

High level of extracellular fermentation and alternative purification of *Escherichia coli* Asparaginase II

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Abstract. L-Asparaginase II (ansB) from *Escherichia coli* is an approved therapeutic substance against diverse forms of leukemia. New application for this enzyme in fried food processing as an acrylamide reducing agent has substantially increased the demand for this enzyme. To expand the supply for ansB, we have developed a new fermentation system in combination with a straightforward purification method to obtain higher amounts of enzyme with adequate purity. The extracellular expression system was constructed by replacing the ansB native signal sequence with PelB leader sequence. The fermentation medium was also optimized to obtain the highest extracellular expression. The effects of concentration of IPTG, exact OD_{600nm} for IPTG implementation, and duration of induction were investigated. These modifications increased the expression levels up to 130 U/mL. Finally, two purification methods, column-based and precipitation-based, were compared for their efficiency and purity. Our results demonstrate the ansB production could be increased significantly by manipulating fermentation parameters and by applying innovative purification processes.

Key words: L-Asparaginase, Extracellular Expression, Optimization, PelB leader sequence, Fermentation, Purification.

Introduction

The secretory production of recombinant proteins has several advantages, such as simplicity of purification, avoidance of protease attack and better chance of correct protein folding (Choi & Lee 2004). Generally, secretory proteins are synthesized in the cytoplasm as premature proteins containing a short (15-30 amino acid residues) signal sequence that allows proteins to be exported outside the cytoplasm. A number of signal sequences have been used for efficient secretory production of recombinant proteins in *E. coli*, including PelB, OmpA, PhoA, endoxylanase and STH (Choi & Lee 2004). The efficiency of protein secretion also depends on the genetic properties of the host strain, the expression vector, the nature of the protein being secreted, the co-expression of facilitator proteins and physical factors such as temperature, medium pH, specific growth rate and percentage of dissolved oxygen (Khushoo et al. 2005). Extracellular expression of L-asparaginase II from *E. coli* facilitates its purification and increases its specific activity due to higher percentage of correctly folded polypeptides. L-asparaginase (EC 3.5.1.1) is found in many animal tissues, bacteria, plants and in the serum of certain rodents except human (Cornea et al. 2002). It catalyses the conversion of L-asparagine to aspartic acid and ammonia. There are two isozymes of L-asparaginase; type I is produced by many kind of bacteria such as *E. coli* (Mercado & Arenas 1999), *Erwinia carotovora* (Aghaiypour et al. 2001), *Enterobacter aerogenes* (Mukherjee et al. 2000), *Candida utilis* (Kil et al. 1995), *Staphylococcus aureus* (Muley et al. 1998), *Thermus thermophilus* (Prista & Kyridio 2001) and *Pisum sativum* (Siechiechowicz & Ireland 1989) and it is a cytosolic enzyme with low affinity for L-asparagine. L-asparaginase II is a periplasmic enzyme in Gram-negative bacteria such as *E. coli* and *Erwinia carotovora* with high affinity for substrate so it is widely used as a therapeutic agent. The most common applications of this enzyme are in acute lymphoblastic leukemia (ALL), Hodgkin disease, acute mye-

locytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarcoma and melanosarcoma (Khushoo et al. 2004). Tumor cells have high level of protein synthesis so they need high amount of necessary amino acids such as L-asparagine. Effective depletion of L-asparagine by L-asparaginase results in cytotoxicity for leukemic cells (Basha et al. 2009). Furthermore, new studies have revealed potential application of this enzyme in prevention of acrylamide formation in fried potatoes and similar food products. Therefore introduction of new fermentation and purification protocols for production of L-asparaginase II will be mandatory to satisfy these demands.

In this investigation, we have used PelB leader sequence to obtain extracellular expression of recombinant L-asparaginase II and we have compared its efficiency to that of the ansB signal sequence as a native leader sequence. The majority of this research has been focused on attaining an efficient system for high-level extracellular production of recombinant L-asparaginase in shake-flask level and scale-up to the bioreactor level. To achieve the highest secretory expression, we tested several factors such as different vectors, the effect of time induction, concentration of IPTG and different culture media in *E. coli* fed-batch fermentation.

