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Research Article

SOLUBLE EXPRESSION OF SYNTHETIC *CSF3syn* GENE FUSED WITH THIOREDOXIN IN *Escherichia coli* BL21(DE3) THROUGH AUTOINDUCTION METHOD AND PURIFICATION

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ABSTRACT

A synthetic human gene of CSF3 (*CSF3syn.Ec3*), coding for hG-CSF was succesfully subcloned into pET32a(+) expression vector and fused with thioredoxin (Trx) at its N-terminal as fusion partner. The obtained fusion gene of *Trx-CSF3syn* within the recombinant plasmid pET32a(+)_*CSF3yn.Ec3* was verified by PCR, plasmid restriction, and DNA sequencing analysis. In order to investigate the fusion gene expression, we transformed *Escherichia coli* BL21(DE3) as the host with the recombinant plasmid. The gene was succesfully expressed within the cytosol as fusion protein of Trx·tag, His·tag, S·tag, EK-site, and hG-CSF moieties. By the auto induction method, 49% of the protein was found in the soluble fraction and the other 51% was found in the insoluble fraction. The soluble fraction was subsequently purified by IMAC method (Ni-NTA) and characterized.

Key words *:* hG-CSF, thioredoxin, autoinduction, IMAC, *E.coli*.

INTRODUCTION

Granulocyte colony stimulating factor (G-CSF) or colony stimulating factor 3 (CSF3) is a hematopoietic cytokines that activates and regulates the production of mature neutrophile, acts on cell survival and differentiation (Metcalf, 1987; Nagata, 1986). It has been studied to have indications in post chemotherapy neutropenia, bone marrow transplantation, severe chronic neutropenia, and in hematologic malignancies (Dale, 1998; Welte, 2012).

Human G-CSF (hG-CSF) is 19.5kDa in molecular weight and *O*-glycosilated at Thr133 (Souza, 1986 and Welte, 2012). Post translational modification is not an issue for hG-CSF because *O*-glycosilated bond does not bind with receptor, so both of the wild-type and non-glycosylated mutant recombinant hG-CSF possess colony stimulating activities (Wingfield, 1988; Hill, 1993). However, the *O*-glycosilated bond has an important role to avoid aggregation, thus, hG-CSF is frequently produced in *E. coli* as inclusion bodies (Yamamoto, 2002; Dasari, 2008).

Thioredoxin (Trx) was used to improve the solubility of various eukaryotic proteins in *E. coli* (Lavielli, 1993; Yasukawa, 1995; Sandhev, 1998). Therefore, we are fascinated to apply Trx as a fusion partner to obtain soluble hG-CSF in *E. coli* BL21(DE3) using auto induction method. This method is more practical and less expensive than the conventional IPTG-induced method (Blommel, 2007, and Li, 2011). It is based on the principle of diauxic growth caused by two types of carbon source, i.e glucose and glycerol as the rapidly metabolized carbon sources and lactose as the slowly metabolized carbon source (Studier, 2005).

The aim of this study is to develope a new approach to express soluble hG-CSF by fusing with Trx and investigate autoinduction as an alternative method for this fusion protein expression. Therefore, the larger or even commercial scale of hG-CSF will be more applicable, more effective and efficient.

MATERIAL AND METHODS

Subcloning of *CSF3syn* into expression vector pET32a(+) and its transformation into expression host *Escherichia coli* BL21(DE3)

A synthetic gene coding hG-CSF was amplified by PCR of recombinant plasmid pTZ57R/T_*CSF3syn.Ec* using Applied Biosystem 2720 Thermal Cycler (Foster City, USA) (Wulandari, 2010). Restriction sites of *Bgl*II and stop codons were added by PCR amplification, this gene is subsequently named

CSF3syn.Ec3. The *CSF3syn.Ec3* and pET32a(+) (Novagen, Darmstadt, Germany) were cut with restriction enzyme of *Bgl*II (Fermentas, Vilnius, Lithuania), then ligated with T4 DNA ligase kit (Fermentas, Vilnius, Lithuania). The ligation product was transformed into a cloning vector *E. coli* XL1-Blue (Fermentas, St. Leon Rot, Germany) by heat shock method (Sambrook, 2001). The recombinant plasmid was verified by plasmid restriction and DNA sequencing analysis. In order to express the gene, the recombinant plasmid was transformed into *E. Coli* BL21(DE3) (Novagen, Darmstadt, Germany) as by heat shock method (Sambrook, 2001).

