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Cloning and expression of Fh8 tag-fused G-CSF recombinant protein

Abstract

Recombinant protein drugs (rPDs) increased much attention in recent years. Optimizing and uniformity of the production process is of great interest. Granulocyte colony-stimulating factor (G-CSF) is a protein used to treatment of neutropenia caused by chemotherapy drugs. The rPD production's low efficiency, low solubility in Escherichia coli (E. coli) as an expression host, and purification are difficulties of these systems. Consequently, this study aimed to clone and express the Fh8 tag-fused G-CSF recombinant protein to investigate the effect of this tag on the solubility of G-CSF. The Fh8 gene and the G-CSF were cloned inside pET28 and pET23 plasmids in previous studies, respectively. Following isolating the G-CSF gene by PCR and transferring it to T Vector, plasmids pET28 and T Vector were cut using BamHI and NotI enzymes. G-CSF gene was inserted into pET28 and next to FH8, with the T4 ligase enzyme assist. The fused gene's presence in the recombinant vector was investigated using the PCR technique; expression of the recombinant proteins was also evaluated using SDS-PAGE and Western blotting. PCR and fragment analysis results on gel electrophoresis indicated that the expression vector was correctly cloned in E. coli. Also, analysis of protein expression using SDS-PAGE, and Western blotting methods indicated that this host can express the desired gene construct. Accordingly initial evaluations, the use of the FH8 tag increases GCSF solubility. E. coli is a proper host for the expression of GCSF-FH8 fused protein. Further examination is needed to confirm FH8's potential to increase GCSF protein solubility.

Keywords; *Cloning and expression- Recombinant protein- The Escherichia coli- Granulocyte-Colony Stimulating Factor- Top10- BL21- T vector- Bam H1- Not1- Biopharmaceuticals- SDS PAGE- Western blotting*

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1. Introduction

The rods provide effective treatments for most life-threatening diseases such as diabetes (insulin), acute renal failure (erythropoietin), viral hepatitis (interferon INF), granulocyte colony-stimulating factor (G-CSF), coagulation disorders (factors VIII, factors VII, IX), and congenital disorders of metabolism (lysosomal enzymes) (1). G-CSF is a hematopoietic growth factor with a vital role in hematopoietic cell proliferation, differentiation of hemopoietic precursor cells, and activation of mature neutrophilic granulocytes. Furthermore, G-CSF has been extensively used for neutropenia treatment caused by cancer chemotherapy (2, 3). The mature G-CSF consists of 174 amino acids predominantly with two intramolecular disulfide bonds, although another minor form composed of 177 amino acids due to alternative splicing of mRNA has also been described (4, 5)

Recent advances in recombinant molecular techniques have made it possible to clone an encoding DNA of a particular protein into an expression vector and express the protein in host systems such as bacteria, yeasts, insects, and mammalian cells. The production of recombinant proteins (rPs) typically has two main stages: Molecular cloning and protein expression (3). The first host to express the rPs, *"Escherichia coli"* is one of the most widely used hosts to produce therapeutic and industrial recombinant proteins commercially, but its restricted capacity for generating extracellular proteins is cited as a disadvantage. On the other hand, its advantages include low costs, simple culture requirements, rapid product accumulation, and a wide range of available genetic information. Despite these advantages, overproduced heterologous proteins in E.coli usually form nonproductive inclusion bodies, and fusion expression using solubility enhancer proteins as fusion partners has emerged as an efficient production approach to overcome the inclusion body formation (9, 10).

In E. coli, the factors influencing recombinant products can be classified into either expression-level factors (host cell genetic factors) or process-level factors (physical and environmental factors at various fermentation scales) (6). E. coli dominates the bacterial expression system and is generally used for expressing rPDs that do not need post-translational modification (PTM) for their bioactivity. Around 30% of certified biologics are generated from various E. coli strains, such as BL21, Top 10, and K- 12 (7, 8). Since G-CSF has only one O-glycosylation site at Thr133, which is not crucial for biological activity; therefore, non-glycosylated recombinant G-CSF has the same specific biological activity as glycosylated G-CSF. The G-CSF, produced by E. coli, lacks glycosylation and has N-terminal methionine. Accordingly, E. coli has been used as a proper host for expressing G-CSF commercially (3).

