

OVERPRODUCTION AND PURIFICATION OF SOLUBLE RECOMBINANT HUMAN GRANULOCYTE COLONY STIMULATING FACTOR in *Escherichia coli* USING THIOREDOXIN AS FUSION

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Abstract

Recombinant human Granulocyte Colony Stimulating Factor (G-CSF) has been produced in a soluble form in *Escherichia coli* BL21 (DE3) as a fusion protein. The open reading frame of G-CSF was synthetically constructed in previous work and was codon optimized for best expression in *E. coli*. In this research, the gene was fused to thioredoxin (Trx) at the N-terminal in pET32 vector. The purpose of this research was to optimize the overproduction and purification processes to obtain high yield recombinant protein in soluble form, and to characterize the Trx-G-CSF fusion protein. Overproduction was performed using IPTG induction method for 3 and 6 hours. The protein was purified by Ni-NTA affinity chromatography and separated using gradient concentration of imidazole. The purified protein was then characterized by SDS-PAGE and Western Blot analysis. Further, enterokinase was used to separate G-CSF from the fusion protein. The purified form of G-CSF was subsequently characterized using Western Blot and mass spectrometry using MALDI-TOF. The results showed that the fusion protein was successfully produced in soluble part as much as 48.25% were obtained after 3 hours of induction. The yield of fusion protein was 67.37% from total protein (229.65 mg protein/L culture). The Western Blot analysis showed the G-CSF band at around 18.6 kDa. Mass spectrometry with MALDI-TOF/TOF revealed that 25.86% of amino acid residue was recognized as part of human G-CSF sequence.

Keywords: G-CSF, Thioredoxin, Soluble protein, *E. coli*

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Introduction

Biopharmaceutical is a biotechnologically manufactured that has largely used to treat many diseases. Nevertheless, biopharmaceuticals are commonly protected by patents for a period of 20 years. This circumstance triggers the emergence of similar product so-called biosimilar. Filgrastim is a recombinant human granulocyte colony stimulating factor (rhG-CSF) produced by Amgen which was developed in 1980's. The rhG-CSF has been used as therapeutic protein for neutropenia and chemotherapy treatment in order to decrease the depression of white blood cell levels produced by cytotoxic agents (Welte *et al.*, 1996). Biosimilar versions of filgrastim are now widely available and also used to induce

peripheral blood stem cell (PBSC) mobilization in patients undergoing autologous stem cell transplantation (AHSCT) (Remenyi *et al.*, 2014).

Meanwhile, hG-CSF is a central neutrophil-based immune defense due to its regulatory role in the growth, differentiation, survival, and activation of neutrophils and their precursors (Callard & Gearing, 1994). hG-CSF is a single polypeptide chain protein that consists of 174 amino acids, five cysteines that form two intramolecular disulfide bridges between Cys36-Cys42 and Cys64-Cys74 and one free cysteine at residue 17 (Lu *et al.*, 1989). hG-CSF crystal structure reveals four-helix-bundle fold and has one O-linked glycosyl group at Thr133, which protects the protein from aggregation and does not

influence the receptor binding (Hill *et al.*, 1993), therefore it is not required for its biological activity (Vanz *et al.*, 2008). The non-glycosylated *E. coli* derived rhG-CSF is biologically active as it is when produced in mammalian cell lines (Bonig *et al.*, 2001).

Currently, commercial rhG-CSF expressed in *E. coli* was produced in inclusion bodies (IB) form. The correct folding of protein which produced in *E. coli* was very important, often challenging due to the over expression protein could lead to aggregation and formation of IB, especially when expressed in the cytoplasm (Khow *et al.*, 2012). Expression of a protein in their soluble part is important for the protein to be biologically active. There are some properties of protein that can affect the formation of inclusion bodies such as presence of disulfide bonds (Lilie *et al.*, 1998), post-translational modifications, hydrophobicity, net charge and secondary structure (Chiti *et al.*, 2003). In order to recover soluble part from the IB, the proteins are solubilized in the presence of strong denaturants such as urea or guanidinium hydrochloride, followed by removing the denaturants under optimal conditions that assist refolding process (Lilie *et al.*, 1998).

Many strategies are applied to improve protein solubility production in the cytoplasm. One of the strategies is expressed the protein as fusion protein, such as glutathione S-transferase (GST), maltose binding protein (MBP) and thioredoxin (Trx). Trx is crucial proteins for oxidative stress defense, redox regulation of cellular function, and different metabolic pathway. Trx as thiol-dependent protein would mediate a reversible disulfide bond formation, this exchange reducing will take the equivalent between their active site cysteines and the cysteines of target proteins that will form the correct folding of the proteins (Ritz & Beckwith, 2001). Trx overproduction to increase the solubility of foreign protein could effect redox reaction of disulfide linkages (Yasukawa *et al.*, 1995). In this research, *E. coli* trx was used as a fusion partner to G-CSF protein in order to circumvent inclusion body formation in *E. coli* cytoplasm.

