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ORIGINAL ARTICLE

Expression and one step intein-mediated purification of biologically active human G-CSF in *Escherichia coli*

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Abstract

Recombinant form of granulocyte colony stimulating factor (G-CSF) was first approved by FDA in 1998 for chemotherapy induced neutropenia. However, despite production of its biosimilars, less expensive production of G-CSF could reduce the overall therapeutic cost. The aim of this study was to evaluate the possibility of producing biologically active recombinant G-CSF via a single step purification procedure mediated by a self-cleavable intein. G-CSF was expressed by *E. coli* BL21 (DE3) through IPTG induction, followed by its purification using pH optimization on a chitin column. Western blotting, ELISA, size exclusion chromatography, circular diachorism, peptide mapping, and in vitro assays were performed to compare the structural similarity and biological activity of the purified G-CSF with Neupogen™. Protein purification was confirmed by revealing a band of approximately 18.8 kDa on SDS-PAGE. Bioactivity and physicochemical assays based on the US pharmacopeia showed almost identical or acceptable ranges of similarities between recombinant G-CSF and Neopogen™. this study, biologically active soluble recombinant G-CSF was successfully produced with high purity without using chaotropic solvents through a one-step procedure. This shorter and more efficient purification procedure can reduce the cost and time of G-CSF production which makes its industrial production more cost-effective and might be also applicable for production of other biopharmaceuticals.

Keywords G-CSF · Intein mediated purification · Biological activity · *Escherichia coli* · pTWIN1

Introduction

Granulocyte-colony stimulating factor (G-CSF), a 24–25 kDa monomeric glycoprotein, regulates proliferation, differentiation and activation of hematopoietic cells in the neutrophilic granulocyte lineage [1, 2]. The most important application of G-CSF is prophylaxis or treatment of chemotherapy induced neutropenia. The first marketed form of recombinant G-CSF approved for treatment of febrile neutropenia was Filgrastim (introduced by Amgen Company in the early 1990s with the brand name of Neupogen™). This form of G-CSF was produced by *E. coli* expression system as a non-glycosylated protein which is about 18.8 kDa [3]. The first biosimilar of Neupogen™, Zarzio™, was approved by FDA in 2015 without clinically meaningful differences with the reference drug [4]. Recently, in July 2018, Nivesym™ was also approved in the United States as the second biosimilar for Neupogen™ [5]. The patent of Filgrastim has expired in 2006, and due to an extended application spectrum of G-CSF, cost-effective production of this protein would be beneficial and impose less expenses to the health systems and patients. The most expensive step in production of a recombinant protein is purification. One of the purification strategies is fusion of a certain affinity tag to the recombinant protein and subsequent application of proper affinity beads. In this case, following purification of the protein, the fused tag must be cleaved and removed by suitable chemical or enzymatic proteases and subsequent purification steps [6, 7]. However, this relatively harsh and multi-step downstream procedure could damage the protein. A less complicated strategy for purification of a recombinant protein is taking advantage of self-cleavability of inteins [8–10]. In this regard, the recombinant protein of interest

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is fused to a proper intein at the genetic level, and upon expression, it is subjected to one step on column cleavage and purification yielding reasonable amount and purity of the expressed protein [11–14]. Hence, the aim of the present study was to produce recombinant G-CSF by a single step purification method to reduce the production complexity and cost for large scale plans. Moreover, in order to authenticate the produced protein, evaluation of its secondary structure and biological activity were also performed.

Materials and methods

Plasmids, bacterial strains and reagents

E. coli TOP 10 and BL21 (DE3) strains were used as hosts for cloning and expression of the protein, respectively. pTWIN1 expression plasmid was from New England BioLabs (Massachusetts, USA). Ampicillin was obtained from Sigma (San Diego, California, USA), and used for selection of recombinant bacterial colonies. FastDigest™ restriction endonucleases were obtained from Thermoscientific (Fermentas; Vilnius, Lithuania). G-CSF (Filgrastim) protein sequence was retrieved from www.drugbank.ca (https://www.drugbank.ca/drugs/DB00099), reverse translated and codon optimized for E. coli (Supplementary Fig. 1). The DNA sequence was synthesized by Gene Ray company (Hong Kong), and received as pGH-G-CSF plasmid. All other chemicals were obtained from commercial sources and were of molecular biology grade. Molecular biology experiments were performed according to standard protocols [15] unless otherwise mentioned.

