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Original Article

Soluble Expression and Purification of Q59L Mutant L-asparaginase in the Presence of Chaperones in SHuffle™ T7 strain

Elham Biglari Goliloo M.Sc., Abdolnabi Tollabi M.Sc., Hossein Zarei Jaliani * Ph.D.

Department of Medical Biotechnology, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

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Background and Aims: Q59L mutant of L-asparaginase enzyme from *Escherichia coli (E. coli)* has been introduced with lower side effects. This version of the enzyme might have potential applications in the treatment of leukemia patients. We utilized *SHuffle T7 strain of E. coli*, to produce the mutant enzyme in the presence of chaperone molecules.

Materials and Methods: Q59LAsp gene was cloned into pET28a expression vector, and two strains of *E. coli* (*BL21 DE3* and *SHuffle T7* strains) were used to produce recombinant protein. In parallel, PG-Tf2 plasmid was cloned into the same strains, and the effect of trigger factor chaperone and groELS chaperonines was studied. The his-tagged recombinant protein was expressed and purified using nickel affinity chromatography. The amount of recombinant protein which is produced in each condition was determined and compared.

Results: The amount of soluble recombinant protein was enhanced in the presence of chaperones in both strains of *E. coli*. *SHuffle T7* strain produced more soluble recombinant protein in the soluble state than *BL21 DE3* strain. So the best condition for the production of soluble recombinant Q59L mutant protein was the use of PG-Tf2 plasmid in the *SHuffle T7* strain.

Conclusion: Application of the new strain *SHuffle T7*, with chaperones simultaneously showed better results in the production of Q59L mutant version of L-Asparaginase.

*Corresponding Author: Department of Medical Biotechnology, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran. **Email:** zarei.j@gmail.com

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Introduction

L-Asparaginase (ASNase) is widely considered the most important biopharmaceutical used in the treatment of various types of leukemia, especially acute lymphoblastic leukemia and lymphosarcoma [1, 2]. This enzyme catalyzes the conversion of L-asparagine (Asn) to L-aspartate (Asp) and ammonia (NH2), leading to asparagine deficiency, inhibiting the synthesis of DNA, RNA, and proteins, and consequently the growth of cancer cells [3, 4]. To date, various iso-enzymes have been recognized in a wide variety of organisms, including bacteria, yeasts, and higher eukaryotes [2, 5, 6]. Isolated iso-enzymes of microorganisms, especially bacteria, have been shown to be more effective [7]. Despite being an extensively used drug, ASNase displays a secondary L-glutaminase activity [8]. This non-specific activity of the enzyme produces side effects such as immunological reactions, pancreatitis, neurotoxicity, coagulation abnormalities, etc., it is necessary to identify the iso-enzymes with fewer complications [9]. Commercial (routine) sources of these biotherapeutics are *Escherichia coli (E. coli)* and *Erwinia Chrysanthemi* that have long been approved in the United States and Europe [2, 10]. Several research studies have been conducted on the production of this enzyme by using recombinant DNA technology [11]. Due to the lack of post-translational modifications in ASNase protein molecule, *E. coli* can be a simple and popular host for the production of recombinant ASNase protein [12]. In general, there are many possible problems for the overproduction of heterologous proteins in *E.coli*, with the most commonly encountered problem being the aggregation of recombinant proteins [13, 14]. Factors that are related to protein folding in *E. coli* have been separated from the cytoplasm of the bacteria, which is the main site of recombinant protein production. Hence, enhancing the efficiency of recombinant protein synthesis in the correct structure and low cost in *E. coli* has remained a challenging issue yet [15]. Many advances have been conducted in the field of strain engineering in the last decades for recombinant protein production in *E. coli*. Recombinant protein synthesis, degradation, secretion, or folding could be improved by host strain engineering [16]. Various problems and issues involved in the protein production in *E. coli* are also pursued in the engineering of *E. coli* strains, including engineering of mRNA stability and translational efficiency, improving protein folding by chaperone coexpression, expression of disulfide-bonded proteins, production of acetylated protein and glycoproteins in *E. coli* [16, 17].

