academicJournals

Vol. 15(31), pp. 1681-1691, 3 August, 2016
DOI: 10.5897/AJB2016.15425
Article Number: B1D21F359843
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Optimization, purification and characterization of recombinant L-asparaginase II in *Escherichia coli*

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Received 21 April, 2016; Accepted 19 July, 2016

We studied optimal L-asparaginase sequence from GenBank accession number X12746 encoding for L-asparaginase from *Erwinia chrysanthemi* NCPPB1125. The expression level of recombinant L-asparaginase was determined as 78% of the total proteins. The purified L-asparaginase had a molecular mass of 37 kDa with specific activity of 312.8 U/mg. Kinetic parameters, K_m, V_{max}, K_{cat} and K_{cat}/K_m of purified enzyme were found to be 0.5 mM, 500 U/mg, 14.9×10^3 s^{-1}, and 29.9×10^3 $mM^{-1}s^{-1}$, respectively. Temperature and pH optimum were observed at 45ºC and pH 7.5, respectively. The enzyme exhibited about 20 and 60% retention of activity following 100 min incubation at 55 or 40°C, respectively. The activity of enzyme was inhibited by EDTA, Hg^{2+}, Cu^{2+}, Ni^{2+}, and enhanced by Mg^{2+}. Detergents (Tween 20, Tween 80, Triton X-100, and Triton X-114) decreased enzyme activity. DTT and DMSO at appropriate concentrations enhanced enzyme activity. *In vitro* anti-cancer activity was performed using different tumor cell lines. Concentration of recombinant L-asparaginase at 50 µg/ml inhibited 45.32, 48.22, 53.68, 51.22% with HL-60, P388, P3X63Ag8, SP2/0-Ag14 cell lines. Recombinant L-asparaginase was expressed successfully in *Escherichia coli* with high expression level, had a high specific activity and antiproliferative effect on several tumor cell lines.

Key words: Characterization, *Erwinia chrysanthemi*, L-asparaginase, purification, tumor cell line.

INTRODUCTION

Acute lymphocytic leukemia (ALL) is a type of blood cancer that results when abnormal white blood cells (leukemia cells) grow quickly and crowd out the bone marrow preventing the normal red blood cells, white blood cells, and platelets that body needs. ALL incidences occur most frequently in people under the age of 15 or over 45. L-asparaginases are a cornerstone of treatment protocols for ALL (Silverman et al., 2001; Pieters and Carroll, 2008). L-asparaginase is also used in treatment of acute myeloid leukemia (AML) (Emadi et al., 2016; Tagde et al., 2016b). Beside, L-asparaginase induced significant growth inhibition and apoptosis in K562 and KU812 cells so it might be a promising new therapeutic strategy for chronic myeloid leukemia (CML) (Song et al., 2015). Normal cells can synthesize L-asparagine by asparagine synthetase. In contrast, tumor

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cells slowly synthesize L-asparagine and are dependent on an exogenous supply. So L-asparaginase destroys extracellular source of L-asparagine, inhibits protein synthesis in lymphoblasts inducing their apoptosis (Duval et al., 2002). L-asparaginase (EC 3.5.1.1, L-asparagine amidohydrolase) catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonium. The enzyme is considered to play a significant role in L-asparagine metabolism in normal cells. There are two types of L-asparaginases: L-asparaginase I, was used to reduce the level of acrylamide in food industry (Friedman, 2003; Yano et al., 2008). Whereas, L-asparaginase II was used to treat leukemia. There are many sources of L-asparaginase such as bacteria, fungi, yeast, actinomycetes, algae and plants (Verma et al., 2007). To date, L-asparaginase gene had been cloned from variety of host such as *Escherichia coli* (Wang et al., 2001), *Erwinia chrysanthemi* (Kotzia and Labrou, 2007), *Erw. carotovora* (Pourhossein and Korbekandi, 2014), *Yesinia pseudotuberculosis* (Sidoruk et al., 2011), *Thermococcus kodakarensis* (Hong et al., 2014), *Saccharomyces cerevisiae* (Ferrara et al., 2010), and expressed in different expression systems including *E. coli* (Kotzia and Labrou, 2007; Magdy and Mohammed, 2008), *Bacillus subtilis* (Jia et al., 2013), and *Pichia pastoris* (Ferrara et al., 2006). At present, clinically useful L-asparaginases are obtained from either *E. coli* or *Erw. chrysanthemi.* In 2002, Duval compared *E. coli*-asparaginase with Erwinia-asparaginase in the treatment of childhood lymphoid malignancies, the study showed that *E. coli*–asparaginase can be recommended for first-line therapy reserving Erwinia-asparaginase for high sensitive *E. coli*-asparaginase patients (Duval et al., 2002). Here, we examined optimization of rASPG expression, along with the purification and characterization of the recombinant L-asparaginase from *Erw. chrysanthemi* in *E. coli.* We used pET21a+ vector with highly-inducible T7 promoter and induced by isopropyl-β-D-thiogalactopyranoside (IPTG) to express L-asparaginase. The results suggest that rASPG was purified with high activity and had high potential for antiproliferative application.

MATERIALS AND METHODS

Plasmid, bacterial strains and cell lines

The L-asparaginase gene based on L-asparaginase sequence from GenBank accession number X12746 was optimized codon for expression in *E. coli*, produced and inserted into vector pUC57 (pUaspg) by GenScript (USA). The DNA fragment (981 bp) encoding the mature L-asparaginase (without the signal peptide of 21 N-terminal amino acids) from pUaspg was inserted into vector pET21a(+) resulting in plasmid pEaspg to express in *E. coli*.

