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A simplified purification procedure for recombinant human granulocyte macrophage-colony stimulating factor from periplasmic space of *Escherichia coli*

F. Rastgar Jazii *, A.A. Karkhane, B. Yakhchali, S.S. Fatemi, A. Deezagi

National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

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Abstract

Cytoplasmic expression is commonly used for production of recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF) which most often comes with inclusion body formation. We expressed rhGM-CSF in periplasmic space of *Escherichia coli* and optimized its extraction by osmotic shock and purification by anion exchange chromatography. Our works show that MgCl2 at 2 mM in osmotic shock buffer improves extraction of the protein and reduces contamination with other proteins. To achieve a simplified purification procedure for rhGM-CSF, efforts were focused on the adjustment of pH of the buffers and application of proper concentration of salt. Following to measurement of the p*I* of 5.4 for rhGM-CSF by isoelectric focusing, the pH of dialysis buffer and buffers used in anion exchange chromatography were adjusted to 6.5 for optimal binding of the protein to the column and removal of proteins with higher p*I*s during washing of the column. In addition, it was found that appliance of NaCl at a concentration of 20 mM in dialysis and column washing buffers prior to elution with elution buffer containing 120 mM NaCl significantly improves purification of the protein. Starting with specific amount of total proteins obtained by osmotic shock, it was possible to recover 95% of which following to purification with a purification yield of 72% for rhGM-CSF along with appropriate biological activity.

Keywords: Ion exchange chromatography; Granulocyte macrophage colony-stimulating factor; Periplasmic; rhGM-CSF; Purification

1. Introduction

Cytokines is a family of growth factors which plays important role in viability, proliferation, differentiation, and activity of hematopoietic cells [1]. Granulocyte macrophage-colony stimulating factor (GM-CSF) regulates proliferation and differentiation of hematopoietic progenitor cells and modulates function of the mature hematopoietic cells [2–4]. GM-CSF induces leukocyte chemotaxis and augments expression of adhesion molecules in granulocytes and monocytes [5–6]. Either alone or in combination with other therapeutic agents, GM-CSF enhances immunogenicity of tumor cells by facilitating tumor antigen presentation [7–9] which is essential for cancer treatment. Recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF) has formerly been cloned and expressed in mammalian, bacterial and yeast cells [10–12].

A big challenge in biotechnology is purification of recombinant proteins which demands procedures to endow the purified protein with structural integrity and biological activity without contamination. Most of the formerly published purification protocols for rhGM-CSF have presented multi-step procedures requiring application of multiple chromatography and chemical and physical manipulations in order to achieve the desired level of purity. Each step in such processes was possible to come with some loss of product and damage to the protein of interest. In addition, employment of urea for dissolution of inclusion bodies was a cause for unfolding, loss of intact protein and contamination with other proteins due to possible co-purification [11–13]. Inclusion bodies which are formed during cytoplasmic expression of recombinant proteins are dense, amorphous and insoluble protein deposits which demand isolation, solubilization and proper refolding. These processes require application of strong denaturating agents at high concentration, detergents, extreme

* Corresponding author.
E-mail address: rastgar_jazii@yahoo.com (F. Rastgar Jazii).

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pH along with addition of reducing and chelating agents. On the other hand, proper refolding of protein following to unfolding necessitates several treatment strategies. Altogether these manipulations increase complexity of the process and might lead to the disruption of protein structure (unfolding) or misfolding in the subsequent processes resulting to reduction of active protein [14].

To avoid problems associated with cytoplasmic expression of rhGM-CSF, formerly an expression vector for periplasmic expression of rhGM-CSF was constructed [15–16]. Expression and partitioning of proteins in the periplasmic space is associated with several advantages among which reduction of contamination with cytoplasmic proteins, endotoxins and DNA. In addition, due to porous structure of outer membrane, proteins could easily be extracted by simple osmotic shock. The presence of molecular chaperones, different proteases along with oxidizing environment promote proper protein folding, removal of signal peptide and elimination of misfolded polypeptides [17–21].