In recent years, efforts have been made to increase the solubility of a recombinant protein and reduce the amount of protein deposited in the cell during production to achieve a properly folded structure, such as modification in the rate of protein synthesis, using fusion proteins, mutagenesis in the target protein, and coexpression with molecular chaperones [13]. These engineered strains include ClearColi®, SHuffle® and etc, in which the genetically engineered *SHuffle* cells resolved the problem of solubility and proper disulfide bond formation to some extent by expressing DsbC enzyme in the cytoplasm, which

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helps isomerization of disulfide bonds in recombinant expressed proteins [15].

Molecular chaperones, which are also classified as heat shock proteins, have been identified in any living organism. The *E. coli* chaperone system consists of ribosomal-dependent trigger factor, KEJ and GroELS systems [18, 19]. Protein folding and the formation of protein multi-subunit complexes require the participation of chaperone molecules. They attach to the hydrophobic portions on the target protein and prevent them from accumulating in the insoluble and inactive form of inclusion bodies, helping to obtain the natural form of proteins [20, 21].

The aim of this study was to obtain higher amounts of soluble enzyme in bacterial cytoplasm by coexpression of a chaperone set with *E. coli* asparaginase. This would prevent the formation of recombinant proteins in the form of inclusion bodies and produce recombinant asparaginase with better quality and much lower cost.

Materials and Methods

Cloning of pET-28a-Q59LAsp

Recombinant pET28a-Q59LAsp construct (Biomatik Comp), the commercial vector PG-Tf2 (Takara bio inc.) expressing molecular chaperone trigger factor and groELS chaperonin system has been purchased. By the technique of calcium chloride, *E. coli DH5α*, *BL21* (*DE3*), and *SHuffle T7* competent cells were prepared and used for transformation. *DH5α* strains were used as hosts for proliferative purposes, and *BL21 (DE3)* and *SHuffle T7* strains for expression purposes. Briefly,70 µl of *E. coli BL21 (DE3)* and *SHuffle T7* competent cells (once with a chaperone and a second time without a chaperone) were added to 2 µl of the expression construct and continued with ice incubation, heat shock at 42°C, and again incubation for 30 min. Then, 1 ml of LB media was added to the tube and incubated for 90 min at 37°C followed by spreading cell pellets on LB agar plates consisting of 50 µg/ml of kanamycin and incubated at 37 °C overnight.

It should be noted that agar plates including both kanamycin and chloramphenicol (20 µg/ml) were used for bacterial strains containing chaperone plasmids. Pursuing bacterial overnight cultures, 5 colonies are haphazardly selected from agar plates. Using these colonies as a template, primers designed specifically for the inserted gene, colony polymerase chain reaction (PCR) was performed to confirm the orientation of the inserted gene in the expression construct in selected colonies.

Expression and purification of pET-28a-Q59LAsp

A single and fresh bacterial colony harboring the expression construct pET-28a- Asp was employed to inoculate 5 ml of LB medium, including 50 µg/ml of kanamycin. Until reaching to the optical density of 0.6, the culture is incubated at 37° C with a strong shake. In the next step, 2 ml of the same culture was added to 200 ml LB medium. When optical density reached about 0.6, IPTG with a final concentration of 250 µM was added to the large culture medium and incubated at 37° C overnight. Then, bacteria expressing L-Asp were collected and resuspended in 15 ml lysis buffer (50 mM Tris, pH:8.0, 300 mM NaCl) and bead-beaten on an ice water bath (30s pulses disrupted with cooling on ice) using 0.1 mm beads. By centrifugation at 4° C for 20 min at 10000 g, the supernatant containing the soluble protein L-asp was separated from the insoluble fraction.

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Purification of recombinant his-tagged asparaginase using Ni-NTA spin column

At First, column washing with 10 ml of distilled water and then equilibration adding10 ml of lysis buffer was carried out. The second step is to load the cell supernatant into the Ni-NTA column and wash the column with 10 ml of wash buffer (the same as lysis buffer except for 20 mM imidazole). After that, elution Buffer (50 mM Tris buffer, pH:8.0 containing 300 mM imidazole) was employed to elute the recombinant L-Asp protein. Eventually, the quality of purified L-Asp protein was assessed by 10% Sodium dodecyl sulphate–polyacrylamide gel electrophoresis stained with Coomassie Brilliant Blue. Protein concentration was determined by Bradford assay using bovine serum albumin as a standard.