Material and Methods

Bacterial strains, plasmids and DNA techniques

The ansB gene was kindly provided in pHM100 plasmid by Dr. Hassan Motejadded, University of Stuttgart, Germany. Bacterial strains and vectors used in this study are listed in Table 1 and the primers used in this study are listed in Table 2.

Primers ansBF1 and ansBR1 were used to PCR-amplify the complete ansB gene with native signal sequence and primers ansBF2 and ansBR1 were used for isolation of mature ansB gene without native signal sequence. In other part, primers ansBF2 and ansBR2 were applied to amplify the mature ansB gene with C-terminal 6×histidine tag.

Table 1. Bacterial strains and vectors.

Bacterial strains:		
DH5α	supE44 ΔlacU169 (Φ80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	
BL21(DE3)	F-ompT hsdSB(rB-mB-) gal dcm lon (DE3)	
Vectors:		
pGEM-5Zf	3.0 kb, Amp Resistance	Promega
pET3a	4.6 kb, Amp Resistance	Novagen
pET21a	5.4 kb, Amp Resistance	Novagen
pET23a	3.6 kb, Amp Resistance	Novagen
pET26a	5.3 kb, Kan Resistance	Novagen
Recombinant Vectors:		
pAsp21	pET21a + ansB Complete	
pAsp23	pET23a + ansB Complete + rrnB Terminator	
pAsp3	pET3a + ansB Complete + rrnB Terminator	
pPAsp26	pET26a + PelB Leader sequence + ansB Mature	
pPAsp3	pET3a + PelB Leader sequence + ansB Mature	
pPAspH26	pET26a + PelB Leader sequence + ansB + C-Terminal 6×His Tag	

Table 2. Primers used in this research.

ansBF1	5'- T TAGTGCTGCAGCCATGGCATTAC -3'	Forward NcoI for complete ansB gene
ansBF2	5'- CAGCATGGCATTACCCAATATCAC -3'	Forward for complete ansB gene
ansBR1	5'-TAGAAGCTTTTAGTACIGATTGAAGATCIGCTG-3'	Reverse HindIII for ansB gene
ansBR2	5'-TTAGTGGTGATGGTGATGGTGGTACTGATTGAAG -3'	Reverse for ansB + C-terminal his tag

The complete *ansB* gene was cloned in the *NdeI* and *HindIII* restriction sites in pET21-a vector (pAsp21) and complete *ansB* gene with *rrnB* terminator were cloned in *NdeI* and *SspI* sites in pET3-a (pAsp3) and pET23-a (pAsp23). Furthermore, *ansB* gene, excluding its signal sequence, was cloned inframe into pET26-a under PelB leader sequence by *NcoI* and *HindIII* restriction enzymes (pPAsp26). The *ansB* gene with PelB fragment was replaced by the *ansB* gene in pAsp3 to produce pPAsp3 vector. Finally, a synthetic fragment consisting of the PelB signal sequence, *ansB* gene and C-terminal His tag was constructed and cloned in *SacI* and *NcoI* sites of pET26-a to produce pPAspH26 vector.

L-asparagine, ammonium sulfate, ampicillin, kanamycine, IPTG and acryl amide were purchased from SIGMA, USA. Nessler's reagent and agar were obtained from Merck, Germany. Restriction and modifying enzymes were purchased from Fermentas and Roche. Culture media and other chemicals were prepared of analytical grade and obtained from local suppliers.

Growth conditions for expression stat in shake-flask

First, BL21(DE3) cells harboring pAsp3 plasmid were grown in 15 mL Luria Broth (LB) (containing 5 gr yeast extract, 10 gr tryptone and 10 gr NaCl per liter) in 50 mL flask at 37°C with shaking at 220 rpm overnight with ampicillin (100µl/mL). Then 100 mL of each of the seven different culture media (listed in Table 3) containing 100 microgram ampicillin per mL, were inoculated with 15 mL of overnight culture in 1000 mL flasks at 37°C with agitation at 220 rpm.

Table 3. Culture media used in this research.