Small scale overexpression of *CSF3syn* through autoinduced method

Pre-inoculum was prepared from 50µL glycerol stock of BL21(DE3)_pET32a(+)_*CSF3syn.Ec3* in 1mL Luria Bertani medium (Sambrook, 2001) and incubated at 37°C, 150rpm, 18h (Series 2G Incubator Shaker, New Brunswick Scientific, Edison, New Jersey, USA). The pre-inoculum was added into 10mL ZYP-5052 autoinduction expression medium (Studier, 2005), then incubated at 20°C, 170rpm, 24h (Heidolph Unimax 1010 Incubator Orbital Shaker, Germany). The cells were resuspended in lysis buffer contains of 100mM Tris-Cl pH 7.4; 3mM EDTA, and 2mM PMSF (8mL/g wet cells pellet) and then lysed by freezing at -20°C and thawing in water bath at room temperature, app. 30min respectively, for ten cycles. Soluble protein was obtained by centrifugating the lysed cells at 12,000 rpm for 15min, ±4°C (Heraeus Sepatech Biofuge 13, Germany). Chemical reagents and other components for medium were purchased from Merck, Sigma, Biobasic, and Caisson Labs.

Purification and characterization hG-CSF

The soluble crude protein was added to Ni-NTA agarose matrix (1 : 1) (Qiagen, Hilden, Germany), then incubated on a rotator for 18h at 4°C. The slurry was washed using 20mM and 30mM imidazole (Biobasic, Markham, Canada), meanwhile the target fusion protein was recovered by elution with 100mM imidazole in 15 fractions. The imidazole solution was prepared in 0.16M Tris.Cl pH 7.4 buffer enriched with 4M NaCl. The first to the fourth elution fraction were collected and dialysed in 0.05M Tris.Cl pH 8.0 buffer for 24h at 4°C with three times of buffer changing. The dialyzed protein was cut with enterokinase (EK) (Invitrogen, Carlsbad, USA), 1U enzyme for 100µg fusion protein, then was incubated at 4°C and 20°C for 16h, respectively. hG-CSF and 6His-Trx·tag were separated with IMAC as well. Both proteins were concentrated using NanoSep® centrifugal filter column (Pall Life Sciences, East Hills, New York, USA) from 300µL to 50µL. The proteins were characterized by western blot using mouse anti-His monoclonal primary antibody (GE Healthcare, Buckinghamshire, UK), goat anti-mouse IgG-AP secondary antibody (Santa Cruz Biotech, Santa Cruz, USA), and detected with Stabilized Substrate for Alkaline Phosphatase (Promega, Madison, USA).

RESULTS AND DISCUSSION

Subcloning of *CSF3syn* into expression vector pET32a(+) and its transformation into *E. coli* BL21(DE3)

The target gene was constructed to be expressed as fusion protein of hG-CSF with Trx·tag and 6×His·tag provided at the backbone of pET32a(+). The enterokinase (EK) site was added at N-terminal of hG-CSF which is functional towards separation of hG-CSF and Trx·tag (Figure 1).

Thirty three transformants of XL1-Blue_pET32a(+)_*csf3syn.Ec3* were gained, but there were only 14 transformants positively carry the insert of *csf3syn.Ec3* (Figure 2.A); three transformants had proper orientation (Figure 2.B), and two recombinant plasmid were succesfully cut with *Bgl*II (Figure 2.C). Those two transformants were analyzed by DNA sequencing and both of them had the correct DNA sequence (data not shown). To express the target gene, the recombinant plasmid was transfered from *E.coli* XL1-Blue to *E.coli* BL21(DE3), resulting 238 transformants. Confirming by colony PCR, five of 20 randomly selected transformants were positively carrying the target gene. (Figure 2.D).