Coexpression and purification of Q59LAsp with chaperone set

To evaluate the effects of chaperones on solubility of Q59L-Asp, the chaperone plasmid pG-Tf2 was transformed into *E. coli BL21* and *SHuffle T7* cells containing pET-28a-Q59LAsp expression cassette. Transformant bacteria were cultured in LB medium containing 20 µg/ml chloramphenicol and 50 µg/ml kanamycin as the selective marker and tetracycline with a 5 ng/ml concentration to induce the expression of chaperones. After 4 h incubation at 37° C, 250 µM IPTG was added to the culture media, and the solubility of recombinant Q59LAsp was analyzed in the same procedure as described above.

Enzyme assay of Q59Lasp

Enzyme assay for the Q59L mutant L-asparaginase enzyme was performed according to the following method. The samples were mixed with 189 mM L-asparagine in 50 mM Tris buffer, pH=8.6. To perform the reaction of the enzyme, 200 µl of the mixture was incubated at 37° C for ten minuts. By the addition of 10 µl of 1.5 M trichloroacetic acid, the enzymatic reactions were stopped. Nessler's reagent was added to determine the ammonia concentration that is released during the enzymatic L-asparagine hydrolysis. Measurements were done at 436 nm using a Unico 2100-UV spectrophotometer. Each experiment was done in triplicate. The calibration curve of the enzymatic assay was prepared using ammonium sulphate solution. The enzyme needed to produce 1 mM of ammonia was defined as one unit of the recombinant enzyme. This study was approved by the Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

Results

Q59L-Asp expression in different *E. coli* hosts

The Q59L-Asp mutant synthetic sequence contains a mutation in the *asnB* gene of *E. coli* that encodes a nucleotide sequence consisting of 1005 bp with 335 amino acids purchased as sub-cloned into pET-28a vector. This protein, which has a molecular mass of approximately 38 kDa, has yielded both soluble and insoluble products in both strains, *E. coli BL21* (*DE3*) and *SHuffle T7*. As shown in Fig. 1, the highest amount of soluble recombinant Q59L-Asp was identified in the *SHuffle T7* strain, whereas it was insignificant in the *E. coli BL21* (*DE3*). Therefore, it seems that *SHuffle* is a suitable strain for producing soluble recombinant L-asparaginase.

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Soluble expression of Q59L-Asp with chaperones usage

The PG-Tf2 plasmid controls the expression of GroES, GroEL, and trigger factor chaperones using the Pzt-1 promoter, and it was employed to investigate the simultaneous effect of the presence of these chaperones with mutated L-asparaginase. Prior to the use of molecular chaperones, most of the Q59L-Asp was detected in the precipitated cell pellet, indicating the low expression of soluble protein. Hence, simultaneous expression of pG-Tf2 and recombinant protein was applied to increase solubility. As shown in Fig. 2, the results showed an improvement in the solubility of recombinant Q59L-Asp in both strains by coexpression of chaperones. The results also demonstrated that the increase in the solubility of the recombinant Q59L-Asp in the *SHuffle T7* strain was more considerable following chaperone usage.

Purification of the recombinant Q59L-Asp enzyme

Recombinant Q59L-Asp enzyme was purified as mentioned in the methods section. Using 300 mM imidazole as elution agent, resin-bounded enzyme molecules were purified from the chaperone expressing *SHuffle* strain bacteria. The concentration of purified Q59L-Asp determined 0.3 mg/ml by Bradford protein assay As shown in Fig. 3, over 80% purity of the recombinant Q59L-Asp enzyme has been achieved. The enzyme activity was obtained 210 unite/mg protein. This activity (210 unite /mg protein) was close to that of pure asparaginase (200 unite /mg protein).

