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, $14.9 \times 10^3$ s$^{-1}$, and $29.9 \times 10^3$ mM$^{-1}$s$^{-1}$, respectively. Temperature and pH optimum were observed at 45°C 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
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-asparaginase 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 kodakarenis (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-asparaginas 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 rASP gene 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 rASP 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 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 pUC57 as template and two oligonucleotides, 21 ASP-F (5'- GCC ATA TGG ATA AAC TGC CGA -3') and LASP-R (5'- AAG CTC GAG TCA GTA GGT AGA G -3') were designed as primers for introduction of the underlined NdeI and Xhol 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 MgCl₂; 1 μl plasmid pUC57 (50-100 ng); 0.25 μl 5 unit Taq polymerase and 1 μl each primer (10 pmol), supplemented with 14.75 μl distilled 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 NdeI and Xhol and purified using Gel Extraction Kit (Qiagen) in accordance with the manufacturer’s instructions. It was followed by ligation of the underlined NdeI and Xhol 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 rASP gene 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 (OD600 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 4°C 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 OD600nm 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 were inoculated, inoculum of different sizes were then added to each of flasks. The culture was cultivated at 37°C with agitation at 220 rpm until an OD 0.8 was reached (for approximately 4 h) then IPTG was added. After 6 h of induction, bacterial cells were harvested and analyzed 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 broth were prepared. Six flasks 100 ml contain 25 ml pLSB broth were prepared. Half ml of the overnight culture were inoculated, inoculum of different sizes were then added to each of flasks. The culture was cultivated at 37°C with agitation at 220 rpm until an OD 0.8 was reached (for approximately 3 h) then IPTG was added. After 6 h of induction, bacterial cells were harvested and analyzed for the enzyme specific activity. The best Amp concentration before induction was selected and applied for the next stage.

**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 (Km, Vmax, Kcat and Kcat/Km) 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 (Ca2+, Cu2+, Fe2+, Fe3+, Mg2+, Ni2+, Zn2+, K+, Mg2+, Pb2+), 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.
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).
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-asparaginase 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
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* BL21(DE3) was 190 U/mg (Khu-shoo 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).

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

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

**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.

**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 45°C 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 thermostolerant 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
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
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 Actinomyces (Basha et al., 2009) and L-asparaginase from Thermococcus kodakarenisis 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⁺ > Fe³⁺ > Ca²⁺ > Ni²⁺ > Al³⁺ > Cu²⁺ > Hg²⁺ while Mn²⁺ enhancer activity of the enzyme by 123%. And Pb²⁺, Mg²⁺, Ba²⁺ showed a slightly enhance effect on rASPG activity, the residual activity was accounted for 108% -109% of the original activity and Zn²⁺ 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 Zn²⁺, Fe³⁺, Cu²⁺, Hg²⁺ and Ni²⁺ (Raha et al. 1990) and the activity of rASPG from Thermococcus kodakarenisis KOD1 was inhibited by Ca²⁺, Co²⁺, Cu²⁺, Ni²⁺, enhanced by Mg²⁺ (Hong et al., 2014). Warangkar and Khobragade, (2010) reported that L-asparaginase form Erw. carotovora was loss of activity with Hg²⁺, Ni²⁺, Cd²⁺, Cu²⁺, Fe³⁺, and Zn²⁺, too, but Na⁺ and K⁺ acting somewhat as an enhancer and Mg²⁺ 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 also 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).

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

<table>
<thead>
<tr>
<th>Additives⁰</th>
<th>Residual activity (%)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no additive)</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>Al₂(SO₄)₃</td>
<td>82 ± 1.7</td>
</tr>
<tr>
<td>Ba(NO₃)₂</td>
<td>109 ± 7</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>88 ± 6</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>77 ± 6</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>91 ± 6</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>115 ± 2</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>72 ± 2</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>88 ± 6</td>
</tr>
<tr>
<td>NaCl₂</td>
<td>108 ± 3</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>123 ± 3</td>
</tr>
<tr>
<td>Pb(NO₃)₂</td>
<td>92±7</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>86 ± 6</td>
</tr>
<tr>
<td>Al₂(SO₄)₃</td>
<td>107 ± 2</td>
</tr>
<tr>
<td>Ba(NO₃)₂</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>72 ± 7</td>
</tr>
</tbody>
</table>