Methods

Sub-cloning of G-CSF coding sequence

The synthesized nucleotide fragment was sub-cloned into the *Nco*I and *BamH*I restriction sites of pTWIN1 plasmid (Fig. 1a), as such it was fused to the C-terminus of the intein1 fragment. The fidelity of cloning was verified by restriction digestion of the obtained recombinant plasmid with the mentioned enzymes, and finally by DNA sequencing. The authenticated plasmid was named as pTWIN-GCSF (Fig. 1b).

Expression of Intein1-G-CSF fusion protein

pTWIN-GCSF plasmid was used to transform the *E. coli* BL21 (DE3) cells, followed by selection of recombinant colonies on LB-agar plates containing 100 µg/mL ampicillin. The selected colonies were then cultivated overnight and used to inoculate fresh cultures to reach an OD_{600} of 0.4 to 0.6. Next, expression of the Intein1-GCSF was induced

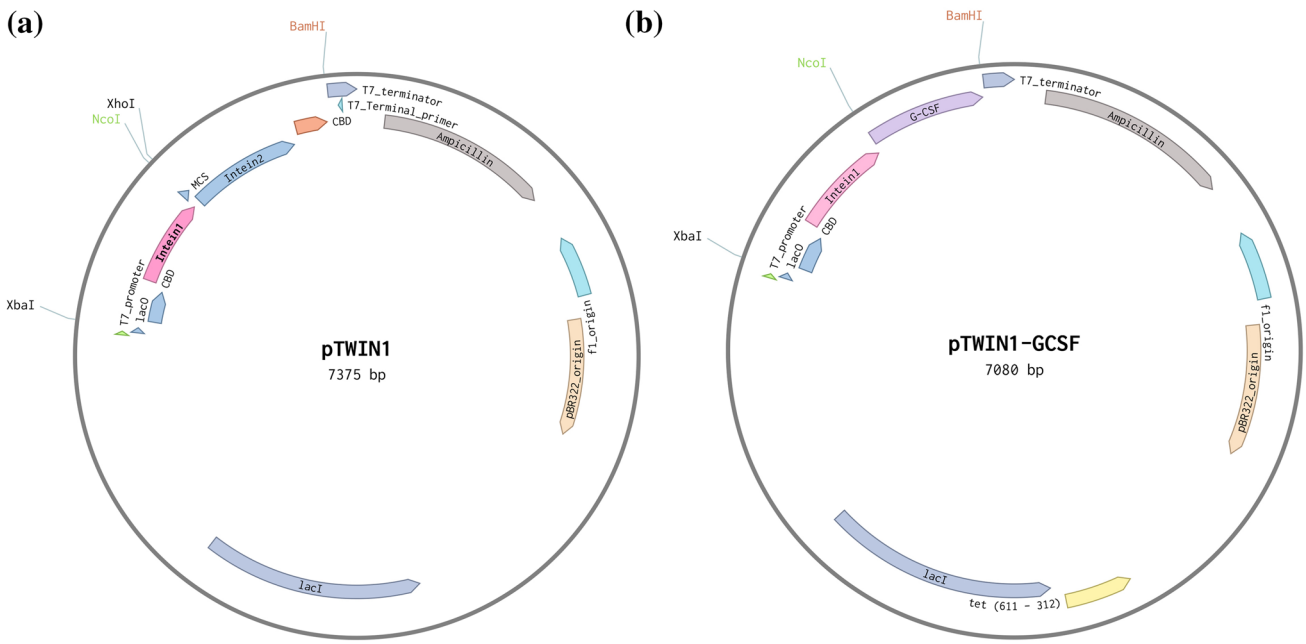

Fig. 1 Schematic representation of the pTWIN1 and recombinant pTWIN1-GCSF plasmids. **a** pTWIN1 plasmid with the ORF expressing fused Intein1, Intein 2, and their corresponding chitin binding domain (CBD). **b** recombinatn pTWIN1-GCSF plasmid in which G-CSF has been fused to the C-terminus of Intein1. As it is shown, Intein 2 and its corresponding CBD is removed in the cloning procedure

by 0.4 mM IPTG for 6 h at 15 °C. Then, the cells were harvested via centrifugation at 7000×*g* for 10 min at 4 °C. Finally, evaluation of the protein expression was performed by 12% SDS-PAGE.