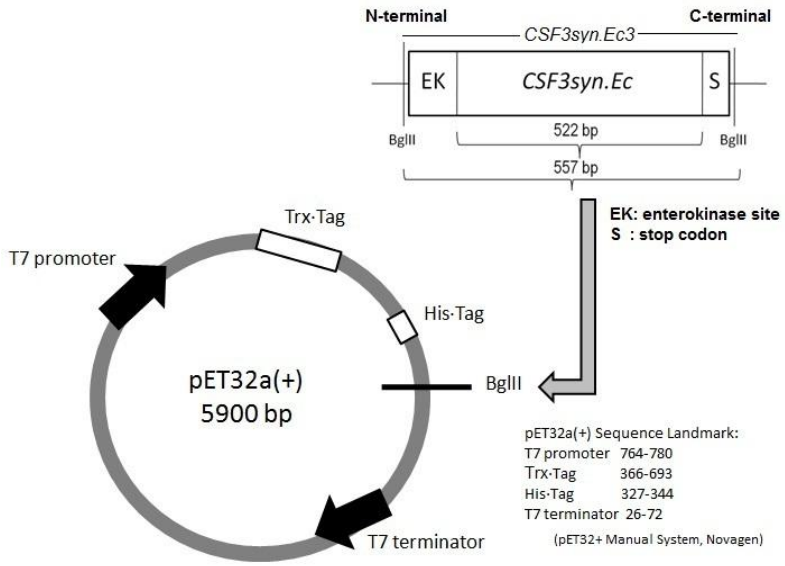

Figure 1. The construction of *CSF3syn.Ec3* and pET32a(+)

