

Cloning, Optimization of Periplasmic Expression and Purification of Recombinant Granulocyte Macrophage-Stimulating Factor in *Escherichia coli* BL21 (DE3)

Abstract

Background: Molgramostim, a nonglycosylated version of recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF), can be produced in a high level by *Escherichia coli*. However, overexpression of GM-CSF in bacterial cells usually leads to formation of inclusion bodies and insoluble protein aggregates which are not biologically active. The aim of the present study was to improve the expression of soluble and biologically active GM-CSF in periplasmic space of *E. coli* BL21 (DE3). **Materials and Methods:** The codon-optimized GM-CSF gene was subcloned into pET-22b expression vector, in frame with the *pelB* secretion signal peptide for periplasmic secretion. Cultivation conditions including as isopropyl β -D-1-thiogalactopyranoside (IPTG) concentration, incubation temperature, and presence of sucrose were optimized to improve periplasmic expression of GM-CSF. The expressed protein was purified using Ni-NTA affinity column. Biological activity of GM-CSF on HL-60 cells was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. **Results:** The amount of soluble protein for periplasmic expression was more when compared with one of the cytoplasmic expressions. The optimum condition for periplasmic expression of GM-CSF was expression at 23°C, using 1 mM IPTG as inducer and in the presence of 0.4 M sucrose. The biological activity of purified GM-CSF on HL-60 cell line was assessed by MTT assay, and the specific activity of produced GM-CSF was determined as 1.2×10^4 IU/ μ g. **Conclusion:** The present work suggests that periplasmic expression and optimization of cultivation conditions could improve soluble expression of recombinant proteins by *E. coli*.

Keywords: *Escherichia coli*, granulocyte-macrophage colony-stimulating factor, inclusion bodies, periplasmic expression

Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a member of CSFs family which promotes proliferation and differentiation of hematopoietic myeloid progenitors.^[1] It is secreted by different cell types including macrophages, T-cells, mast cells, NK cells, endothelial cells, and fibroblasts on receiving immune stimuli.^[2] Clinical use of GM-CSF has been approved for treatment and prevention of neutropenia due to myelosuppressive chemotherapy in cancer patients or in patients after bone marrow transplantation.^[3] The pharmaceutical analogs of GM-CSF are called Sargramostim, a glycosylated protein expressed in *Saccharomyces cerevisiae*, and Molgramostim, a nonglycosylated protein expressed in *Escherichia coli*.^[4]

E. coli as one of the most common hosts for expression of recombinant protein has some well-known advantages. First, *E. coli* grows fast and easily reaches to high-density culture in inexpensive media. Second, it can be easily manipulated as its genetics and biochemical properties are well characterized. Finally, it has a high-level expression of recombinant protein and an easy scale-up process.^[5] However, there are some drawbacks using *E. coli* expression system such as formation of inclusion bodies (IBs), the main problem with expression of recombinant proteins in the cytoplasm of *E. coli*.^[6,7] IBs are dense, amorphous, and insoluble protein aggregates which are not biologically active, unless they can be isolated and resolubilized for proper folding. These processes require

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use of high concentration of strong denaturing agents, detergents, and extreme pH along with addition of reducing and chelating agents. All of these processes can result in production of biologically active protein by *E. coli*, although they might lead to the interruption of protein structure or misfolding. In other words, the quantity and quality of protein recovered from IBs can significantly decrease. To improve soluble expression of recombinant proteins the following strategies can be applied: 1) modifying the fermentation parameters (e.g., temperature, aeration, pH and content of medium), the promoter strength and the level of inducer, 2) coexpression with chaperones (e.g., GroES/EL and DnaK/DnaJ), folding modulators (e.g., peptidyl-prolyl isomerases or protein-disulfide isomerases [PDIs]), 3) genetic engineering to create fusion protein or to eliminate amino acid sequences responsible for aggregation, and 4) secretion of protein to periplasmic space.^[8]

Oxidative environment and presence of folding modulators such as PDIs and PPI in periplasmic space, facilitate disulfide bonds formation; therefore, periplasmic expression can increase proper protein folding. In addition, expression in periplasmic space has other advantages over the cytosolic one such as simple downstream processing and purification as periplasmic fraction is less contaminated with cytoplasmic proteins, endotoxins and DNA compared to cytoplasmic one.^[9-12] Recent studies have shown that periplasmic expression of human growth hormone, single chain antibody fragment against human epidermal growth factor receptor 2, human cystatin C, and tumor necrosis factor (TNF)-related apoptosis-inducing ligand could significantly increase the amount of soluble proteins.^[13-16] However, there are few reports on periplasmic expression of GM-CSF which usually lead to the very low yield of protein expression. Oloomi and Bouzari reported that periplasmic expression of GM-CSF using a pMAL-p2 construct led to a yield of only 4% of total cell protein.^[17] The aim of the present study was to improve the expression of soluble and biologically active GM-CSF in periplasmic space of *E. coli* BL21(DE3).

