Full Length Research Paper

Recombinant expression, purification of L-asparaginase-II from thermotolerant *E. Coli* strain and evaluation of its antiproliferative activity

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Repeated use of L-asparaginase II enzyme, in the treatment of acute lymphoblastic leukaemia, is commonly needed because of the enzyme's instability and relatively short half-life which leads to more serious side effects on patients. In the present study, we report on the cloning and expression of L-asparaginase from a thermotolerant strain of *Escherichia coli* (KH027) which was isolated from camel manure and could grow at 45°C. Expression of recombinant asparaginase was conducted by fusion asparaginase gene to pelB leader sequence and 6His residues at the C-terminus under the inducible T7 promoter in DH5α cells. Induction of the cells with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at late log phase of growth resulted in 0.6-fold (2111 UI) higher to that obtained in early log phase induction (1319 UI) and 0.3-fold compared with mid log phase induction (1623 UI). The recombinant asparaginase protein was purified from the culture supernatant through nickel affinity chromatography. The apparent molecular weight of the tetramer enzyme was found to be ~141 kDa. Overall yield (87 mg/L) of the purified recombinant asparaginase was achieved at the shake flask level. The purified protein showed optimum activities at a temperature of 43°C and pH 6. The K_m and K_{cat} parameters were 3.8 mM^{-1} and $2.92 \times 10^{3s-1}$, respectively. The enzyme retained around 57 and 30% of its initial activity after 30 and 60 min of incubation at 50°C, respectively. Recombinant L-asparaginase was evaluated for its antiproliferative effect in the leukemia cell lines of RS4; 11 and HL60 after 96 and 72 h of incubation. The doses of 100 µg/mL and time-response effect of 96 h caused a reduction value of 50% in cell viability of RS4. However, cell viability of 50% in the leukemic cells HL-60 was noticed with a concentration of 200 µg/mL with an incubation period of 72 h. *In vitro* antiproliferative results in the leukemia cell lines encourage for making *in vivo* investigation to increase the possibility of using this thermostable enzyme in leukaemia therapy.

Key words: L-Asparaginase II, leukemia cell, recombinant asparaginase.

INTRODUCTION

Asparaginases are a cornerstone in the treatment proto-cols for acute lymphoblastic leukemia (ALL) (Pieters et al., 2011; Muller and Boos, 1998; Keating et al., 1993). L-asparaginase (ASNase) is an enzyme which hydrolyzes amino acid L-asparagine (Asn) to L-aspartic acid and ammonia resulting in the systemic depletion of L-Asn (Horowitz et al., 1968; Goldberg, 1992; Capizzi, 1993; Baran et al., 2002) which in turn induces apoptosis of ALL lymphoblasts (Avramis and Panosyan, 2005; Story et al., 1993; Ueno et al., 1997). Unlike normal cells, cancer cells are unable to synthesize L-Asn due to the absence of L-asparagine synthetase activity (Prager and Bachynsky, 1968; Vieira et al., 2006) where they have L-Asn from extracellular sources (Duval et al., 2002; Keating et al., 1993; Wriston, 1985). Thus, depletion of the circulating pools of l-Asn byl-ASNase leads to the destruction of the tumour cells, since they are unable to complete protein synthesis (Kotzia and Labrou, 2007).

Therapeutic response to those asparaginases of *Erwiniachrysanthemi* and *E. coli* rarely occurs without some evidence of toxicity (Duval et al., 2002; Pavelic et al., 2011) and hypersensitivity reactions (Panosyan et al., 2004; Woo et al., 2000; Larson et al., 1998). Treatment of ALL with L-asparaginase may lead to thrombotic complications and transient protein inhibition in the liver and pancreas (Nowak-Göttl et al., 2003) pancreatitis (Barry et al., 2007; Alvarez and Zimmerman, 2000; Kearney et al., 2009), diabetes, leucopoenia, neurological seizures are associated with the treatment course of ALL with L-asparaginase (Chabner and Loo, 1996; Duval et al., 2002).

Because of many L-asparaginase naturally available, aspraginases are usually not optimally suited for thera-peutic purposes, many homologous L-aspraginases have been cloned and characterized to find enzymes with fewer side effects and less toxicity (Kotzia and Labrou, 2005; 2007). Hence, the ideal enzyme for therapeutic use should persist in the circulatory system for long time with reduced antigenic properties. In order to meet these qualities, many attempts have been made to solve this problem by attaching the asparaginase enzyme with chemicals like polyethylene glycol (Burnham, 1994; Veronese and Pasut, 2005; Fernandes and Gregoriadis, 1997), encapsulation to RBCs (Godfrin et al., 2006), deimmunization by combinatorial T-cell epitope removal using neutral drift (Cantor, 2011). Trypsin resistance L-asparaginase with increased stability was achieved by immobilization to natural silksericin protein (Yu-Qing, 2004).

