA by restriction enzymes was carried out according to the manufacturer's instructions. The reaction digestion was terminated by heating at 70°C for 15 min and adding 1/6 volume DNA loading dye.

Polymerase Chain Reaction (PCR)

Oligonucleotides DNA primers (forward) (5'ATAATG-CGTAGGGATCCCGTAACT3') and (reverse) (5'TGGCAATGGATCCGAGTCTGAAGT3') were designed with defined *Bam*HI restriction site (under line) to facilitate the cloning process. DNA primers were designed in frame to amplify the asnII gene from the chromosomal DNA of *E. coli* W3110. The PCR was carried out in a total volume of 50 μL containing 2.5 μL of each primer (50 ng μL^{-1}), 2.5 μL (2 mM) deoxynucleoside triphosphate mix, 3 μL Mg^{++} ion (25 mM), 5 μL buffer (10X buffer provided with the pfu DNA polymerase enzyme), 1 μL template DNA (~0.1 ng), 5 μL dimethyl sulphoxide (DMSO), 1 μL of pfu DNA polymerase and the reaction was completed to 50 μL with distilled water and mixed gently. To each reaction tube two drops of mineral oil were added. The reaction mixture was incubated at 94°C for 4 min. The following PCR cycle was repeated 30 times: denaturation 94°C for 1 min, annealing of primers at 55°C for 1 min and DNA synthesis at 72°C for 2 min. This was followed by 4 min incubation at 72°C before the mixture was stored at 4°C.

Cloning the *asn*II Gene into pGEX-2T DNA Plasmid

The PCR product of the amplified *asn*II gene was treated with *Bam*HI restriction enzyme and purified by low melting point agarose as described by Sambrook *et al*. (1989). A plasmid pGEX-2T DNA vector was purified and linearized with *Bam*HI restriction enzyme. The 5' phosphate ends of the plasmid were eliminated by treatment with calf intestine alkaline phosphatase (CIAP). The *asn*II gene digested with *Bam*HI restriction enzyme was ligated into the plasmid previously treated with both *Bam*HI restriction enzyme and CIAP. The ligation mixture was transformed into competent cells of *E. coli* DH5 and plated onto LBA plates and incubated at 37°C overnight. Individual colonies were examined by plasmid mini prep with restriction enzyme digestion to identify the recombinant plasmids. The recombinant plasmid designated pASNII contains the whole *asn*II gene in-frame with the GST fusion protein. *Escherichia coli* BL21(DE3) cells were transformed with pASNII DNA plasmid to express the GST-asparaginase II (GST-ASNII) fusion protein. The generated *E. coli* strain designated *E. coli* ASNII.

Time Course of Overexpression of GST-ASNII Fusion Protein

Escherichia coli ASNII was streaked onto LBA plates and incubated overnight at 37°C. A single colony was used to inoculate 10 mL of LB broth supplemented with 100 μg mL^{-1} ampicillin and grown overnight at 37°C, 200 rpm in a shaking incubator. The overnight cultures were used to inoculate 100 mL LBA media. The cultures were incubated at 37°C and 200 rpm, until they reached to the mid-logarithmic growth phase $OD_{650\ nm}$ of 0.4-0.6, at which point isopropyl-1-thio-β-galacto-pyranoside (IPTG) was added to a final concentration 1 mM. At various times 1 mL samples were removed and the cells were pelleted by centrifugation at 6,000 rpm f or 5 min. Cells were then resuspended in 100 μL of 1X SDS gel loading buffer [100 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200 mM dithiothreitol (DTT)], boiled for 4 min, sonicated three times for 5 sec and analysed by SDS-PAGE.