M1	12 gr/L trypton, 14 gr/L yeast extract
M2	LB medium
M3	12gr/L trypton, 14 gr/L yeast extract, 2.5% glycerol, Gly 0.7%
M4	12 gr/L trypton, 14 gr/L yeast extract, 5% glycerol
M5	12 gr/L trypton, 14 gr/L yeast extract, 4% glycerol
M6	M9 medium, yeast extract 5 gr/L, 1% glycerol
M7	TB (12g tryptone/ 24g yeast extract/4ml glycerol/K2Hpo4/KH2po4)+ Gly 0.7%

Asparaginase II expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at late log phase of cells growth. Then samples were taken at several intervals from each different culture. To separate extracellular fractions, cells were precipitated by centrifugation at 8000 rpm for 10 min at 4°C. Periplasmic fractions

were separated by osmotic shock in ice-cold 5mM MgSO₄ (Sambrook & Russell, 2001) of resuspended cell pellet.

Culture conditions in bioreactor

Cells were grown in TB medium with glycine in a 2 liter fermentor (New Brunswick Scientific company, Model Bioflo 3000) at 40% dissolved oxygen (DO) and agitation rate of 500-700 rev/min at 37°C under both batch and fed-batch conditions. The pH was constantly adjusted at 7.00 using 2N HCl/NaOH. In fed-batch cultivation, concentrated nutrient solution containing 12% yeast extract, 10% trip-ton and 13% glycerol was added into the fermentor, using a variable speed pump to support a specific growth rate of 0.3 h⁻¹. Expression of asparaginase II was induced by addition of IPTG at late log phase at 0.25 mM and 2 mM concentrations in batch and fed-batch conditions, respectively.

Asparaginase purification

Asparaginase purification was performed by two different methods. In one method, the supernatant was precipitated by addition of 0.4 volume of EtOH. The remaining supernatant was pelleted with 0.2 volume of EtOH. Then the pellet was resuspended in 0.1 volume of second supernatant and was loaded on a Sephacryl-300 column equilibrated with the same buffer. The eluted fractions were assayed for asparaginase activity and protein content (Bradford method).

In the second method, the supernatant was heated at 55°C for 35-45 min, then the sample was centrifuged at 13000 rpm for 15 min. pH of the supernatant was adjusted to 7.0 with acetic acid. Next, 0.4 volume cooled EtOH 95% was added and stirred for 5 min at 4°C, then centrifuged at 13000 rpm 15 min. 0.2 volume cooled EtOH was added to the supernatant and again centrifuged (13000 rpm 15 min). pH of the remaining supernatant was adjusted to 5.0 using acetic acid. Then ammonium sulfate content was adjusted to 80% and centrifuged at 13000 rpm for 5 min. The precipitate was dialyzed against PBS and pH reduced to 5.00 (pI of asparaginase) prior to second precipitation step. Then ammonium sulfate crystals were added to the supernatant to reach 80% saturation and incubated at 4°C for 2 hours. Following centrifugation at 13000 rpm for 15 min at 4°C the precipitate was dissolved in 1x PBS and dialyzed overnight in the same buffer at 4°C.

Asparaginase Assay

Crude cell extract or purified asparaginase was assayed for asparaginase II activity. 0.1 mL of the sample, 0.1 mL Tris-HCl 6mM (pH 7), 0.1 mL asparagine (189 mM), and 1.8 mL H₂O were put in 2.5 mL

vials and mixed thoroughly. Following 30 min incubation at 37°C, the reaction was stopped by addition of 0.1mL TCA 1.5 mM. The solution was pelleted by centrifugation at 13000 rpm for 20min. Then, 0.2 mL supernatant was added to 4.3 mL H₂O and 0.5mL Nessler solution (Shifrin et al. 1974) to start the color reaction for 1 min, then the concentration of released NH₄⁺ was determined by spectrophotometer.

Results

Effect of IPTG induction on asparaginase expression

For optimization, pAsp3 cells were used. These cells were induced with different concentration of IPTG in order to determine the optimum condition for asparaginase expression. For this purpose, pAsp3 cells were grown in LB medium and induced with 0.05, 0.075, 0.1, 0.2, 0.5 and 1mM of IPTG. Cells were grown for 24 hours post-induction, then the supernatant of collected samples was analyzed for asparaginase activity.

During induction, 1mM IPTG led to highest level of enzyme production versus 0.05 mM IPTG which had the most modest effect on enzyme production (1/3 of 1 mM IPTG) (Table 4). Our results showed that 0.1 mM and 1 mM concentration of IPTG have approximately the same effect on asparaginase expression. Because of IPTG toxicity, in this project 0.1 mM concentration of IPTG was used.