The situation becomes even worse when asparaginase is used repeatedly because of the short half-life and instability that many leads to more serious toxic effects on patients. However, chemical modification usually leads to reduced enzyme activity (Making and Inada, 1975). Therefore, improving the stability of asparaginase without affecting its activity becomes an urgent problem. Some progress has been achieved in protein thermal stability through different trials. These trials give a promising way to enhance thermostability and strengthen the protein's resistance to proteolysis and improve whole stability of the enzyme (Daniel et al., 1982; Imanaka et al., 1992; Li et al., 2007; Kotzia and Labrou, 2009). In a trail to have a thermostable asparaginase enzyme, our work was directed to have a thermotolerant strain of *E. coli*. We have isolated one from camel's dung in a farm at the desert of A-lkharj governorate in the Kingdom of Saudi Arabia (KSA). In this study we have isolated the (*Ans*B) gene from this strain (KH027) and cloned it in pET20b vector with a C-terminal 6His tag. This is followed by expression, purification, characterization and evaluation of the proliferative activity of the cloned gene.

MATERIALS AND METHODS

Plasmids and bacterial strains

A thermotolerant strain of *E. coli* (KH027) was isolated from camel dung. It was isolated by serial dilution of the dung and its plating into EMB agar medium plates for selecting colonies of the green metallic sheen. Plates were incubated for 24 h at 48°C. Bacterial identification was confirmed by routine biochemical and microscopic tests and API 20 E system (BioMerieux, France). This strain was numbered as KH027 and overnight subcultured in Luria-Bertani (LB) medium and used as a genomic DNA source for cloning step. Cells of the strain of *E. coli* DH5α were used for the cloning of plasmids and as a host in expression studies. The plasmid of pET20b (Novagen) was used as expression vector.

Cloning of L-asparaginase II Gene (AsnB)

The gene coding for the mature region of L-asparaginase II (AnsB) was PCR amplified from the genomic DNA of *E. coli* (KH027) using the two oligonucleotide primers of AsBf (5′-gcggaattcgttacccaatatcacca-3′) and AsnBr (5′-ggcgaagcttgtactgattgaaga-3′). *Eco*RI and *Hind*III restriction sites were incorporated in the primers to facilitate cloning of the asparaginase gene (without its native signal sequence) in the vector pET20b in fusion with by 6His residues at its C-terminus to facilitate the purification step of the recombinant asparaginase protein.

Program of the PCR was conducted as follows: initial denaturation

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at 94°C for 4 min, denaturation at 95°C for 40 s, annealing at 58°C for 30 s, extension at 72°C for 90 s for 35 cycles, and a final extension at 72°C for 10 min. Band of the amplified fragment was 988 bp and extracted from the gel using Qiagen extraction kit and cloned as EcoRI/HindIII digest in pET20b. Ligated product was introduced to competent DH5α cells and the ampicillin resistant transformants were selected and right clones were confirmed by double digestion and insert release. The resultant recombinant plasmid pET20b-Asn-9 His6was sequenced using NCBI BLAST to confirm the asparaginase gene insert.

Expression of L-asparaginase II Gene (AsnB)

Transformed cell by clone pET20b-Asn-9 His6 were tested for expression of cloned L-asparaginase II Gene. For this step, 5 mL of LB broth containing 50 µg/mL ampicillin was inoculated by a single colony and grown at 37°C overnight. Fifty milliliters of the LB broth was inoculated with 0.5 mL of the overnight grown culture, and 10 µM of inducer (IPTG) was added after it attained an OD_{600} of 0.6 and grown for a post induction period of 6 h. Cells were harvested by centrifugation at 5000 rpm for 10 min and resuspended in 50 mMTris buffer pH 7 containing lysozyme and the soluble proteins were collected by centrifugation at 11000 rpm for 15 min at 4°C. Extracted protein fractions were mixed with SDS PAGE loading buffer and analyzed on a 12% SDS-PAGE and then stained with Coomassie Brilliant blue R-250. Stained gel was detained to visualize protein bands.

Purification of His-tagged L-asparaginase II Gene (AsnB)