Many approved rPs are produced in E. coli due to well-known genetic characteristics and its rapid growth and production with high protein yield. E.coli BL21 strain is the widely used and effective host in recombinant expression applications,

grows in minimal environments, is non-pathogenic, cannot survive in host tissues, or causes disease (9). BL21's significant advantage is the lack of ompT and Lon proteases that interfere with the isolation of recombinant proteins. This strain, which has mutated protease genes, can produce large amounts of rPs inside the cell and minimize its breakdown during purification (10). Moreover, the top strain with high transformation and replication power in vitro and suitable replication potential of foreign plasmid in bacteria is one of the suitable prokaryotic alternatives to investigate the efficiency of vectors (11, 12).

Moreover, short peptide tags have also been revealed to increase recombinant protein expressions, including poly-Arg, poly-Lys, Fh8, and histidine tags (13, 14). Previous studies have shown that the Fh8 works as a purification tag and enhancer solubility partner to improve protein expression and solubility. In addition, it confirmed that small fusion tags allowed a more straightforward evaluation of the target protein solubility (15, 16). For example, the novel Fh8 tag, a small (8 kDa) calcium-binding protein separated from Fasciola hepatica, has shown significant combined purification and solubility potential in E. coli (17). Thus, the Fh8 tag, compared to larger fusion tags, is a promising alternative for soluble protein production in Escherichia coli due to its low molecular weight and adequate solubility-enhancing effect (18, 19). Accordingly, the study conducted here aimed to clone and express the Fh8 tag-fused G-CSF recombinant protein to investigate the effect of this tag on the solubility of G-CSF and the reduction of protein aggregates.