Table 4. Enzyme activity (unit/mL) at different concentration of IPTG (in M7).

Conc./Hours	3hr	6hr	9hr	12hr	24hr
0.05 mM	2±1	9±1	21±1	29±1	41±1
0.075mM	7±1	20±1	34±1	46±1	79±1
0.1 mM	10±1	23±1	42±1	55±1	96±1
0.2 mM	10±1	26±1	43±1	55±1	107±1
0.5 mM	12±1	30±1	45±1	58±1	112±1
1 mM	13±1	31±1	47±1	61±1	124±1

In another case, pAsp3 cells were grown in LB medium and induced with 0.1 mM IPTG at early log phase (OD₆₀₀ = 1.2), mid log phase (OD₆₀₀ = 2.2) and late log phase (OD₆₀₀ = 4.4) of growth. After 24 hours post-induction growth, the samples and supernatants were collected and analyzed to determine the asparaginase activity.

It was observed that the maximum secretion of recombinant asparaginase was obtained by induction at late log phase. After 12 hours of IPTG induction in late log phase the asparaginase activity was 45.2 U/mL. It was 1.3 fold higher than in mid-log phase (34.5 U/mL) and 1.58 fold higher compared with early log phase induction (28.6 U/mL). Also total volumetric specific productivity went up to 904 UI/OD₆₀₀ for late log phase, which was 1.07 fold higher than that of mid- log phase (841 UI/ OD₆₀₀) and 1.3 fold of early log phase.

The measurement of specific activity showed increasing in late log phase (86U/mg), compared with mid-log phase (65U/mg) and early log phase (51U/mg). These results suggest that specific secretion of recombinant asparaginase takes place when late log phase induction is done.

Effect of different culture media on extracellular expression

pAsp3 cells were grown in 7 different media (listed in Table 3) and then induced with 0.1 mM IPTG in late log phase. Af-

ter 24 hours post-induction, samples were collected and supernatants were analyzed for asparaginase activity.

Asparaginase activity in M7 medium (TB + Gly 0.7%) was 1.3-fold higher in compare with M1 medium (without Gly). It was showed that the secretion of recombinant asparaginase into the culture medium increased due to Glycine presence. In addition, it was observed that extracellular expression of recombinant asparaginase increased with the complexity of medium and increasing the glycerol up to 2.5% in medium inhibited cell growth and extracellular secretion. Inhibition of asparaginase secretion at high amounts of glycerol was due to its high viscosity.

It was observed that M7 medium had the most enzyme activity (96U/mL) and M6 medium had the highest specific activity (65% of pure asparaginase) and second one was M7 medium with 51% specific activity. Therefore, M7 medium was selected for further studies in order to improve the secretion of asparaginase (Fig. 1).

Comparing periplasmic expression and extracellular secretion of various recombinant clones

The asparaginase encoding gene (*ansB*) was cloned in various commercially expression vectors (Table 1), and in different assemblies (containing native signal sequence, PelB leader sequences and His-tag) as explained in Materials and Methods. The recombinant expression plasmids were transformed in BL21 (DE3) cells and checked for protein expression. Therefore, different expression clones were compared to determine the asparaginase specific activity at secretory level. At first, transformed cells were cultured in M7 medium and induced with 0.1 mM IPTG in late log phase. 12 h post-induction, 30 mL of bacterial culture was precipitated, and then extracellular and periplasmic expressions were compared (Fig. 2).

The efficiency of native sequence and PelB leader sequence on the total secretory expression was analyzed. It was observed that the total secretory expression with PelB leader sequence was higher than using native signal sequence. In addition, the efficiency of extracellular export of asparaginase was increased by PelB leader sequence with ~ 65% of recombinant proteins being exported to culture medium. Using of C-terminal His-tag (pPAspH26) led to decrease of extracellular export. This declined to 39% in comparison with the case with non-tag protein (pPAsp26).

The results showed that pPAsp3 recombinant plasmid had the highest level of total secretory product and extracellular expression, so the pPAsp3 was used for expression studies in batch and fed-batch cultivation in fermentor.

