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Original Article

Evaluation of soluble expression of recombinant granulocyte macrophage stimulating factor (rGM-CSF) by three different *E. coli* strains

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Abstract

Background and purpose: Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine with a wide range of therapeutic applications although expression of GM-CSF in *Escherichia coli* (*E. coli)* usually leads to formation of insoluble aggregates mostly lack biological activity. The aim of this study was to compare the soluble expression level of GM-CSF in three *E. coli* strains, BL21 (DE3), SHuffle® T7 and Origami™ 2 (DE3).

Experimental approach: The effect of different temperatures and inducer concentrations on soluble expression of GM-CSF was evaluated. The soluble GM-CSF was subjected to endotoxin removal and purification using nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography, ultrafiltration. The biological activity of produced GM-CSF was evaluated based on its growth promotion effect on TF-1 cell lines by MTT assay method.

Findings / Results: A significant improvement of the soluble yield of GM-CSF (about 30% of GM-CSF was expressed as soluble proteins) was observed when protein expression was induced at 30 °C with 0.5 mM isopropyl β- d-1-thiogalactopyranoside (IPTG) in *E. coli* Shuffle T7. The soluble GM-CSF with a high purity up to 95 % and specific activity of 1.25×10^4 IU/μg was obtained.

Conclusion and implications: The proposed strategy here can be used to improve the soluble expression of other hard-to-express proteins with similar structural properties (*i.e.*, containing disulfide binds or cysteine).

Keywords: Disulfide bond isomerase; Expression; GM-CSF; Shuffle T7; Soluble.

INTRODUCTION

The colony stimulating factors (CSFs) are glycoproteins are locally produced at the site of an infection or systemically by multiple tissue types and able to respond rapidly to changing demands imposed by microorganisms (1). There are four distinct types of CSF named after the major types of colony formation stimulated by their action-granulocyte-macrophage CSF (GM-CSF), granulocyte CSF (G-CSF), macrophage CSF (M-CSF) and multipotential CSF (interleukin-3) (2). Among these four CSFs, GM-CSF has a special role in immune responses. It is known as the most important cytokine to stimulate dendritic cell formation and to the enhancement of dendritic cell activity (3).

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Native GM-CSF is glycosylated and has 127 amino acids. It can be expressed in yeast, bacteria, or mammalian cells. Expression in each system leads to different proteins from native GM-CSF. Yeast-expressed GM-CSF is also glycosylated but differs from native GM-CSF in molecular mass and in the substitution of leucine for proline at position 23. GM-CSF expressed in *Escherichia coli* (*E. coli*) is not glycosylated and has six fewer amino acids than the native protein, and an extra methionine at position 1. This variety of expression systems result in different pharmacokinetics, biologic activity, and immunogenicity of recombinant GM-CSF (4).

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The advantages of using *E. coli* as the host system are well known. (a) It has unique fast growth kinetics (5); (b) it can easily grow to high cell densities (6); (c) its media is inexpensive and easily available; (d) its transformation and genetic manipulation is easy and fast (7). However, the expression of foreign proteins in *E. coli* usually leads to the formation of inclusion bodies, accumulated insoluble aggregated proteins mostly without biological activities. Therefore, there is a need for solubilization, refolding, and purification procedures to recover functionally active products which is a difficult and challenging task (8). Alternatively, researchers tried to improve the soluble expression of proteins in *E. coli* by application of different approaches such as optimization of cultivation conditions, co-expression with chaperons, and expression in genetically modified strains (9).

E. coli BL21 (DE3) is the most widely used prokaryotic expression host which is deficient for two main proteases OmpT and Lon (10). Origami™ 2 host strains have mutations in both the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes, which greatly enhance disulfide bond formation in the *E. coli* cytoplasm. This strain is ideal to express proteins that require disulfide bond formation for proper folding (11). SHuffel® T7 is an *E. coli* strain engineered for expression of disulfide-bonded recombinant proteins controlled by T7 promoter. In SHuffle® strains, glutaredoxin reductase and thioredoxin reductase are deleted (*Δgor ΔtrxB*), and a version of the periplasmic disulfide bond isomerase DsbC which lacks its signal sequence is expressed in the cytoplasm. Studies showed that this strain can express recombinant proteins with multiple disulfide bonds and also correct the mis-oxidized bonds and promote proper folding (12). The aim of this study was to compare the soluble expression level of GM-CSF in three *E. coli* strains, BL21 (DE3), SHuffle® T7, and Origami™ 2 (DE3). The biological activity of the soluble and purified GM-CSF was evaluated based on its proliferative effect on TF-1 cell line.