2. Material and method

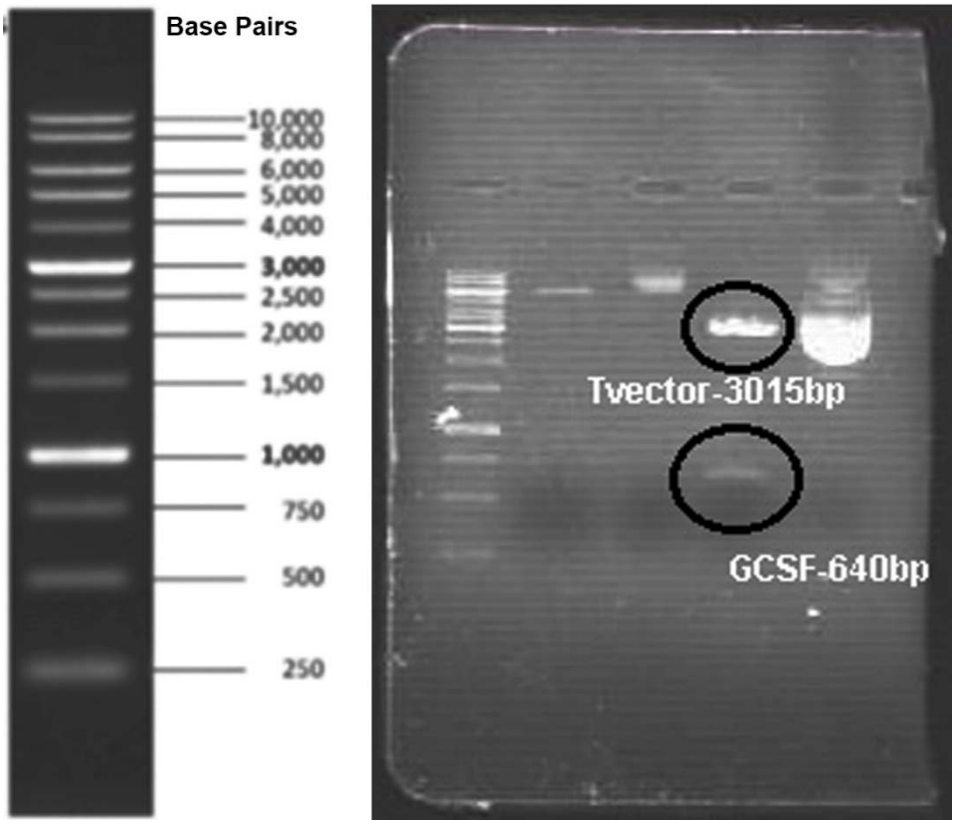
Chemicals such as Isopropyl β- d-1-thiogalactopyranoside (IPTG), Glycerol, Agar, Ammonium persulfate (APS), Coomassie blue, Tetramethylethylenediamine (TEMED), X_GAL, 2_Mercaptoethanol, Anti-6X His tag antibody, and Monosodium phosphate (NaH2PO4) were purchased from Sigma-Aldrich. Also, Glycine, Ethylenediaminetetraacetic acid (EDTA), Tris-HCL, Agarose, Calcium chloride (CaCl2), Ethanol, N, N′-methylene bis (acrylamide), Acrylamide, sodium dodecyl sulfate (SDS), Skim Milk Powder, were prepared from Merck. Top10 and BL21 (DE3) strains of Escherichia coli were used as hosts during the cloning process and expression. pET23, pET28 and T vectors were used. Cutting enzymes and T4 DNA ligase were prepared from Roche. Also, a Lysogeny broth (LB) medium was used to grow and replicate recombinant bacteria. The antibiotics tetracycline (final concentration 30 µg / ml), kanamycin (final concentration 30 µg / ml), and ampicillin (final concentration 100 µg / ml) were used in the culture medium. IPTG one mM and X-gal 40 g / ml were used to fabricate the selected medium for screening recombinant clones in addition to suitable biotics. Molecular methods used in this design, including enzymatic cutting, attachment, and transgenic, were performed according to standard methods. The produced PET23 vector containing the G-CSF gene was amplified using the PCR method. To transfer the plasmid using E.coli's TOP 10 strain, the cells were transformed into susceptible cells and receptive to plasmids using calcium chloride and heat shock. One of the clones grown in the previous step was transferred to 5 ml of liquid LB medium containing ampicillin at a 100 µg / ml concentration and placed in an incubator shaker overnight at 37 ° C. Then, the recombinant plasmid was extracted according to the protocol of the Favorgene kit. The accuracy of vector extraction was investigated by electrophoresis on the agarose gel. The primers were designed using Generunner software and synthesized by Abtin Gene Company. The G-CSF gene was then cloned into T Vector using T4 DNA Ligase, and the ligation product was transferred to the Top 10 bacteria for replication. The grown clones appeared in blue or white colors. White clones received the T Vector containing the G-CSF gene. A matrix was prepared from these white colonies, and PCR was performed to ensure that the gene entered the vector. Enzymatic digestion was used to evaluate the efficacy of GCSF gene entry into T Vector. The Fh8 gene was cloned into the plasmid pET28 as a urate oxidase fuse in the previous study. Aimed to remove the G-CSF gene from T Vector and urate oxidase gene from pET28, an enzymatic digestion reaction was performed on T Vector containing G-CSF gene and PET 28 with FH8 gene using two enzymes Bam HI and Not I. Following confirming enzymatic digestion reaction by electrophoresis, G-CSF and FH8 pET28 bands were cut from the gel, and gene fragments were analyzed. The binding reaction of G-CSF and FH8-pET28 was performed using the T4 DNA Ligase enzyme. To transfer the pET28 vector containing G-CSF.FH8 fusion to the Top10 strain, the result of the ligation reaction, was transferred to the Top 10 after OverNight. Accuracy of G-CSF.FH8 gene construct was assessed using PCR, and ten clones were evaluated. One of the approved colonies was selected, and the plasmid was extracted. Sequentially, the expression and solubility of Fh8 tag-fused G-CSF recombinant protein compared to G-CSF were assessed using SDS-PAGE gel and Western blotting.

3. Results

3.1. Isolation and amplification of G-CSF gene using PCR

The Pasteur Institute of Iran provided the pET23 vector comprising the G-CSF gene. Behind amplifying this recombinant vector in the coil's TOP10 strain and extracting the amplified vector from this host, the G-CSF gene was isolated from the pET23 vector using PCR. The reaction result

was run on agarose gel 1%. The 640 bp band, confirms the success of the G-CSF gene isolation process.



3.2. Isolation of G-CSF gene from agarose gel

A nucleic acid extraction kit was used to isolate the G-CSF gene amplified by PCR from agarose gel. The product was run on 1% agarose gel. The 640 bp band and the successful progress.