Materials and Methods

Bacterial strain, vector and growth media

Two *E. coli* strains were used in the experiment, DH5 α for sub cloning and plasmid amplification, and BL21(DE3) for protein expression. The pET32a(+) vector (Novagen, Darmstadt, Germany) was linearized with *Bgl*III enzyme (Fermentas, Vilnius, Lithuania). The G-CSFsyn ORF was previously constructed by dual asymmetrical-overlap extension PCR and clone into pTZ57R vector (Fuad *et al.*, 2013). Luria-Bertani (LB) broth and agar containing 50 μ g/ml of ampicillin were used for growing recombinant *E. coli* and protein production.

Plasmid Construction

The G-CSF ORF contained *Bgl*III restriction sites obtained by PCR amplification from pTZ57R-G-CSF as DNA template. The DNA insert was separated by electrophoresis on 1% of agarose gel, then purified with DNA fragment extraction kit (Geneaid, Taipei, Taiwan). The DNA insert was inserted to pET-32a(+) vector at corresponding restriction sites, then transformed into *E. coli* DH5 α . Analysis of transformant was performed by isolating the recombinant plasmid from single colony resistant to ampicillin on selection plate. The recombinant plasmid was analyzed using restriction with *Bgl*III enzyme. For further analysis, DNA sequencing using T7 promoter and T7 terminator were used to confirm the DNA sequences. Template for DNA sequencing was produced by PCR amplification of recombinant plasmid using a pair of T7 promoter and T7 terminator primers. pET32(+)-G-CSF plasmid which was confirmed by DNA sequencing, subsequently transformed into *E. coli* BL21(DE3) cells.

Protein Overproduction

A single colony of *E. coli* BL21(DE3) harboring the pET32a(+)-G-CSF plasmid was inoculated into selective LB broth with agitation at 150 rpm at 37 °C overnight. Five percent v/v of this overnight culture was inoculated into fresh selective LB broth with agitation at 150 rpm at 37 °C until OD₆₀₀ 0.6-0.8 was reached. The protein expression was induced by Isopropil- β -D-1-Thiogalactopyranoside (IPTG) with the final concentration of 0.4 mM. Cells were harvested

after 3 hours by cold centrifugation at $4500 \times g$ for 15 min.

Protein Isolation, Purification and Cleavage

Wet cell pellets were washed with phosphate buffer pH 7.4 (20 mM sodium phosphate and 0.5 M NaCl) and recovered by cold centrifugation at $4500 \times g$ for 10 min. The cells were resuspended in lysis buffer (50 mM Tris HCl at pH 7.4, 3 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF)) with ratio of 8 ml lysis buffer per gram pellet. The cell suspension was frozen at -20°C for 15 min, and then thawed at room temperature for 30 min, this freeze-thaw process was repeated 10 times. The crude cell lysates were recovered by cold centrifugation at $9600 \times g$ for 10 min at 4°C .

The Trx-G-CSF protein was produced as a fusion protein with histidine tag at its amino terminus and purified using Ni-NTA His-Bind Resin according to manufacturer's protocol (Qiagen, Hilden, Germany). Crude lysates were added to Ni-NTA agarose matrix at 1 : 1 v/v ratio. The binding of protein was done using rotator at 4°C for 18 h. The matrix washed with washing buffer (20 mM imidazole in phosphate buffer pH 7.4) for five washing fraction and 50 mM imidazole for another five washing fractions. The elution of bound protein was performed by using phosphate buffer consisting of gradient concentration of imidazole start from 50 mM to 500 mM. Total and purified Trx-G-CSF fusion proteins was each analyzed using 15% Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and stained with coomassie blue.

Purified protein was dialyzed against 0.5 M Tris HCl of pH 8.0 for 48 h with buffer changing every 6 h. The fusion protein was then proteolytically cleaved with Enterokinase (EK) enzyme as described in manufacturer's protocol (EKMax, Invitrogen, California, USA). The protein was incubated with EK at 20°C for 16 h using various amount of enzyme. Ni-NTA agarose affinity chromatography was used to separate G-CSF protein fragment from fusion. The cleavage product was analyzed with 17% SDS-PAGE.