Purification of GST-ASNII Fusion Protein

E. coli ASNII was prepared from 500 mL culture. The cell pellet was resuspended in buffer A (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM DTT, 1.0 mM phenyl methyl sulphonyl fluoride [PMSF], 0.1 mM benzamidine and 10% (v/v) glycerol and then placed in an ice-water bath and sonicated. The lysate was cleared by centrifugation at 10,000 rpm for 30 min, 4°C. The resulting supernatant was applied to a 10 mL DEAE-Sepharose fast flow column at 0.5 mL h^{-1} previously equilibrated with at least ten bed volumes of buffer A. The unbound proteins were removed from the column by passing 100 mL buffer A through the column. The bound proteins were eluted from the column by using 100 mL gradient of 50-500 mM NaCl in buffer A. Five millilitre fractions were collected and assayed for the GST-ASNII fusion protein by SDS-PAGE. The best fractions containing the GST-ASNII fusion protein as judged by SDS-PAGE were collected and dialysed overnight against 2 L of buffer B (25 mM phosphate, 125 mM NaCl, 1 mM PMSF, 0.1 mM benzamidine and 10% (v/v) glycerol). The dialysed proteins were applied on to a 5 mL glutathione S sepharose 4B column previously equilibrated with 50 mL buffer B with flow rate 30 mL h^{-1}. The unbound proteins were washed away from the column with 50 mL buffer B. The bound protein was eluted from the column with a 25 mL buffer B containing 10 mM reduced glutathione ~30 mL h^{-1} and 2 mL fractions were collected for GST-ASNII protein and then assayed by l asparaginase assay and SDS-PAGE.

The GST tag was cleaved off and this was performed by using thrombin (1 U/5 mg of fusion protein) at 22°C for 2 h in buffer C (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2.5 mM $CaCl_2$ and 1 mM DTT). After thrombin digestion, the cleaved mixture was applied to 1.5 mL Glutathione S sepharose 4B column previously equilibrated with 30 mL buffer B with flow rate 20 mL h^{-1}. The unbound ASNII protein was washed away from the column with 15 mL buffer B. The bound GST protein was eluted from the column with a 10 mL buffer B containing 10 mM reduced glutathione ~15 mL h^{-1}. Three mL fractions were collected for ASNII protein and then assayed by l-asparaginase assay. The purified l-ASN II protein was stored at -20°C after the addition of 10% glycerol.

Immobilization of L-asparaginase II in calcium alginate-gelatin composites

Combinations of gelatin-alginate mixtures were prepared by adding gelatin (3%) to sodium alginate solution (5%) in water and then cross-linking with glutaraldehyde. Typically, sodium alginate (500 mg) and gelatin (300 mg) were added to distilled water (8 mL) in a conical flask which autoclaved for 15 min at 120°C. The hot solution was allowed to cool to room temperature with constant stirring with a magnetic bead. The purified L-asparaginase II enzyme (3 mL) was then added and the mixture was stirred for 15 min. Glutaraldehyde (0.3 mL of 25% solution in water) was added and the contents were stirred for an additional 15 min. This slurry was then transferred to a dropping funnel with a plastic tip and allowed to fall into cold $CaCl_2$ solution (4°C) drop wise. The beads were left in the $CaCl_2$ solution for 30 min to harden. The supernatant was decanted and the beads were then washed with distilled water and stored in a refrigerator.

Effect of pH and Temperature on Enzyme Activity

The free and immobilized L-asparaginase II enzyme activities were measured in the pH range from 6 to 10. Buffer used was 100 mM Tris-HCl (pH 6-10). To test the effect of temperature on free and immobilized purified L-asparaginase II enzyme activities, the reactions were performed over the range of 20-80°C in a temperature controlled water bath and at their optimum pH values.

Enzyme and Protein Assay

The L-asparaginase activity was measured in terms of rate of hydrolysis of L-asparagine by measuring the amount of ammonia released in the reaction. The enzyme samples were mixed with 10 mM l-asparagine dissolved in 50 mM Tris-HCl, pH 8.6. The enzyme substrate mixtures were incubated at 37°C for 10 min, after which the reaction was stopped by addition of 100 μL of 1.5 M TCA. Samples were centrifuged and then used for estimation of ammonia, the amount of ammonia released was determined by Nessler's reagent using ammonium sulfate solution as standard. An international unit (UI) of l-asparaginase is defined as the amount of enzyme required to release one micromole of ammonia per minute under the conditions of the assay at saturating substrate concentration (Wriston, 1985). Protein concentration was determined using Bradford dye method with BSA as a standard (Bradford, 1976).

