Human G-CSF synthesis using stress-responsive bacterial proteins

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Abstract

We previously reported that under the stress condition caused by the addition of 2-hydroxyethyl disulfide, a thiol-specific oxidant, to growing cultures of *Escherichia coli* BL21(DE3), a population of stress-responsive proteins [peptidyl-prolyl cis–trans isomerase B (PpiB), bacterioferritin (Bfr), putative HTH-type transcriptional regulator yjdC (YjdC), dihydrofolate reductase (FolA), chemotaxis protein cheZ (CheZ), and glutathione synthetase (GshB)] were significantly upregulated when compared with the nonstress condition. When those stress-responsive proteins were used as fusion partners for the expression of human granulocyte colony-stimulating factor (hG-CSF), the solubility of hG-CSF was dramatically enhanced in *E. coli* cytoplasm, whereas almost all of the directly expressed hG-CSF were aggregated to inclusion bodies. In addition, the spectra of circular dichroism measured with the purified hG-CSF were identical to that of standard hG-CSF, implying that the synthesized hG-CSF has native conformation. These results indicate that the bacterial stress-responsive proteins could be potent fusion expression partners for aggregation-prone heterologous proteins in *E. coli* cytoplasm.

Introduction

Human granulocyte colony-stimulating factor (hG-CSF) is a hematopoietic growth factor that plays an important role in hematopoietic cell proliferation, differentiation of hematopoietic precursor cells, and activation of mature neutrophilic granulocytes (Metcalf, 1985; Nomura *et al*., 1986). In addition, hG-CSF has been widely used for treating neutropenia caused by cancer chemotherapy. The mature hG-CSF is an 18.7-kDa glycoprotein that predominantly consists of 174 amino acids with two intramolecular disulfide bonds, although another minor form comprised of 177 amino acids as a result of alternative splicing of mRNA has also been described (Nagata *et al*., 1986; Lu *et al*., 1992; Hill *et al*., 1993). Furthermore, native hG-CSF has only one single O-glycosylation site at Thr133, which is not essential for biological activity (Kubota *et al*., 1990). Therefore, nonglycosylated recombinant hG-CSF has the same specific biological activity as natural glycosylated hG-CSF (Souza *et al*., 1986; Oh-eda *et al*., 1990). Because hG-CSF is synthesized in *Escherichia coli* as inclusion bodies, solubilization and renaturation steps are necessary to obtain the native conformation (Misawa & Kumagai, 1999), which means that highly complicated downstream processes with low-recovery yield are required.
a proteome-wide analysis of E. coli proteins under 2-hydroxyethyl disulfide (2-HEDS)-induced stress condition (Han et al., 2008). Using the six stress-responsive proteins of E. coli, we successfully produced hG-CSF with correct conformation as well as demonstrated using circular dichroism (CD).

Materials and methods

Bacterial strain and plasmid vectors

Escherichia coli strain BL21(DE3) [F'ompT hsdSB(rB'CmB')] was used for the hG-CSF synthesis. After PCR amplification using appropriate primers, hG-CSF gene and its various fusion mutants (YjdC::hG-CSF, GshB::hG-CSF, FolA::hG-CSF, Bfr::hG-CSF, CheZ::hG-CSF, PpiB::hG-CSF) were inserted into the NdeI–HindIII site of plasmid pET28a (Novagen) to construct the fusion expression vector (Fig. 1). After complete DNA sequencing of all gel-purified plasmid vectors, the E. coli BL21(DE3) was transformed with the plasmid expression vectors, and kanamycin-resistant transformants were subsequently selected using Luria–Bertani (LB) agar plates supplemented with kanamycin (50 mg L⁻¹).

Recombinant E. coli culture, gene expression, and recombinant hG-CSF purification

For shake flask experiments, 250-mL Erlenmeyer flasks containing 50 mL LB media with 50 mg L⁻¹ kanamycin were incubated at 37 °C and 200 r.p.m. When the culture turbidity (OD₆₀₀ nm) reached 0.5, gene expression was induced by the addition of isopropyl-β-D-thiogalactoside (1 mM), and after an additional 4 h of cultivation all of the recombinant cells were harvested by centrifugation (MICRO17TR, Hanil Science Industrial, Korea); 16 609 g for 5 min. The cell pellets were then resuspended in 5 mL lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA) and were disrupted using a Branson Sonifier (Branson Ultrasonics Corp., Danbury, CT). The cell-free supernatant and insoluble protein aggregates were then separated at 16 609 g (MICRO17TR, Hanil Science Industrial) for 10 min. The isolated inclusion bodies, if any, were washed twice with 1% Triton X-100. The cell-free supernatants and the washed inclusion bodies were then subjected to polyacrylamide gel electrophoresis (PAGE) analysis, and Coomassie-stained protein bands were scanned and analyzed by densitometry (Duoscan T1200, Bio-Rad, Hercules, CA).