⁰ The final concentration of additive (EDTA or inorganic salt) in the reaction mixture was 10 mM. ² 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.**

<table>
<thead>
<tr>
<th>Detergent</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1%</td>
<td>5%</td>
</tr>
<tr>
<td>Tween 20</td>
<td>87.65 ± 0.8</td>
</tr>
<tr>
<td>Tween 80</td>
<td>54.35 ± 0.2</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>47.91 ± 0.5</td>
</tr>
<tr>
<td>Triton X-114</td>
<td>45.76 ± 0.2</td>
</tr>
</tbody>
</table>

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.**

<table>
<thead>
<tr>
<th>Concentration of DTT (mM)</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>98.10 ± 1.39</td>
</tr>
<tr>
<td>0.5</td>
<td>109.89 ± 4.17</td>
</tr>
<tr>
<td>1</td>
<td>146.57 ± 11.58</td>
</tr>
<tr>
<td>5</td>
<td>119.06 ± 9.73</td>
</tr>
</tbody>
</table>

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

**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.

<table>
<thead>
<tr>
<th>Concentration of DMSO (%)</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>101.70 ± 0.93</td>
</tr>
<tr>
<td>0.5</td>
<td>108.25 ± 0.93</td>
</tr>
<tr>
<td>1</td>
<td>152.46 ± 10.65</td>
</tr>
<tr>
<td>1.5</td>
<td>122.01 ± 12.97</td>
</tr>
<tr>
<td>2</td>
<td>112.84 ± 7.41</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>NIH/3T3</th>
<th>HL-60</th>
<th>P388</th>
<th>P3X63Ag8</th>
<th>SP2/0-Ag14</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>-0.22</td>
<td>-2.05</td>
<td>0.98</td>
<td>0.56</td>
<td>0.98</td>
</tr>
<tr>
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Substrate were 0.5 mM, 500 U/mg, 14.9 × 10<sup>3</sup> s<sup>-1</sup>, 29.9 × 10<sup>3</sup> mM<sup>-1</sup>s<sup>-1</sup>, respectively. The K<sub>m</sub>, V<sub>max</sub>, K<sub>cat</sub>, and K<sub>cat</sub>/K<sub>m</sub> 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<sup>-1</sup>, and 266.9 mM<sup>-1</sup>s<sup>-1</sup>, 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<sup>-1</sup>, and 48 mM<sup>-1</sup>s<sup>-1</sup> respectively (Onishi et al. 2011). The K<sub>m</sub>, K<sub>cat</sub>, and K<sub>cat</sub>/K<sub>m</sub> value of rASPG from Erw. chrysanthemi 3937 in E. coli BL21(DE3) pLysS were 0.058 mM, 23.8 × 10<sup>3</sup> s<sup>-1</sup>, and 411.8 × 10<sup>3</sup> mM<sup>-1</sup>s<sup>-1</sup> respectively (Kotzia and Labrou, 2007). It was found that rASPG had low K<sub>m</sub> value, high V<sub>max</sub>, K<sub>cat</sub> and K<sub>cat</sub>/K<sub>m</sub> value. So rASPG is highly specific for the substrate L-asparaginase.

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 acrmycin NGP (130 U/mg) inhibits the gastric stomach cancer cells with an IC<sub>50</sub> of 49.11 µg/ml (Selvam and Vishnupriya, 2013). L-Asparaginase from bean (Vicia faba) and white kidney bean (Phaseolus vulgaris) seeds (2.75 and 1.47 U/mg) had low IC<sub>50</sub> values 217.71 µg/ml and 187.66 µg/ml with Hep-G2 cells (Sanaa et al., 2012). IC<sub>50</sub> 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<sup>2+</sup>, Pb<sup>2+</sup>, Mg<sup>2+</sup>, Ba<sup>2+</sup> and inhibited by EDTA, Na<sup>+</sup>, K<sup>+</sup>, Fe<sup>3+</sup>, Ca<sup>2+</sup>, Ni<sup>2+</sup>, Al<sup>3+</sup>, Cu<sup>2+</sup>, Hg<sup>2+</sup> 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 as the concentra...
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.

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