RESULTS

Cloning the *asn*II Gene

The *asn*II gene was amplified by PCR from *E. coli* W3110 chromosomal DNA with the designed forward and reverse oligonucleotides primers, yielding the expected 1.2 kbp DNA product (Fig. 1) containing the 1049 bp *asn*II gene with flanking DNA. The PCR product was ligated into *Bam*HI restriction site under the control of the IPTG-inducible *tac* promoter and the *lac*I repressor in

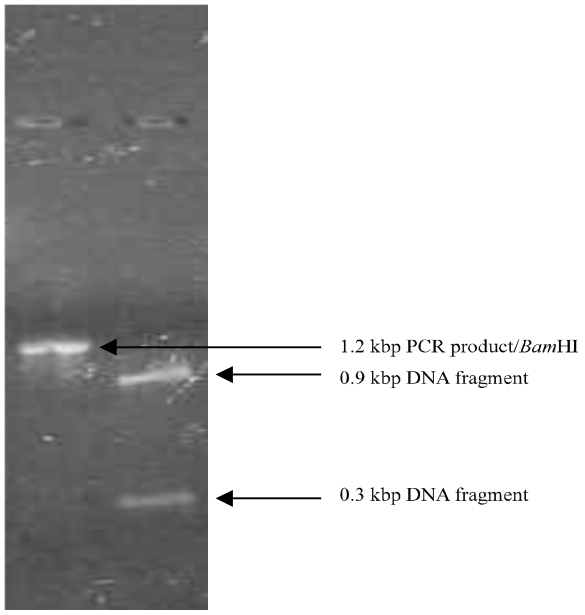

Fig. 1: Agarose gel electrophoresis representing the restriction digestion, of the 1.2 kbp PCR products containing the whole *asn*II gene of *E. coli* W3110, with *Bam*H1(Lane 1) or *Ac*lI (Lane 2) restriction enzymes. The fragments were analyzed on a 1.2% TAE agarose gel

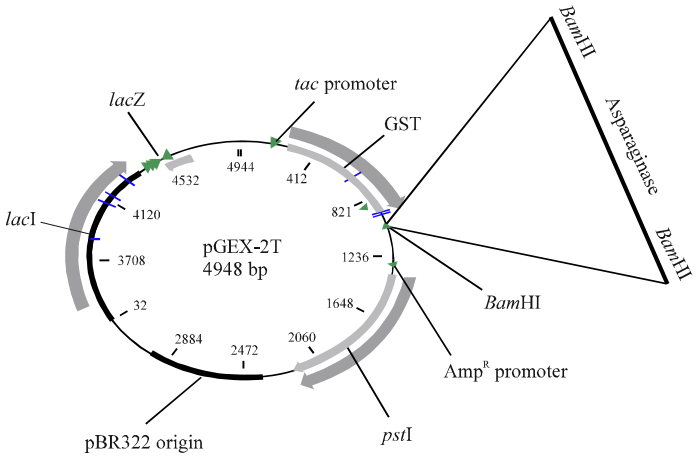

Fig. 2: Schematic diagram of the construct used for overexpression of recombinant L-asparaginase II. The L-asparaginase II, *asn*II gene fused to GST sequence and was cloned downstream of the *tac* promoter in pGEX-2T DNA expression vector, which also contained the genes for *lac*I and *lac*Z repressors, pBR322 origin and ampicillin resistance