E. coli BL21(DE3) cells (F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) (Fermentas). Luria-Bertani medium (LB) containing 1% (w/v) bacto tryptone; 0.5% (w/v) yeast extract; 1% (w/v) NaCl; pH 7-7.5 was used for cultivation of *E. coli*. The LB agar plates contained additionally 2% (w/v) agar and100 μg ampicillin/ml. Four tumor cell lines: human promyolecytic leukemia HL-60, mouse lymphocytic leukemia P388, mouse myeloma P3X63Ag8 and Sp2/0-Ag14 and a normal cell line mouse mus musculus NIH/3T3 were obtained from the Bioassay group (Institute of Biotechnology, Vietnam).

Chemicals

L-asparagine, Nessler reagent and RPMI-1640 media were from Sigma (Louis, USA). IPTG, trichloroacetic acid, bactotryptone and yeast extract were from Bio Basic Inc (New York, USA). DEAE-sepharose and Sephacryl S200 were supplied by Pharmacia Co. (GE Healthcare. SDS was supplied Sigma Aldrich Co. (St, Louis, USA). Tween 20 and Tween 80 were from Bio Basis Inc. (New York, NY, USA), and Triton X-100, Triton X-114 and EDTA by Merck (Darmstadt, Germany). All chemicals were used in the experiments in their purified forms.

Plasmid construction

The L-asparaginase gene based on L-asparaginase sequence (1044 bp) from GenBank accession number X12746 was optimized codon for expression in *E. coli*, synthesized and inserted into vector pUC57 (pUaspg) by GenScript. The DNA fragment (981 bps) encoding the mature L-asparaginase (without the signal peptide of 21 N-terminal amino acids) from pUaspg was amplified using pUaspg as template and two oligonucleotides, 21 ASP -F (5'- GCC ATA TGG ATA AAC TGC CGA -3') and LASP-his R (5'- AAG CTC GAG TCA GTA GGT ATG GAA G -3') were designed as primers for introduction of the underlined *Nde*I and *Xho*I restriction sites, respectively. The PCR mixture contained 2.5 μl 10 × PCR buffer; 2 μl of 2 mM dNTP; 2.5 μl of 25 mM $MgCl_2$; 1 μl plasmid pUaspg (50-100 ng); 0.25 μl 5 unit *Taq* polymerase and 1 μl each primer (10 pmol), supplemented with 14.75 μl distillated water to fulfill 25 μl. The thermocycler conditions were as follows: 95°C/4′; 30 cycles of (95°C/45″, 55°C/45″, 72°C/45″); 72°C/10′. The polymerase chain reaction (PCR) products amplified from the pUaspg with both primer 21 ASP-F and L-ASP-his R were digested with *Nde*I and *Xho*I and purified using Gel Extraction Kit (Qiagen) in accordance with the manufacturer's instructions. It was followed by ligation of the *Nde*I-*Xho*I digested *aspg* products with pET21(a+) linearized by the same enzymes, resulting in pEaspg under the control of the T7-promoter induced by IPTG (isopropyl-β-D-thiogalactopyranoside) and possessing the ampicillin marker. The L-asparaginase encoded by the plasmid pEaspg contains the mature L-asparaginase without the 6 × histidine-tag and no leader sequence. The pEaspg plasmid was transformed in *E. coli* DH5α and BL21 (DE3) cells by heat shock method as described previously (Quyen et al., 2007).

Soluble rASPG expression

The transformant *E. coli* BL21/pEaspg was cultivated in 5 ml of LB medium with 100 μg/ml ampicillin at 37°C with agitation at 220 rpm overnight. This culture was used to inoculate 250 ml of the same media, and grown to an optical density at 600 nm ($OD_{600\ nm}$) 0.4 - 0.6 at 37°C with shaking at 220 rpm. IPTG was then added to 1 mM final concentration, the culture was continuously incubated at 28°C with agitation of 220 rpm for 6 h of induction. Cells were harvested by centrifugation at 8000 rpm and 4ºC for 5 min.

Enzyme assay

Activity analysis of L-asparaginase II was performed according to Chung's report (Chung et al. 2010) comprising the following steps: The 100 μl samples were mixed with 900 μl 0.01 M L-asparagine in 50 mM Tris buffer, pH=8.6, and 1 mL of assay mixture were incubated for 10 min at 37°C for enzymatic reaction. The reaction

was interrupted with 100 µL of 1.5 M trichloroacetic acid and the samples were centrifuged before the addition of 100 µL Nessler's reagent to measure the released ammonia after L-asparagine hydrolysis. All the measurements were done spectrophotometrically at 480 nm. The enzyme activity of recombinant protein was determined using an ammonium sulphate calibration curve. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µM of ammonia per minute.

Effect of IPTG concentration

IPTG control T7 *lac* promoter so that IPTG concentration may be affected expression level of recombinant protein. To assess the effects of IPTG concentration on the enzyme specific activity, eight flasks 100 ml contain 25 ml per flask of the recombinant clone culture was grown to OD_{600nm} of about 0.4 - 0.8 (for approximately 4 h), and induced by adding IPTG in final concentrations of 0; 0.2; 0.4; 0.6; 0.8; 1; 1.2; 1.4 mM, respectively. After 6 h of induction, bacterial cells were harvested and analyzed for the enzyme specific activity. The best IPTG concentration was selected and applied for the next stage.