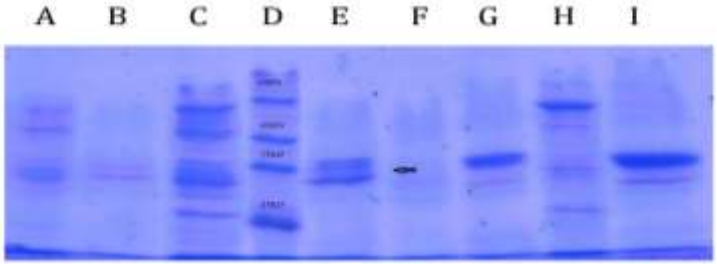

Fig. 1. Q59L-Asp expression in different *E. coli* hosts. A, B; Soup and pellet of the *SHuffle T7* cells, C, E; Soup and pellet of the *SHuffle T7* cells harboring chaperone expression plasmid, F, G; Soup and pellet of the *BL21 DE3* cells, H, I; Soup and pellet of the *BL21 DE3* cells harboring chaperone expression plasmid, D; protein molecular weight marker(10-245KB).

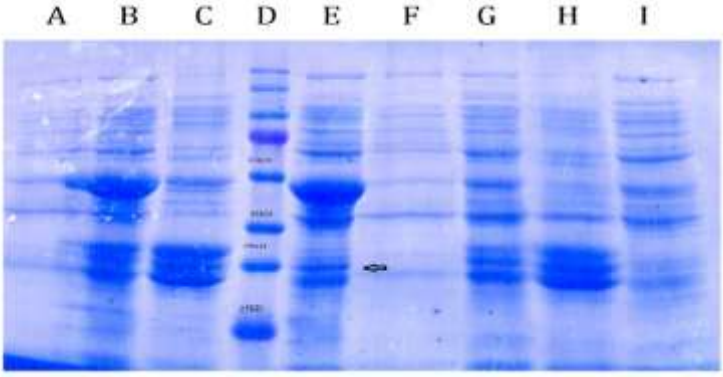

Fig. 2. Effect of chaperone coexpression in the solubility of Q59L-Asp expression in SHuffle T7. A, B; *SHuffle T7* cells before and after the induction of the recombinant Q59L-Asp enzyme. C, E; pellet and soup of the induced *SHuffle T7* cells. F, G; *SHuffle T7* cells harboring chaperone expression plasmid before and after the induction of the recombinant Q59L-Asp enzyme. H, I; pellet and soup of the induced *SHuffle T7* cells harboring chaperone expression plasmid, D; protein molecular weight marker.

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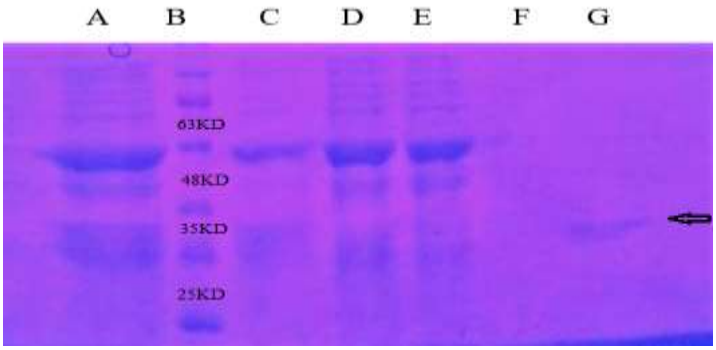

Fig. 3. Purification of the recombinant Q59L-Asp enzyme. Recombinant Q59L-Asp enzyme expression was induced in *SHuffle T7* cells harboring chaperone expression plasmid. A: Pellet, B: Protein marker, C: Soup D; Flow Throw E: Wash and F: Late wash, G: eluted fraction of the *SHuffle T7* cells after the induction.

Discussion

Various agents influence the expression of recombinant proteins. Growth condition, type of *E. coli* strain used, codon optimization, and toxicity of the target protein are among the most significant factors affecting the desired protein expression [17]. Even though *E. coli* is the most helpful expression system in the production of heterologous proteins. However, the main problem observed during the production process is the accumulation of recombinant proteins in the form of an inclusion body with an inappropriate tertiary structure which strongly affects the folding and solubility of recombinant proteins. The high speed of the *E. coli* protein production machinery will almost lead to the aggregation of the heterologous proteins[13]. In the present study, we used two procedures to reduce the amount of protein aggregation: 1) usage of different *E. coli* hosts, 2) coexpression of recombinant protein with a chaperone set.

Various expression systems have been used to produce recombinant His-tagged L-ASNase, as shown by the following studies. In 2011L-Asparaginase II-encoding gene *ansB* was isolated by from *E. coli* MTCC 739, cloned in frame with pelB leader sequence of prokaryotic expression vector pET20b and expressed in *E. coli* DE3 cells[22]. In 2016 L-ASNase II gene was subcloned into pQE-30 expression vector and expressed in *E. coli* M15.

