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The Structural Characterization of Recombinant Human Granulocyte Colony Stimulating Factor

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***Abstract*—The biochemical and biophysical characterization of recombinant protein is required, when they are developed for human clinical use. A number of techniques can be used to determine the biophysical properties of protein and to examine their biochemical and biological integrity. The results of these experiments are compared with those obtained using naturally occurring proteins in order to be confident that the recombinant protein has the desired characteristics of the naturally occurring one. In this study, the purified protein was characterized by using Neupogen® and PDgrastim as reference standards.**

This research investigates the characterization of final product of rh-GCSF as characterization analysis: Bacterial endotoxin test, CD measurement, Disulfide bond analysis, Analysis of monomer and aggregates form of rh-GCSF. Also purity was measured by SDS-PAGE, Western blotting and quantified by Bradford .

An efficient, scalable and cost-effective procedure for production and purification of rh-GCSF in E. coli were used. The quantitative analysis shows that the purified protein yield was 400 mg from 1 g of cell dry mass (40%) by Bradford, SDS-PAGE (gel densitometry) and Western blotting and the purity was more than 99%. According to the inspection chromatogram, obtained peak conforms to molecular weight of rh-GCSF. Disulfide bonds are in correct position, rh-GCSF and reference standard chromatograms overlap with each other.

The obtained results approved that the rh-GCSF protein isolated in this study was highly pure and comparable with the innovator products, Neupogen® and PDgrastim. Based on the above results, the product has been found to be adequate for preclinical studies.

***Index Terms* — rh-GCSF, recombinant protein, *E*. coli.**

I. Introduction

Human granulocyte colony-stimulating factor (h-GCSF) is a single chain polypeptide with a total molecular size of 18.8 kDa and is composed of 174 amino acid residues. It has a free cysteine at position 17 and two intramolecular disulfide bonds, Cys36-Cys42 and Cys64-Cys74, which are necessary for GCSF bioactivity [1].

This factor stimulates proliferation and differentiation of neutrophil precursor cells as well as activation of mature granulocytes for more efficient immune responses [2],[3]. Furthermore, this factor applied for treatment of neutropenia arising from chemotherapy and radiotherapy and in patients who have undergone bone marrow transplantation and synthesized by biotechnological methods [4].

Analytical characterization of proteins and recombinant proteins is necessary according to the ICH Q6B Guideline. Test Procedures and Acceptance Criteria for Biotechnological/Biological Products, when proteins are developed for human clinical use, are necessary. Many quality control tests are essential when working with protein expression, purification and functional in-vitro and in-vivo assays. It can be improved and speed up the process development with analytical characterization of recombinant protein products and protein contaminants [5].

Initially we investigated the effects of medium composition on the production of rh-GCSF in batch culture and then a simple, scalable and cost-effective downstream process for the economical production of rh-GCSF is developed.

This research investigates the characterization of final product of rh-GCSF as characterization analysis: Bacterial endotoxin test, CD measurement, Disulfide bond analysis, Analysis of monomer and aggregates form of rh-GCSF. The rh-GCSF purity was measured by SDS-PAGE, Western blotting and quantified by Bradford [6].

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II. Materials and methods

A. Microorganism and vector system

Escherichia coli strain BL21 (DE3) (Novagen, Inc.) was used as the host for rh-GCSF expression. This strain was transformed with a commonly available plasmid, pET23a inducible expression vector (Novagen, Inc.), in which the rh-GCSF gene (Biotechnology Research Center, Tehran, Iran) was inserted into the NotI and NdeI sites. Host cells were transformed with the plasmid using the calcium chloride procedure. Transformed cells were spread on several LB agar plates containing 100 mg/l ampicilin .

B. Media and Solutions Batch Cultivation

All batch fermentations were performed twice with the M9 modified medium. Batch culture was started by adding 100 ml of an overnight-incubated seed culture (OD600 = 0.7-1) into the bioreactor containing 900 ml of medium. The pH was

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controlled at 7± 0.05 by the addition of 25% (w/v) NH4OH or 3M H3PO4. Dissolved oxygen was controlled at 30-40% of air saturation by controlling both the inlet air and agitation rate. Foaming was controlled by adding silicon-antifoaming reagent. In batch culture, cells were induced by the addition of IPTG (1mmol/l) when initial DCW (2.2 g DCW/l) reached a considered level [7]. Then, the production phase continued until the growth ceased [8].