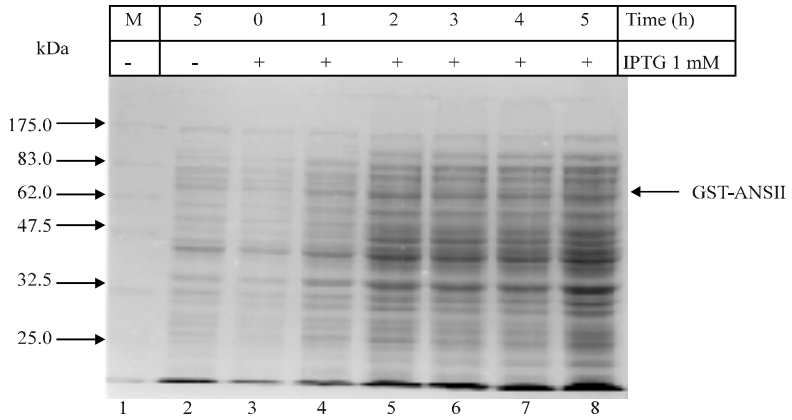

Fig. 3: Induction time course for overexpression of GST-ASNII fusion protein. Early to mid-log cultures of *E. coli* ASNII were induced at time 0 h with IPTG to final concentration 1 mM and samples were taken and analyzed by 10% SDS-PAGE gel at times indicated (Lanes 3-8), (Lane 2) in absence of IPTG, (Lane 1) protein marker

pGEX-2T DNA plasmid (Fig. 2). A resulting plasmid, pASNII contained *asn*II gene in-frame and correct orientation with respect to the plasmid *tac* promoter. The resulting plasmid, pASNII, was used to generate *E. coli* ASNII.

Time Course of Overexpression of GST-ASNII Fusion Polypeptides

A time course of the appearance of the putative induction of GST-ASNII fusion polypeptides is shown in Fig. 3. Following the addition of 1 mM IPTG to *E. coli* ASNII at time 0 h and samples were taken every 1 h. Overproduction of the 68 kDa GST-ASNII fusion polypeptide was obvious after 2 h of IPTG induction (Fig. 3, lane 5) and maximal expression was reached after 5 h (Fig. 3, lane 8). This was most obvious that the maximum expression of 68 kDa GST-ASNII fusion polypeptide occurs after 5 h IPTG induction.

Table 1: Purification of the l-aspraginase II from *E. coli* ASNII

Purification step	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification fold
Crude extract	65.0	2.31	1.69	0.732	100.0	1.0
DEAE-sepharose	20.0	611.00	1.57	2.57	92.8	3.5
Glutathione S sepharose 4B	10.0	6.34	556.00	87.69	32.8	119.8
Thrombin	3.0	1.96	342.00	174.49	20.2	238.4