In the present work, recombinant pET28a-Q59LAsp construct was cloned and overexpressed in different *E. coli* hosts [23]. Our experiments indicate that the expression of recombinant Q59L-Asp in *E. coli BL21* (*DE3*) was not successful. As in the cytoplasm of common *E. coli* strains, there are elements such as thioredoxin, glutaredoxin, and glutathione that always maintain intracellular cytosol in a reducing state, resulting in the reduction of disulfide bonds during aerobic growth. Therefore, these strains are not an appropriate host for the production of cytoplasmic proteins containing disulfide bonds. Previous studies have confirmed that engineered bacterial strains can provide a desirable opportunity for proper protein synthesis and folding. One of the strains, known as *shuffleT7*, contains mutations in both thioredoxin reductase and glutathione

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reductase genes (trxB /gor), in addition to a cytoplasmic version of DsbC for the isomerization of disulfide bonds [15, 24].

This strain has revealed good results for the production of disulfide-bonded recombinant proteins in soluble form, such as the Fab antibody [25] and bovine fibroblast growth factor (BbFGF) [26].

In the present study, the expression levels of recombinant Q59LAsp were compared between the two strains, *SHuffle T7* and *E. coli* BL21 (DE3). The results showed that *SHuffle T7* strain had the highest production of recombinant Q59LAsp in the soluble form, which was consistent with previous reports with other recombinant proteins [15, 27].

Since the heat shock protein family plays an important role in stress tolerance and proper folding of proteins, their coexpression with proteins is considered an important strategy to improve folding and increase the solubility of recombinant proteins. But despite good performance, selecting an inappropriate chaperone may even have adverse effects on product solubility as well as host cell survival [28, 29]. The effects of coexpression of molecular chaperones have been previously reviewed. In 1998, GroEL-ES and DnaK chaperones were co-expressed with Cryj2 protein, which resulted in Cryj2 stability and reduced inclusion body formation[30]. In 1999, the effect of the coexpression of GroES/GroEL/Dnak/Dnaj/GrpE/TF chaperones on murine endostatin, ORP150, and human lysozyme protein solubility was studied, and it was found that when expressed alone, most of these proteins were insoluble. Nevertheless, the expression of these chaperones alone or together has increased the amount of soluble recombinant proteins [31]. In 2007, a study on the simultaneous expression of chaperones with recombinant hbFGF protein revealed that neither of the two GroELS and DnaKJ/GrpE chaperone systems alone could prevent the formation of protein aggregation. But when both systems work together, they can even lead to the dissolution of the inclusion body [32]. Experiments on increasing the solubility of single human chains (hscFv), using various molecular plasmid chaperones (PG-tf2, ptf16, pGro7) were performed in 2015 by a team of Iranian researchers, who showed that PG-tf2, in comparison with the two other plasmids, has a better efficiency in raising the solubility of protein [31].

In the present project, the effects of coexpression of GroELS/TF chaperone system on increasing soluble recombinant Q59LAsp were investigated and compared. The results of this study exhibit that the presence of GroELS and TF chaperones expressed from PG-Tf2 expression cassette increased the amount of soluble recombinant Q59LAsp protein in both *SHuffle T7* and *E. coli BL21* (*DE3*). In addition, the amount of soluble Q59LAsp protein produced in the *SHuffle T7* strain was obviously higher in the presence of chaperones than in *E. coli BL21* (*DE3*).

It seems that the existence of extra mutations in the shuffle and possibly extra DsbC isomerization function in the E. coli cytoplasm improves protein solubility and decreases protein aggregation. Therefore, the outcoming of this study indicates that the highest amount

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of Q59LAsp protein could be yielded by engineered strain (*SHuffle*) in the presence of appropriate chaperone composition.

Conclusion

the results of the current study indicate that simultaneous application of new *E.coli* strains such as *SHuffle* with chaperones can be a suitable candidate in order to produce more amounts of the soluble Q59L mutant version of L-Asparaginase.

Conflict of Interest

The authors declare that there is no conflict of interest.

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