Western Blot Analysis

The protein separated by 15% SDS-PAGE was analyzed by Western blot analysis as described in protein blotting guide (Biorad). The acrylamide gel was transferred into nitrocellulose membrane. The protein on nitrocellulose membrane, incubated with primary antibody. The mouse anti-His monoclonal antibody (GE healthcare, Buckinghamshire, UK) was used as primary antibody for detection of Trx-G-CSF fusion protein or Trx-His tag fragment, diluted 1:3500 in TBS-T. The other was rabbit anti-G-CSF polyclonal antibody (Santa Cruz Biotechnology, California, USA) used for detection of G-CSF protein after separation from Trx fusion, dilutes 1:3500 in TBS-T. Secondary antibody that used was goat anti-mouse IgG AP conjugate and goat anti rabbit IgG AP conjugate (Santa Cruz Biotechnology, California, USA) (1:3500) performed for each primary antibody. The signal was developed using Western Blue® substrate for AP (Promega, Wisconsin, USA).

Protein analysis using MALDI-TOF/TOF MS

MALDI-TOF spectrometry allows the identification of protein based on the amino acid sequence in polypeptide chains. Most typically, analyzed protein is processed enzymatically (e.g., by trypsin digestion), then selected peptides to undergo further fragmentation in spectrometer, and sizes (m/z values) of fragmentary ions are determined in the so called tandem mass spectrometry (ms/ms) (Pietrowska and widlak, 2012).

Peptides were extracted according to standard techniques (Bringans, *et.al.*, 2008), analyzed by MALDITOF-TOF (*Matrix assisted laser desorption/ionization time of flight*) mass spectrometer using a 41800 Proteomics Analyzer (AB Sciex). Spectra were analyzed to identify protein of interest using Mascot sequence matching software (Matrix Science) with Ludwig NR Database. This analysis was performed by Proteomic International Laboratory Ltd, Australia.

Results and Discussion

Construction of Recombinant Expression Vector

The ORF was inserted downstream of DNA encoding for *trxA* and *6x-His-tag* of pET-32a(+) at *Bgl*III restriction site. DNA insert was of 567 base pairs (bp) in size, which correlated to the theoretical size of G-CSF ORF, 567 bp (Fig. 1, lane 1). After transformation, several putative recombinant plasmids were characterized by migration and single digestion analyses. Single digestion of a recombinant plasmid using *Eco*RI generated a linear plasmid of 6467 bp (Fig. 1, lane 3) which was in accordance with the theoretical size of recombinant pET-32a(+)-G-CSF.

The insert DNA of a recombinant plasmid was analyzed by DNA sequencing. The sequencing result showed that the insert DNA contained the sequence of codon-optimized ORF of G-CSF gene without any mutation. The confirmed recombinant plasmid was designated as pET-32a(+)-G-CSFsyn (Fig. 2).

Overproduction, Purification and Characterization of Trx-hG-CSF Fusion Protein

The expression of Trx-hG-CSF fusion protein was induced by addition of 0.4 mM IPTG in LB liquid medium in a shake flask with vigorous shaking (150 rpm) at 37 °C. The Trx-hG-CSF fusion protein was produced mostly in soluble form (96.6%) and the remaining was as insoluble protein (3.38%) (Fig. 3). The calculation was using image J software. Results obtained by other research showed the rhG-CSF mostly in the form of Inclusion Body (IB) and to be solubilized in order to become an active protein (Fallah, *et al.*, 2003, Vanz, *et al.*, 2008, Babaeipour, *et al.*, 2010, Dehaghani, *et al.*, 2010, Rao, *et al.*, 2008). hG-CSF codon optimization that was done to improve protein expression by reducing the GC content at 5'end (Alrokayan, S., 2011) resulting rhG-CSF in soluble and insoluble form. Jeong and Lee, 2001, successfully expressed rhG-CSF on periplasmic space using fusion with *Bacillus* sp endoxylanase signal peptide. As previously reported by Song *et al.*, 2009, rhG-CSF expressed with Pyr-B and GST as N-terminus fusion partner was effectively protected the

protein from proteolysis and capable of increasing solubility. Native hG-CSF gene was difficult to be transcribed in *E. coli* because of the high (G + C) contents (Devlin *et al.*, 1988). Therefore, in this research codon optimization was carried out to decrease (G + C) content of hG-CSF. Native hG-CSF had 65.7% of (G + C) content whereas rhG-CSF resulted in this research had 60.7% of (G + C) content, respectively. rhG-CSF solubility was expected to increased significantly using N-terminus Trx fusion and decreasing of (G + C) content. The result of Trx mechanism to helps fusion protein produced in soluble form was not clear. In oxidoreductase activity, Trx control the activity target protein via reversible thiol-disulfide exchange reaction (Kumar, *et al.*, 2003).