Batch and fed-batch cultivations

After optimization, *E. coli* BL21 (DE3) cells transformed with pPAsp3 recombinant expression vector were cultured in M7 medium in fermentor. Then the growth curve was drawn to determine the best time for induction in late log phase and it was in OD₆₀₀ value of 10 (Fig. 3). After induction of 0.25 mM IPTG in late log phase (OD₆₀₀ = 10) was done, samples were taken at different post-induction times (3, 5, 7, 9 and 12).

Samples were collected and analyzed with SDS-PAGE and Nessler's assay. It was observed that the asparaginase activity increased with passing time, up to 152 U/mL, the specific activity at 3 hours post- induction was 126.6 U/mg,

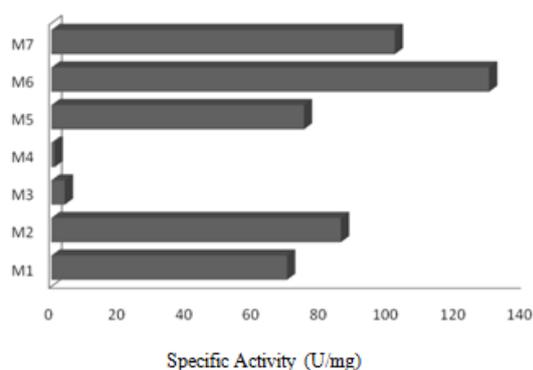


Figure 1. Effect of different culture media on total secretory expression of recombinant asparaginase.

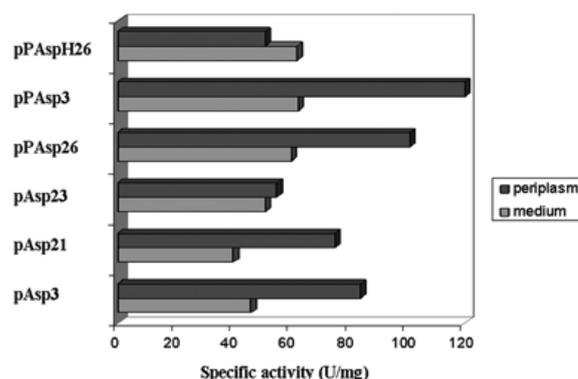


Figure 2. Specific activity of periplasmic expression and extracellular secretion of various recombinant clones.

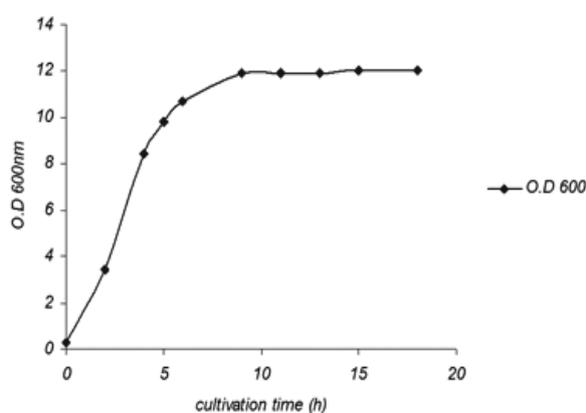


Figure 3. The cells growth curve in batch cultivation.

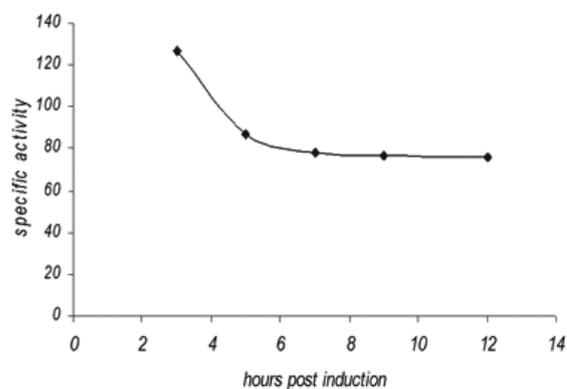


Figure 4. Specific activity in batch cultivation

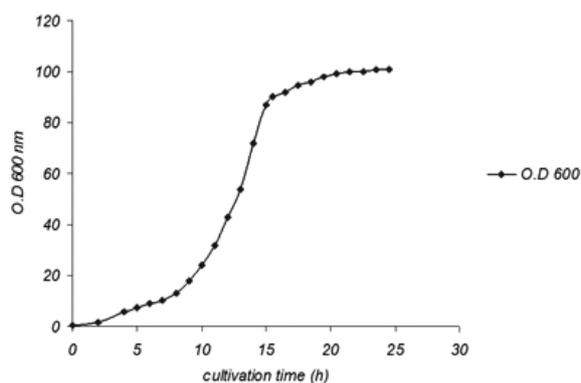


Figure 5. The cells growth curve in fed-batch cultivation.