Purification of the recombinant protein

On column cleavage and purification of G-CSF was mediated by the IMPACT™ purification system (New England BioLabs, Massachusetts, USA) as instructed by the manufacturer. Briefly, the harvested cell pellets were resuspended in B1 buffer (Tris–HCl 20 mM, NaCl 500 mM and EDTA 1 mM, pH 8.5), followed by sonication, cell disruption, and 30 min of centrifugation at 7000×*g* and 4 °C. Then, the supernatant containing soluble Intein1-GCSF protein was loaded on chitin column. The flow through was collected and replaced with B2 buffer (Tris–HCl 20 mM, NaCl 500 mM and EDTA 1 mM, pH 6.5), and the column was incubated for 24 h at room temperature. Then, different fractions were collected and analysed by 15% SDS-PAGE and Western blotting.

Size exclusion chromatography

As a complementary purification step, Size exclusion chromatography was carried out by HILOAD 16/60 Superdex™ 200 prep grade column (GE Healthcare,Germany) using FPLC. The mobile phase was PBS with pH adjusted to 7.4. Flow rate was maintained at 1.5 mL/min and fractions were verified for their absorbance at 280 nm.

Western blot analysis

In order to authenticate the purified protein, Western blot analysis was performed. In this regard, the protein bands were resolved by 15% SDS-PAGE, and then transferred to nitrocellulose membrane (Sigma, Germany). Afterwards, the membrane was blocked overnight with blocking buffer containing 3% non-fat dry milk (skimmed milk) in Tris Buffer (pH 8.8) at 4 °C. Then, the membrane was incubated at room temperature with HRP-conjugated anti-human G-CSF antibody (R&D Systems, Minneapolis, Minnesota, US) (1:1000 in TB-Tween buffer [0.1%]) for 1.5 h, followed by three times of 10 min washing with wash buffer (TB-Tween buffer, pH 8.8). Finally, the bands were visualized by DAB (3,3-Diaminobenzidine) solution (Sigma, Germany).

Peptide mapping

In order to verify the primary structure of the purified G-CSF, it was subjected to peptide mapping. Hence, the purified G-CSF and also Neupogen™ were prepared as 200 µg/mL in PBS and the peptide mapping analysis was performed by Ark zist Azma Company (Tehran, Iran).

Circular dichroism (CD)

To structurally compare the purified recombinant protein with the commercial standard (Neupogen™), CD evaluation was performed. In this regard, 500 µg/mL of the sample and standard proteins were prepared in water for injection at room temperature. Then, the samples were analysed by a Jasco J-810 circular dichroism spectropolarimeter.

ELISA assay

For further authenticating the structure of the purified protein, it was subjected to ELISA alongside the Neupogen™ using Quantikine® ELISA Human G-CSF kit (R&D Systems, Minneapolis, Minnesota, US). In this regard, a solution of 40 ng/mL of the recombinant G-CSF or the standard was assayed according to the manufacturer's instructions.

Sample preparation for in vitro assay

Lipopolysaccharide (LPS) removal of the purified G-CSF was performed using Triton X-114 method [16]. Briefly, Triton X-114 was added to the protein solution to a final concentration of 1%. The sample was shaken gently at 4 °C for 30 min and then incubated at 37 °C for 10 min. Subsequently, the sample was centrifuged at 20,000×*g* and 25 °C for 10 min to form two liquid phases, and the upper phase containing G-CSF was collected. This extraction procedure was repeated three times, and the endotoxin free protein solution was dialyzed against phosphate buffered saline (PBS, pH 7.4). Finally, the protein concentration was determined by Bradford method against various concentrations of BSA (Bovine serum albumin) as a standard solution.

Biological assay

As it is instructed by the US Pharmacopeia [17], in order to evaluate the biological activity of G-CSF as a biosimilar, its proliferative effects on M-NFS-60 cell line must be verified and compared to the standard. Hence, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to compare the proliferative effect of the purified G-CSF and Neupogen™. In this regard, 180 µL of RPMI 1640 medium containing 0.05 mM 2-mercaptoethanol, 62 ng/mL M-CSF, and 2×10^5 cells/mL of M-NFS-60 was poured in each well of a 96-well plate and kept in a CO_2 incubator at 37 °C overnight. Then, the cells were treated with 20 µL of various (to set up the experiment, data not shown) or certain (4 µg/mL, to compare the bioactivity) concentrations of Neupogen™ or the purified G-CSF. Following

48 h of incubation, 20 µL of MTT solution (5 mg/mL) was added to each well, and the plate was further incubated for 3 h, and subjected to absorbance read at 570 nm using a microplate reader.