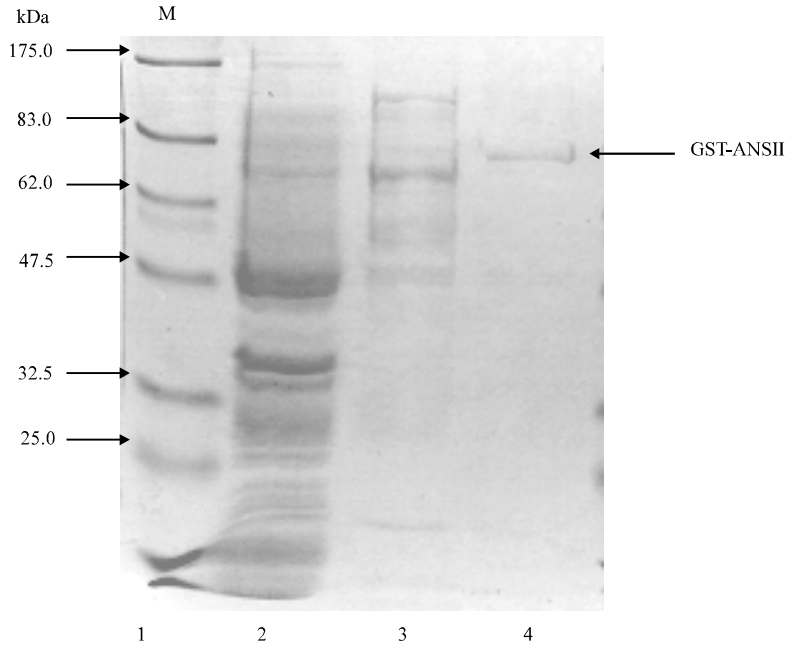

Fig. 4: SDS-PAGE showing the purification profile of GST-ASNII fusion protein. Lane 1: Protein marker. Lane 2: Crude extract of *E. coli* ASNII. Lane 3: GST-ASNII protein eluted from DEAE-Sepharose fast flow column. Lane 4: GST-ASNII protein eluted from Glutathione S sepharose 4B column

Purification of L-Aspraginase II as GST-ASNII Fusion Protein

L-asparaginase activity and SDS-PAGE were used to monitor the purification of GST-ASNII fusion protein. A summary of the purification procedure and the results obtained are shown in Table 1 and Fig. 4. *Escherichia coli* ASNII cells induced for 5 h by 1 mM IPTG were thawed overnight at 4°C then suspended buffer A. Cells were then placed in an ice bath, sonicated and centrifuged at 10,000 rpm for 30 min to remove cellular debris. The purified GST-ASNII fusion protein was then obtained from the extract using the two-step procedure described in Materials and Methods. The purified GST-ASNII fusion protein migrated as a single protein band on SDS-PAGE and was homogeneous (Fig. 4, lane 4). The GST-ASNII fusion protein molecular masses was determined to be about 68 kDa (by SDS-PAGE: Fig. 4).

Treatment of GST-ASNII Fusion Protein with Thrombin

Thrombin was used to cleave the GST-ASNII fusion protein. The products of the digestion are two bands one for A SNII polypeptides (40 kDa) and the other for GST polypeptides (28 kDa)

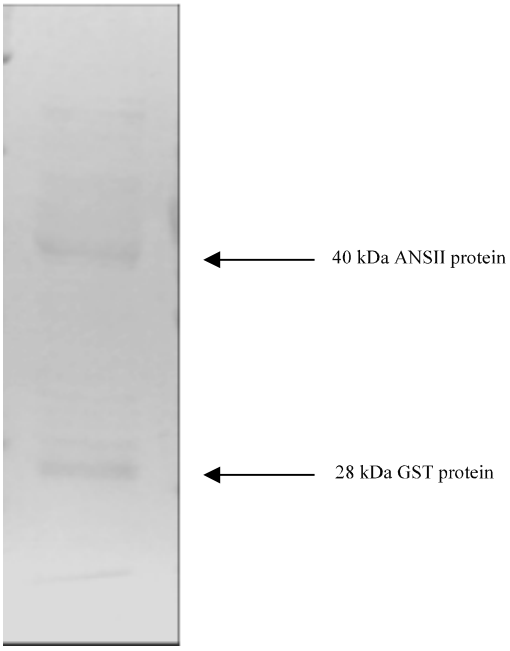

Fig. 5: SDS-PAGE showing the thrombin effect on GST-ASNII fusion protein. ASNII-GST fusion proteins was incubated for 30 min with thrombin to cleave the ASNII (40 kDa) from the GST carrier (28 kDa)

appeared as expected molecular weight (Fig. 5). The l aspraginase II was purified about 238.4-fold with a yield of 20.2% (Table 1).

Immobilization of L-Asparaginase II

L-aspraginase II enzyme can be efficiently immobilized in calcium alginate gelatin composites in the presence of glutaraldehyde. The immobilized enzyme showed about 78% of the activity of the native enzyme (data not shown), indicating that the method of immobilization is particularly suitable for L-aspraginase II enzyme isolated from *E. coli* W3110.