The culture supernatant was collected, by centrifuging the cells at 8000 *g* for 10 min at 4°C, 24 h post-induction, and this was used for purification of recombinant asparaginase by Ni-NTA affinity chromatography. The column was packed with 8 ml of 50% Ni-NTA resin (Qiagen) and equilibrated with equilibration buffer (50 mM potassium phosphate, pH 7.8, 200 mM NaCl, 0.5 mM PMSF, and 20 mM imidazole). The culture supernatant was treated so as to contain 200 mM NaCl, 1 mM PMSF, and 20 mM imidazole and the pH was adjusted to 7.8 by 1M K_2HPO_4. One hundred milliliter of the treated culture supernatant was passed through 0.45 µm filter and loaded on to the Ni-NTA column. The column was washed with 60 ml of equilibration buffer (50 mM phosphate buVer, pH 7.8, 200 mM NaCl, 0.5 mM PMSF, and 20 mM imidazole) and the protein was eluted with elution buffer (50 mM phosphate buffer, pH 8.0, containing 250 mM imidazole and 1 mM PMSF). Fractions of 1 ml each were collected and analyzed by SDS-PAGE and the protein concentration was measured using Bradford dye method (Bradford, 1976) with BSA as a standard. Fractions containing recombinant asparaginase were pooled and dialyzed against 50 mM Tris-HCl, pH 8.0. The protein solution was concentrated by Ultrafiltration using Centricon MWCO 10kDa, Amicon, Germany.

Enzyme assay of L-asparaginase II

Activity assay enzyme was performed according to Imada et al (1973). Samples were mixed with 0.04 M L-asparagine in 0.05 M Tris buffer pH 7.0. 200 µL of assay mixture were incubated at 37°C for 10 min. and the reaction was stopped by adding 50 µL of 1.5 M trichloroacetic acid followed by centrifugation. Nessler's reagent was added to measure the released ammonia after L-asparagine hydrolysis and spectrophotometric measurements were done at 450 nm. Ammonium sulphate calibration curve was used to determine the enzyme activity of recombinant protein and one unit of enzyme activity was defined as the amount of enzyme required

to release 1 µM of ammonia per minute under the conditions of the assay at saturating substrate concentration (Wriston, 1985).

Temperature and pH profiles

Activity assay of the purified L-asparaginase enzyme was carried out at different temperatures from 25 to 60°C. For optimum pH, assay of the purified L-asparaginase was conducted with acetate buffer for pH for 4-6, Tris-HCl pH for 7-8 and glycine NaOH for pH 9. Thermostability of the Purified L-Asparaginase: Purified protein of the L-asparaginase was tested for thermal stability at 50°C at different time intervals (10, 20, 30, 40, 50, 60, 70, 80 and 90 min). For this purpose, the pure enzyme was incubated at 50°C for the previous mentioned intervals followed by keeping the enzyme at 4°C. Enzyme activity was calculated at optimum conditions of temperature and pH by L-asparaginase assay. The residual activity was determined by keeping untreated enzyme as control.

Kinetic properties of the purified enzyme

Determining the K_m of the recombinant AsnB Enzyme was performed by incubating the enzyme with different substrate concentrations at different time intervals in 50 mMTris buffer of pH 7.3. Substrate concentrations were taken at the range from 0.2 to 10 mM of L-asparagine. Kinetic parameters Lineweaver-Burk plot (David and David, 1972).

Cell culture and cell preparation

The human leukemia cell lines HL-60 (pre-myeloid leukemia) and RS4;11 (leukemia with lymphoid and myeloid characteristics, Stong et al. (1985) were purchased from the American Type Culture Collection and were maintained in RPMI (GIBCO, USA) supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin. Human peripheral blood mononuclear cells (PBMC) were purified from heparinized venous blood drawn from healthy donors. PBMC were isolated by centrifugation on Ficoll-Paque (Pharmacia-LKB, Uppsala, Sweden) density gradients (1.077 g/mL) at 1000 rpm for 15 min at room temperature and subsequently re-suspended in RPMI-1640. All cell cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere. The counting and cell viability tests were determined using the test of Try-pan blue exclusion.

Proliferation assay

Cells were seeded in 96-well plates at 1×10^4 cells per well. After 24 h, L-asparaginase was added at concentrations of 15, 25, 50, 100 and 200 µg/mL. At different time points (48, 72, and 96 h) of continuous drug exposure, 10 µl of XTT (XTT II; Roche Molecular Biochemicals, Indianapolis, IN) dye (3 mg/mL) was added in each well. The plates were incubated for 2 h at 37°C and the formazan product was measured at 450 nm by using an iMarkmicroplate reader (Bio-Rad Laboratories). The experiments were performed in triplicate in three independent sets. Values are shown as mean ± SD. Cell survival was calculated by subtracting the background absorbance of media alone and then dividing the absorbance of test wells by the absorbance of the control (untreated) wells.