The purification of recombinant hG-SCF was accomplished using metal affinity chromatography. Briefly, polyhistidine-tagged hG-CSF fusion mutants [(His)₆::fusion partner::(D₄K)::hG-CSF] were loaded onto a ProBond resin (Ni²⁺⁺ column). Before sample loading, the resin was washed twice with 10 column volumes of binding buffer (50 mM potassium phosphate, 300 mM KCl, 20 mM imidazole, pH 7.0), which contained 20 mM imidazole to minimize nonspecific binding of untagged protein contaminants. Binding was then conducted in batch mode at 4 °C, after which the resin was washed twice with 5–8 mL Tris-HCl (10 mM Tris, pH 8.0) before the enterokinase digestion step. Enterokinase digestion was carried out in batch mode at 4 °C for 10 h using 5 U of enterokinase (Invitrogen, CA).

![Fig. 1. Plasmid vectors used for the direct (a) and fusion (b) expression of hG-CSF. As shown in (b), the plasmid vectors for the fusion expression of hG-CSF contain hexa-histidine tag (His)₆ and the specific sequence for enterokinase cleavage (DDDDK).](image-url)
Next, the proteolytic product was collected and centrifuged (MICRO17TR, Hanil Science Industrial); 16 609 g for 10 min, and the supernatant fraction was then analyzed by sodium dodecyl sulfate (SDS)-PAGE and Western blotting. Removal of the enterokinase was conducted following the manufacturer’s protocol (EK-Away™ resin, Cat. No. R180-01, Invitrogen, Germany).

**SDS-PAGE and Western blot analysis**

hG-CSF digests were resuspended in 0.5 M Tris-HCl, pH 6.8, 10% SDS, 10% glycerol, 0.05% bromophenol blue, 5% 2-mercaptoethanol, and then subjected to reducing 12% SDS-PAGE using Tris-glycine gel. Peptides were transferred onto a nitrocellulose membrane (Schleicher & Schuell BioScience GmbH, Germany) for 2 h at 4°C in transfer buffer (glycine 2.9 g L⁻¹, Tris base 5.8 g L⁻¹, SDS 0.37 g L⁻¹, MEOH 200 mL, distilled water 800 mL). Nonspecific antibody-binding sites on the membrane were blocked by incubating for 1 h at room temperature with blocking buffer [1% nonfat skim milk in phosphate-buffered saline (PBS)]. After washing three times with PBS, the membrane was incubated for an additional 1 h at room temperature with 1:1000 diluted hG-CSF primary antibody (mouse anti-human G-CSF monoclonal antibody; Clone 3D1, Santa Cruz Biotechnology Inc., CA). The membrane was washed again as described above, after which it was incubated with 1:1000 diluted goat anti-mouse immunoglobulin G-conjugated horseradish peroxidase (HRP) secondary antibody (Santa Cruz Biotechnology Inc.). After being washed, the membrane was developed using an HRP conjugate substrate kit (Sigma-Aldrich, MO).

**CD**

The CD spectra of commercial standard hG-CSF [Grasin Prefilled-Syringe (Filgrastim), Kirin, Japan] and the purified hG-CSF (74 mg L⁻¹) were measured using a JASCO J-710 spectropolarimeter (Korea Basic Science Center, Ochang, Korea) at room temperature.

**Results and discussion**

**Direct expression of hG-CSF gene in the cytoplasm of E. coli**

For the direct expression of mature hG-CSF gene in E. coli cytoplasm, the plasmid vector, pT7-GCSF was constructed by inserting the mature hG-CSF gene into the NdeI–HindIII site of the pT7-7 vector. However, as previously reported by Devlin et al. (1988), the native hG-CSF gene is hardly transcribed in E. coli because of the high (G+C) contents at 5' end of coding strand. Therefore, the 5'-end coding sequences of hG-CSF gene were modified by changing the first five codons (5' ACC CCC CTG GGC CCT 3') to the high-frequency codon of E. coli (5' ACT CCG TTA GGT CCA 3') (Jeong & Lee, 2001). As shown in Fig. 2, the expression of the modified hG-CSF gene resulted in the synthesis of a large amount (> 40% of total E. coli proteins) of recombinant hG-CSF. However, most of the synthesized hG-CSF was present in the form of insoluble inclusion bodies.