Effect of amp concentration

Six flasks 100 ml were contained 25 mL LB broth were prepared. Amp with final concentrations of 25; 50; 100; 150; 200 and 250 µg/ml were added to each flask, respectively. Half ml of the overnight culture was inoculated into each of flasks. The culture was cultivated at 37°C with agitation at 220 rpm until an $OD_{600\ nm}$ of 0.4 - 0.8 was reached (for approximately 3 h) then IPTG was added. After 6 h of induction, bacterial cells were harvested to analyze for the enzyme specific activity. The best Amp concentration before induction was selected and applied for the next stage.

Effect of inoculum size

To evaluate the effect of inoculum size on the enzyme expression, the overnight culture were inoculated, inoculum of different sizes 0.5%; 1; 2, and 5% (v/v) into four flasks 100 ml which contained 25 ml LB, the recombinant clone culture was grown at 37°C in LB medium for 3 h, and then induced by adding IPTG. After 6 h of induction, enzyme specific activity was evaluated. Inoculum size with higher protein production was determined.

rASPG purification

The rASPG was expressed in *E. coli* BL21(DE3). To purify rASPG, 0.7 g cells from a 100 ml culture in LB medium were harvested by centrifugation at 8000 rpm and 4°C for 5 min, and resuspended in 8 ml of 50 mM Tris HCl buffer pH 8.6, sonicated and centrifuged at 12000 rpm and 4°C for 15 min.

Gel filtration

The supernatant cell free extract containing the crude L-asparaginase was loaded into Sephacryl S-200 column (2.6 × 6 cm) equilibrated with 50 mM potasium phosphate (pH 8) and eluted with the same buffer at the flow rate of 0.5 ml per minute. Fractions showing L-asparaginase activity were pooled and concentrated with bench top protein concentrator at 4°C. The homogeneity of the protein was checked by SDS -PAGE.

DEAE chromatography

The concentrated enzyme solution was added on the top of Diethylaminoethyl Sepharose ion exchange column (DEAE - Sepharose) (2.6 × 6 cm) equilibrated with 50 mM Tris HCL (pH 8.6). The column was washed with 2 volumes of starting buffer and the protein was eluted with linear gradient of NaCl (0 - 1 M) prepared in 50 mM Tris HCL (pH 8.6) at the rate of 30 ml per hour. The eluate was collected with 1.5 ml per fractions. The fractions showing L-asparaginase activity were stored at 4°C.

The molecular mass of the rASPG was determined by 12.5% SDS polyacrylamide gel electrophoresis with Biometra equipment (Laemmli, 1970). Proteins were visualized by staining with 0.1% (w/v) Coomassie Brilliant Blue R-250. Protein concentrations were estimated by the method of Bradford with the bovine serum albumin as standard (Bradford, 1976).

Kinetic parameters determination

Aliquots of 100 µl of reconstituted enzyme were prepared and added with different concentrations of L-asparagine ranging from 1 mM to 4.5 mM prepared in 50 mM Tris HCl. The apparent kinetic parameters (K_m, V_{max}, K_{cat} and K_{cat}/K_m) of enzyme for L-asparagine were determined by Lineweaver-Burk plots method.

Temperature and pH optimum

The pH and temperature optimum of rASPG were determined by measuring the activity as described above using 100 mM potassium acetate buffer (pH 4-6), potassium phosphate buffer (pH 6.5-8), and Tris HCl buffer (pH 8-10) at 37°C for 30 min, and in the temperature range of 20 - 65°C at pH 8.6 for 30 min.

Temperature and pH stability

For the determination of temperature and pH stability, the purified enzyme (0.7 µg for each reaction) was incubated at 40 and 50°C, and pH 6; 7; 8 the activities were measured at various time intervals of 20; 40; 60; 80 and 100 min. Percentage of residual activities was calculated based on the untreated control activity, which is taken as 100%.

Effect of metal ions and EDTA, detergents, DTT, DMSO

The purified enzyme (0.7 µg protein for each reaction) was preincubated in presence of 10 mM of various metal ions (Ca^{2+}, Cu^{2+}, Fe^{3+}, Fe^{2+}, Mg^{2+}, Ni^{2+}, Zn^{2+}, K^{+}, Hg^{+}, Pb^{2+}), ethylenediamine tetraacetic acid (EDTA), in presence of 1-5% (w/v) of various detergents (Tween 20, Tween 80, Triton X-100, and Triton X-114), in presence of 0.1-5 mM dithiothreitol (DTT) and in presence of 0.1-2% (w/v) dimethyl sulfoxide (DMSO) at 37°C for 1 h. The residual activity was then determined at pH 8.6 and 37°C.

Cell culture and proliferation assay

Cells were routinely cultured in RPMI 1640 media. It were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate and 50 units/ml penicillin, 50 µg/ml streptomycin. Cells were cultivated in a humid atmosphere (5% CO_2, 37°C) (Takahashi et al., 2015; Hasegawa et al., 2016; Rajabi et al., 2016; Tagde et al., 2016a). Cells were seeded in 96-well plates at 1 × 10^4 cells per well. rASPG was added at concentrations of 0.4; 2; 10 and 50 µg/ml. After 72 h of continuous enzyme exposure, 10 µl of MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was added in each well. The plates were incubated for 1 - 4 h at