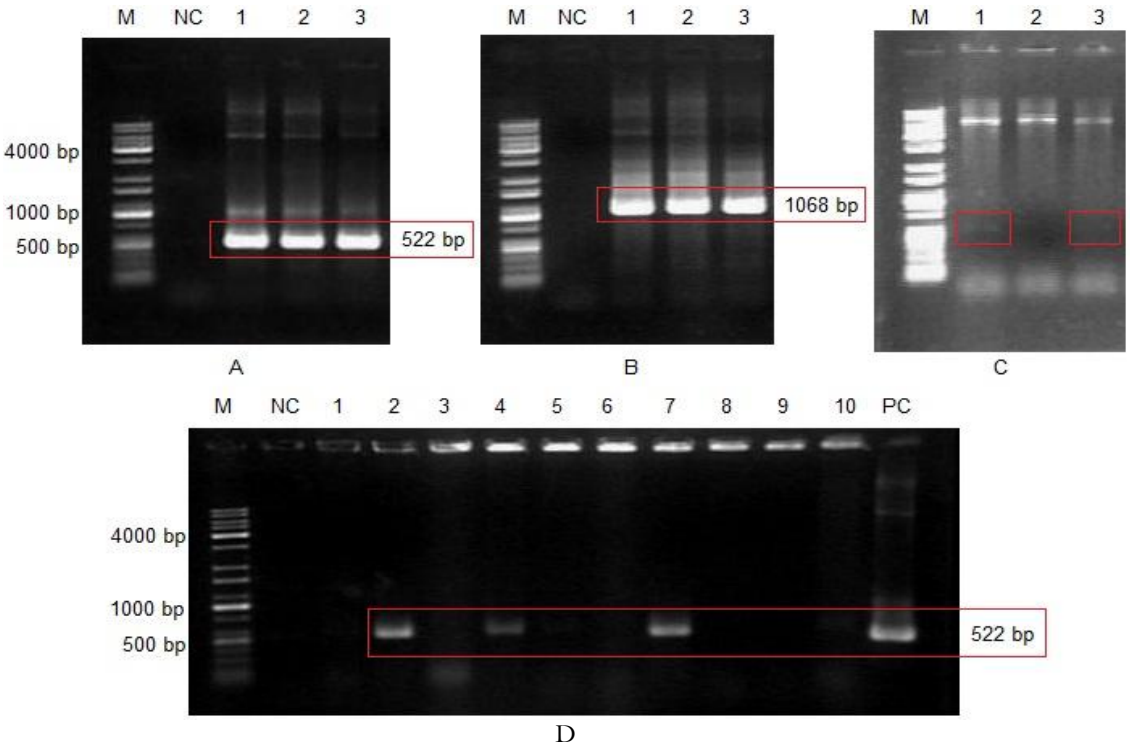

Figure 2. DNA Electrophoresis A. PCR product of pET32a(+)_*CSF3syn.Ec3* with R- and F- insert gene primers. B. PCR product of pET32a(+)_*CSF3syn.Ec3* with T7 promoter and R-insert gene primer C. pET32a(+)_*CSF3syn.Ec3* cut with BglII, lane 1 and 3 shows *CSF3syn.Ec3* as restriction product D. Colony PCR of BL21(DE3)_pET32a(+)_*CSF3syn.Ec3* with R- and F- insert gene primers. M = 1 kb DNA Marker; NC = negative control; PC = positive control or recombinant plasmid pET32a(+)_*CSF3syn.Ec3*

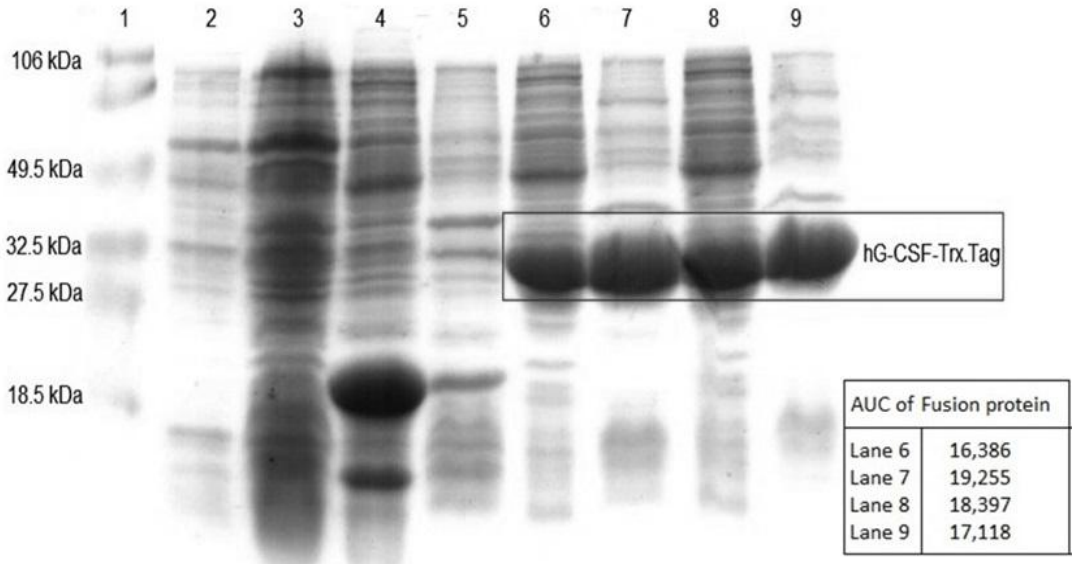

Figure 3. Crude protein profile on 15% SDS-PAGE
Lane 1) Protein Marker; Lane 2)Non transforman BL21(DE3) soluble protein, Lane 3) Non transforman BL21(DE3) insoluble protein, 4) Transforman without insert gene BL21(DE3)_pET32a(+) soluble protein, Lane 5) Transforman without insert gene BL21(DE3)_pET32a(+) insoluble protein, Lane 6 and 8) Transforman BL21(DE3)_pET32a(+)_*CSF3syn.Ec3* soluble protein, 7 and 9) Transforman BL21(DE3)_pET32a(+)_*CSF3syn.Ec* insoluble protein