C. Purufication

Cell lysis and IB recovery: The fermented broth was centrifuged and the obtained pellet was washed twice with phosphate buffer (pH 7.4). The wet cells (50 g) were suspended in lysis buffer. The cells were broken by passing the medium through a homogenizer three times (NIRO-SOAVI). The cells were cooled to 4°C between each pass. The cell homogenate was centrifuged, the supernatant was discarded and the inclusion bodies recovered.

IBs washing: The IBs pellet obtained in previous step was resuspended in wash buffer and incubated and recovered by centrifugation. In the second washing, the IBs pellet was resuspended in wash buffer and incubated and recovered by centrifugation.

IB solubilization and refolding: Washed inclusion bodies were dissolved , The solution was incubated and spun down to get rid of insoluble cell debris and recovered by centrifugation and then solubilized inclusion bodies was refolded by refolding buffer .After completion of refolding, the protein pH was adjusted with 2 Molar citric acid and centrifuged at 10,000 g for 20 minute at 4°C.

Anion Exchange Chromatography: The pH of Refolded protein was adjusted to 5-6 by adding 2 Molar Acetic acid and then loaded in mono Q column in FPLC (SYKAM-S2100). The column temperature and flow rate were maintained at 20 °C and 1 ml/min respectively throughout the process. The column was equilibrated with 3 bed vol. of 25 mMolar sodium acetate buffer (pH= 4.5). The refolded protein sample was directly loaded on to the column at the same flow rate. The column was extensively washed with 3 beds vol. of the same buffer but with 1 Molar NaCl.

D. Process analytical methods

D.1. Bacterial endotoxins test

Residual endotoxin levels (LPS) were analyzed by Limulus Ameboecyte Lysate (LAL) kit [9].

D.2. SDS-PAGE

In the reduced SDS-PAGE 15% gel and three standards of rh-GCSF (Neupogen®, Roche, Germany), (PDgrastim, Pooyesh Darou, Iran) and a molecular weight marker (#SM0431, Fermentas) were used. Sample buffer [0.5 M Tris pH 6.8, 50% (v/v) glycerol, 100 g l^{-1} SDS, 20 g l^{-1} bromophenol blue and 50 g l^{-1} 2-mercapto ethanol (2-ME)] was added to three samples and standards before boiling for 5 min. The samples were loaded on to the gel and ran at a constant voltage of 120 V for 100 min. Gels were stained with Coomassie brilliant blue R250 [10].

D.3. Western blotting

For confirmation of rh-GCSF band in gel, western blotting with polyclonal human GCSF antibody was performed. Separated proteins on the SDS-PAGE gels were transferred into a poly-vinylidene fluoride (PVDF) membrane (Roche Diagnostic, Germany) for recognizing the exact existence of rh-GCSF. PVDF sheet was blocked with 3% BSA in TBS-T solution (20 mMolar Tris-HCl, pH 7.5, 150 mg NaCl, and 0.05% Tween 20). Then, anti-GCSF polyclonal antibody was added at a dilution of 1:500 in TBS-T solution for 1 h. A second incubation with HRP anti-rabbit (1:1000) in TBS-T was carried out and the third incubation of 5-10 min was done with Diaminobenzine (DAB) solution. (0.5 mg/ml DAB, 0.1% H2O2) [11].

D.4. CD measurement

The purified rh-GCSF alongside innovator product (Neupogen®) were analyzed with 10 mg l^{-1} protein in citrate buffer (20.5 ml citric acid, 29.5 ml sodium citrate), pH 5, at 22˚C by Jasco J 715 spectropolarimeter with the protein solution contained in 2 mm path length cylindrical cell.

D.5. Analysis of monomer and aggregates

The SEC was carried out using TSK-GEL G3000SWXL (300 mm × 7.8 mm, TOSOH, Japan) column chromatography system with photodiode array (PDA) detector. The mobile phase consisted of K2HPO4-Na2HPO4 1 mMolar in water pH 6.2. Flow rate was maintained as 0.6 ml/min and analysis was carried out at a wavelength of 280 nm.

D.6. Analysis of disulfide bond

The RP-HPLC was carried out by using a C4-OL5-36071 (4.6 mm × 150 mm, Capital HPLC Ltd) column chromatography system with photodiode array (PDA) detector. The mobile phase consisted of solvent A and B, solvent A was 0.1% (v/v) TFA in water and solvent B was 0.1% (v/v) TFA in acetonitrile. Flow rate was maintained as 0.5 ml/min using a linear gradient of A to B from 90:10 to 10:90 in 65 min and analysis was carried out at a wavelength of 280 nm.

III. Results and Discussion

The purified protein was characterized by using Neupogen® (Roche, Germany) and PDgrastim (Pooyesh Darou, Iran) as reference standards.