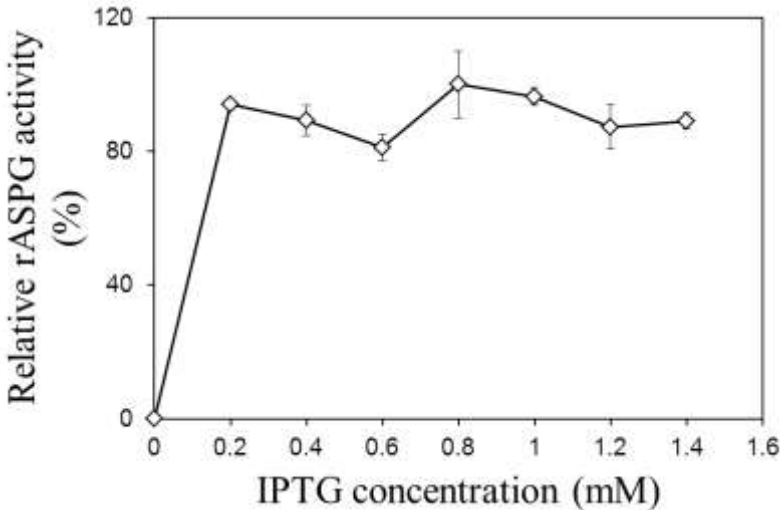


Figure 1. Effect of IPTG concentrations on rASPG activity. Relative activity was expressed as a percentage of control (100% rASPG activity was 31.7 U/mg).

37°C and the formazan product was measured at 490 nm. The experiments were performed in triplicate in three independent sets. Cell survival was calculated by subtracting the background absorbance of media alone and then dividing the absorbance of experimental wells by the absorbance of the control (untreated) wells (Neisius and Moll, 1989; Sonawane et al., 2014; Tagde et al., 2014).

RESULTS AND DISCUSSION

Optimization of rASPG expression

In previous studies, we expressed a plasmid pET22b(+) containing the full-length *aspg* gene in *E. coli* BL21(DE3) but no expression was detected by SDS-PAGE analysis, and recombinant plasmid pEaspg containing *aspg* gene and expression vector pET21a(+) was previously constructed without signal peptide with his tag, pEaspg was transformed in *E. coli* BL21(DE3) and expressed at 28°C, recombinant protein was higher levels expressed but had low activity (data not show). In this study, we constructed plasmid without the 6 × histidine-tag and no leader sequence. The DNA fragment encoding the mature L-asparaginase with stop codon, truncated 21 N-terminal amino acids was inserted into pET21(a+) vector resulting in the recombinant plasmid pEaspg. The transformant *E. coli* BL21/pEaspg was grown in LB medium for the rASPG production. After IPTG induction, the cells were collected used for enzyme activity assay. The *E. coli* BL21/pEaspg transformant was showed high production of L-asparaginase (data not show). To increase the specific as well as volumetric yield of recombinant L-asparaginase, a variety of independent cultivation parameters such as inducer concentration, ampicillin concentration and inoculum size were optimized.

Effect of IPTG concentration

IPTG concentration did not affect enzyme activity. Although, IPTG concentration increase from 0.2 to 1.4 mM but there are significant changes observed in enzyme activity (Figure 1). The maximum enzyme activity was at 0.8 mM IPTG (100%), but no significant decrease at 0.2 mM IPTG (95%). Thus, 0.2 mM IPTG was selected for the next stages. The similar results were reported in the study of Sidoruk et al. (2011) and Bahreini et al. (2014). In 2011, Vidya demonstrated that enzyme activity was decrease with the increase of IPTG from 10 µM to 50; 100 and 400 µM (Vidya et al., 2011)

Effect of Amp concentration

Amp was supplemented in culture medium to prevent the overgrowth of plasmid-free cells. Amp also affects the number of plasmid per cell. Chong reported that increase in the concentration of Amp in cultures causing the increase of the plasmid copy number in cells (Chong et al., 2003). Bahreini assumed that the increase in plasmid copy number corresponding to the rise of protein expression, but Bahreini's research has demonstrated that the higher levels of Amp had no effect on the L-asparaginase activity (Bahreini et al., 2014). We have found a similar result. It seems that the increase of Amp level higher than 25 µg/ml slightly decrease specific enzyme activity (Figure 2).

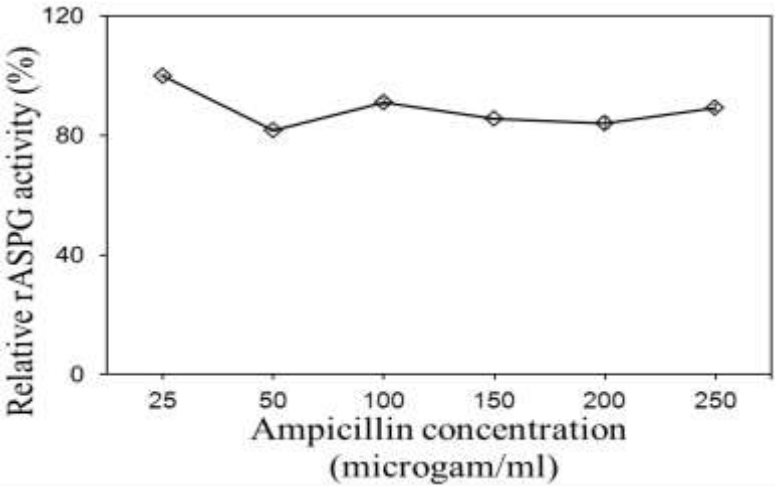


Figure 2. Effect of Amp concentration on rASPG activity. Relative activity was expressed as a percentage of control (100% rASPG activity was 35.9 U/mg).