Statistical analysis

The MTT assay was performed in three independent experiments of four replicate wells for each concentration. PBS-treated cells were considered as negative control and results were expressed as cell viability percent ± SD. SPSS software version 22 was used for statistical analysis. Independent T-test was used to distinguish the differences between groups and the significance was assumed as $P < 0.05$.

Results

Cloning and expression of recombinant G-CSF

Successful sub-cloning of the G-CSF coding sequence from pGH-G-CSF plasmid to the pTWIN1 plasmid as a C-terminal fusion to the Intein1 coding sequence (as represented by Fig. 1b) was confirmed by endonuclease digestion and finally by DNA sequencing (Data not shown).

Expression of the Intein1-GCSF fusion protein was evaluated by 12% SDS-PAGE. Figure 2a, lanes 1, 3, and 5 represent non induced *E. coli* BL21(DE3) cells having either pTWIN1-GCSF plasmid, pTWIN1 plasmid, or no plasmid, respectively. Since the non-recombinant pTWIN1 plasmid codes for a fusion of Intein1, Intein2, and their corresponding chitin binding domains, a band of about 55 kDa was expected, which is seen in lane 4. Furthermore, nothing was observed following IPTG induction of the non-recombinant *E. coli* BL21(DE3) bacteria (lane 6). Finally, a band of about