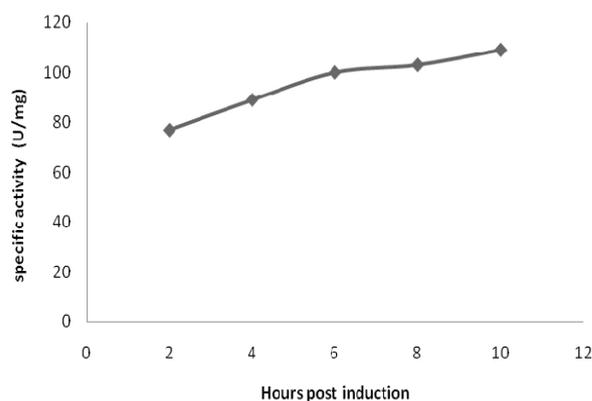


Figure 6. Specific activity in fed- batch cultivation.

but at 5 hours post- induction decreased to 76 U/mg (Fig. 4).

In fed-batch cultivation, the growth curve was also drawn, as described before. It was observed that the best time for induction in late log phase was in OD₆₀₀ value of 88 (Fig. 5). Transformed cells were grown at specific growth rate (0.3 h⁻¹) by an exponential feed of concentrated substrate and induced with 2 mM IPTG in late log phase, and at varying post-induction times, the samples were analyzed. The results showed that the enzyme activity went up to 109 U/mg after 10 hours (Fig. 6).

Purification of Recombinant Asparaginase

The recombinant asparaginase secreted into the culture supernatant was purified with two methods as described in Materials and Methods. In gel filtration method, purified asparaginase eluted from gel filtration chromatography column within fractions 75-92, then the SDS-PAGE analysis was done and protein concentration was determined by Bradford method (Fig. 7). The measurement of the specific activity of purified asparaginase (210 U/mg) revealed a value similar to that of native asparaginase (200±10 U/mg) (Table 5).

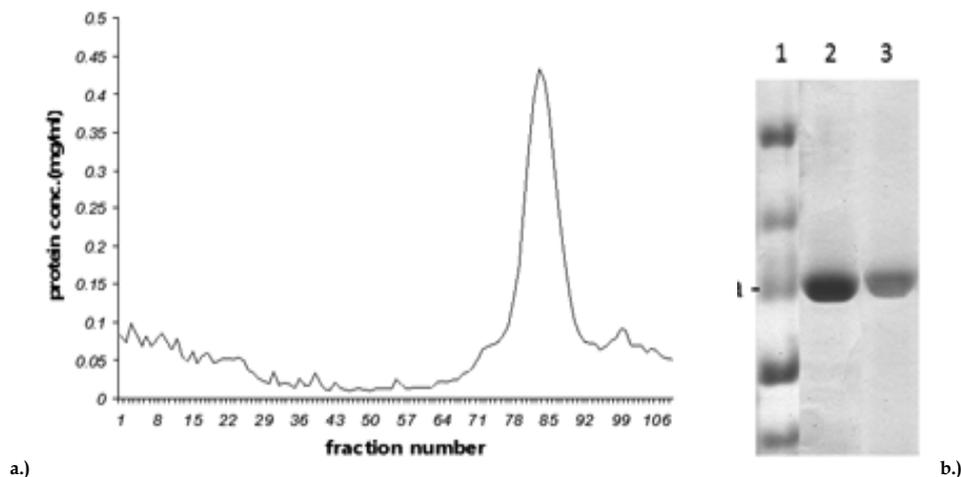


Figure 7. Protein purification of recombinant L-asparaginase II. **a)** chromatogram of different fractions in gel-filtration chromatography column, **b)** SDS-PAGE analysis of purified recombinant L-asparaginase II after ethanol precipitation (lane 2) and gel-filtration (lane 3).

Table 5. Purification of recombinant asparaginase by gel-filtration.