Materials and Methods

Subcloning of granulocyte-macrophage colony-stimulating factor

The recombinant pET28a-GM-CSF plasmid constructed in our previous study^[18] for cytoplasmic expression of GM-CSF was subjected to digestion with *Nco*I and *Xho*I enzymes. The resulting fragment was gel-extracted (QIAquick gel extraction kit, Qiagen, USA) and then was subcloned into pET-22b (Novagen, USA) expression vector, in frame with the pelB secretion signal peptide [Figure 1]. *E. coli* XL1-Blue (Stratagene, USA) was transformed with the ligation mixture and grown on Luria-Bertani (LB) agar plates containing ampicillin to screen positive colonies. The constructed pET22b-GM-CSF was subjected to sequencing to ensure the correct expression frame.

Expression of granulocyte-macrophage colony-stimulating factor

Chemically competent cells of *E. coli* BL21 (DE3) were transformed with pET22-GM-CSF using heat shock method. A single positive colony was transferred to 5 ml of LB broth supplemented with 100 µg/ml ampicillin and incubated at 37°C and 180 rpm for approximately 16 h. This culture was added to 100 ml of fresh LB medium at ratio of 1:10 and incubated at the same condition until the optical density at 600 nm (O_{D600}) reached to 0.4–0.6. To induce protein expression, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture and incubated for further 2 h. Bacterial cells were separated from culture using centrifugation at 7500 g for 10 min, and the pellets were stored at –70°C for further analysis.

Optimization of cultivation conditions for periplasmic expression of granulocyte-macrophage colony-stimulating factor

E. coli BL21 (DE3) containing pET22-GM-CSF cells were grown overnight. This culture was added to fresh medium and incubated at 37°C and 180 rpm until reaching exponential phase. Then, the expression of GM-CSF was induced under various conditions including incubation at different temperatures (37°C, 30°C, and 25°C), addition of different IPTG concentrations (0.25, 0.5, and 1 mM), and supplementation of the medium with 0.4 M sucrose.

Periplasmic extraction and purification

To extract GM-CSF expressed in periplasm, an osmotic shock approach was applied. First, the bacterial pellets were resuspended in the cold hypertonic buffer (50 mM Tris-HCl, 20% sucrose, 1 mM ethylenediaminetetraacetic acid, pH 8.0). The suspension was incubated on ice and shaken for 45 min. Then, the mixture was subjected to centrifugation to separate the cells and the supernatant-containing periplasmic proteins (fraction 1). Second, the pellet was resuspended in the cold hypotonic buffer (5 mM MgSO₄) and shaken for 30 min on ice and then centrifuged at 9000 g for 10 min at 4°C. The supernatant (fraction 2) was added to the previous one to make the whole extracted periplasmic proteins. The obtained soluble protein was subjected to purification using an Ni-NTA affinity column under native condition, as described previously.^[18]

Analytical methods

The protein samples were analyzed by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting using anti-His-HRP antibody (Abcam, USA). The concentration of purified GM-CSF was measured using Bradford method.^[19] The biological activity of obtained GM-CSF was determined by evaluation of its proliferative effect on HL-60 cells using

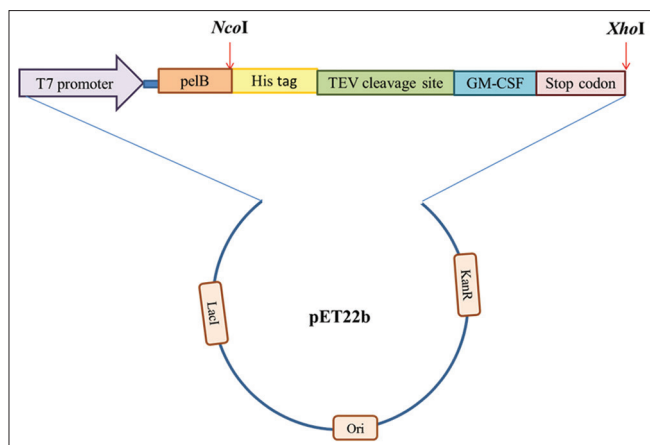


Figure 1: Schematic diagram of the pET22b-granulocyte-macrophage colony-stimulating factor expression vector. The positions of enzymatic cleavages are indicated by the red arrow. The gene encoding granulocyte-macrophage colony-stimulating factor protein was inserted into the pET22b vector under the control of the *T7* promoter, in frame with a *pelB* signal peptide and an hexa-histidine tag