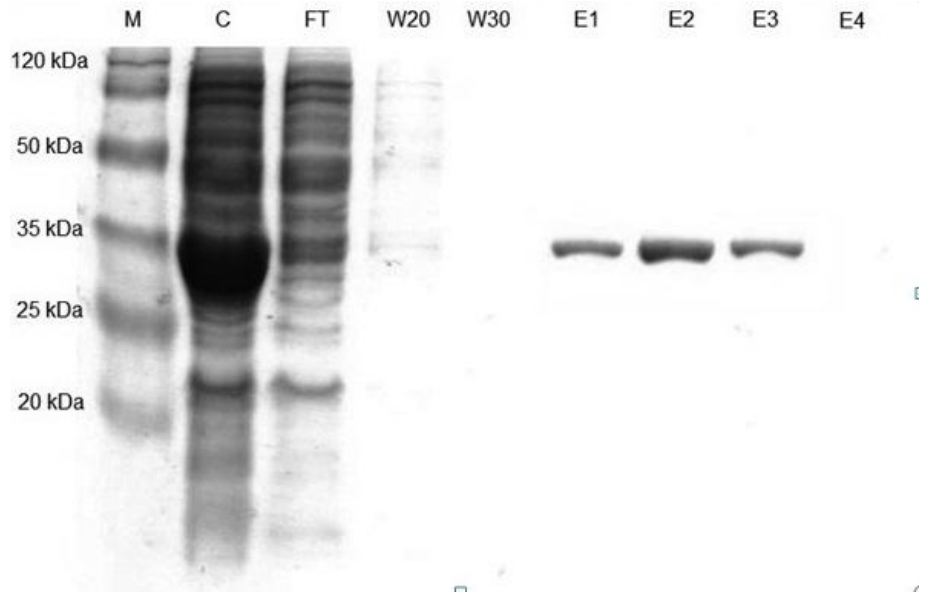

Figure 4. IMAC (Ni-NTA) purified protein profile on 15% SDS PAGE
M) Protein Marker, C)Crude or unpurified protein, FT) Flow through, protein that not binds to the matrix, W20) Washing fraction with 20mM imidazole, W30) Washing fraction with 30mM imidazole, E1-E4) 1st to 4th elution fractions with 100mM imidazole

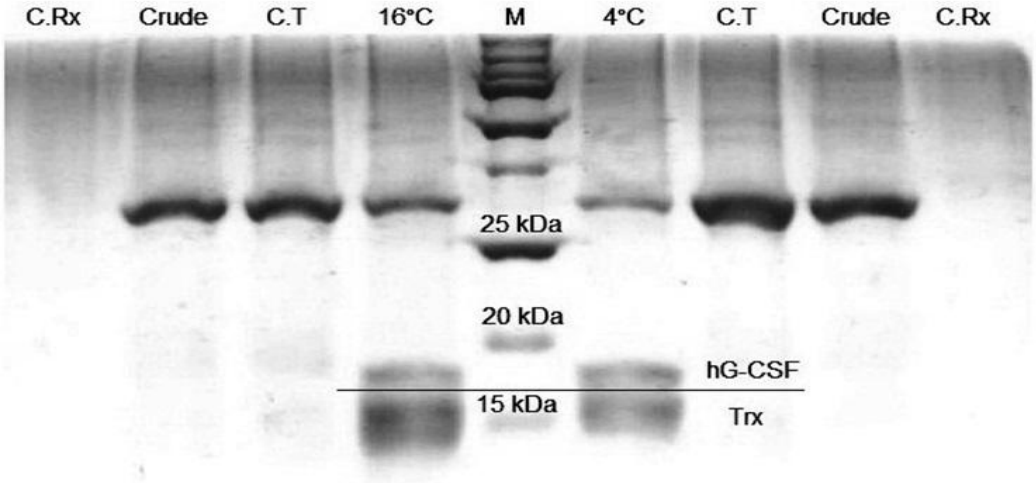

Figure 5. Enterokinase cleaved fusion protein profile on 18% SDS PAGE
Lane C.Rx) Control of reaction, contains of EK and EK buffer, Lane Crude) Dialysed purified fusion protein, Lane C.T) Control of temperature, contains of dialysed fusion and EK buffer, Lane 16°C) Cleaved fusion protein incubated at 20°C, Lane 4°C) Cleaved fusion protein incubated at 4°C, M) Protein marker