3.3. Cloning the G-CSF gene into T Vector

Following isolation through a ligation reaction, the G-CSF gene was inserted into the T Vector. The ligation reaction result entered the susceptible cells of the TOP10 strain of E. coli to amplify the recombinant vector. To evaluate the accuracy of GCSF gene entry into T Vector among grown clones, PCR and enzymatic digestion were used. The products of both methods were run on 1% agarose gel. The expected size for the PCR product is 640 bp, and the enzyme cutting product with Eco RI is equivalent to 3015 and 640 bp.

3.4. Extract the G-CSF gene from T-vector

Aimed to extract the G-CSF gene from T Vector, the enzymatic digestion reaction was performed using Bam HI and Not I enzymes. Expected components were 3015 bp for T Vector and 640 bp for GCSF, respectively. The results are shown in **Figure 1**.

Figure 1. Electrophoresis of T vector-GCSF enzymatic digestion product with BamHI and NotI. The product runs on 1% agarose gel. Of left, respectively: DNA Ladder 1kb, digested plasmid (including two pieces of 640 and 3015 bp), undigested plasmid.

3.5. Enzymatic digest of pET28 plasmid

The pET28 plasmid containing recombinant Fh8 and urate oxidase genes, following amplification in the TOP10 strain and plasmid extraction, was cut to remove the urate oxidase gene using BamHI and NotI enzymes. The product of the enzymatic digestion was run on 1% agarose gel. The expected fragment sizes are 1000 bp for urate oxidase and 5595 bp for pET28.FH8, respectively. The image reveals the plasmid digestion and urate oxidase gene release.

3.6. Extraction of G-CSF and pET28.FH8 genes from agarose gel

G-CSF and pET28.FH8 genes were transferred on 1% agarose gel, and following cutting the relevant bands, fragments were extracted from the gel. The extraction product was analyzed on 1% agarose gel. The results are exhibited in **Figure 2**.

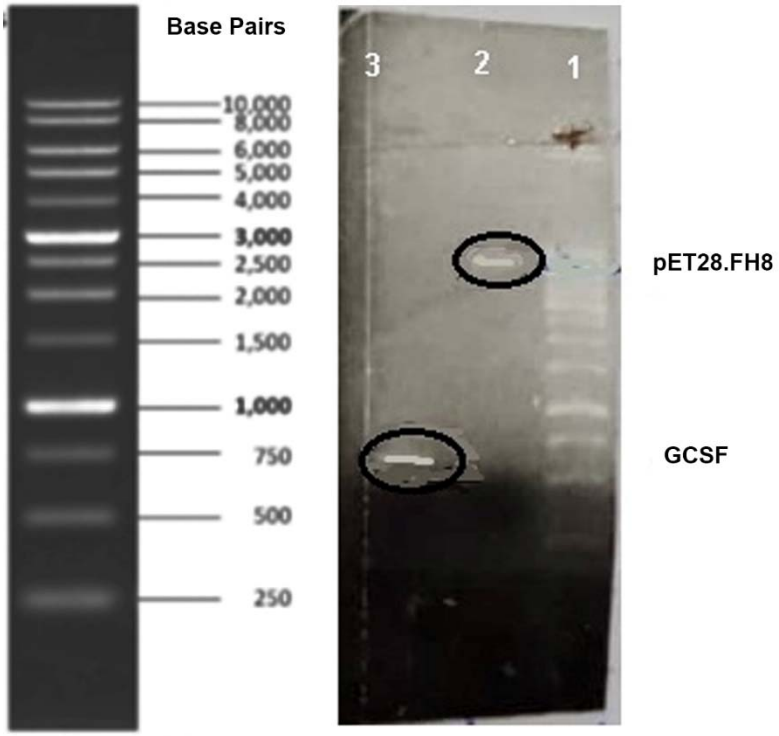


Figure 2. Outcomes of G-CSF and pET28.FH8 gene electrophoresis following extraction from agarose gel. Column 1: DNA Ladder 1kb, Column 2: pET28.FH8 fragment, Column 3: G-CSF fragment.