MATERIALS AND METHODS

Bacterial strains and plasmids

The following strains were used for expression of GM-CSF: (1) *E. coli* BL21 (DE3) F^- ompT $hsdS_B(r_B^- m_B^-)$ gal dcm (DE3) (Novagen, Germany), (2) *E. coli* Origami™ 2 (DE3), genotype: Δ(ara–leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL F'[lac^+ $lacI^q$ pro] (DE3) gor522::Tn10 trxB (Str^R, Tet^R) (Novagen, Germany), and (3) *E. coli* SHuffle® T7, genotype: F- lac, pro, $lacI^q$/ Δ(ara-leu)7697 araD139 fhuA2 lacZ::T7 gene1 Δ(phoA)PvuII phoR ahpC* galE (or U) galK λatt::pNEB3-r1-cDsbC ($Spec^R$, $lacI^q$) ΔtrxB rpsL150(Str^R) Δgor ΔmalF3 (NEB, Germany). pET28-GM-CSF plasmid was generated in our previous study according to standard genetic engineering protocols (13).

Expression of GM-CSF

The transformation of the pET28-GM-CSF plasmid into *E. coli* BL21 (DE3), Origami™ 2 (DE3), and SHuffle® T7 cells were performed using heat shock method and positive colonies were selected on Luria-Bertani (LB) plates with antibiotics. A single positive colony from each strain was inoculated in 5 mL of LB broth containing appropriate antibiotics (25 µg/mL tetracycline (Sigma, Germany) + 34 µg/mL kanamycin (Sigma, Germany) for Origami™ 2 (DE3), 34 µg/mL kanamycin + 30 µg/mL spectinomycin (Sigma, Germany) for SHuffle® T7, 34 µg/mL kanamycin for BL21 (DE3) and grown overnight. The next day, this culture was used to inoculate (10% v/v) 25 mL of fresh LB broth in 100 mL shaker flask and incubated at 37 °C and 180 rpm until mid-log phase (*i.e.,* OD_{600} reached 0.4 to 0.6). The expression of the protein was induced by the addition of 0.5 or 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated at 37, 30, and 23 °C for further 3, 4.5 and 18 h, respectively (14). The cultures were centrifuged at 3000 *g* and 4 °C for 10 min and the pellets were stored at -70 °C for future analysis.

Purification of GM-CSF

Nickel-nitrilotriacetic acid affinity chromatography under native condition

The pellet was resuspended in native binding buffer (50 mM NaH_2PO_4 and 0.5 M NaCl, pH 8.0) and then subjected to probe sonication on ice. The lysate was centrifuged at 3000 *g* and 4 °C for 10 min to separate the soluble and insoluble fractions. The soluble fraction was loaded to a chromatographic column containing

nickel-nitrilotriacetic acid (Ni-NTA) agarose (Invitrogen, USA) and incubated at room temperature for 1 h by gentle agitation. The column was washed with native wash buffer (50 mM NaH_2PO_4 and 0.5 M NaCl and 20 mM imidazole, pH 8.0) and then GM-CSF was eluted with native elution buffer (50 mM NaH_2PO_4 and 0.5 M NaCl and 200 mM imidazole, pH 8.0).

Ni-NTA affinity chromatography under hybrid condition

The pellet was resuspended in 6 M guanidin hydrochloride buffer and slowly rocked for 10 min at room temperature to ensure thorough cell lysis.

The cell lysate was sonicated on ice and then centrifuged at 3000 *g* for 15 min and the supernatant transferred to a fresh tube. The solubilized protein was applied to a chromatographic column containing Ni-NTA agarose (Invitrogen, USA) and incubated at 25 °C for 1 h by gentle agitation. The column was washed twice with the denaturing binding buffer (8 M urea, 20 mM NaH_2PO_4, 500 mM NaCl, pH 7.8), and twice with denaturing wash buffer (8 M urea, 20 mM NaH_2PO_4, 500 mM NaCl, pH 6.0). Finally, the resin was washed five times with native wash buffer and GM-CSF was eluted from the column using native elution buffer (50 mM NaH_2PO_4 and 0.5 M NaCl and 200 mM imidazole, pH 8.0).

Endotoxin removal and ultrafiltration

Triton® X-114 extraction was performed to remove endotoxins from the purified GM-CSF, as described previously (15). The samples were more purified and concentrated with ultrafilters (Millipore, USA) having cutoff molecular weights of 10 and 20 kDa.