Effects of pH on L-Asparaginase II Activity

The effect of pH on activities of free and immobilized L-asparaginase II is shown in Fig. 6. The free and immobilized L-asparaginase II activities was determined over a wide pH range from 6 to 10. The optimal pH for the free L-aspraginase II activity was registered at 7.5 (Fig. 6). It is clear that purified L-asparaginase II is active in alkaline solutions. The optimum pH for the immobilized enzyme is recorded at the pH 8.5 (Fig. 6) whereas, the, optimal pH of the immobilized L-aspraraginase is higher than that observed with the free enzyme.

Effects of Temperature on L-Asparaginase II Activity

The optimum temperature for L-asparaginase II activities were determined after pre-incubation of the enzyme for 30 min over a wide temperature range 20-80°C. The optimum temperature for free L-asparaginase II activity was 40°C and pH 7.5 (Fig. 7). The optimum temperature for the immobilized L-asparaginase II is shifted to higher temperature and recorded at 50°C which is higher than that of the free enzyme.

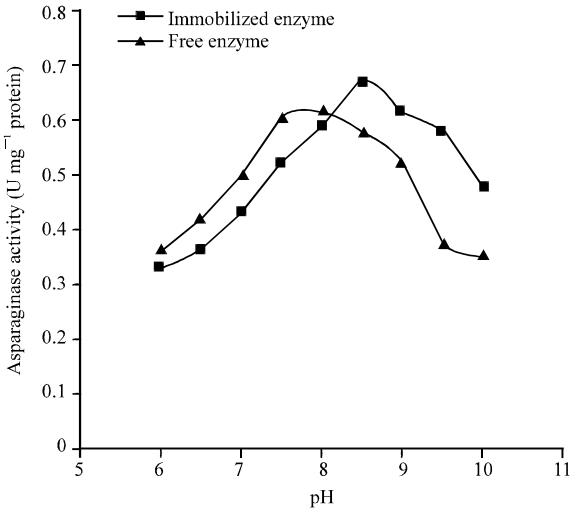

Fig. 6: Effect of pH on the activity of free and immobilized L-asparaginase II in 100 mM Tris-HCl buffer. Results are the Mean±SD of triplicate assays

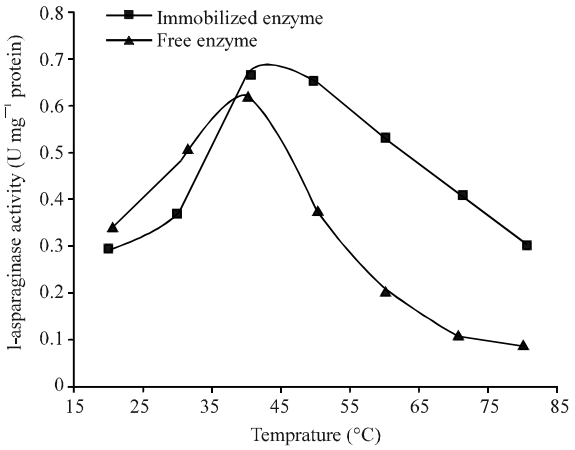

Fig. 7: Effect of temperature on the activity of free and immobilized L-asparaginase II in 100 mM Tris-HCl buffer (pH 7.5 for free L-asparaginase II and pH 8.5 for immobilized L-asparaginase II). Results are the Mean±SD of triplicate assays

Heat Stability of Free and Immobilized L-asparaginase II

The heat stability of the immobilized L-asparaginase II was compared with that of the free enzyme at 60°C for the specified times (30 min). The results shown in Fig. 8 indicate that the heat stability of immobilized enzyme was greatly enhanced. For example, the free enzyme retained 22.4% of its initial activity after heat treatment at 60°C for 30 min, while the remaining activity of the immobilized enzyme after similar treatment was 66.8%, approximately a 2-fold increase of heat stability under the specified conditions.