3.7. Assessment of G-CSF.FH8 gene construct accuracy using PCR

The binding reaction of G-CSF and FH8-pET28 was performed using the T4 DNA Ligase enzyme. Following the transfer of ligation product to TOP10 strain and recombinant plasmid amplification, the accuracy of the construct of the G-CSF.FH8 and its entry into the pET28 expression vector were evaluated using PCR, run on 1% agarose gel. The desired fragment size is 860 bp. The results are shown in **Figure 3**.

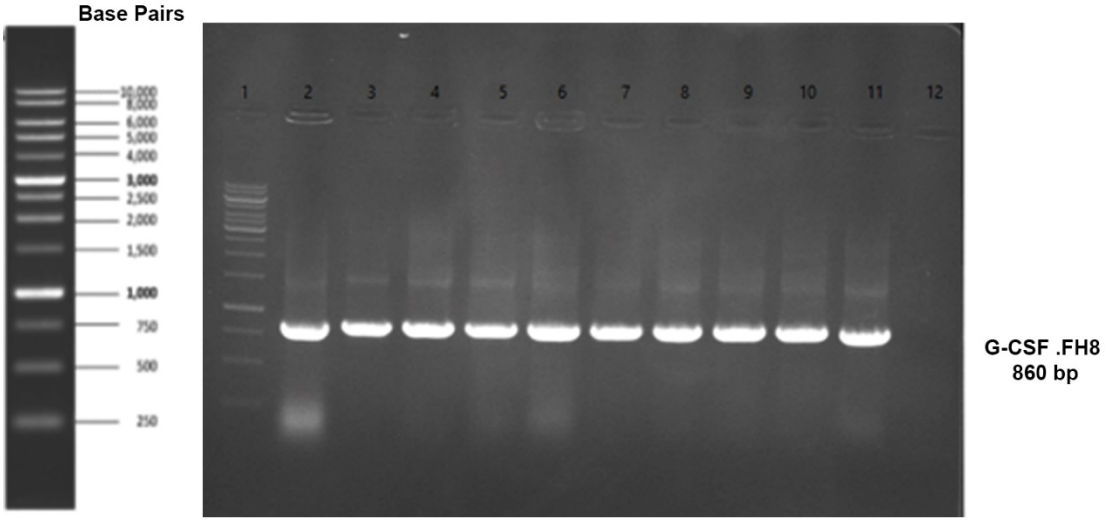


Figure 3. Electrophoresis of PCR colony on TOP10 clones transformed with recombinant pET28.FH8.G-CSF vector. Column 1: DNA Ladder 1kb, Column 2 to 12: are related to the understudy clones.

3.8. FH8 G-CSF protein fusion expression

One of the colonies approved in the previous step was chosen to extract a plasmid containing the FH8 G-CSF gene. The extracted plasmid was transferred to the BL 21 strain as an expression host. Following selecting a clone, replicating, and inducing expression, the cell pellets before and after induction

were analyzed using SDS-PAGE and Western blotting. The expected size is 26.8 kDa for this protein fusion. The results are exposed in Figures 4 and 5.

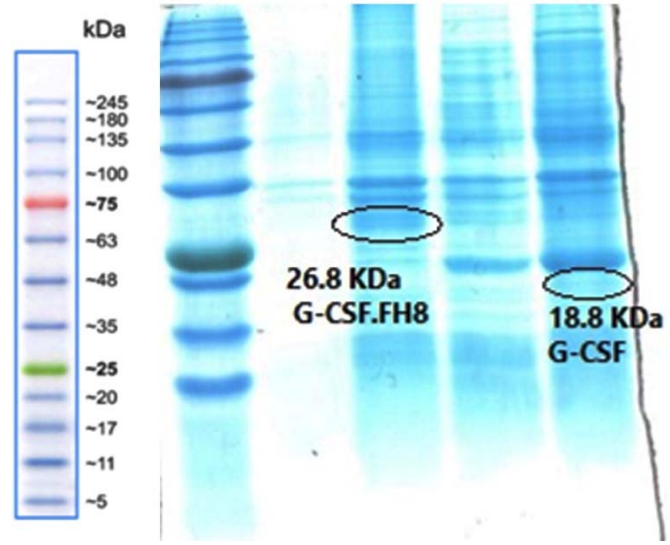


Figure 4. SDS-PAGE gel related to G-CSF.FH8 protein fusion expression analysis, dyed using Kumasi blue. Specimens were run on 12% polyacrylamide gel. Of left: protein marker, total samples before induction correspond to FH8.G-CSF protein fusion expression host, total samples after induction related to FH8.G-CSF protein expression fusion host, total samples before induction related to the G-CSF protein expression host, total samples after induction correspond to the G-CSF protein expression host.