Miscellaneous

All DNA manipulation protocols, restriction analysis, electro-elution of DNA fragments, ligation, and DNA sequence determination were
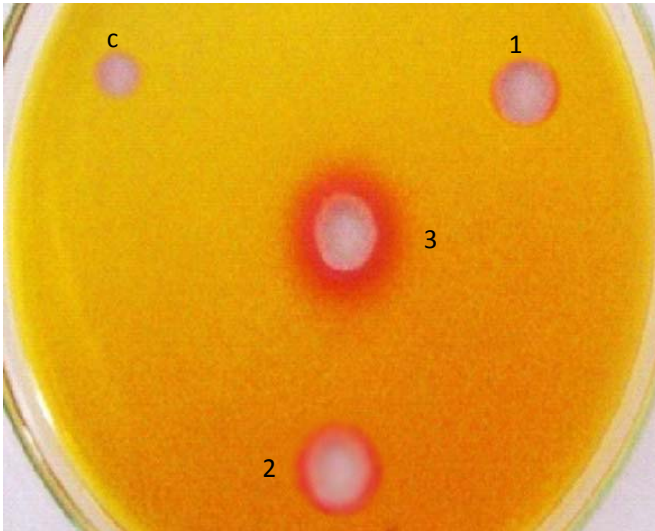
Figure 1. Effect of induction time on the secretion of asparaginase using a rapid plate test for screening L-aspraginase secretion with phenol red as an indicator.cells were grown and induced with 1 mM IPTG at early log phase (1), mid log phase (2), and late log phase (3) of growth and monitored by secretion at zero time (c) as a negative control. Cells were grown for 24 h post-induction and equal amount from each sample (100 µl) was applied in each well.
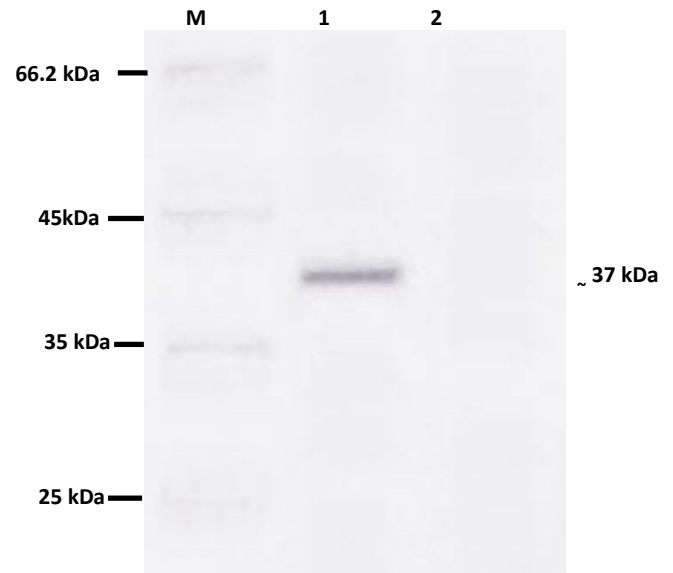

Figure 2. Immunoblotting of the recombinant asparaginase gene. Transformed IPTG-induced cells of *E. coli* strain (KH027) were grown for 24 h on LB medium. Total protein was extracted subjected to 12% SDS-PAGE and Western analysis using anti-His6 antibody. Lane, M: protein marker; Lane, 1: *expressed Asn from clone* pET20b-*Asn*-9; Lane, *3:* proteins of transformed cells with empty pET20b vector as a negative control

performed as described in Sambrook et al. (1989). Polyacrilamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). Protein concentrations were determined by the Bradford method (1976). Molecular masses of L-asparaginase were determined by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Garfin (1970). The prestained protein markers (Biolabs, England), broad range 14.4 to 116.2 kDa were used in detection of molecular masses. Immuno-detection of proteins was conducted according to Towbin et al. (1979). Screening of the ability of isolates to produce L-asparaginase was carried out according to Gulati et al. (1997).

RESULTS

Cloning and expression of asparaginase Gene

Genomic DNA of the *E. coli* strain (KH027) was used to amplify the mature part of asparaginase gene by PCR. The amplified fragment (988 bp) was ligated as EcoRI/HindIII digest in pET20b downstream to the T7 promoter and pelB leader sequence. The cloned gene showed 99% similarity with L-asparaginase type II of *E. coli* (K011). IPTG-induced *E. coli* cells, that harboring the recombinant clone pET20b-Asn-9, were grown in LB medium for 24 h post induction. Samples were collected at different stages of growth in order to determine the optimum conditions for asparaginase secretion. For this purpose, cells were induced with 1 mM IPTG at early log phase (OD600:0.6), mid log phase (OD600: 2.0), and late

log phase (OD600:4.0) of growth. It was noticed that induction during late log phase resulted in maximum secretion of the recombinant asparaginase (Figure 1). At this stage the total volumetric activity jumped up to (2111 UI) which was 0.69-fold higher to that obtained with early log phase induction (1319 UI) and 0.27-fold compared with mid log phase induction (1623 UI). Also, the specific productivity (total activity/OD$_{600}$) increased 3-fold with late log phase induction (336UI/OD$_{600}$) in comparison to early log phase induction (112 UI/OD$_{600}$). More interestingly the specific activity was also higher. These results show that secretion of the recombinant asparaginase takes place when late log phase induction is done. Therefore, induction during late log phase was chosen in the next experiments of recombinant asparaginase in this study. Also, the molecular mass of the subunit was ˷37 kDa consistent with a homodimeric enzyme expression of recombinant asparaginase gene as monitored by immunoblotting using anti-His6 antibody (Figure 2, lane 1).