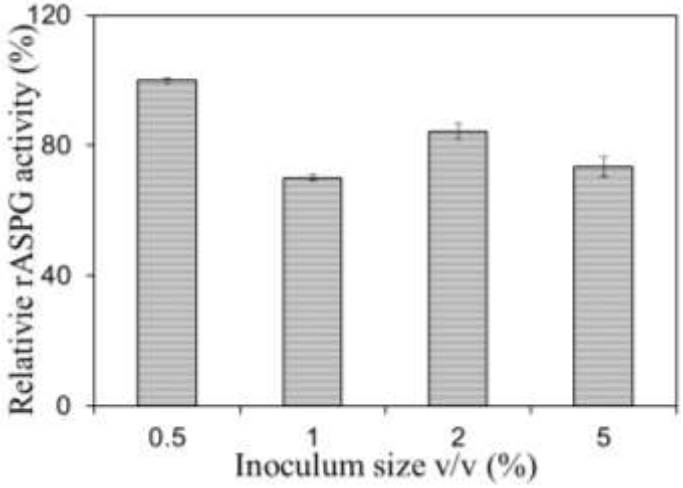


Figure 3. Effect of inoculum size on rASPG activity. Relative activity was expressed as a percentage of control (100% rASPG activity was 37.2 U/mg.

Effect of inoculum size

After 4 h culture, four flasks with different inoculum size 0.5; 1; 2 and 5% (v/v) and the value of $OD_{600\ nm}$ reached 0.5; 0.6; 0.7; 0.8, respectively. Our study showed that the inoculum size of 0.5% were found to be the most suitable condition for maximum enzyme activity, the increase in inoculum size is the reason for the decrease in enzyme activity (Figure 3). In general, the increase in cell density of bacterial expression enhances recombinant protein production. Khushoo et al. (2004) reported that induction IPTG during late log phase ($OD_{600\ nm}$= 4.5) resulted in maximum secretion of the recombinant asparaginase and specific activity (Khushoo et al., 2004). Later, Kenari et al. (2011) optimized inoculum size of 10% (Kenari et al., 2011). Bahreini et al. (2014) reported that in L-asparagi nase production level with the maximum production at the highest cell density of $OD_{600\ nm}$= 10 (Bahreini et al., 2014). A simple explanation of these findings that inoculum sizes can be attributed to decrease in the concentration of the medium components, such as O_2 level, pH, and nutrients.

Purification of rASPG

The expression level of rASPG in optimized conditional expression was 78% of the total cellular protein by

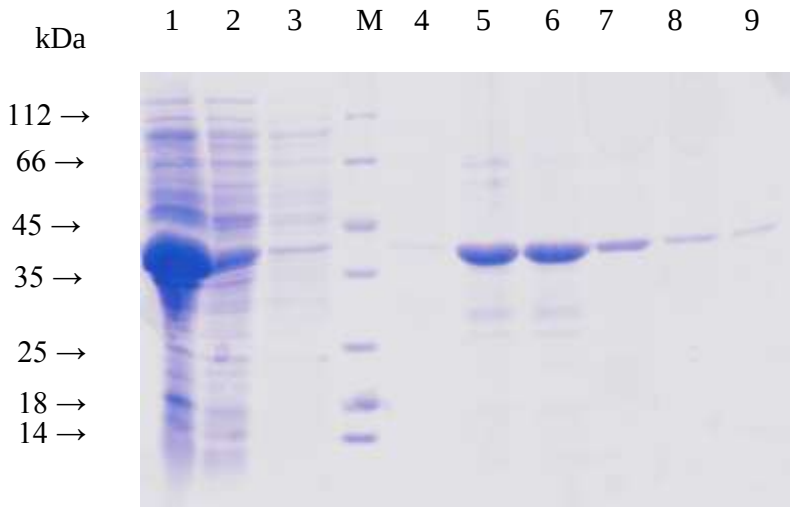


Figure 4. (A) SDS-PAGE of the overexpressed and purified of rASPG in *E. coli* BL21(DE3) (Lane M: molecular mass of standard proteins (Fermentas, Thermo Fisher Scientific Inc.,Waltham, USA); lane 1-2: *E. coli* pEaspg cell lysate after (1) and before (2) optimal conditional expression; lane 3: *E. coli* pEaspg cell lysate before IPTG induction lane 4-5-6: fractions of purified rASPG eluted from Sephacryl S-200, lane 7-8-9: fraction of purified rASPG eluted from DEAE-sepharose.

Table 1. Purification procedure of rASPG from the cell lysate of *E. coli* BL21/pEaspg.

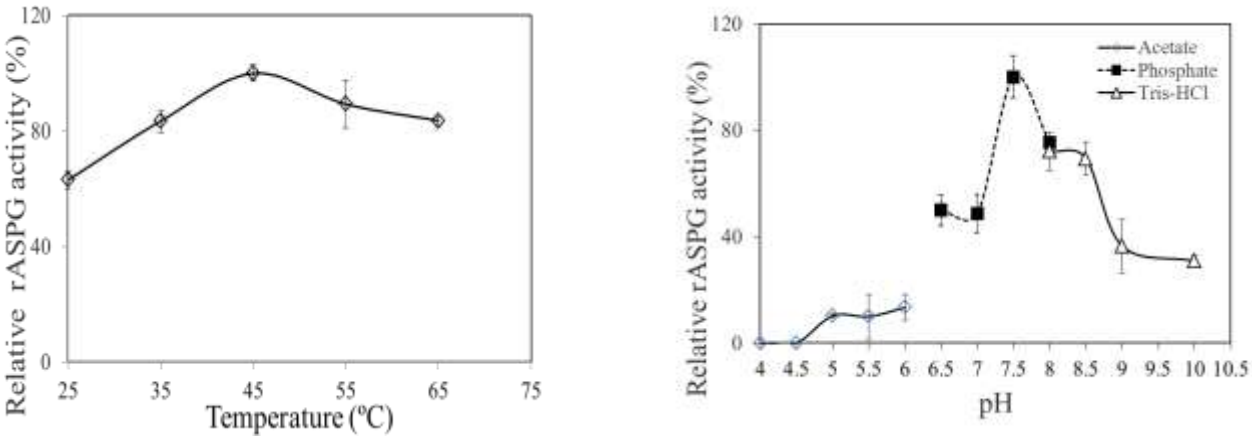
Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Cell lysis	385.1	9.6	39.9	100	1
Sephacryl S-200	360.1	2.5	141.3	93.5	3.5
DEAE-Sepharose	68.5	0.2	312.8	17.8	7.8