**Strategy to enhance solubility of recombinant hG-CSF using E. coli stress-responsive proteins**

The stress induced by overexpression of recombinant proteins resembles environmental stress conditions such as heat shock or amino acid starvation (Sørensen & Mortensen, 2005). Similarly, when proteins are exposed to oxidative stressors such as oxygen radicals and hydrogen peroxide (H₂O₂), E. coli decreases the protein synthesis rate and enhances the protein degradation pathway (Koo et al., 2004). Previously, VanBogelen et al. (1987) reported that the expression level of molecular chaperones such as DnaK and GroES increased in the presence of H₂O₂-derived oxidative stress. It has also been reported that addition of 2-HEDS, a thiol-specific oxidant, dramatically reduced the NADPH/NADP⁺ ratio consistently in response to severe oxidative stress (Halleck et al., 1997; Ayene et al., 2002). In our recent report (Han et al., 2008), we investigated the E. coli proteome under 2-HEDS-induced stress conditions.
and found stress-responsive proteins that are significantly upregulated by the stressor through the comparative and quantitative analysis of *E. coli* proteomes under nonstress and stress conditions. Because many proteins were aggregated by 2-HEDS-induced stress, it seems reasonable to assume that such stress-responsive proteins have an intrinsically higher folding efficiency than other proteins that are aggregated in response to the same stress conditions. Among the stress-responsive proteins that we previously found (Han et al., 2008), we were interested in the six stress-responsive proteins [peptidyl-prolyl cis–trans isomerase B (PpiB, 18 kDa), bacterioferritin (Bfr, 19 kDa), putative HTH-type transcriptional regulator yjdC (YjdC, 22 kDa), dihydrofolate reductase (FolA, 18 kDa), chemotaxis protein cheZ (CheZ, 24 kDa), and glutathione synthetase (GshB, 36 kDa)] that have relatively lower molecular mass compared with the other stress-responsive proteins. We found that these relatively small stress-responsive proteins were highly effective in enhancing the solubility of recombinant hG-CSF when used as fusion partner, which will be discussed next.

**Fusion expression of hG-CSF using *E. coli* stress-responsive proteins as fusion partners**

The six stress-responsive proteins (PpiB, Bfr, YjdC, FolA, CheZ, and GshB) were used as N-terminus fusion expression partner to synthesize the hybrid proteins, i.e. NH$_2$-[fusion partner]::[D$_4$K]::[hG-CSF]-COOH in *E. coli* cytoplasm. Proteins with high molecular mass are usually not used as fusion partner because the big fusion partners may reduce the actual amount of target protein that is obtained after the fusion partner is removed. PpiB (18 kDa), Bfr (19 kDa), YjdC (22 kDa), FolA (18 kDa), CheZ (24 kDa), and GshB (36 kDa) are relatively small proteins compared with the other proteins above and were also the most effective in enhancing the solubility of hG-CSF. As shown in Fig. 3a and b, the N-terminus fusion of six stress-responsive proteins led to a significant increase in both the expression level and solubility of hG-CSF. Inclusion body formation presumably occurs due to protein overexpression in a heterologous environment in which nascent polypeptide chains are continuously synthesized and then aggregate because of nonspecific hydrophobic interactions among the partially folded intermediates of the recombinant proteins, various host proteins, or a combination of the two within the cytoplasm. In many cases, broad exposure of hydrophobic surfaces on newly synthesized polypeptides results in intermolecular aggregation and misfolding. To prevent such occurrences, molecular chaperones (DnaK/DnaJ/GrpE system, GroEL/GroES, etc.) that recognize and interact with hydrophobic patches of nascent proteins can be used (Maier et al., 2005). Hydrophobic amino acids are usually buried within the core of folded proteins but become exposed in the partially folded polypeptide intermediates (Rüdiger et al., 1997; Suh et al., 1999). Because of this, the solubility-enhancing function of stress-responsive proteins may be similar to that of cis-acting molecular chaperones in the context of fusion proteins (Kapust & Waugh, 1999) or as a ‘chaperone magnet’ (Fox et al., 2001; Ahn et al., 2005) that effectively recruits chaperone binding and prohibits undesired and nonspecific protein–protein interactions.

**Purification and characterization of fusion partner-free hG-CSF**

Because the addition of a fusion partner to the native peptide can influence the biological activities and the
physical properties of the protein (Esposito & Chatterjee, 2006), the removal of the fusion tags is of crucial importance especially when the recombinant protein is a human therapeutic reagent. In order to purify the fusion partner-free hG-CSF in E. coli, we already inserted the hybrid gene encoding NH₂-[fusion tag]:[D₄K]:[hG-CSF]-COOH into the NdeI–HindIII site of the pET28a(+) plasmid vector.