Purification of recombinant asparaginase

Secreted recombinant asparaginase into the culture supernatant was obtained with IPTG concentrations (5, 10, 25, 50 and 100 µM). Maximum expressed protein in soluble fraction appeared upon induction with 10 µM IPTG (Figure 3, lane 3). The extracellular recombinant
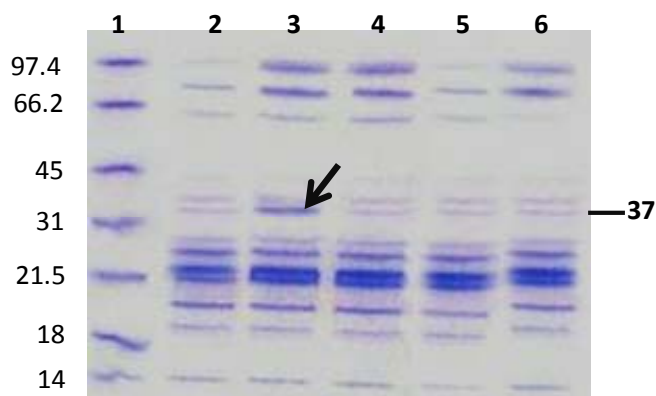
Figure 3. SDS-PAGE analysis of the proteins from soluble fraction after expression. Lane, 1: protein marker; Lanes, 2-6: soluble protein fractions from induced cells with 5, 10, 25, 50 and 100 µM IPTG.
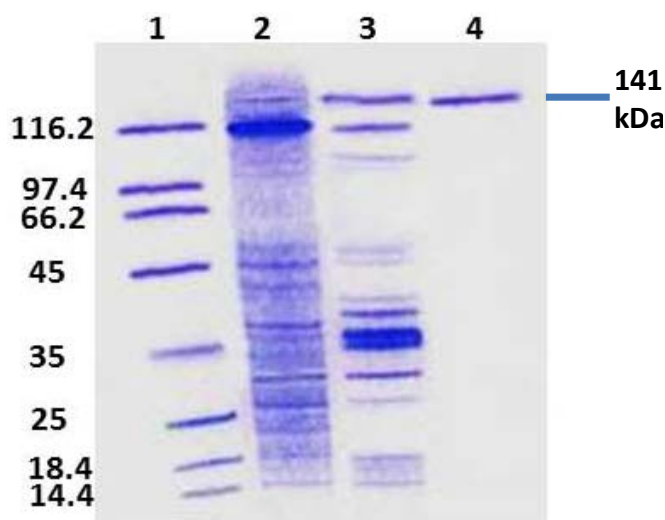

Figure 4. Purification of recombinant asparaginase by SDS-PAGE. Protein samples were analyzed on a 12% polyacrylamide gel. Lane, 1: molecular weight marker; lane, 2: supernatant of the induced transformed culture of *E. coli* BL21 (DE3) by pET20b-Asn-9; lane, 3: supernatant collected after passing through Ni-NTA column; lane, 4: eluted protein from Ni-NTA column after size exclusion chromatography.

protein constituted most of the total extracellular protein. The recombinant protein from the soluble fraction was purified through Ni-NTA column. A single protein band was developed by SDS-PAGE analysis (Figure 4). The apparent molecular weight of the oligomeric protein was ~141 kDa as determined by polyacrylamide gel electrophoresis. An increase of 2.94 fold was achieved upon the purification step where the specific activity of the recombinant asparaginase jumped from 64 to 188
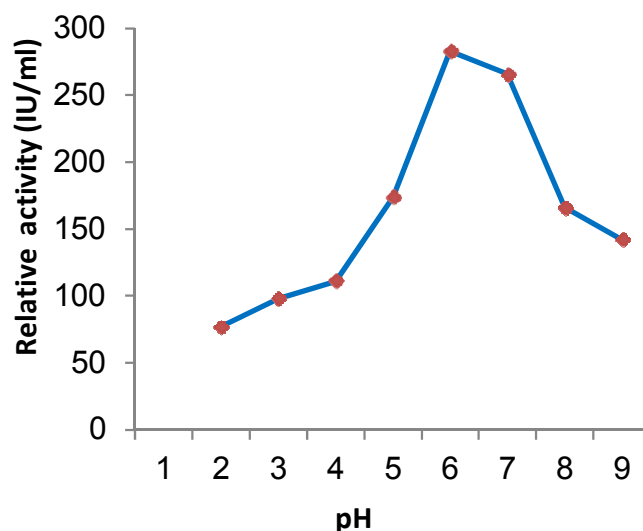

Figure 5. pH profile of the purified recombinant asparaginase. Buffer systems were acetate buffer for pH 4-6, Tris-HCl for pH 7-8 and glycine NaOH for pH 9.