Considering the fact that commonly used commercial ion exchangers are designed to adsorb a wide range of proteins with different charges, a more targeted adsorption, immobilization and purification conditions for proteins were the subject of several recent studies. Guisan and Fernandez-Lafuente's research group have shown that applying tailor made ion exchangers provide a condition for adsorption and immobilization of large proteins such as β-galactosidase from other proteins [22,23]. This group has achieved this goal by reducing surface charge of ion exchanger and thus was able to purify or immobilize large proteins as a result of multipoint interaction with ion exchanger. Here, we report that application of an optimized salt concentration along with pH adjustment close to p*I* of protein plays more or less the same role and thus reduces adsorption of most undesired proteins to the exchanger. The present report represents an extension of our further efforts on the improvement of preparation of rhGM-CSF from periplasmic space by addition of divalent cation Mg^{2+} and purification procedure by optimizing ionic strength along with adjusted pH as determined based on p*I* of rhGM-CSF.

2. Experimental

2.1. Seed preparation

Escherichia coli strain BL21 (DE3) (Novagen Inc.) bearing the inducible expression vector; pBZY and efficiently directs the expressed protein into periplasmic space, was cultivated in LB (Luria Bertani) agar containing 30 μg/ml kanamycin and subsequently a single colony of which was cultured in 200 ml of F medium (Table 1) at 30 °C for 12 h for seed preparation [15–16].

2.2. High cell density cultivation: fed-batch fermentation

Fed-batch cultivation was carried out in 2.5 l Bioflo 3000 bioreactor (New Brunswick Scientific) containing 2 L of F medium inoculated with 200 ml of the 12 h seed culture (1–1.5 g dry cell weight/l). The pH of fermentation was adjusted to 6.9 with aqueous ammonia (25%, w/w) and HCl (20%, v/v). Dissolved oxygen (DO) was maintained at 40% air saturation with agitation speed change between 500 and 900 r.p.m. Foaming was controlled by using sterilized viscose silicon oil. Following to consumption of glycerol, as observed by increase in DO and pH, feeding was started in a fixed volume operation with exponential feeding strategy. IPTG (3 mM) was used for induction of rhGM-CSF expression [15].

Table 1
Composition of the semi defined F medium (developed in NIGEB) for batch and feeding solutions

Components	Batch medium (g/l)	Feeding solution (g/l)
Glycerol[a]	40	500
Peptone	10	200
KH_2PO_4	19.9	–
$(NH_4)_2HPO_4$	4.0	–
$MgSO_4{\cdot}7H_2O$	1.2	20.0
EDTA	14.1×10^{-3}	13.0×10^{-3}
$CoCl_2{\cdot}6H_2O$	2.5×10^{-3}	4.0×10^{-3}
$MnCl_2{\cdot}4H_2O$	15.0×10^{-3}	23.5×10^{-3}
$CuCl_2{\cdot}2H_2O$	1.5×10^{-3}	2.3×10^{-3}
H_3BO_3	3.0×10^{-3}	4.7×10^{-3}
$Na_2MoO_4{\cdot}2H_2O$	2.1×10^{-3}	4.0×10^{-3}
$Zn(CH_3COO)_2{\cdot}2H_2O$	33.8×10^{-3}	16.0×10^{-3}
Fe(III) citrate	100.8×10^{-3}	40.0×10^{-3}

[a] Initial concentration of glycerol for shake flask and seed culture was 10 g/l.

2.3. Osmotic shock

Osmotic shock was applied according to the Neu and Heppel [24] with modifications. Bacterial culture was centrifuged at 4000 r.p.m (J-6 centrifuge, rotor # 4.2, Beckman) for 10 min at 4 °C, the pellets was suspended in 15 ml TES buffer (0.2 M Tris–HCl pH 8.0, 0.5 mM EDTA, 0.5 M sucrose) per liter of the original culture and was shacked for 20 min on ice. Subsequently, cells were subjected to osmotic shock by adding 22.5 ml cold water containing 2 mM $MgCl_2$ and 1 mM PMSF, incubated on ice for 30 min, centrifuged at 20,000 rpm (Sigma) for 20 min and the periplasmic fraction (supernatant) was collected.