densitometry scanning, resulting in 10% increase in the production compared to the original condition. The expression level of rASPG in *E. coli* system was reported to be approximate 50% in JM105, TG1, DH5α, AS1.357 and 75% in JM109 (Wang et al., 2001). The rASPG was purified from the cell lysis of *E. coli* BL21(DE3) by filter chromatography Sephacryl S-200 and DEAE Sepharose showed only one protein band about 37 kDa on SDS-PAGE (Figure 4, lane 7-9). The specific activity of recombinant L-asparaginase after two step purification obtained by 312.8 U/mg with a yield of 17.8% and purification factor of 7.8 (Table 1). The specific activity was very different: The activity of purified recombinant L-asparaginase II from *E. coli* K-12 express in *E. coli* BLR(DE3) was 190 U/mg (Khushoo et al., 2004), recombinant L-asparaginase II from *Erw. chrysanthemi* 3937 express in *E. coli* BL21(DE3) pLysS was 118.7 U/mg (Kotzia and Labrou, 2007), L-asparaginase II from *B. subtilis* express in *E. coli* JM109 (DE3) was 45.5 U/mg (Onishi et al., 2011) L-asparaginase from *Rhizomucor miehei* express in *E. coli* was 1,985 U/mg (Huang et al., 2014) and activity of purified L-asparaginase from *B. licheniformis* was 697.09 U/mg (Mahajan et al., 2014).

Characteristic of rASPG

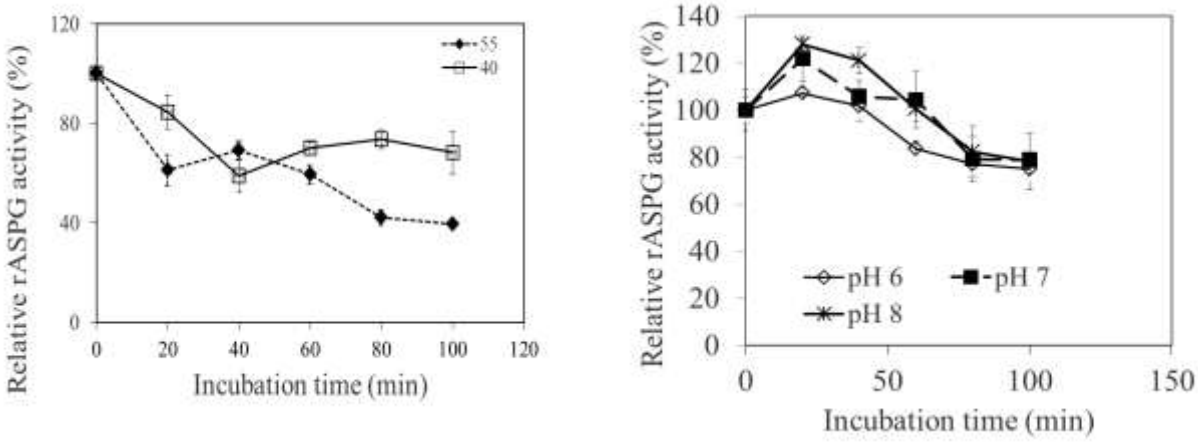
Temperature and pH optimum

The recombinant L-asparaginase from *Erw. chrysanthemi* had optimum temperature of 45°C (Figure 5A) and optimum pH of 7.5 in 100 mM Tris-HCl buffer (Figure 5B). It was similar to recombinant L-asparaginase II from the *B. subtilis* B11−06 in *B. subtilis*168 which had an optimum temperature of 45ºC and pH 7.5 (Jia et al., 2013). It was different from that of E. coli MTCC739 in *E. coli* BL21(DE3), which were 37°C and pH 6 (Vidya et al., 2011). The optimal temperature and pH of rASPG from a thermotolerant strain *E. coli* KH027 in *E. coli* DH5α was 43°C and pH 6 (Muharram et al., 2014). It was 37°C and pH 7.5 for rASPG from *E. coli* W3110 in *E. coli* BL21 (DE3) (Magdy and Mohammed, 2008) and that of rASP



A B

Figure 5. Temperature optimum of rASPG (A) and pH optimum of rASPG (B).



A

Relative activity was expressed as a percentage of control (100% rASPG activity was 293.7 U/mg)

B

Relative activity was expressed as a percentage of control (100% rASPG activity was 321.4 U/mg

Figure 6. Temperature stability of rASPG (A) and pH stability of rASPG (B).