UI/mg. This activity (188UI/mg protein) was close to that of pure asparaginase (200 UI/mg protein), which showed that fairly pure asparaginase preparation has been obtained. Overall yield of 87 mg/L (i.e., 16356 UI/L) of the purified recombinant asparaginase was achieved at the shake flask level.

Characteristics of Purified L-Asparaginase

The purified enzyme was studied for its temperature and pH and profile where it showed activity across a wide pH range with an optimum in the range of 6 to 7 (Figure 5). The activity of the purified enzyme was studied at different temperature degrees (25 to 60°C), exhibited its maximum value at 43°C and decreased beyond 50°C (Figure 6). The thermostability of the purified enzyme was analyzed at 50°C for 90 min and the enzyme retained around 47% of its initial activity even after 30 min of incubation and 24% after 60 min of incubation at 50°C (Figure 7). A sharp decrease was noticed in the thermostability where it retained only 6% of the purified enzyme at 90 min. The affinity of the recombinant L-asparaginase towards the natural substrate L-asparagine was studied and the K_{cat} and K_m values of were investigated as shown in Table 1.

Antiproliferative activity of the purified recombinant L-asparaginase II

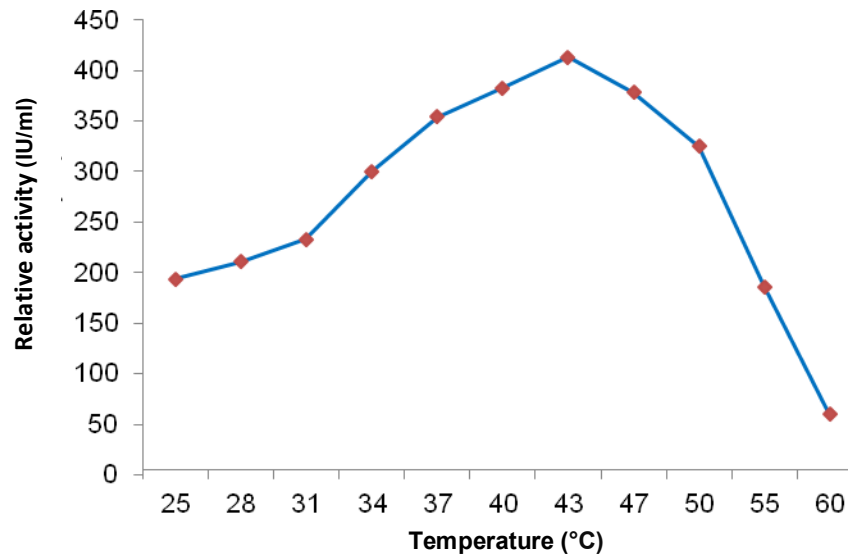Antiproliferative effects of the purified recombinant

Figure 6. Temperature range for the activity of the recombinant asparaginase. The activity for enzyme was measured at different temperature range (25∘C-60∘C). Asparaginase enzyme showed an optimum temperature at 43°C.
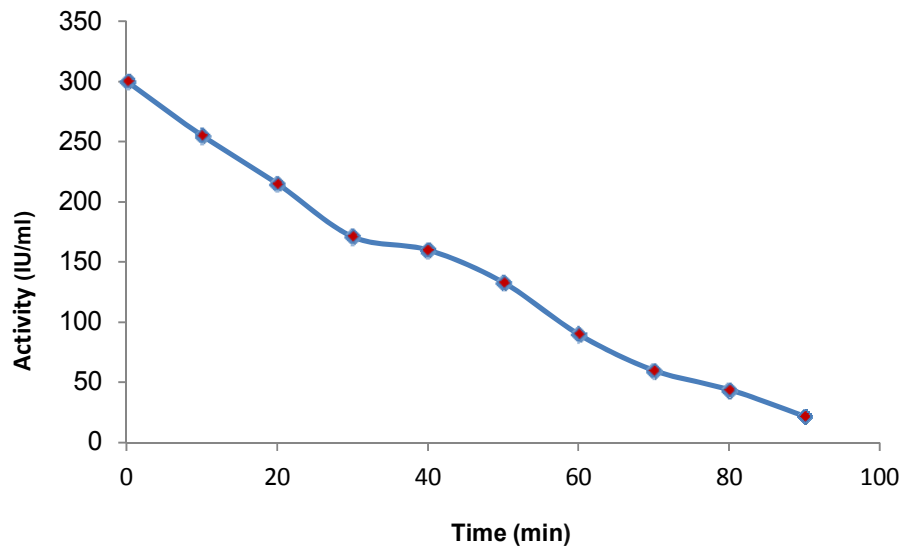

Figure 7. Thermostability of the purified recombinant asparaginase enzyme. At 50°C, a percentage activity retention at various time intervals of (10-90) minutes of incubation.