2.4. Ammonium sulfate precipitation

The osmotic shock preparation was adjusted to 70% ammonium sulfate (0.42 g/ml) and left on ice for 2 h. The precipitate was centrifuged at 5500 rpm (Rotor #3047, Heraeus) for 20 min at 4 °C and dissolved in dialysis buffer (50 mM Tris–HCl pH 6.5, 20 mM NaCl), dialyzed against the same buffer overnight at 4 °C.

2.5. Anion-exchange chromatography

The dialyzed ammonium sulfate preparation was loaded on 3.6 cm × 20 cm DE-52 column (manufactured by Wattman Company) equilibrated with binding buffer (50 mM Tris–HCl, pH 6.5, 20 mM NaCl) and washed extensively. Subsequently, protein was eluted applying elution buffer (50 mM Tris–HCl,

pH 6.5, 120 mM NaCl) and monitoring the process at 280 nm. Samples were collected (as 3.5 ml fractions), concentrated and subjected to the next step.

2.6. Gel filtration chromatography

Partially purified protein from the former step was concentrated (bench top vacuum freeze dryer), dialyzed (50 mM Tris–HCl, 150 mM sodium chloride, pH 7.5) overnight at 4 °C with three changes of buffer and loaded on Sephacryl S-100 (2.6 cm × 100 cm Amersham-Pharmacia Biotech) column equilibrated with dialysis buffer. Elution of protein was done at flow rate of 12 ml/h as 3.5 ml fractions along with monitoring protein elution at 280 nm. Fractions containing rhGM-CSF were pooled and protein concentration was assessed [25].

2.7. Electrophoresis (SDS-PAGE)

Electrophoresis was carried out according to Laemelli [26] using a 13% separating and 4% stacking gels. Gels were stained with R-250 Coomassie blue (BioRad) or silver nitrate [27–28].

2.8. Antibody preparation

Anti *E. coli* antibody was prepared according to Bouksaim et al. [29]. Briefly, 1–5 mg of freeze dried bacterial homogenate was dissolved in phosphate buffered saline (8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na2HPO4, 0.24 g/l KH_2PO_4, pH 7.4), emulsified with complete Freund's adjuvant (Difco) at a 1:1 (v/v) ratio and injected subcutaneously into white female New Zealand rabbits at four different sites in the intramuscular area. Another injection was done with the same amount of bacterial homogenate in the incomplete Freund's adjuvant 28 days later subcutaneously. Fifteen days after second injection, blood was collected and serum was recovered. For preparation of anti-rhGM-CSF antibody 0.5 mg of pure rhGM-CSF was used according to the above protocol.

2.9. Immunoblotting

Polypeptides were transferred onto nitrocellulose membrane using a semidry electroblotting apparatus (Pharmacia), blocked with TTBS (100 mM Tris–HCl, pH 7.4, 0.9% NaCl, 0.1% Tween 20) overnight and exposed to rabbit anti-rhGM-CSF antibody at 1:1000 dilutions in TTBS for 1–1.5 h at room temperature. Membrane was washed three times with TTBS, each for 10 min and incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody at 1:1000 dilutions in TTBS for 1–1.5 h. Following to three washes of the membrane with TTBS and once with TBS (10 min), development was carried out with 4-chloro-α-naphtol (30 mg/10 ml methanol) [30].