Fraction	total activity(UI)	total protein(mg)	Specific activity(U/mg)	Recovery
Crude extract	7595	37	76	100
After gel-filtration	5082	25	210	76

Table 6. Purification of recombinant asparaginase by thermochemical method.

Fraction	Activity(U/mL)	Protein conc.(mg/mL)	Specific activity(U/mg)	Recovery
Crude extract	12	0.145	82	100
After gel-filtration	32	0.156	205	47

In the second method, since the asparaginase enzyme was stable at 55°C and the other proteins that were extracted in culture media were unstable and degraded, the purification of asparaginase was done on the base of thermochemical properties. Different analyses such as SDS-PAGE, Ness-lers' assay and Bradford assay showed that this method had high efficiency for purification of asparaginase with a 205 U/mg specific activity close to that of pure enzyme (Table 6 and Fig. 8).

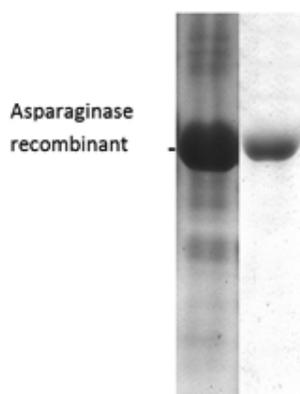


Figure 8. Purification of recombinant asparaginase by thermochemical method. (lane 1 first step of purification, lane 2 final step of purification)

Discussion

By this study, we developed an extracellular expression system for Asparaginase II from *E. coli* and performed simplification on downstream processing of this enzyme. Together, these modification increased yield, and reduced

these modification increased yield, and reduced cost of production for ansB II. In previous reports (Khushoo et al. 2005), this enzyme was expressed and purified using its native signal sequence. Expression of recombinant enzyme with PelB signal sequence increased substantially both the extracellular and total amount of enzyme. Existence of C-terminal His-tag lead to the decrease of extracellular export (Khushoo et al. 2005), although Khushoo and colleagues reported that asparaginase activity in culture medium is lower than periplasmic. Our result indicated that C-terminal His-tag causes a 28% reduction of extracellular protein, but the enzyme activity in culture medium was more than periplasmic enzyme, unlike results of Khushoo.

Researchers demonstrated that induction of recombinant *E. coli* cells during late log phase lead to increasing of recombinant asparaginase expression yield (Galloway et al. 2003). Our investigation also confirmed that induction in the late log phase causes an increase of recombinant protein expression and cytoplasmic proteolysis reduction. It seems that recombinant protein extraction was facilitated because metabolic flow mainly changes to recombinant protein production.

Previous studies revealed that the existence of glycine in the culture media has a critical role in the biosynthesis of the cell wall. Glycine replaces L- and D-alanine in the peptide bridges of peptidoglycan membrane and increases the permeability (Hammes et al. 1973). Kaderbhai and colleagues (1997) showed that concentrations of 1 and 1.5 percent of glycine in the medium lead to a growth decrease of 20 and 50 percent, respectively. However our results indicated that

use of 0.7% glycine in the medium just decreased the growth of recombinant *E. coli* about 11 percent but the secretion of asparaginase into the medium was three times higher. Our results demonstrated that the timing of glycine addition to the medium is critical, with addition at the start of the bacterial growth (in early log phase) having the highest effect on the rate of secretion of recombinant protein in *E. coli* (Kaderbhai et al. 1997). This clearly shows that the increase of membrane permeability by glycine is not a physical event and it has a physiological mechanism.

Downstream processing share on final cost of biopharmaceuticals is more than 50%. Innovative purification processes in this field are required. Yield comparison between two purification processes showed that the specific activity in both thermochemical purification and gel filtration chromatography method was equal (205 versus 210 U/mg). On the other hand, the thermochemical purification method is more economical, so it seems that this method is more suitable and cost effective especially for food processing applications. However, in pharmaceutical applications there are required higher levels of purity; therefore, we suggest the utilization of gel filtration method to ensure approval from corresponding agencies.

Annual revenue in recombinant pharmaceutical products is expanding rapidly and constitutes a large fraction of health care budget in all countries especially from developed world. To reduce cost and expand coverage of the population for these products, cost effective and efficient methods for expression and downstream processing are under development.

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