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously.^[19] Briefly, different concentrations (1–100 pg/ml) of expressed and standard hGM-CSF (R and D systems, USA) were added to cells, and after 48 incubation, the viability of cells was evaluated. The percentage of cell survival was plotted against concentrations, and based on the obtained equation, EC₅₀ (the concentration of GM-CSF exhibiting 50% of the maximal proliferative effect) was determined. Specific activity was calculated using the following equation:

$$\text{Specific activity (IU/}\mu\text{g)} = 1/\text{EC}_{50} \text{ (pg/ml)} \times 10^6 \text{ (1 } \mu\text{g} = 106 \text{ pg)}$$

Results

Subcloning and expression of granulocyte-macrophage colony-stimulating factor

As expected, a 440-pb band containing GM-CSF gene was observed [Figure 2] after digestion of pET22-GM-CSF plasmid with *NcoI* and *XhoI* enzymes.

The correct molecular weight of GM-CSF on SDS-PAGE and western blotting confirmed the expression of His-tagged full-length protein. The amount of soluble protein for periplasmic expression (*E. coli* BL21 (DE3) containing pET22-GM-CSF plasmid) was more when compared with one of the cytoplasmics (*E. coli* BL21(DE3) containing pET28a-GM-CSF plasmid) expression. For periplasmic expression, we used *pelB* signal peptide (2.45 kDa), and as shown in Figure 3, two bands were observed, the larger one corresponds to unprocessed protein (17.5 kDa) and the smaller one belongs to processed protein (15 kDa). Accordingly, 30% of the expressed GM-CSF could be translocated into the periplasm (processed protein without signal peptide) while a significant part of it remained in the cytosol [Figure 4].

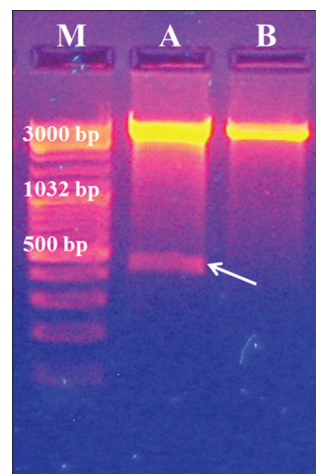


Figure 2: Double digestion of pET-22b- granulocyte-macrophage colony-stimulating factor with DNA samples were digested with *NcoI* and *XhoI* enzymes. M: DNA ladder; Lane A: digested DNA sample; lane B: undigested DNA sample. As expected, a 440-bp band is seen in lane A (shown by the arrow)

Optimization of granulocyte-macrophage colony-stimulating factor expression conditions

To evaluate the effects of temperature and different concentrations of IPTG in the presence or lack of sucrose (0.4 M), variety of experiments were performed. SDS-PAGE analysis showed that the maximum expression of GM-CSF occurred when 1 mM IPTG was used at 23°C [Figure 5]. Then, the effect of addition of sucrose to the medium was also evaluated when protein expression was induced with different concentrations of IPTG at 23°C. Results show that the presence of sucrose can improve the periplasmic expression of GM-CSF [Figure 6].

Purification and bioassay of expressed granulocyte-macrophage colony-stimulating factor

Purification of GM-CSF was successfully performed by using Ni-NTA affinity column under native condition. SDS-PAGE analyses verified the purification [Figure 7]. The biological activity of purified GM-CSF on HL-60 cell line was assessed by MTT assay, and the specific activity of produced GM-CSF was calculated as 1.2×10^4 IU/ μ g when compared with standard GM-CSF (R and D systems, USA).

Discussion

GM-CSF as a hematopoietic growth factor is used for different clinical applications including mobilization of hematopoietic stem cells after bone marrow transplantation, neutropenia induced by chemotherapy, oral mucositis, and immunotherapy of cancer.^[20,21] GM-CSF was approved by the Food and Drug Administration in 1991; the generic name of this drug is sargramostim; and it is demonstrated to acceleration of myeloid recovery in selected patients.^[22] Hence to the wide range clinical applications of GM-CSF, various studies were done about soluble and active