The crude intracellular proteins were obtained by cold sonication and freeze thaw methods. Protein obtained from freeze thaw method was better than that of sonication (Fig. 4). The appropriate isolation protein method can be selected based on the protein location. The *E. coli* Trx position was on the cytoplasmic facing the adhesion zones that exist between the inner and outer membranes of the *E. coli* cell envelope (Bayer, 1968). Since the Trx-hG-CSF fusion protein contained 6x-His tag at its amino terminus, Ni-NTA affinity chromatography was applied to purify the protein of interest. The quality of protein purification was influenced by matrix binding capacity and quality of crude protein. The protein of interest found on flow through (FT) phase from sonication crude indicates that the protein unattached well on the matrix (Fig. 4b lane 4). The result also showed that the protein obtained from freeze thaw method was better than sonication method, there was no target protein band on FT phase indicating that protein is attached well on the matrix (Fig. 4a lane 4). The protein isolated using freeze thaw, would tear out the cell gently, and entirely separated by centrifugation.

The yield of purified Trx-hG-CSF fusion protein was approximately 228.5 mg protein per L of cell culture (data were taken from 3 repetitions). The protein concentration was estimated by Image J software. The Image J software can determine concentration of a protein in SDS-PAGE gel bands. The protein of interest was applied in SDS-PAGE with some concentration of BSA as standard

protein. A standard curve would be created by plotting intensity of BSA as a function of mass, then the concentration of protein interest can be defined (Ferreira and Rasband, 2012). SDS-PAGE analysis showed the presence of a protein band with the apparent size of 36 kDa which was close to the theoritical size, 37.6 kDa (Fig. 4, lanes 6, 7, 8). The identity of the 36 kDa protein was confirmed by a Western blot analysis using mouse monoclonal antibody against His-tag peptide (GE healthcare, Buckinghamshire, UK).

The fusion protein also contained enterokinase (EK) cleavage site (Fig. 1). The EK cleavage site was introduced into the sequence to separate the G-CSF protein from Trx fusion. Fig. 5A shows that the Trx-G-CSF fusion was cleaved specifically at EK cleavage site, resulting two protein fragments, a 19 kDa product corresponding to the size of G-CSF and 17 kDa product consisting of Trx and 6xHis-tag (Fig. 5, lane 2). Uncleaved protein fusion and the Trx portion were removed by Ni-NTA affinity chromatography, the G-CSF protein was recovered from the unbound fraction (Fig. 5, lane 3). Western blot analysis using rabbit polyclonal antibody against human G-CSF (Santacruz, Texas, USA) confirmed that the 19 kDa product to be G-CSF protein (Fig. 5B) and the 17 kDa product was identified as Trx by mouse monoclonal antibody against His-tag (Fig. 5C).

MALDI-TOF/TOF analysis used trypsin in order to digest the recombinant protein. Trypsin is the most commonly used enzyme in mass spectrometry, it was used due to it highest specificity protease. Trypsin is a serine protease, it cleaves peptide chains mainly at the carboxyl side of the amino acids lysine or arginine, except when either is followed by proline (Rodriguez, *et al.*, 2008) rhG-CSF characterization can be improved by using another protease, such as Chymotrypsin. In silico study using Chymotrypsin-high specificity to digest hG-CSF protein, showed 13 cleavage sites i.e. at position 20, 25, 43, 72, 91, 116, 118, 146, 151, 173, 177, 193, and 198. Chymotrypsin cleavage sites at amino acid 91, 116, 118, 146, and 151 can digest the hG-CSF fragment which was can not be digested by trypsin enzyme.

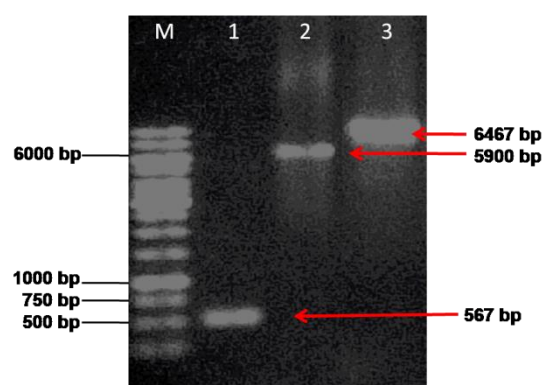


Fig. 1 Agarose gel electrophoresis of DNA. M. 1 kb DNA ladder, 1. ORF of CSFsyn gene, 2. Plasmid pET-32a(+) digested with *Bgl*II, 3. Recombinant pET-32a(+)-CSFsyn digested with *Eco*RI