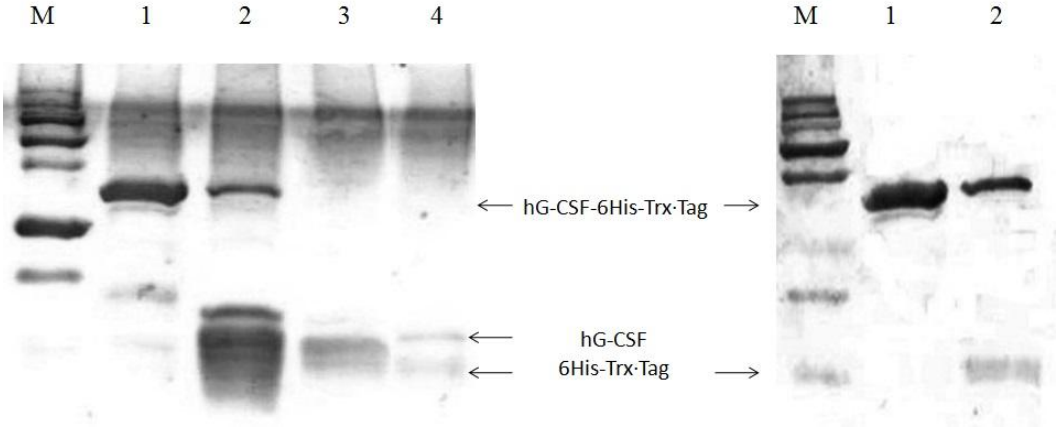

Figure 6 A. The second purified protein profile on 18% SDS PAGE; Lane M) Protein marker, Lane 1) Trx-hG-CSF fusion protein before EK cleavage, Lane 2) Trx-hG-CSF fusion protein after EK cleavage, Lane 3) Concentrated flow through fraction, Lane 4) Concentrated elution fraction with 100 mM imidazole.Figure 6.B. Western Blot with anti-His primary antibodyLane M) Protein marker, Lane 1) Trx-hG-CSF fusion protein before EK cleavage, Lane 2) Trx-hG-CSF after EK cleavage

Small scale overexpression of *CSF3syn* through autoinduced method

hG-CSF is prone to form inclusion bodies (IB) in *E.coli* because of its hydrophobicity and disulphide bonds (Dehaghani, 2010). This protein has five Cys residues that form two disulphide bonds between Cys37-Cys43 and Cys65-75, and another Cys18 a free Cys residue which is not involved in any disulphide bond formation (Wingfield, 1988). It is complicated to recover soluble protein from IBs related to initial recovery, solubilization, and renaturation steps, hence, fusion partners are commonly considered to obtain the soluble protein (Thatcher, 1990).

Several fusion partners have been successfully investigated to enhance the solubility of hG-CSF with IPTG induced method, e.g. EDA or *E.coli* 2keto-3-deoxy-6-phosphogluconate aldolase (Kang, 2014) and ArsC or arsenate reductase (Song, 2011). Das (2012) carried out an experiment with Trx·tag by IPTG induced method to increase the solubility of hGM-CSF, another colony stimulating factor which is useful to maintain post transplantation leukocytes level. In addition, Trx has the ability to be spesifically released from the *E.coli c*ytoplasma by freeze-thaw methods or osmotic shock in the presence of EDTA, which facilitates to isolate the protein from the cells (LaValielli, 1993).