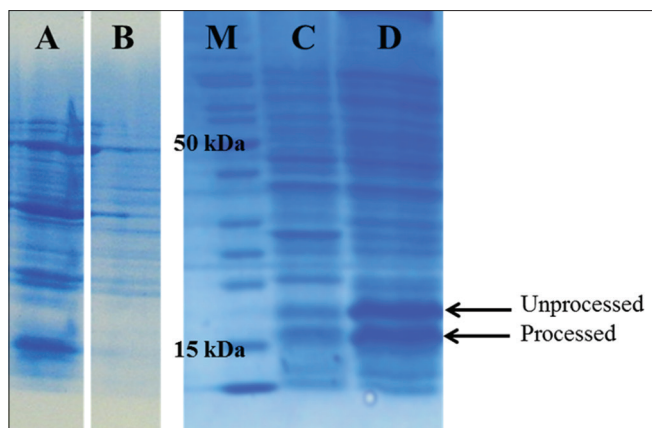


Figure 3: Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of granulocyte-macrophage colony-stimulating factor expression in *Escherichia coli* BL21(DE3) containing pET28a-granulocyte-macrophage colony-stimulating factor plasmid (cytoplasmic construct) (lane A and B) and *Escherichia coli* BL21(DE3) containing pET-22b-granulocyte-macrophage colony-stimulating factor plasmid (periplasmic construct) (lane C and D). Lanes A and E belong to total granulocyte-macrophage colony-stimulating factor, and lanes B and D belong to soluble granulocyte-macrophage colony-stimulating factor. M: protein marker. The processed and unprocessed proteins are shown by arrows

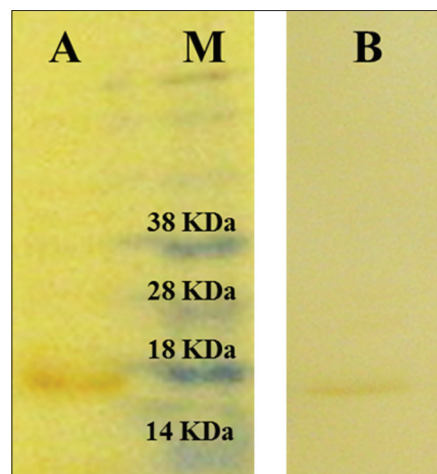


Figure 4: Western blot analysis with anti-His antibody: granulocyte-macrophage colony-stimulating factor expressed in *Escherichia coli* BL21(DE3) containing pET22b-granulocyte-macrophage colony-stimulating factor plasmid (lane A) and in *Escherichia coli* BL21(DE3) containing pET28a-GM-CSF plasmid (lane B)

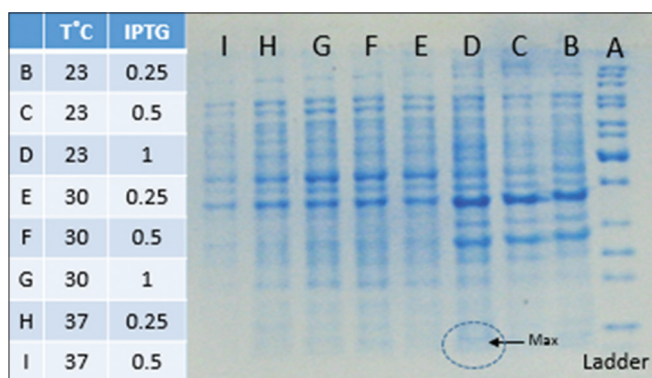


Figure 5: Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of periplasmic expression of granulocyte-macrophage colony-stimulating factor with different concentration of isopropyl β-D-1-thiogalactopyranoside (0.25 mM, 0.5 mM, and 1 mM) at various temperatures (23°C, 30°C, and 37°C). Description of corresponding lanes is presented in the table

production of GM-CSF in cytoplasm and periplasm. However, previous studies usually obtained very low yield of protein periplasmic expression and most of protein could not be translocated into periplasm space.^[23] There are several advantages for secretion of recombinant protein to the periplasmic space including oxidizing environment (i.e. the presence of two foldase disulfide oxidoreductase (DsbA) and disulfide isomerase (DsbC) which all help to the correct disulfide bonds formation), diminished proteolysis, and decreasing of contamination with other proteins or contents in the cytoplasm.^[24] To secrete the protein to the extracytoplasmic space, a signal peptide is needed that its sequence must be added to the N-terminal of target protein. Five signal peptides including *OmpA*, *pelB*, *Lpp*, *PhoA*, and *MalE* had been broadly used for efficient secretion of recombinant proteins in