Product impurity profile shows that endotoxins were within the acceptable limits.

The qualitative analysis of the rh-GCSF shows that the purity was more than 99% by SDS-PAGE (Fig. 1), SEC (Fig. 2).

According to the inspection chromatogram in inspection data of TSK-GEL column, obtained peak (peak 1) conforms to molecular weight of rh-GCSF (Fig. 2).

Disulfide bonds are in correct position, rh-GCSF and PDgrastim chromatograms overlap with each other (Fig. 3).

The purified protein was analyzed by CD alongside innovator sample and the overlaid CD spectra shows that the rh-GCSF was on par to the Neupogen® (Fig. 4).

The quantitative analysis shows that the purified protein yield was 400 mg from 1 g of cell dry mass (40%) by Bradford, SDS-PAGE (gel densitometry) and Western blotting (Fig. 1) validated by using Neupogen® and PDgrastim as reference standards.

Based on the above results can be found that the rh-GCSF protein isolated in this study was highly pure and comparable with the innovator products, Neupogen® and PDgrastim.

Based on the above results, the product has been found to be adequate for preclinical studies.

Fig. 1

A

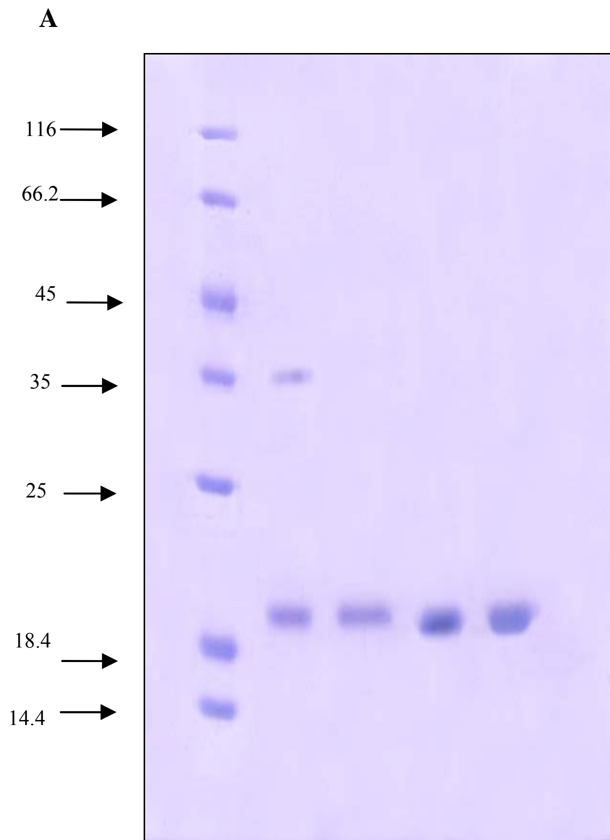

Fig. 1: The purity profile of rh-GCSF expressed in *E. coli*. (A) SDS-PAGE (15%) analysis of purified rh-GCSF showing a single protein band. Lane-1: Molecular weight marker (#SM0431, Fermentas which contain β-galactosidase 116.0, Bovine serum albumin 66.2 ,Ovalbumin 45.0, Lactate dehydrogenase 35.0, REase Bsp981 25.0,β-lactoglobulin 18.4, Lysozyme 14.4), lane-2: refolded rh-GCSF and lane-3: purified rh-GCSF, lane- 4: reference standard (Neupogen®), lane-5: reference Standard (PDgrastim).

Fig. 1

B

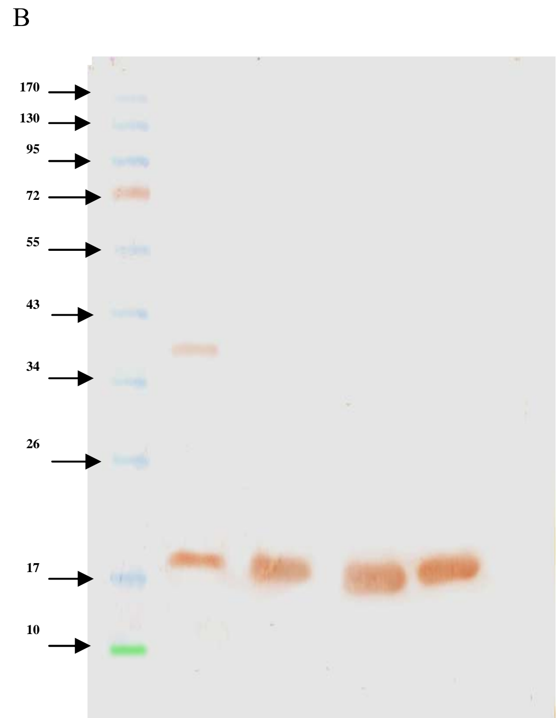

Fig. 1: The purity profile of rh-GCSF expressed in *E. coli*. (B) Western blot of purified rh-GCSF showing a single protein bond . Lane-1: molecular weight Marker (#SM0671, Fermentas), lane-2: refolded rh-GCSF and lane-3: purified rh-GCSF, lane-4: reference standard (Neupogen®), lane-5:

Fig. 4

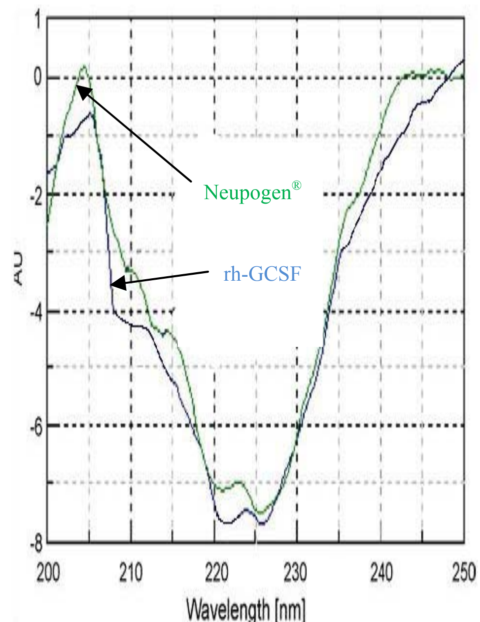

Fig. 4: The conformation form of rh-GCSF by CD. The sample concentration was 10 mg/ml.

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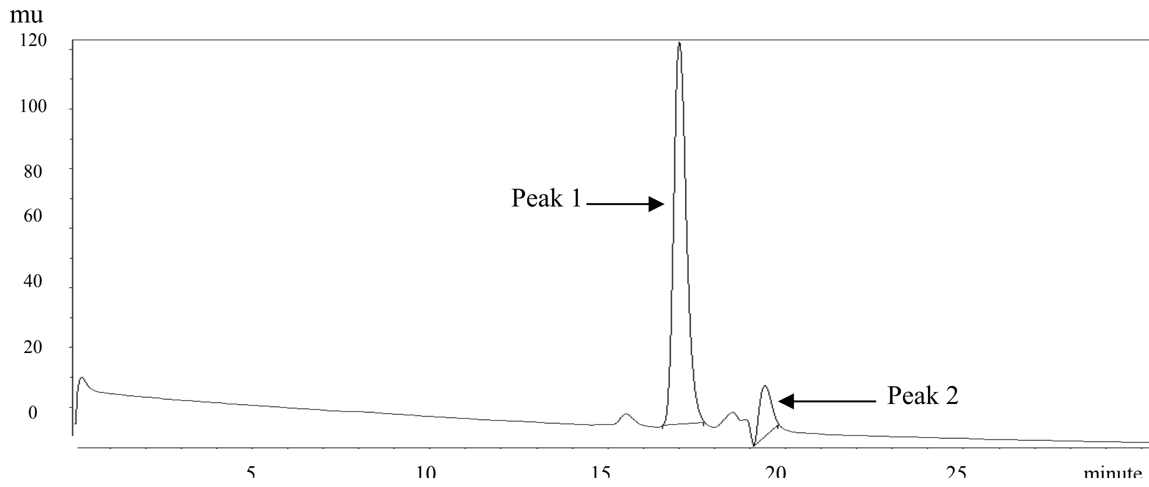
Fig. 2