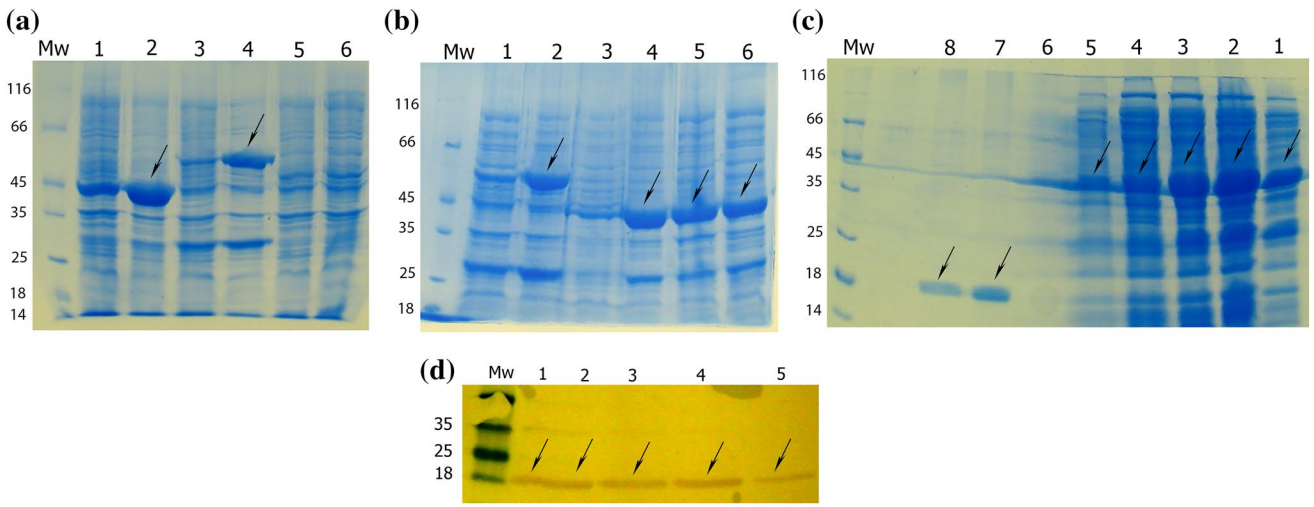

Fig. 2 **a** 12% SDS-PAGE analysis of Intein1-G-CSF expression. Mw: protein molecular weight marker; lane 1: un-induced *E. coli* BL21 (DE3) containing pTWIN1-G-CSF; Lane 2: induced *E. coli* BL21 (DE3) containing pTWIN1-G-CSF; Lane 3: un-induced *E. coli* BL21 (DE3) containing non-recombinant pTWIN1; Lane 4: induced *E. coli* BL21 (DE3) containing non recombinant pTWIN1; Lane 5: un-induced non-recombinant *E. coli* BL21 (DE3); Lane 6: induced non-recombinant *E. coli* BL21 (DE3). As it is shown in lane 2, a band of about 45 kDa confirmed the successful expression of Intein1-GCSF, while in lane 4 a band of about 55 kDa represents the expression of CBD-Intein1-Intein2-CBD cassette. **b** 12% SDS-PAGE analysis of soluble expression of G-CSF induced with 0.4 mM IPTG, and at 15 °C for 6 h as the best condition to yield soluble expression**.** Mw: protein molecular weight marker; lane 1: un-induced *E. coli* BL21 (DE3) containing non-recombinant pTWIN1; Lane 2: induced *E. coli* BL21 (DE3) containing non-recombinant pTWIN1;. Lane 3: un-induced *E. coli* BL21 (DE3) containing pTWIN1-G-CSF; Lane 4: total cell lysate of the induced *E. coli* BL21 (DE3) containing pTWIN1-G-CSF; Lane 5: Cell lysate supernatant of the induced *E. coli* BL21 (DE3) cells containing pTWIN1-G-CSF. Lane 6: The remained cell pellets after sonication of the induced *E. coli* BL21 (DE3) cells containing pTWIN1-G-CSF. It must be noted that although reasonable amount of soluble protein (lane 5) was obtained at the mentioned condition, however, almost similar amount of the protein is still found in the remained insoluble fraction (lane 6). **c** 15% SDS-PAGE analysis of the fractions obtained throughout purification procedure. Mw; Mw: protein molecular weight marker; Lane 1; cell pellets following induction of expression. Lane 2; sonicated sample before centrifugation. Lane 3; cell lysate supernatant; Lane 4: Column flow through after protein binding. Lane 5; Column flow through after column wash by the B1 buffer; Lane 6: column wash by the B2 buffer; Lane 7: First fraction collected following induction of self-cleavage; Lane 8: Second fraction collected following induction of self-cleavage. **d** Western blotting of the purified G-CSF. Mw: prestaiend protein molecular weight marker; Lane 2–6: the purified G-CSF authenticated by anti-G-CSF antibody

45 kDa pertaining to the molecular weight of Intein1 fused to G-CSF confirmed the successful expression of Intein1-G-CSf protein (lane 2), and the study was proceeded to the next step.

In order to obtain the highest amount of the soluble protein, many attempts were made, and finally the best condition for soluble protein production was determined to be 0.4 mM IPTG, and incubation at 5 °C for 6 h. As it is shown by Fig. 2b, induction of expression at the mentioned condition yielded reasonable amount of the expressed protein in the cell lysate supernatant as soluble fraction (lane 5). However, still significant amount of the protein is insoluble (lane 6). This ratio was much better when compared to other expression conditions (Data not shown).

On-column purification of the recombinant G-CSF

As it is shown by Fig. 2c, SDS-PAGE of the fraction collected 24 h following incubation of the chitin column at room temperature, revealed a band of about 18.8 kDa, corresponding to the purified G-CSF. In addition, the purified protein was further authenticated by Western blotting using HRP-conjugated anti human G-CSF antibody (Fig. 2d). Moreover, the extra step of size exclusion chromatography gave a purified form after 103 min with no dimer formation (Supplementary Fig. 2). The final yield of the purified G-CSF was calculated to be 4.7 mg of bioactive protein per 1000 mL of cell culture.

Circular dichorism (CD) analysis

As it is shown by Fig. 3, circular dichorism spectra confirmed similarity of the secondary structure of the recombinant G-CSF and Neupogen™.

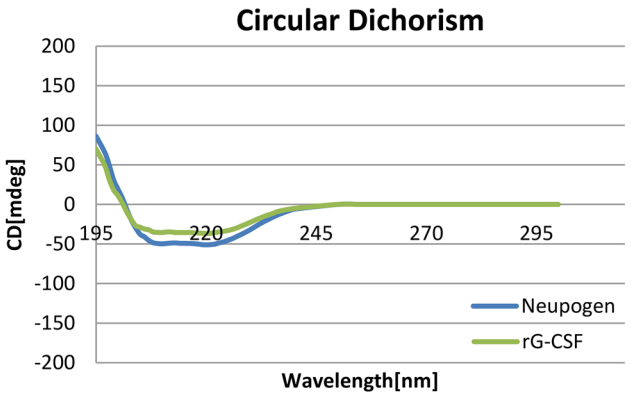

Fig. 3 Circular Dichorisem spectra of G-CSF and Neupogen™. The CD diagram of Neupogen® showed 68% alpha-helix content and recombinant G-CSF indicated an alpha-helix content of 64%. This confirms similar secondary structure of the two proteins

Peptide mapping

As it shown by Fig. 4, peptide mapping confirmed the similarity between the Intein-mediated purified G-CSF and Neupogen™ by revealing similar patterns.