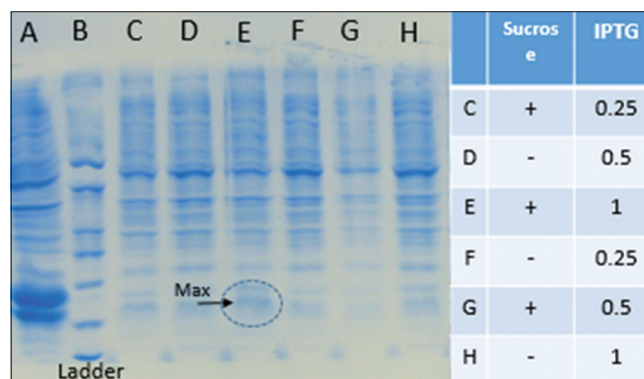


Figure 6: Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of periplasmic expression of granulocyte-macrophage colony-stimulating factor with different concentration of isopropyl β-D-1-thiogalactopyranoside (0.25 mM, 0.5 mM, and 1 mM) at 23°C in presence (+) or absence (-) of sucrose (0.4M). Description of corresponding lanes is presented in the table

E. coli.^[25] It is supposed that each signal peptide may have different behaviors when used for the secretion of different proteins.^[26] *pelB* is a 22 N-terminal leader sequence from pectatelyase B of *Erwinia carotovora* CE.^[27] Based on our results, the secretion of GM-CSF to periplasm was well driven by *pelB* signal sequence. However, still, the significant fraction of proteins were accumulated in the cytosol; this maybe due to high-level expression of GM-CSF and limited capacity of the translocation system and the signal peptidase to direct and process the expressed protein. Pan *et al.* reported effectively translocation of penicillin G acylase by *pelB* signal sequence in comparison the other signal peptide (*OmpA*, *Lpp*, *PhoA*, and *MalE*).^[28] Borjaliloo *et al.* assessed the expression of *pelB*-GM-CSF in two different expression system, and they reported that some part of the expressed protein could be secreted into the periplasmic space using T7 promoter.^[23] The choice of promoter is one of the important parameters in recombinant

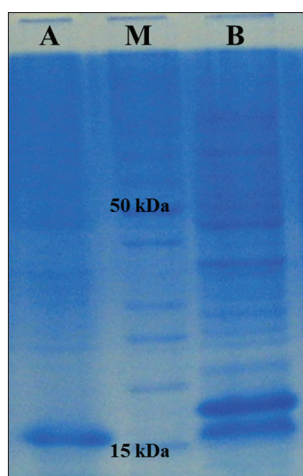


Figure 7: Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of concentrated purified granulocyte-macrophage colony-stimulating factor (lane A) and concentrated periplasmic granulocyte-macrophage colony-stimulating factor (lane B). M: protein marker

protein expression. In this study, the expression of GM-CSF was performed under T7 promoter control. At the present study, the T7 RNA phage polymerase, one of the most commonly used prokaryotic protein-producing systems that distinguishes the T7 phage promoter, was also used.^[29] This system causes very effective and high levels of target protein expression that can be reached up to 50% of the total cellular protein production.^[30] In some of the previous studies, this system was used for GM-CSF expression.^[19,23,31,32] In one study, the expression of GM-CSF was assessed under the control of lac and T7 promoter. According to the results, expression under the control of T7 promoter was more efficient than lac promoter.^[29] In the present study, the amount of expressed GM-CSF was significantly high in total protein expressed. Furthermore, according to the obtained SDS-PAGE results, two bands were observed in periplasmic expression GM-CSF; this can indicate that because of high level of protein expression, the signal peptide could be removed for some parts of periplasmic protein and not be removed from another part. In other words, the level and rate of protein expression and secretion were higher than protein possessing by signal peptidase. Based on the result of expression optimization, the maximum expression occurred at 23°C, with 1 mM IPTG and in the presence of sucrose. The optimum concentration of IPTG was in accordance with Hajinia *et al.*'s study results. They reported that 1 mM IPTG was the optimal concentration of this inducer for expression of hGM-CSF.^[33] Borjaliloo *et al.* evaluated the effect of temperature on rhGM-CSF expression. They reported that the optimum temperature for production of recombinant protein under control of T7/lac systems was 30°C.^[23]

Conclusion

Considering the important clinical applications of GM-CSF, production of this drug in a cost-effective and

efficient method seems necessary. Variety studies are performed about GM-CSF production. In the present study, periplasmic expression of GM-CSF in an optimized method was done. According to the results, the best temperature for expression of GM-CSF was 23°C in the presence of 0.4M sucrose and 1 mM IPTG. Biological activity of purified GM-CSF was checked and verified based on its effect of HL-60 cell lines proliferation. According to the advantages of periplasmic expression in comparison of cytoplasmic expression, it seems that optimized method in this study can help in effective expression of GM-CSF.

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Conflicts of interest

There are no conflicts of interest.

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