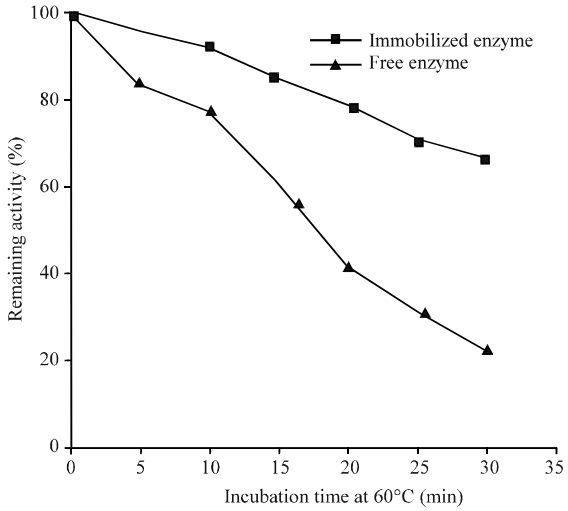

Fig 8: Thermal stability of free and immobilized l-aspraginase II with incubation time at 60°C

DISCUSSION

The commercial availability of L-asparaginase has revolutionized the molecular therapy of acute lymphocytic leukemia and melanosacroma. Therefore, the necessities of new purified and effective recombinant L-asparaginase are not only of academic importance but also of practical interest (Avramis and Panosyan, 2005). In the present study, we have described procedures for successful cloning, expression and purification of L-asparaginase II enzyme, from *E. coli* W3110, as well as immobilization and its biochemical characterization. The expression levels of GST-ASNII fusion protein in *E. coli* cells were high. Typically high expression levels are correlated with high specific growth rates (Shokri *et al*., 2002). However, stationary phase protein overexpression is an important characteristic of *E. coli* and fairly high-level expression of the target protein has been obtained previously with induction in the stationary phase (Ou *et al*., 2004). Likewise higher solubility of the recombinant protein and lower proteolytic rates were reported with late log phase induction which resulted in enhanced protein yields (Galloway *et al*., 2003). In the present study, It is observed that the induction in the late log phase led to protein overexpression. An *E. coli* ASNII was constructed and used to overexpress the GST-ASNII fusion polypeptides from the generated DNA plasmid pASNII containing the *asn*II gene, encode functional L-asparaginase II enzyme, under the control of the strong *tac* promoter and *lac*I repressor (Fig. 1). Following 5 h of IPTG induction, the GST-ASNII fusion polypeptides were expressed.

Purification of ASNII protein often require multi-steps procedure while introduction of a GST sequence upsteam the ASN II, to generate GST-ASNII fusion protein, reduce the purification procedure steps. A purification of GST-ASNII fusion proteins were achieved by using both DEAE-Sepharose fast flow column and glutathione S sepharose 4B column. The specific activity increased from 0.732 to 174.49 U mg^{-1} for the crude extract and the final preparation, respectively. The final preparation was examined using SDS-PAGE which revealed that it contained one protein band with *Mr* of 40 kDa. In this respect, the specific activity of the enzyme was more than that obtained from *Corynebateriun glutamicum* (Mesas *et al*., 1990).

Other bacterial species produced more than one component of L-asparaginase with variable molecular weights (Rozalska, 1989). However, in *E. coli* K-12, the *ans*I and *ans*II genes, which code for L-asparaginase I and L-asparaginase II, respectively, are located in separate cistrons