Enzyme linked immunosorbent assay

The ELISA assay results showed similar absorbance for the same concentrations of recombinant G-CSF and the standard of the kit (40 ng/mL), confirming the similarity in their antibody binding capability. In fact, the independent T-test did not show any significant difference between the absorbance of the recombinant G-CSF and standard at the same concentration ($P > 0.05$) (Fig. 5a).

Biological assays

Assessment of biological activity of the purified G-CSF revealed similar survival rates at equal concentrations for both recombinant G-CSF and Neupogen™ on M-NFS-60 reference cell line (Fig. 5b). The results of independent T-test analysis did not show any significant difference between the survival caused by the purified G-CSF and Neupogen® on these cells at the same concentration (4 µg/mL) ($P > 0.05$).

Discussion

Purification of recombinant therapeutic proteins is one of the most challenging steps which affects the yield and cost of any obtained active protein. Traditional purification strategies with routine tags, for example 6x-His, includes several complicated steps for isolation of target protein from whole cell debris followed by addition of competitive chemicals for elution of the tagged protein from an affinity column. Furthermore, in most cases, especially for biopharmaceuticals, it is necessary to remove the affinity tag from target protein by chemical or enzymatic cleavage which makes the purification procedure much more complicated. Therefore, application of tags that are self-cleavable makes the purification step simpler [7]. In a previous study, MBP, PDI, Nus A, PDIa´b´, GST, 6x-His and Trx tags were fused to G-CSF via a TEV cleavage site to facilitate the purification process. MBP, PDI, Nus A, PDIa′b′ solubilized the fusion protein at 30 °C, while the optimum temperature was 18 °C for GST and Trx tags. However, 6x-His-tgged protein was not soluble in any temperatures [18]. In addition, the process of purification of the tagged G-CSF in the mentioned study consisted of at