G from *Withania somnifera* in *E. coli* BL21(DE3) was 37°C and pH 8 (Oza et al., 2011). The optimum temperature and pH of the wild L-asparaginase from *Cladosporium sp.* were 30°C and 6.3, respectively (Kumar and Manonmani, 2013). Consequently, the optimal temperature and pH of the recombinant enzyme is not same with different bacterial sources and different expression host.

pH and thermo stability

The thermal stability of the purified enzyme at 40 and 55°C was studied to find out the extent of temperature resistance of the enzyme. Around 60 and 30% of the initial activity was retained by the purified enzyme after 100 min of incubation at 40 and 55°C, respectively (Figure 6A). The earlier reports on the thermostability of different L-asparaginase preparations indicate that the native enzymes were unstable at high temperatures. Wild L-asparaginase from *Erw. chrysanthemi,* which expressed in *E. coli* BL21(DE3) retains ~40% of its initial activity after 7.5 min of incubation at 50°C and on mutagenesis, around 20% increase in activity retention was achieved (Kotzia and Labrou, 2009). The wild enzyme from *E. coli* W3110 that expressed in *E. coli* BL21 (DE3) retained about 22% of its activity at 60°C after an incubation of 30 min, the remaining activity of the

Table 2. Effect of metal ions on the rASPG activity.

Additives[a]	Residual activity (%)[b]
Control (no additive)	100 ± 6
$Al_2(SO_4)_3$	82 ± 1.7
$Ba(NO_3)_2$	109 ± 7
$CaCl_2$	88 ± 6
$CuSO_4$	77 ± 6
$FeSO_4$	91 ± 6
$HgCl_2$	115 ± 2
$MgSO_4$	72 ± 2
$MnSO_4$	88 ± 6
$NaCl_2$	108 ± 3
$NiCl_2$	123 ± 3
$Pb(NO_3)_2$	92+7
$ZnSO_4$	86 ± 6
$Al_2(SO_4)_3$	107 ± 2
$Ba(NO_3)_2$	100 ± 5
$CaCl_2$	72 ± 7

[a] The final concentration of additive (EDTA or inorganic salt) in the reaction mixture was 10 mM. [b] Relative activity was expressed as a percentage of control (100% rASPG activity was 307.4 U/mg).

Table 3. Effect of detergent on the rASPG activity.

Detergent	Residual activity (%)	
	1%	5%
Tween 20	87.65 ± 8	0
Tween 80	54.35 ± 2	0
Triton X-100	47.91 ± 5	31 ± 12
Triton X-114	45.76 ± 2	0

Relative activity was expressed as a percentage of control (100% rASPG activity was 207 U/mg).

Table 4. Effect of DTT on the rASPG activity.

Concentration of DTT (mM)	Residual activity (%)
0.1	98.10 ± 1.39
0.5	109.89 ± 4.17
1	146.57 ± 11.58
5	119.06 ± 9.73

Relative activity was expressed as a percentage of control (100% rASPG activity was 203.67 U/mg).

immobilized enzyme after similar treatment was 66.8% (Magdy and Mohammed, 2008). rASPG showed pH stability at a pH range 6-8. The residual rASPG activity was above 80% in comparison to the original activity after 100 min of treatment (Figure 6B).

Effect of metal ions and EDTA, detergents, DTT, DMSO

The rASPG activity was inhibited by EDTA (Table 2). This results is in agreement with results reported for L-asparaginase from *Actinomycetes* (Basha et al., 2009) and L-asparaginase from *Thermococcus kodakarensis* KOD1 in *E. coli* (Hong et al. 2014). But several researches reported that EDTA enhanced the enzyme activity (Raha et al., 1990; Warangkar and Khobragade, 2010).

Metal ions showed that rASPG inhibitory activity of 92-72% in order of $Na^+ > K^+$, $Fe^{3+} > Ca^{2+} > Ni^+ > Al^{3+} > Cu^{2+} > Hg^{2+}$ while Mn^{2+} enhancer activity of the enzyme by 123%. And Pb^{2+}, Mg^{2+}, Ba^{2+} showed a slightly enhance effect on rASPG activity, the residual activity was accounted for 108% -109% of the original activity and Zn^{2+} was not effect enzyme activity (Tab.2). The same results was also found in the report that the activity of native L-asparaginase from *Cylindrocarpon obtusisporum* MB-10 was inhibited by Zn^{2+}, Fe^{2+}, Cu^{2+}, Hg^{2+} and Ni^{2+} (Raha et al. 1990) and the activity of rASPG from *Thermococcus kodakarensis* KOD1 was inhibited by Ca^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, enhanced by Mg^{2+} (Hong et al., 2014). Warangkar and Khobragade, (2010) reported that L-asparaginase form *Erw. carotovora* was loss of activity with Hg^{2+}, Ni^{2+}, Cd^{2+}, Cu^{2+}, Fe^{2+} , and Zn^{2+}, too, but Na^+ and K^+ acting somewhat as an enhancer and Mg^{2+} inhibited enzyme activity. All detergents (Tween 20, Tween 80, Triton X-100, and Triton X-114) showed an inhibitory effect on rASPG activity. The higher concentration, the detergents are more inhibited enzyme activity (Table 3).

Interestingly, the addition of DTT at low concentration of 0.1 - 0.5 mM was not significantly effected enzyme activity, but at higher concentration of 1 - 5 mM enhanced the enzyme activity by 46% and 19%, respectively (Table 4). L - asparaginase of activity from *Erw. carotovora* was aslo reported to enhance in presence of thiol protecting reagents like DTT and it has been explained by asparaginase possesses the thiol group binding domain with high affinity towards free-SH group containing effectors (Warangkar and Khobragade, 2010). The addition of lower concentration DMSO (0.1 and 0.5%), enzyme activity was not effect, but the rASPG activity remained much higher 152% by the addition of DMSO at higher concentration (1 mM). When concentrations of DMSO was higher (1.5; 2%), the level of increased enzyme activity were decreased (Table 5).