Most of the expressed fusion protein, i.e. NH₂-(His)₆-[fusion tag]:[D₄K]:[hG-CSF]-COOH was present in the soluble fraction of the cell lysates, and then the soluble fraction was subjected to metal affinity purification using an Ni²⁺–NTA column (Wülfing et al., 1994). After loading the soluble fraction containing the fusion proteins onto the Ni²⁺–NTA column, we subsequently added enterokinase to the column to remove the fusion tags. Because the sequence D₄K for enterokinase cleavage is downstream of the fusion tag, the cleaved fusion tags as well as any uncleaved fusion proteins still have the (His)₆ tag and hence remains bound to the Ni²⁺–NTA column, while the released hG-CSF is found in the flow-through from the Ni²⁺–NTA column. The flow-through containing the released hG-CSF was centrifuged, and the insoluble pellet and supernatant were then analyzed by SDS-PAGE and Western blot (Fig. 4). Figure 4 clearly indicates that the recombinant hG-CSF released from the fusion protein was still present in the form of soluble protein.

With the purified hG-CSF released from all the six fusion proteins, the spectra of CD were analyzed and compared with the CD spectrum of commercial standard hG-CSF [Grasin Prefilled-Syringe (Filgrastim), Kirin]. CD is very useful for the analysis of secondary structures of polypeptides and proteins (Purdie et al., 1989). Recent studies also verified the proper folding of recombinant hG-CSF using CD spectra (Bae et al., 1998, 1999; Jeong & Lee, 2001). In the other recent studies using CD spectrum, properly folded conformations of recombinant human and viral proteins were structurally analyzed (Leopoldino et al., 2006; Brucz et al., 2007; Tolun et al., 2007; Vamvakas et al., 2007). The CD spectra of the purified fusion-free hG-CSF (Fig. 5a) were identical to the spectrum of commercial hG-CSF standard.

![Fig. 4](image)

**Fig. 4.** (a) Results of SDS-PAGE analyses showing the solubility of recombinant hG-CSF purified after the removal of fusion partner by enterokinase treatment. M, molecular markers; 1, soluble fraction of recombinant cell lysates; 2, soluble fraction of purified hG-CSF; 3, insoluble fraction of purified hG-CSF, each arrow indicates the purified hG-CSF. (b) Results of Western blot analyses of purified hG-CSF that were run from YjdC::hG-CSF (lane 1), GshB::hG-CSF (lane 2), FolA::hG-CSF (lane 3), CheZ::hG-CSF (lane 4), PpiB::hG-CSF (lane 5), and Bfr::hG-CSF (lane 6).

![Fig. 5](image)

**Fig. 5.** CD spectra measured with the purified recombinant hG-CSF that were run from PpiB::, Bfr::, YjdC::, FolA::, CheZ::, and GshB::hG-CSF (a) and CD spectrum of commercial standard of hG-CSF (b).
Consequently, the six stress-responsive proteins had a conformation with the correct secondary structure. It is also assumed that the recombinant hG-CSF possesses biological activities similar to native hG-CSF based on the proper conformation of recombinant hG-CSF, even though the biological activity was not directly determined (Bae et al., 1998, 1999; Jeong & Lee, 2001).

Conclusion

We used the upregulated stress-responsive proteins as N-terminus fusion expression partners upon the synthesis of hG-CSF, and then the hybrid proteins designated as NH2-[fusion partner]-[hG-CSF]-COOH were synthesized in E. coli cytoplasm. The synthesized hG-CSF was aggregated and formed inclusion bodies when directly expressed without the N-terminus fusion tag. However, the solubility of hG-CSF in E. coli cytoplasm dramatically increased when the stress-responsive proteins were used as fusion partners, indicating that the fusion expression partners were highly effective solubility enhancers. In addition, even after the fusion partner was removed by enzymatic cleavage, the released, i.e. fusion-free recombinant hG-CSF was present in the form of soluble protein. The CD spectra of the affinity-purified hG-CSF was investigated, and the results showed that the recombinant hG-CSF had a conformation with the correct secondary structure. Consequently, the six stress-responsive E. coli proteins were highly effective cis-acting solubility and folding enhancers for the production of hG-CSF and could be used for the bacterial synthesis of other aggregation-prone heterologous proteins in the form of soluble and active proteins.

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