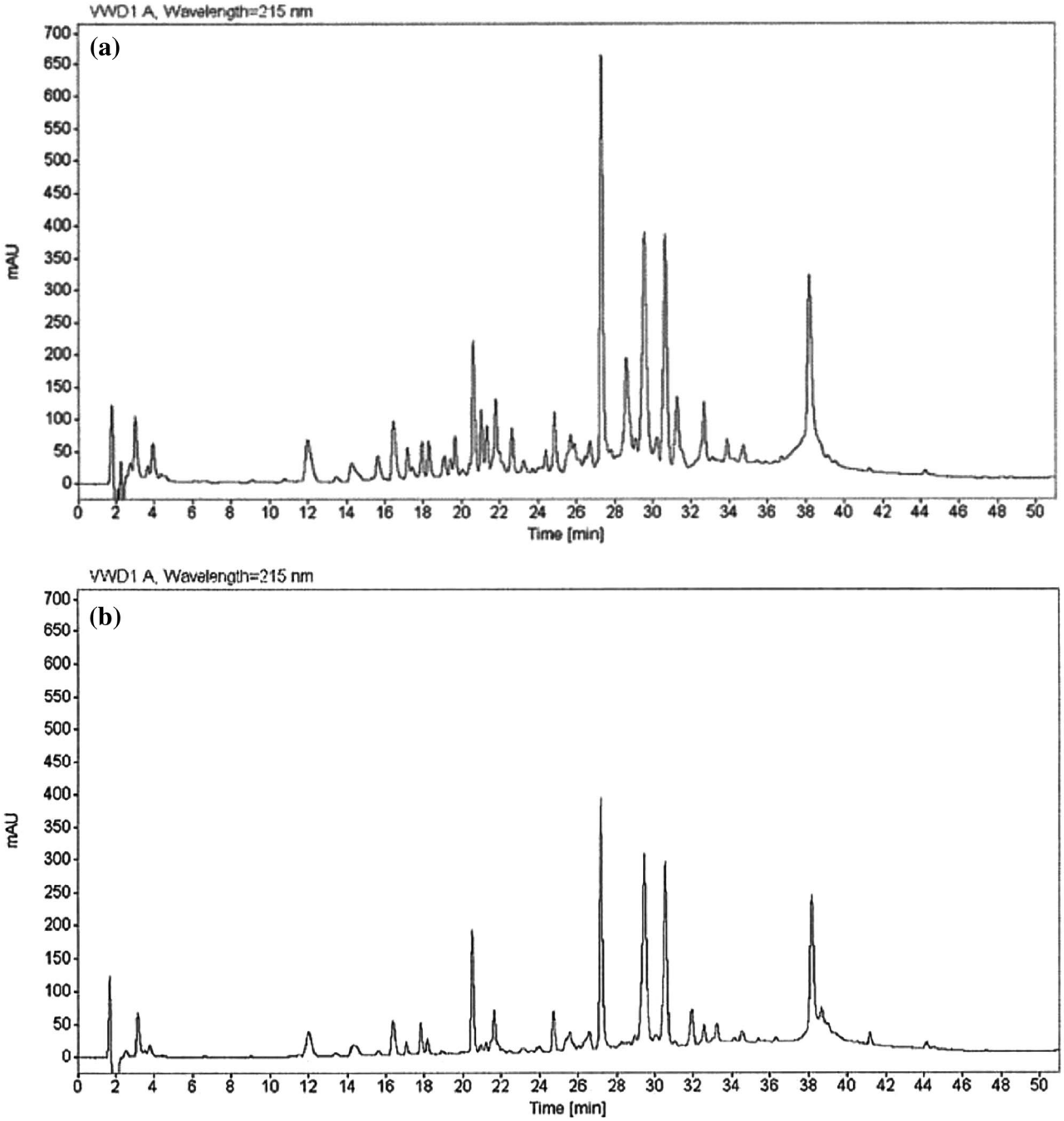

Fig. 4 Peptide mapping spectra of the purified G-CSF (**a**) and Neupogen™ (**b**). Similar patterns of protein fragments confirmed the similarity between the intein-mediated purified G-CSF and Neupogen™

least two steps, and further cleavage and purification steps was needed to obtain un-tagged protein.

IMPACT® system has been used for production of many recombinant proteins with the ability to produce the recombinant proteins in fusion to inteins [19]. For example, GM-CSF was fused to the intein of pTYB11 vector and successfully purified on a chitin affinity column by cleaving intein with 50 mM DTT [20]. However, DTT is a reducing agent and could remove disulfide bonds of the purified protein. G-CSF has two disulfide bonds which are necessary for its full biological activity. Therefore, here we used an intein which its activity is not induced by a reducing agent. In fact, since the Ssp DnaB intein of the pTWIN1 plasmid is activated by pH and temperature adjustments and not a reducing

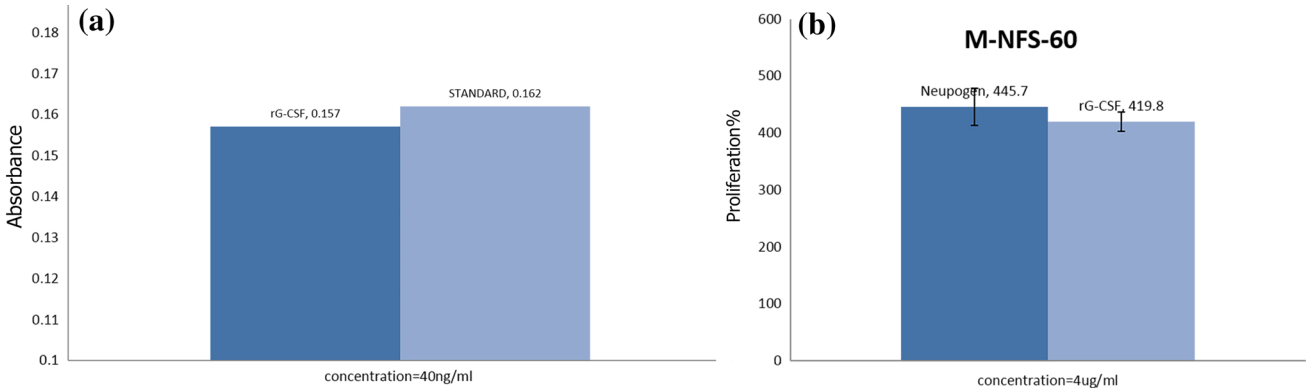

Fig. 5 **a** Comparison of the purified G-CSF and standard by ELISA at the same concentration. No differences in specific antibody binding capabilities of the recombinant G-CSF and Neupogen™ were observed (P-value = 0.953). **b** Comparison of the proliferative effect of Neupogen™ and the purified G-CSF on M-NSF cells. The purified G-CSF and Neupogen™ showed similar proliferative effects on the M-NSF-60 cells at the same concentration. Results are the mean of three independent triplicates and error bars represent mean ± SD. P-value = 0.287

agent, we obtained a fully functional protein without need to further refolding of the protein. Moreover, after purification and separation of the intein, this fusion retains the N-terminus of the G-CSF protein intact, i.e. without addition of any extra amino acid to the protein [21].