Kinetic parameters

The K_m, V_{max}, K_{cat} and K_{cat}/K_m obtained for rASPG *Erw. chrysanthemi* expressed in *E. coli* with L-asparagine

Table 5. Effect of DMSO on the rASPG activity.

Concentration of DMSO (%)	Residual activity (%)
0.1	101.70 ± 0.93
0.5	108.25 ± 0.93
1	152.46 ± 10.65
1.5	122.01 ± 12.97
2	112.84 ± 7.41

Relative activity was expressed as a percentage of control (100% rASPG activity was 203.67 U/mg).

Table 6. Cell death of four tumor cell lines after treatment with rASPG.

Concentration (µg/ml)	Cell death (%)				
	NIH/3T3	HL-60	P388	P3X63Ag8	SP2/0-Ag14
0.4	-0.22	-2.05	0.98	0.56	0.98
2	1.76	6.91	8.23	12.79	8.23
10	4.92	16.09	19.45	27.18	19.45
50	18.02	45.32	48.22	53.68	51.22
IC_{50}	> 50	> 50	> 50	41.67	48.09

substrate were 0.5 mM, 500 U/mg, 14.9×10^3 s^{-1}, 29.9×10^3 $mM^{-1}s^{-1}$, respectively. The K_m, V_{max}, K_{cat} and K_{cat}/K_m value of the recombinant L - asparaginase from *Thermococcus kodakarensis* KOD1 expressed in *E. coli* BLR(DE3) were found to be 2.6 mM, 1121 U/mg, 694 s^{-1}, and 266.9 $mM^{-1}s^{-1}$, respectively (Hong et al. 2014), of recombinant L-asparaginase from *B. subtilis* in *E. coli* were 2.06 mM, 45.5 U/mg, 98.6 s^{-1}, and 48 $mM^{-1}s^{-1}$ respectively (Onishi et al. 2011). The K_m, K_{cat} and K_{cat}/K_m value of rASPG from *Erw. chrysanthemi* 3937 in *E. coli* BL21(DE3) pLysS were 0.058 mM, 23.8×10^3 s^{-1}, and 411.8×10^3 $mM^{-1}s^{-1}$ respectively (Kotzia and Labrou, 2007). It was found that rASPG had low K_m value, high V_{max}, K_{cat} and K_{cat}/K_m value. So rASPG is highly specific for the substrate L-asparagine.

Anti-cancer activity of the rASPG

Abakumova et al. (2013) provided convincing evidence that L-asparaginase induced apoptosis as its principal process of causing death of leukemic and solid tumor cells (Abakumova et al., 2013). In our study, four tumor cell lines with increasing amounts of rASPG resulted in a significant increase in the number of dead cells (Table 6). At dose 0.4 µg/ml, rASPG did not significantly affect the growth of cells but at dose 50 µg/ml, rASPG inhibited HL-60 (45.32%), P388 (48.22%), P3X63Ag8 (53.68%), and SP2/0-Ag14 (51.22%). Normal cells were not significantly affected by rASPG at dose 50 µg/ml rASPG inhibited 18.02 % for NIH/3T3.

Muharran et al. (2014) indicated that recombinant L-asparaginase in *E. coli* caused a reduction of 50% in cell viability of RS4 at a dose of 100 µg/ml after 96 h of incubation, and a reduction of 50% in cell viability of HL-60 at a dose of 200 µg/ml after 72 h of incubation (Muharram et al. 2014). L- asparaginase from *Streptomyces acrimycini* NGP (130 U/mg) inhibits the gastric stomach cancer cells with an IC_{50} of 49.11 µg/ml (Selvam and Vishnupriya, 2013). L-Asparaginase from bean (*Vicia faba)* and white kidney bean (*Phaseoulus vulgaris)* seeds (2.75 and 1.47 U/mg) had low IC_{50} values 217.71 µg/ml and 187.86 µg/ml with Hep-G2 cells (Sanaa et al., 2012). IC_{50} value of L-asparaginase from *Aspergillus flavus* on MCF-7 cells was 120.875 µg/ml (Rani et al., 2011)

Conclusion

In conclusion, L-asparaginase was expressed in *E. coli* BL21 (DE3) at high level expression. After purification, a single band indicative of purified protein was recorded. The purified enzyme was 7.8 folds with a final specific activity of 312.8 IU/mg protein and about 17.8% yield recovery. Temperature and pH optimum for rASPG were 45°C and 7.5, respectively. The activity of enzyme was enhanced by Mn^{2+}, Pb^{2+}, Mg^{2+}, Ba^{2+} and inhibited by EDTA, Na^{+}, K^{+}, Fe^{3+}, Ca^{2+}, Ni^{+}, Al^{3+}, Cu^{2+}, Hg^{2+} and detergents (Tween 20, Tween 80, Triton X-100, and Triton X-114). Also, DTT at a concentration of 1 mM and DMSO at a concentration of 1% enhanced the enzyme's activity. Recombinant enzyme had high anti-cancer activity. The number of apoptotic cells significantly

increased after rASPG treatment experimental wells by the absorbance of the control (untreated) wells.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This study was supported by Vietnam Academy of Science and Technology (project VAST02.03/13-14: “Study on the production of recombinant L-asparaginase to inhibit cancer cell lines and treatment of acute lymphoblastic leukemia”. The authors are thankful to Prof. Quyen Dinh Thi for guidance. We also extend our thanks to Dr. Do Thi Thao (Bioassay group, Institute of Biotechnology) for tumor cell lines.

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