Analytical methods

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used for analyzing the protein samples and the amount of proteins was estimated based on the band intensity determined using TotalLab TL120 software (Nonlinear Inc, Durham NC, USA). Bradford method was used to measure the concentration of total purified protein as described previously (16).

Bioassay

The biological activity of GM-CSF refolded during purification under hybrid condition was determined based on the ability of GM-CSF to induce cell proliferation. Human erythroleukemic, TF-1, cell line was supplied by Pasteur Institute of Iran. TF-1 cells (1×10^4) were seeded in a 96-well plate (17). After 24 h, various concentrations (10-10000 pg/mL) of the purified GM-CSF and standard GM-CSF (R&D Systems, USA) were added to different wells and further incubated for 48 h. Then, 20 µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (5 mg/mL) was added to each well and incubated for further 4 h at 37 °C. Finally, the medium was replaced with 150 µL DMSO to dissolve formazan crystals and the absorbance was read at 570 nm by a microplate reader (Bio-Tek©, USA).

RESULTS

Expression of GM-CSF in different hosts

The ability of BL21 (DE3), Origami™ 2 (DE3), and SHuffle® T7 for expression of GM-CSF were evaluated using SDS-PAGE analysis. Our results confirmed the expression of the recombinant protein by all three hosts. However, SHuffle® T7 yielded the highest level of soluble GM-CSF (induction with 0.5 mM IPTG at 30 °C).

Furthermore, the effect of inducer concentration and induction temperature on soluble expression of three strains was evaluated. As shown in Fig. 1, the best condition for soluble expression of GM-CSF in BL21 (DE3) was induction with 1 mM IPTG at 30 °C (about 15% of GM-CSF was expressed as soluble proteins).

The optimum condition for soluble production of GM-CSF in SHuffle® T7 was induction with 0.5 mM IPTG at 30 °C (Fig. 2) (about 30% of GM-CSF was expressed as soluble proteins).

According to SDS-PAGE analysis (Fig. 3), Origami™ 2 (DE3) induction by 0.5 mM IPTG at 37 °C exhibited the highest yield of soluble GM-CSF in comparison with the other induction temperatures (about 10% of GM-CSF was expressed as soluble proteins).

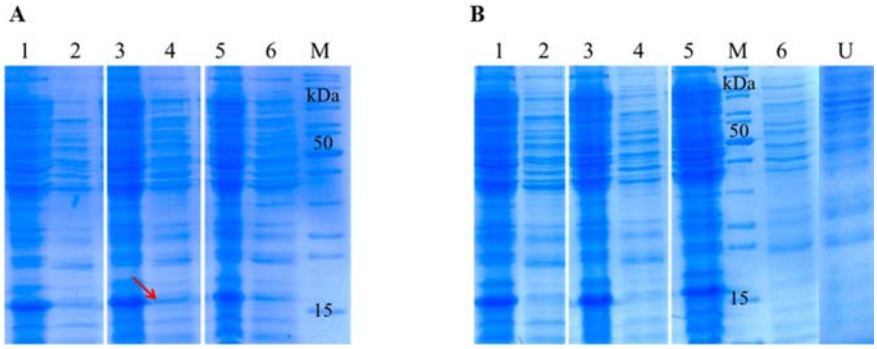

Fig. 1. GM-CSF expression in *Escherichia coli* BL21 (DE3). Protein expression was induced with (A) 1 mM and (B) 0.5 mM isopropyl-β-D-thiogalactopyranoside. Insoluble (lanes 1, 3, and 5) and soluble (lanes 2, 4, and 6) fractions of protein expresed at 37 °C, 30 °C and 23 °C, respectivly. Lane M, molecular weight protein marker and lane U, uniduced sample. GM-CSF (15 kDa) is indicated by arrow.