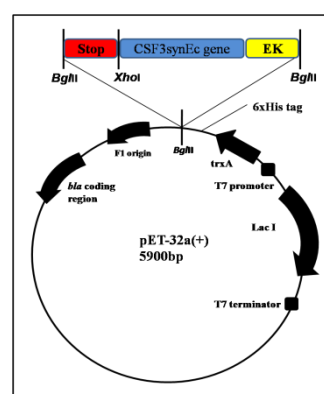


Fig. 2 Plasmid map of vector pET-32a(+)-G-CSFsyn

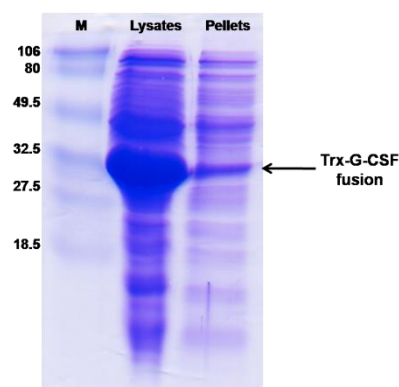


Fig. 3 SDS-PAGE analysis of overproduction of Trx-hG-CSF fusion protein in *E. coli* BL21(DE3). M. Marker 1. Soluble form in the protein lysate. 2. Insoluble form in cell pellet.

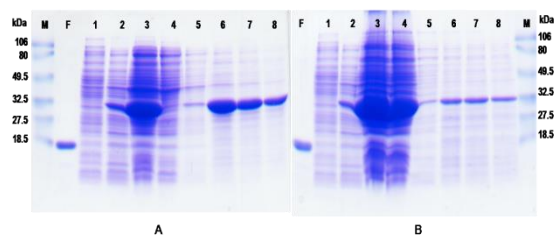


Fig. 4 Analysis of *E. coli* crude lysate and purified Trx-hG-CSF fusion protein using SDS-PAGE. Analysis of crude lysate obtained using freeze thaw method (a) and that obtained using sonication method (b). Lanes: 1. Crude lysate from non-recombinant *E. coli*, 2. Crude lysate from recombinant *E. coli* obtained from non-induced condition, 3. Crude lysate from recombinant *E. coli* from the induced condition, 4. Flow Trough fraction, 5. Washing fraction, 6 - 8. Eluted fraction, M. Unstained protein marker.

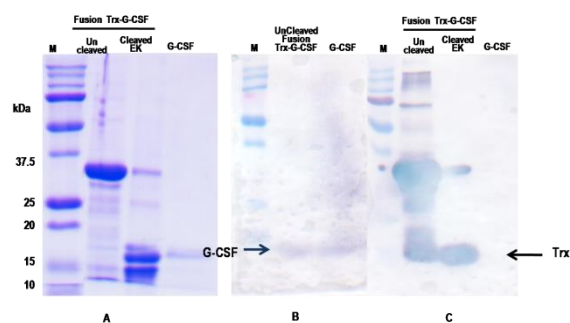


Fig. 5 Analysis of Trx-hG-CSF fusion protein cleavage by EK. (A) SDS-PAGE analysis of affinity-purified hG-CSF protein (B) Western blot analysis using rabbit polyclonal antibody against human hG-CSF, and (C) Western blot using mouse polyclonal antibody against 6xHis-tag.

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1  TPLGPASSLP QSFLLKCLEQ VRKIQGDGAA LQEKLCATYK
41  LCHPEELVLL GHSLGIPWAP LSSCPSQALQ LAGCLSQLHS
81  GLFLYQGLLQ ALEGISPELG PTLDTLQLDV ADFATTIWQQ
121 MEELGMAPAL QPTQGAMPAF ASAFQRRAGG VLVASHLQSF
161 LEVSYRVLRH LAQP

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Fig. 6 Amino acid sequence of hG-CSF protein as determined by MALDI TOF/TOF MS analysis. Letters in red: amino acid residues identified in the analysis, letters in blue: 207 amino acid residues with no tryptic digestion site, letters in black: amino acid residues not identified in the analysis.

Conclusion

The Trx-G-CSF fusion protein was successfully produced as a soluble protein and confirmed by anti-His antibody. The G-CSF protein was obtained by EK digestion, followed by separation using affinity chromatography. The identity of G-CSF protein was confirmed by anti-G-CSF antibody and MALDI TOF/TOF analysis.

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