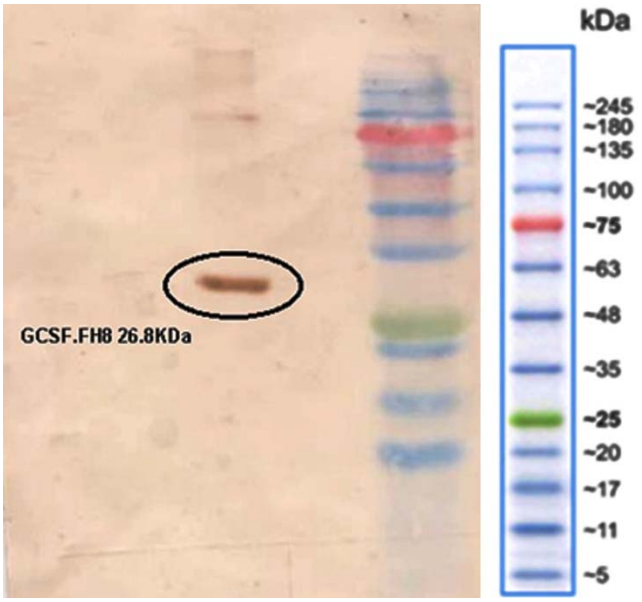


Figure 1. Analysis of Recombinant FH8.G-CSF protein expression using Western blotting. Samples were run on 12% polyacrylamide gel. From right: protein marker, total samples before induction correspond to FH8.G-CSF protein fusion expression host, total samples after induction related to FH8.G-CSF protein expression fusion host, total samples before induction related to the G-CSF protein expression host, total samples after induction corresponds to the G-CSF protein expression host. The presented band shows a triumphant expression of the FH8.G-CSF fragment using the express host. Since the Anti-6X His tag antibody is used in Western blotting, the GCSF band, which lacks a histidine tag, is not observed.

3.9. Comparison of G-CSF solubility and FH8.G-CSF

Expressing G-CSF and FH8.G-CSF Colonies were amplified and induced. Ultrasonic pulses lysed the resulting pellets. Pre-lysis samples, pellets, and lysed cell soup were run on 12% polyacrylamide gel and analyzed after dyeing by Kumasi Blue. Also, solubility analyses were confirmed using Western blotting. The expected sizes for these proteins are 18.8 and 26.8 for G-CSF and G-CSF.FH8, respectively.

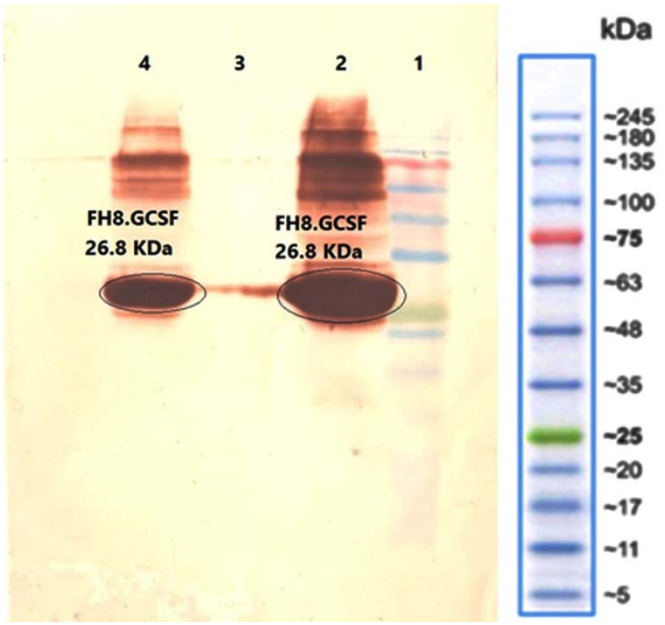


Figure 6. Analysis of produced FH8-GCSF protein solubility using Western blotting method. Column 1: Protein marker, Column 2: Pellet sample after sonication, Column 3: Soup sample after sonication, Column 4: Total sample before sonication. Anti-6X His tag antibody has been used. The results indicate the presence of FH8-GCSF in total, pellet, and soup samples.