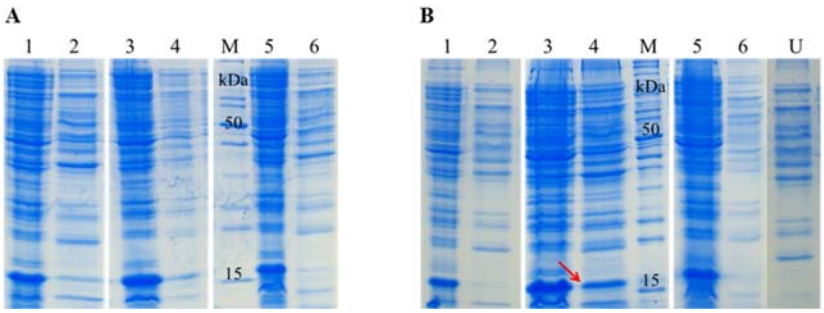

Fig. 2. GM-CSF expression in *Escherichia coli* Shuffle T7. Protein expression was induced with (A) 1 mM and (B) 0.5 mM isopropyl-β-D-thiogalactopyranoside. Insoluble (lanes 1, 3, and 5) and soluble (lanes 2, 4, and 6) fractions of protein expresed at 37 °C, 30 °C and 23 °C, respectivly. Lane M, Molecular weight protein marker and lane U, uniduced sample. GM-CSF (15 kDa) is indicated by arrow.

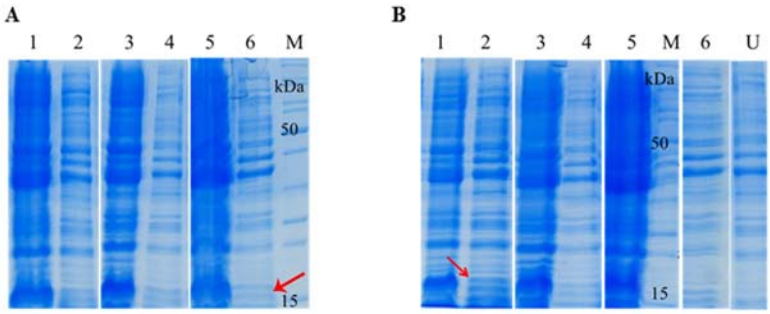

Fig. 3. GM-CSF expression in *Escherichia coli* Origami 2 (DE3). Protein expression was induced with (A) 1 mM and (B) 0.5 mM isopropyl-β-D-thiogalactopyranoside. Insoluble (lanes 1, 3, and 5) and soluble (lanes 2, 4, and 6) fractions of protein expresed at 37 °C, 30 °C and 23 °C, respectivly. Lane M, Molecular weight protein marker and lane U, uniduced sample. GM-CSF (15 kDa) is indicated by arrows.

Purification

Purification of GM-CSF (expressed in SHuffle® T7) was performed using Ni-NTA resin by two approaches including hybrid and native. As most GM-CSF expressed as insoluble protein, a very low amount of protein can be purified under the native condition and the purification yield was 35% (Fig. 4A). However, most of GM-CSF can be recovered by Ni-NTA chromatography under hybrid

condition (Fig. 4A) a high purity up to 95% and the purification yield was estimated to be 55%. For further purification, the samples were subjected to ultrafiltration leading to separation of GM-CSF monomeric form (Fig. 4B). SDS-PAGE analysis demonstrated two bands of 15 and 30 kDa representing monomeric and dimeric forms of GM-CSF. Non-reducing and reducing SDS-PAGE of obtained GM-CSF was shown in Fig. 4C. The dimeric form of GM-CSF was observed on non-reducing conditions while in reducing condition disappeared.

Biological activity of GM-CSF

To confirm the biological activity, the proliferative effect of the refolded GM-CSF (expressed in SHuffle® T7) on TF-1 cells was evacuated using MTT assay. As shown in Fig. 5A, the produced GM-CSF promoted the growth of TF-1 cells in a dose-dependent manner. The curve was fitted to a sigmoidal four-parameter logistic model and the half-maximum effective concentration (EC_{50}, the concentration of GM-CSF showing 50% maximal growth of the TF-1 cells), was calculated to be 80 pg/mL. The specific activity of the produced GM-CSF was determined as 1.25×10^4 IU/µg. Furthermore, TF-1 cells were also incubated with different concentrations of standard GM-CSF to estimate relative potency/bioactivity of the produced GM-CSF (Fig. 5B). Based on the parallel-line analysis, the relative potency of produced GM-CSF was found to be 1.1.