A



B

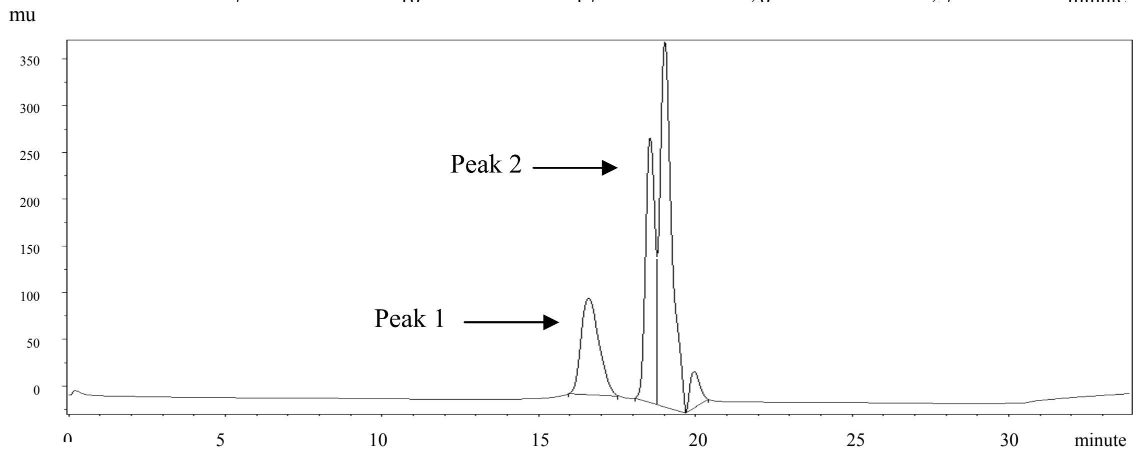


Fig. 3

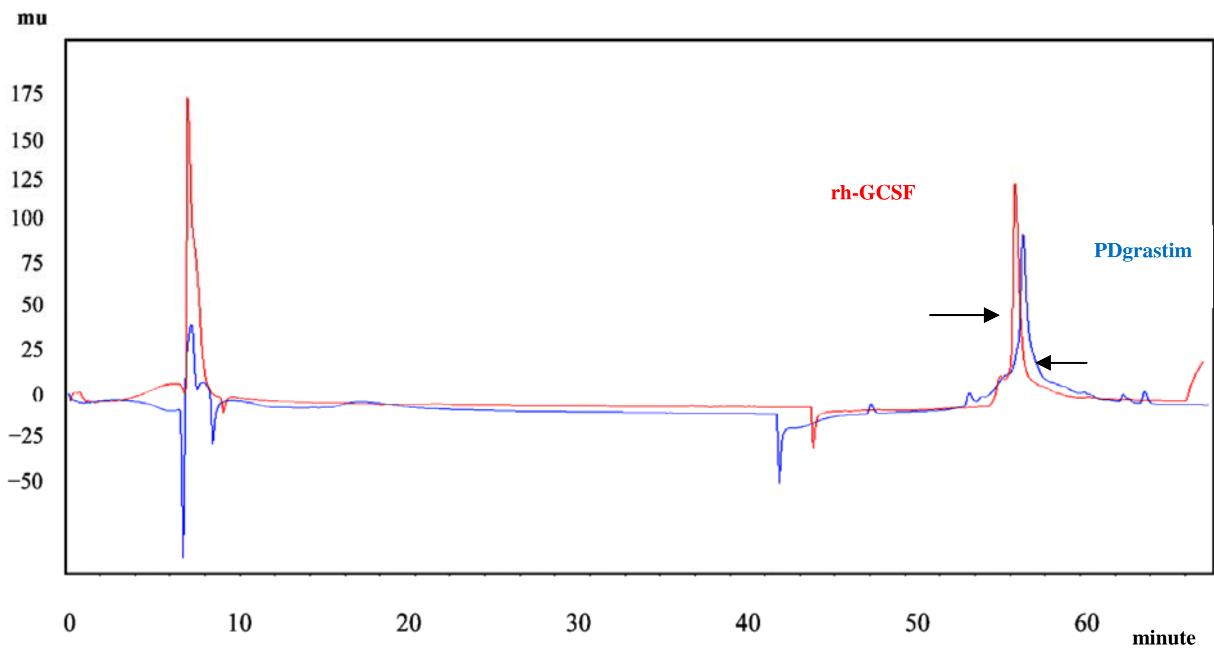


Fig. 2: Size exclusion HPLC analysis of rh-GCSF. 30 µg of either sample or standard was loaded for analysis. SEC was employed to detect monomer and aggregates forms of rh-GCSF. (A): reference Standard (PDgrastim), peak 1- monomer and peak 2- excipient. (B): purified rh-GCSF, peak 1- monomer and peak 2- excipient.

Fig. 3: RP-HPLC analysis of rhG-CSF. 30 µg of either sample or standard was loaded for analysis.

IV. Conclusions

The biochemical and biophysical characterization of recombinant protein is required, when they are developed for human clinical use. In this study, the process control method was used to eliminate the impurities and produce a clinically acceptable grade of rh-GCSF. Based on the above results can be found that the rh-GCSF protein isolated in this study as good as the innovator products, Neupogen® and PDgrastim. These properties serves a basis for comparison reproducibility, for creating the range of conditions to stabilize the protein during production, storage, and for identifying characteristics valuable for monitoring stability during long-term storage.

V. References

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