4. Discussion

G-CSF is a single-chain protein comprising 174 amino acids with a molecular weight of 18.8 kDa. This cytokine is the primary regulator of neutrophilic granulocytes (20). Neutrophils are essential cells in the innate immune system and are very important for clearing pathogenic pathogens (21). Recombinant G-CSF is known as an essential drug for the treatment of neutropenia. Human G-CSF cDNA was cloned in 1996 using amino acid sequences and oligonucleotide probes to screen CHU-2 and 5637 tumor cell line libraries (22). The limitation of natural resources for the biological purification of this protein is one of the main problems. Therefore, recombinant DNA technology to production and the availability of such proteins is of great interest. In this regard, the most critical factor is the protein expression with correct folding and post-translational changeability. E. coli is one of the most widely used expression hosts in the pharmaceutical industry (23). Various strategies and approaches have been

developed to increase the expression and solubility of recombinant proteins. A novel approach is to use tag fusion proteins because of the potential to reduce recombinant proteins' proteolytic degradation, improve protein secondary structure, and proper folding, increase solubility, and facilitate the process of detecting and purifying recombinant proteins (24). Fused tags direct the adjacent protein to the folding path and have a chaperone-like activity. Fused tags direct the adjacent protein to the folding path and have a chaperone-like activity. The Association of the hydrophobic regions of the tag with nearby relatively folded proteins helps proteins fold and prevent them from accumulating spontaneously. Consequently, tags increase the solubility of recombinant proteins by reducing protein aggregation and helping to fold (19). FH8 is one of the beneficial tags in increasing the solubility of recombinant proteins. Furthermore, due to the bearing of two calcium-binding sites, it can be used in affinity chromatography, and protein isolation from the column will be possible using calcium-chelating agents or pH changes. Therefore, FH8 is one of the few tags that increases protein solubility and facilitates the purification process (25, 26). As G-CSF is expressed aggregated (Inclusion bodies) in the E. coli host, the final yield of the protein is usually low, and numerous studies have used various strategies to increase this yield. A study using MBP tags and (PDI) Protein Disulfide Isomerase fused to GCSF increased the protein's solubility. Also, it was shown that decreasing temperature leads to a solubility increase of this protein binds to the thioredoxin and glutathione S-transferase tags. In comparison, histidine tags at average or low temperatures did not affect GCSF solubility (27). Moreover, G-CSF showed approximately 10-fold more excellent bioactivity through the (A (EAAAK) 4ALEA (EAAAK) 4A) helical linker insertion. Also, (Interferon) INF-α2b with HSA (Human serum albumin) fused with the help of a linker showed enhanced half-life under in vivo conditions (28). According to previous studies and the effectiveness of the FH8 tag on increasing the solubility of various proteins, it was predicted that the use of this tag could reduce inclusion bodies and increase the solubility. This study aimed to clone and express the fusion of GCSF-FH8 protein in the E. coli host. After assessing the ability of E. coli to express this protein fusion, the effect of the FH8 tag on GCSF solubility was also compared with alone expressed GCSF.

5. Conclusion

The results of the study showed that the GCSF-FH8 gene construct was completed .Also, the results of protein fusion showed that the bacterial host was well able to express it. In the initial evaluation of GCSF-FH8 solubility compared to GCSF, the results indicated that this tag could increase the solubility of recombinant G-CSF protein. To increase the level of confidence in the conclusion, it seems necessary to repeat the evaluation. On the other hand, standard bacterial growth conditions at 37 ° C and a concentration of inducer (IPTG) were used in this experiment. Since the physicochemical culture parameters such as medium composition, pH, induction temperature, type of culture, amount of inducer, induction time, the expression rate of recombinant protein, bacterial growth rate, and parameters involved in downstream processes all affect the solubility of recombinant protein, it is predicted that better results can be achieved by optimizing these conditions.

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Declarations of interest:

None

Contribution Statement:

Farzaneh sadat Abdollahidemneh: Conceptualization, Methodology, Investigation, Writing - Original Draft, Supervision. **Sosan moghbel:** Investigation, Formal analysis, Writing - Review & Editing.

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