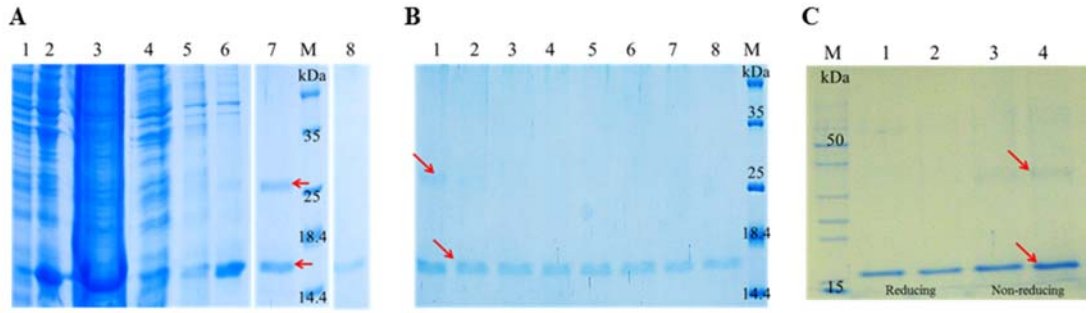

Fig. 4. GM-CSF purification. (A) Purification of GM-CSF using nickel-nitrilotriacetic acid affinity chromatography. Hybrid condition: lane 2, insouble fraction; lanes 3, denaturing wash; lane 4, native wash; lanes 6 and 7, elute 1 and elute 3. Native condition: lane 1, souble fraction; lane 5, wash; lane 8, elute 3. (B) Ultrafiltration: lane 1, the protein fraction before ultrafiltration; lanes 2-8, protein fractions after ultrafiltration. (C) Reducing and non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of GM-CSF. Lane M, molecular weight protein marker. Monomeric and dimeric GM-CSF (15 and 30 kDa) is indicated by arrows. GM-CSF, Granulocyte-macrophage colony-stimulating factor.

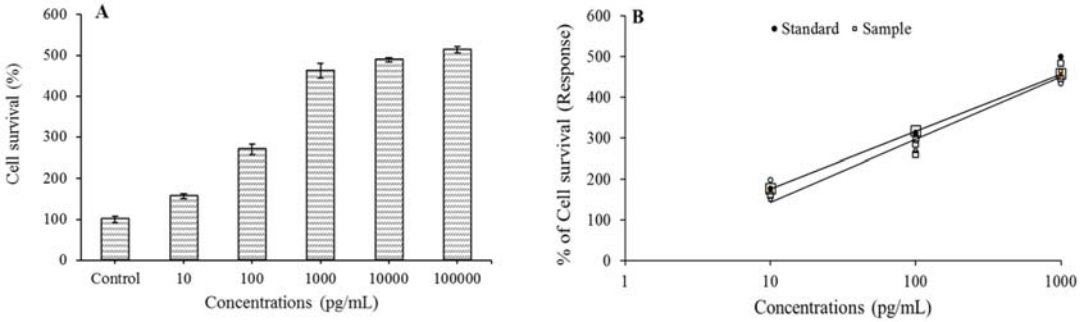

Fig. 5. Biological activity of GM-CSF. (A) Effects of different concentrations of produced GM-CSF on cell viability as determined by MTT assay. TF-1 cells were incubated for 48 h with different concentrations of the protein. Data are presented as mean ± SD, n = 3; (B) parallel line assay of the biological activity of produced and standard GM-CSF on TF-1 cell line. GM-CSF, Granulocyte-macrophage colony-stimulating factor.

DISCUSSION

GM-CSF is a cytokine with a wide range of therapeutic applications and many studies have attempted to improve its expression and purification strategies to reply to the increasing demand for this protein. However, expression of GM-CSF in *E. coli* usually leads to the formation of the insoluble aggregates mostly lack of biological activity (18). Two native disulfide bonds and nine cysteine residues exist in GM-CSF protein (19). This may be the reason for its misfolding and aggregation upon expression in the reducing cytoplasm of *E. coli* (20). Previous studies evaluated different approaches including a periplasmic expression to improve soluble production of GM-CSF in *E. coli* and in many cases very low yield of protein expression could be obtained (21). However, some studies reported successful applications of co-expression with chaperones and fusion to thioredoxin for soluble expression of GM-CSF (14,22). Fusion protein and co-expression strategies are more time-consuming and expensive, therefore, they are not routine approaches in industrial scales (23). Alternatively, genetically modified hosts having a more oxidative cytoplasm can be applied to improve the soluble production of proteins.