L-asparaginas from *E. coli* (KH027) was evaluated after 24, 48, 72 and 96 h of incubation against two leukemia cell lines (RS4,11 and HL-60) and peripheral blood mononuclear cells (PBMC). Purified enzyme did not exhibit any observable effect on proliferation of PBMC. In this test, obtained data indicated that the dose of 100 µg/mL and time-response effect of 96 h of incubation are required to cause a reduction value of 50% in cell viability of RS4; 11 (Table 2). Cell viability of 50% in the leukemic cells HL-60 was noticed with a concentration of 200 µg/mL with an incubation period of 72 h (Figure 8). In case of HL-60, it was noticed that the cell viability increased again when the incubation prolonged from 72 to 96 h of incubation (Table 2).

Table 1. Kinetic parameters of the recombinant l-asparaginase II:

E_a (kJ mol^{-1})	K_m (mM^{-1})	K_{cat} (×10^3 s^{-1})	K_{cat}/K_m (×10^5 M^{-1} s^{-1})
11.4	3.8 ± 0.1	2.92 ± 0.08	7.68

Table 2. Cell viability of leukemic cells after treatment with purified L-asparaginase from *E. coli* (KH027)

Incubation time (h)	Conentration (µg/ml)	Cell viability (%) RS4-11	Cell viability (%) HL-60
	15	96	83
	25	92	90
48	50	88	88
	100	82	75
	200	76	64
	15	80	77
	25	78	73
72	50	70	68
	100	66	54
	200	59	**50**
	15	73	86
	25	71	83
96	50	68	71
	100	**50**	82
	200	43	63

DISCUSSION

One of the main goals of protein engineering is to produce thermostable enzymes. However, the thermal stability of a protein is not readily predictable from its 3D structure. Directed evolution is the best way to alter this enzyme property (Giver et al., 1998; Oh et al., 2002; Hao and Berry, 2004; Bommarius et al., 2006).Some progresses made in the studies of protein thermostability have shown some promising ways through a single amino acid substitution (Imanaka et al., 1986, 1992).In this work, asparaginase gene of a thermotolerant *E. coli* strain (KH027) was expressed as a His-tagged protein.On sequencing and BLAST analysis, thecloned gene showed 98% similarity with L-asparaginase type II of *E. coli* KO11. Theaddition of histidine tag to the C-terminal region of the gene permits the purification of the recombinant protein through nickel affinity chromatography.

There are very few reports on the efficient release of recombinant proteins into the culture medium of *E. coli*. Although extracellular secretion of recombinant proteins has been achieved by co-expression of permeabilizing proteins, it leads to non-specific secretion and also requires controlled low level expression of the co-expressing gene to avoid problems of cell lysis and lethality (Makrides, 1996; Jonasson et al., 2002). There is no space limitation for the accumulation of the protein, moreover the cells need not be lysed or processed for extracting the desired protein. The extracellular compartment is protease deficient which is important for proteins susceptible to proteolysis, the protein exported to the medium is mostly soluble, biologically active, has an authentic N-terminus and also results in minimization of the potentially harmful action of recombinant proteins against the host cells. In this work we have shown that the use of pelBleader sequence, along with induction strategy significantly improves extracellular secretion of asparaginase to yield reasonably high amounts of protein in the culture medium. Induction at late log phase resulted not only in higher volumetric activity but more importantly promoted secretion specificity, where the recombinant protein constituted a major fraction of the total protein present in the extracellular medium.

Upon SDS PAGE and Coomassie staining ∼37 kDa protein band was visible in induced fractions. It was consistent with a homodimeric enzyme expression of recombinant asparaginase gene. The recombinant protein from the soluble fraction was purified through Ni-NTA column and dialysed fractions. After PAGE analysis, the apparent molecular weight of the oligomeric protein was ∼141 kDa. Maintaining a high specific growth rate, while running a high cell-density culture, is required to enhance the production of recombinant protein. This helps to overcome the metabolic stress associated with recombinant protein expression (Sanden et al., 2003). The specific growth rate has a direct effect on the export of periplasmic proteins into the medium (Shokri et al., 2002). The overall yield of the purified recombinant asparaginase was 87 mg/L (that is, 6.5-fold) compared to the previously reported yield of 10-15 mg/L of purified protein when recombinant asparaginase was expressed in the periplasm (Harms et al., 1991). It should be noted that this level of expression for the recombinant asparaginase was achieved under shaking condition. This means that the extracellular environment is the preferred location for the accumulation of recombinant proteins.