Similar to our study, Ssp DnaB intein was used for purification of human Natrieuretic hormone by Sun et al. They reported high yield purification after refolding of the fusion protein and pH adjustment to 7 and 16 h of incubation at room temperature [22]. PorA (the class 1 protein of the outer membrane of *Neisseria meningitidis*) is another protein which reported to be successfully purified by its fusion to the intein1 of the pTWIN plasmid. However, efficient purification of the PorA protein was occurred at 4 °C, pH 7, and after 5 days of incubation [23]. Comparing to the condition we used for G-CSF, the reported purification condition for PorA is much longer and also needed to be incubated in a much lower temperature. This means that in addition to the intein characteristics itself, the characteristics of the protein which is going to be purified also affect the purification condition and must be defined for each protein.

G-CSF has been produced by different host cells like *E. coli*, *Saccharomyces cervisiae* and mammalian cell lines [24–26]. The first recombinant form of commercial G-CSF, Neupogen™, produced in *E. coli* [27]. Neupogen™ is a non-glycosylated protein with a methionine at the N-terminal site with the similar biological effects to glycosylated form [27]. Lenograstim and AVI-014 are two glycosylated forms of G-CSF, produced in CHO cell line and transgenic hens, respectively, with biological activities similar to Neupogen™ [28]. *E. coli* is the most suitable host for G-CSF production [29–31]. However, in this expression host, G-CSF is produced as inclusion bodies [32]. Solubilizing inclusion bodies is a complicated multi-step process, requiring chaotropic solvents which might change the protein structure, and reduce the yield of purification [32]. However, in our study, we were taking the advantage of self-cleavability of the Ssp DnaB intein, and obtained soluble expression by lowering the IPTG concentration to 0.4 mM, decreasing the temperature to 15 °C, and increasing the induction time to 16 h. These parameters, resulted in a final purification yield of 4.7 mg of the purified G-CSF per 1 L of culture.

In the peptide mapping graphs, as required by the USP, chromatographic profile of the purified G-CSF and Neupogen™ were similar, and the relative height difference of the eight major peaks between the sample and the standard was less than 15% which is considered acceptable. Moreover, circular dichroism analysis confirmed the secondary structure of the purified G-CSF to be similar to Neupogen™. Finally, ELISA results showed similar absorbance for the same concentration of the sample and standard with a P-value of 0.953. The results of circular dichroism, peptide mapping and ELISA elicited perfect similarity in structure of the purified G-CSF and Neupogen.

For comparison of biological activity of the purified G-CSF and Neupogen, M-NFS-60 cells were used. These cells were cultivated in a medium containing M-CSF and beta-mercaptoethanol, as differentiation factors which elevate the levels of G-CSF receptor on the surface of these cells. The estimated potency of the recombinant G-CSF on the M-NFS-60 cells was calculated to be 94–106% of the Neupogen™ bioactivity which is considered acceptable by the USP (acceptable range is 80–125%).

Conclusion

In this project, G-CSF was successfully expressed and purified in soluble form and evaluation of its biological activities showed proliferative effects on a standard cell line similar to its commercial counterpart, Neupogen™. In addition, its physicochemical properties were comparable to the standard. Since the produced G-CSF protein was active, with acceptable characteristics in comparison to the standard, the intein-mediated purification system might be applicable for production of recombinant biopharmaceuticals as a single-step purification procedure which can reduce the total cost even further for large scale productions. Detailed studies on further improvement of production aspects and the characteristics of this protein are undergoing.

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Author contributions AJN and FS designed the study. SS performed the experiments. SS, FS and AJN analysed the results. SS and FS prepared first draft of the manuscript. AJN finalized and submitted the manuscript. AJN prepared the revised manuscript according to the reviewers comments and submitted the final revision.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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