(Jeristrom *et al*., 1989; Jennings and Beachem, 1990). L-asparaginase I has low affinity to L-asparagine, is cytoplasmic, its production thought to be constitutive and inactive against animal tumors (Roberts *et al*., 1968; Campbell *et al*., 1967; Khushoo *et al*., 2004). The other enzyme L-asparaginase II has high affinity to L-asparagine, is secreted to the periplasm, its expression is positively regulated and is active against animal tumors (Schwartz *et al*., 1966; Chesney, 1983; Riley, 1984; Jerlstrom *et al*., 1987). Dunlop *et al*. (1978) reported that the expression of extracellular L-asparaginase II is elevated during nitrogen-limited growth. The L-asparaginase II enzyme are regulated by the *fnr* gene product, a positive regulator of anerobic respiration (Jerstrom *et al*., 1987). It has been suggested that asparaginase II in *E. coli* may be implicated in either using asparagine as an anaerobic electron acceptor of simply providing aspartate from exogenous asparagine (Svobadova and Stebanova-Necinova, 1973; Jeristrom *et al*., 1989). The external L-asparaginase II enzyme is not derived from L-asparaginase I by post-translational modifications or by the addition of an alternate subunit to a core L-asparaginase I subunit. The L-asparaginases I and asparaginase II enzymes seem to be biochemically and genetically distinct. In fact other than sharing the ability to hydrolyze L-asparagine, the two enzymes have little in common. They differ significantly in fractionation properties, stability to heat, pH and in sensitivity to sulfhydryl reagents. Asparaginase I is optimally active at pH 6.8, whereas asparaginase II has highest activity at pH 7.5-9.

Interestingly, the amino acid sequence of the *B. subtilis* AnsA enzyme closely resembles the sequence of *E. coli* asparaginase I, while the sequence of AnsZ is more similar to that of *E. coli* asparaginase II (Ye *et al*., 2000). In the present work the maximum activity for L-asparaginase II occurred at pH 7.5. A similar pH value was obtained for l aspraginase II isolated from *E. coli* (Castaman and Rodeghiero, 1993; Liboshi *et al*., 1999), *Pseudomonas aeruginosa* 10145 (Roberts *et al*., 1968) and many other microbial asparaginase activities (Balcao *et al*., 2001).

However, the optimal pH for the immobilized enzyme is shifted to 8.5. There are numerous references that relate the displacement of the pH activity curve of an immobilized enzyme in either direction from that of the free enzyme. Changes in pH activity behavior may due to immobilization of enzyme have been explained on the basis of an unequal distribution of hydrogen and hydroxyl ions between the polyelectrolyte phase on which the enzyme is immobilized and the external solution (Goldstein, 1970). Changes in optimum pH curve are caused by the charge of a water-insoluble carrier, a chemical modification of the enzyme, or certain enzymatic reactions.

A temperature profile showed that the enzyme had maximum activity at 40°C for the free enzyme and 22.4% activity was attained at 50°C after 30 min. Similar results were recorded for L-asparaginases from *Pseudomonas stutzeri* (Manna *et al*., 1995), *E. carotovora* (Maladkar *et al*., 1993) and *Staphylococcus* (Sobis and Mikucki, 1991). Also, Qian *et al*. (1996) reported that *E. coli* L-asparaginase II lost its activity more rapidly at higher temperatures. On the other hand, L-asparaginase II from chrombacteriaceae had maximum activity at 20°C (Roberts *et al*., 1972). Incubation of L-asparaginase at 37°C for different times showed that the activity reached its maximum at 30 min (Qian *et al*., 1996). The optimal temperature for the immobilized enzyme is shifted to 50°C and 66.8% of the enzyme activity attend after incubation at 50°C for 30 min. The production of L-asparaginase from *Pseudomonas aeruginosa* under solid-state fermentation improve the asparaginase II properties (Roberts *et al*., 1968). Economically, this enzyme could be produced from cheap, untreated biomass residues. The excellent properties of this enzyme, such as the activity at the alkaline pH range at 37°C, make it extremely valuable in the chemotherapeutic treatment of leukemia.

In summary, we have amplified the *asnII* gene from *E. coli* W3110 DNA. The gene was cloned into pGEX-2T vector under the *tac* promoter. The L-aspraginase II protein was overexpressed, purified 238.4 fold with yield 20.2%. The purified enzyme was immobilized in calcium alginate gelatin composites. The immobilized enzyme and free enzyme were characterized. The immobilized exhibit temperature stability more than that recorded with the free enzyme.

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