Two transformants of BL21(DE3)_ pET32a(+)_*CSF3syn.Ec3* were randomly selected and gave the similar profile on 15% SDS PAGE (Figure 3). In our previous study, the expression level of soluble hG-CSF-Trx·tag was higher through autoinduction than IPTG-induced method and it was increased by lowering the incubation temperature (Pratiwi, 2012). Therefore, in this experiment, we detect the effectiveness of autoinduction method by comparing the amount of fusion protein from the soluble and insoluble fraction. The soluble fraction was extracted by centrifugating the lysed cells, while the insoluble fraction was prepared from residual cells pellet. To evaluate the amount of soluble and insoluble fusion protein, the thickness of target protein bands were semiquantitatively measured using ImageJ software and figured as area under curve (AUC) (Figure 3). The fusion protein was expressed approximately 49% in soluble fraction and 51% in insoluble fraction. Consequently, the autoinduction condition still needs to be thoroughly optimized particularly for hG-CSF-Trx·tag production. This method depends on the oxygenation state of the culture, the composition of the autoinduction medium, and time – temperature of incubation (Blommel, 2007; Studier, 2005). Tyler (2005) found that 0.6% or more of glycerol is important to get higher yield.

Purification and characterization hG-CSF

Some downstream steps were required to gain the hG-CSF e.g., the first purification step to remove *E.coli* endogenous proteins from fusion protein, and the cleavage of the fusion protein, followed by the second purification step to divide hG-CSF and 6×His-Trx·tag. The purification was conducted through IMAC (Ni-NTA), a method which is widely used to purify the active and soluble recombinant protein based on the covalent binding of target protein-His·tag towards Ni^{2+} matrix (Bartlow, 2011, Awade, 1996). In order to recover the target protein, pH gradient or imidazole is typically used to release the protein target from the matrix (Porath, 1992).

To mitigate endogenous protein contaminants, washing steps were performed in elevated imidazole concentrations prior to elute the fusion protein with an optimized imidazole concentration. The purified fusion protein was acquired from the first to the third elution fractions (Figure 4).

The protein quantification was generated using ImageJ software by calculating the AUC of the protein from Figure 4. The crude protein contained of 10.46% fusion protein. The purification recovery was 83.56%, by comparing the total AUC of elution fractions towards the AUC of fusion protein from the crude protein, or 8.74% towards the overall crude protein.

Each 6×His-Trx·tag and hG-CSF were separated by EK, a serine protease that recognizes the amino sequence -Asp-Asp-Asp-Asp-Lys|X- with high specificity (Hopp, 1988). As a preliminary study, 1U of EK was added to 100 μg fusion protein, then it was incubated at 4°C and 20°C for 16 hours. The fusion protein was succesfully cleaved at both of those temperatures, but not completed yet (Figure 5), thus the ratio of fusion protein and enterokinase has to be optimized.

According to the amount of uncut protein, the cleavage at 4°C was more effective than it was at 16°C. Besides, the degraded proteins which are less than 15 kDa were intensively found after incubation at 16°C. By using ImageJ software, the effciency of cleavage at 4°C was calculated. The AUC of each hG-CSF, Trx, and uncut protein were compared to the AUC of purified fusion protein. The cleavage efficiency was obtained as 21.72% for hG-CSF and 39.31% for Trx. On the other hand, 38.05% protein was unsuccesfully cleaved.

The cleaved protein was subsequently purified using IMAC by trapping 6×His-Trx·tag on the Ni^{2+} matrix. The hG-CSF was eluted in flow through fraction. At this step, flow through fraction and elution fraction were still recovered as mixture of 6×His-Trx·tag and hG-CSF (Figure 6.A). The larger scale experiment with enhanced binding process of protein – matrix is needed. In order to confirm whether the elution fraction possess 6×His-Trx·tag, it was detected by western blot with anti-His primary antibody (Figure 6.B).

CONCLUSION

Human granulocyte colony stimulating factor has been successfully expressed in fusion with thioredoxin in *E.coli* BL21(DE3) through autoinduction method. A number of 49% of this protein was found as soluble fraction, whereas the other 51% was still found aggregated as IBs. It was succesfully purified using IMAC, cleaved from the fusion Trx partner and confirmed by western blot.

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