Recently, different genetically engineered strains like AD494, Origami™ and SHuffle® have been widely used for soluble expression of heterologous proteins (24-26). These modified strains might differ in their intracellular environment, therefore the various soluble yield of recombinant proteins could be achieved for these hosts. Among the strains used in this study, SHuffle® T7 exhibited the highest yield of soluble GM-CSF. Safarpour *et al.* compared the expression of tumor necrosis factor alpha (TNF-α), a protein containing one disulfide bond, in BL21 (DE3) and SHuffle® T7 (27). Despite the higher yield of protein expression in BL21 (DE3), 1.5 fold higher disulfide bind formation was observed in TNF-α expressed in Shuffle T7 (27). It may be attributed to more oxidative cytoplasm of the SHuffle® T7 strain. Interestingly, Nasiri *et al*. compared the expression of recombinant human fibroblast growth factor 1, a non-disulfide-bonded protein containing three free cysteines, in three strains and reported that SHuffle® T7 yielded the highest soluble expression (28). They suggested that chaperone properties of DsbC improve the correct folding of expressed proteins in the cytoplasm of SHuffle® T7 by breaking non-native disulfide bonds (28).

Here, Origami™ 2 (DE3) yielded the lowest soluble expression of GM-CSF among the three strains. This could be explained based on this fact that Origami™ 2 (DE3) is an *E. coli* K-12 derivative. Science *E. coli* B strains like BL21 (DE3) and SHuffle® T7 are deficient for two main proteases, OmpT and Lon, which can degrade expressed protein during down-stream processing (*e.g*. cell disruption) (29), they exhibited a higher yield of soluble expression of GM-CSF than Origami™ 2 (DE3). Our result showed a significant amount of GM-CSF accumulated as inclusion bodies in the cytoplasm of Origami™ 2 (DE3). However, the oxidative cytoplasm of Origami™ 2 (DE3) and SHuffle® T7 could enhance the formation of non-classical inclusion bodies, folding intermediates of proteins with native-like secondary structure. During the refolding procedure, these partially folded intermediates will be more easily transited to native and biologically active proteins (30). Formation of native disulfide bonds are likely to happen in these folding intermediate and therefore, inclusion bodies formed in Origami™ 2 (DE3) and SHuffle® T7 might exhibit higher yield of refolding compared with those obtained from BL21 (DE3).

The effect of fermentation conditions such as the temperature of expression and concentration of the inducer was also evaluated on the soluble expression of GM-CSF in three strains. For SHuffle® T7 strain, decreasing the temperature of expression to 30 °C resulted in a significant improvement of the soluble expression of GM-CSF. Similar to our findings, other studies reported higher soluble expression of proteins at 30 °C which might be due to higher activity and expression of molecular chaperones (*e.g*. DsbC) at this temperature (31). However, the most soluble expression of GM-CSF in Origami™ 2 (DE3) was observed 37 °C. It could be explained based on this fact that low temperature decreases the rate of cell growth and total protein production (both soluble and insoluble protein). In addition to temperature, inducer concentration also could influence the soluble expression of proteins. In agreement

with our present work, other studies reported that decreasing inducer concentration improved soluble expression of proteins probably by reducing the rate of protein production and aggregation (14). Our results showed that the optimal temperature and inducer concentration varied among the three strains. We concluded that environment parameters must be optimized for each strain. In the current work, the expression of GM-CSF in SHuffle® T7 yielded the highest level of soluble and biologically active protein. Based on the results of GM-CSF bioassay, GM-CSF expressed in SHuffle® T7 exhibited reasonable biological properties and was slightly superior to GM-CSF produced in BL21 (DE3) by co-expression with chaperones in our previous study (14). The specific activity of GM-CSF produced in the present study was determined as 1.25×10^4 IU/µg while the specific activity for GM-CSF produced in the previous study was measured as 1.1×10^4 IU/µg. It suggests better quality of expressed proteins in this strain as its ability to fix the mis-oxidized bonds improves the proper and functional folding of recombinant proteins (12).

CONCLUSION

In the present study, we compared the soluble expression of GM-CSF in three *E. coli* strains. Additionally, the effect of inducer concentration and expression temperature on the soluble production of the protein was evaluated. A significant improvement of the soluble yield of GM-CSF was observed when protein expression was induced at 30 °C with 0.5 mM IPTG in *E. coli* SHuffle® T7. The soluble GM-CSF was successfully purified and its biological activity was confirmed based on its growth promotion effect on TF-1 cell lines. Based on the results of the present study, we concluded that genetically engineered *E. coli* strains like SHuffle® T7 can be used to improve the soluble expression of other hard-to-express proteins with similar structural properties (*i.e.* containing disulfide binds or cysteine residues).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest for this study.

AUTHORS' CONTRIBUTION

S. Soheili contributed to the design, experimental studies, data analysis, and manuscript preparation. A. Jahanian-Najafabadi contributed to the concept and manuscript editing. V. Akbari contributed to the concept, design, experimental studies, data analysis, statistical analysis, manuscript preparation and revision.

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