Temperature and pH profiles of the purified enzyme were studied. Results correlate with the earlier reports of
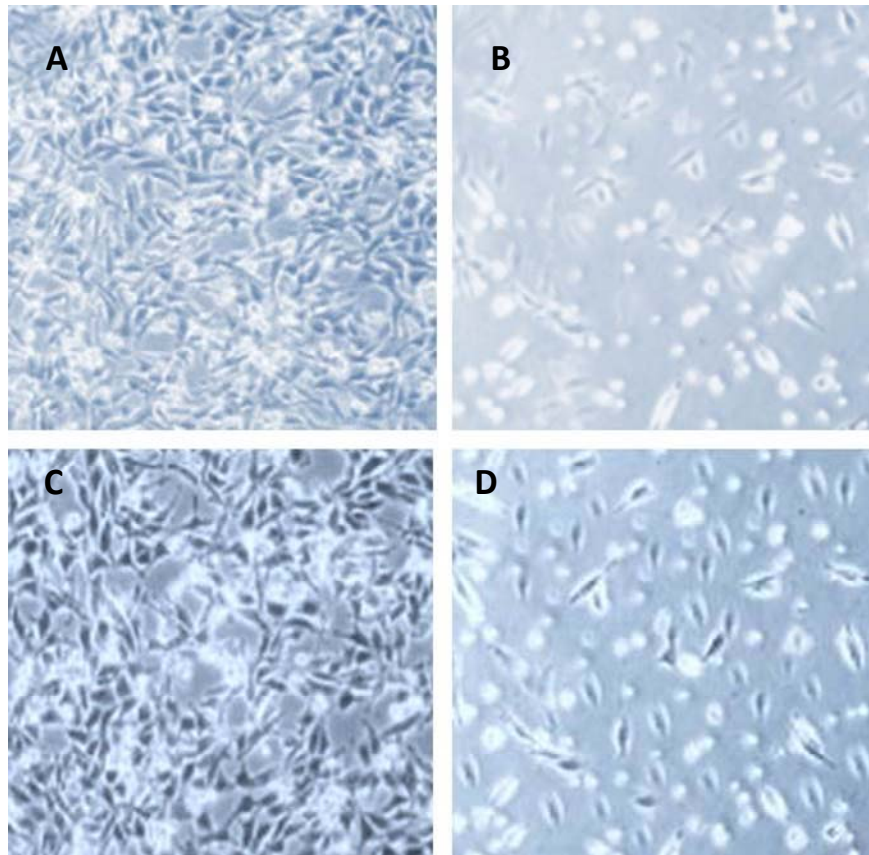
Figure 8. Antiproliferative effect of the recombinant asparaginase enzyme.A and C are cells (1 × 10^4 cell/well) of non-treated leukemic RS4;11 (b) and leukemic HL-60, respectively. B and D are treated cells of RS4;11 HL-60 with of L-asparaginase (5 IU/ml) for 48 h, respectively. Images of were taken after a 48 h-incubation.

L- asparaginase II from *E. coli* and other organisms (Joseph, 1976; Stecher et al., 1999). Based on the control, the enzyme displayed 50 and 23% remaining activity of its initial one even after 60 and 90 min of incubation at 50°C. At 55°C the thermal stability of wild type enzymes of *E. carotovora* and *E. chrysanthemi* displayed 19.9 and 37.2% remaining activity, respectively (Kotzia and Labrou, 2009). Theobtained K_m value is higher than the reported K_m for L-asparagine in the casesof *E. coli* and *Erwinia* enzymes (Kotzia and Labrou, 2009; Christian et al., 2000).

The anti-proliferative effect of the purified enzyme of L-asparaginase produced *E. coli* (KH027) was evaluated. There was no effect on proliferation of PBMC which reflects this activity of L-asparaginase to cancer cells over normal lymphocyte. Reduction in cell viability by 50% in case of HL-60 was achieved after 72 h and after 96 h in case of line RS4;11. Other studies have demonstrated increased asparagine synthetase (AS) expression in cells treated with L-asparaginase which allows these leukemia cells to become resistant to the treatment. Other adaptive processes may provide a substrate to asparagine synthetase (for example, aspartate or glutamine) which comes from intracellular and extracellular sources (Aslanian et al., 2001; Aslanian and. Kilberg, 2001).

Conclusion

In this study, we have isolated L-asparaginase II gene from a thermotolerant *E. coli* strain, cloned in pET20b vector with 6His residues at the C-terminus downstream to the T7 promoter and *pel*B leader sequence, and biochemically characterized. L-asparaginase produced by *E. coli* (KH027) displayed good results on two Leukaemia cell lines. These results prompted for further investigations and can nominate this type of enzymes as candidates in therapy where the pharmaceutical proteins should maintain appropriate serum levels for imparting its complete potency. Apart from the clinical use, this enzyme can be used in fried starchy food preparations to

reduce the acrylamide content, a potent carcinogen formed during the baking process by the reaction of asparagine and sugar at high temperature.

Conflict of interests

The author(s) have not declared any conflict of interests.

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