2.10. Capillary isoelectric focusing

Capillary isoelectric focusing gel was prepared by mixing 0.825 g urea, 0.6 ml distilled H_2O, 0.2 ml 30% acrylamide/1.8% bis-acrylamide, 75 μl ampholines (1 part pH 4–6 and 2 part pH 3.5–10), 30 μl NP-40, 5 μl ammonium persulfate and 1 μl TEMED, poured into glass tubes (1.5 mm internal diameter and 15 cm height) and left to polymerize. Tubes were mounted, upper reservoir of electrophoresis tank was filled with catholyte (20 mM NaOH) and the lower reservoir with anolyte (0.085% phosphoric acid). Prefocusing was carried out at 200 V for 1 h. Ten micrograms of the purified rhGM-CSF in urea solubilization buffer (5.4 g urea, 0.4 ml NP40, 0.2 ml ampholine pH 3.5–10 and 0.2 ml β-mercaptoethanol in 10 ml double distilled H_2O) was applied, covered with overlay buffer (four times dilution of urea solubilization buffer) and focused for 16 h at 500 V [31–32]. Two pairs of separate gels were run, one pair with purified rhGM-CSF and the other without purified protein. Following to focusing one pair was stained while the other two were used for determination of p*I* along the entire length of the focused gel. p*I* was determined by cutting gels into 0.5 cm long pieces, each piece was placed in a vial containing 2 ml of double distilled H_2O, crushed, mixed and incubated for 3 h at room temperature and then the pH of each vial was determined [33]. The other pair gels were impregnated in distilled H_2O till ampholine was completely diffused out, stained and destained as described above.

2.11. High-performance liquid chromatography

The purified rhGM-CSF was subjected to high-performance liquid chromatography applying C18 reverse-phase column (Beckman). The column was eluted with a gradient of 15–60% acetonitrile in 0.1% trifluoroacetic acid at 1.5 ml/min flow rate over 30 min, applying absorbance of 280 nm for monitoring protein elution profile.

2.12. GM-CSF bioactivity assay

In each well of 96 wells plate (Nunc), 10^4 human erythroleukemia TF-1 cells were cultured, supplemented with RPMI-1640 (Gibco) and 10% fetal bovine serum (FBS). Cells were maintained, subcultured two times in a week and treated with increasing concentrations of the purified rhGFM-CSF (0–40,000 pg/ml), incubated at 37 °C, 5% CO_2 and 95% humidity for 96 h. Ten microliters of fresh MTT dye (Sigma) (5 mg/ml in sterile PBS) was added to each well and incubated for 4 h at 37 °C. Subsequently, 100 μl of solubilizing reagent (50% dimethylformamide, 20% SDS) was added and incubated overnight at 37 °C. The absorbance was measured at 620 nm applying multiscan ELISA reader (Labscientific).

3. Results and discussion

To overcome problems associated with cytoplasmic expression of rhGM-CSF an expression vector for periplasmic expression of rhGM-CSF was constructed [15,16]. Subsequently, in the present study, we focused our attempts on the improvement of rhGM-CSF purification. The study was carried out in two steps. Firstly, optimization of protein preparation by osmotic shock on shaken flask culture and purification of rhGM-CSF through a two-step purification process including ion exchange and gel filtration chromatography. Secondly, further

simplification of purification process by applying an optimized single step anion exchange chromatography on fermenter culture which provides a large amount of bacterial culture and thus protein.

For extraction of proteins from periplasmic space, osmotic shock was used along with addition of divalent cation Mg^{2+}. While the presence of EDTA in TES buffer makes outer membrane leaky and thus enhancement of protein extraction by osmotic shock, the presence of Mg^{2+} in subsequent cold-water treatment inhibits the effect of EDTA on plasma membrane as a result of chelation of EDTA. Supports to our suggestion are former studies on the effects of EDTA on Gram-negative bacteria such as *E. coli*. These reports have well documented that EDTA increases permeability of the outer membrane of Gram-negative bacteria by weakening outer membrane and release of proteins [34–35]. However, it should be noted that excess EDTA might also affect the plasma membrane and thus release of cytoplasmic proteins. This effect of EDTA could be observed at approximately 10 mM [34], a concentration much higher than what was used in this study (0.5 mM). The effect of EDTA could be prevented by addition of divalent cations especially Mg^{2+} and Ca^{2+} at appropriate concentration. The antagonistic effect of divalent cations was found primarily to be due to their stabilizing effect on the outer membrane and chelation of EDTA [34–37]. Altogether application of divalent cations in osmotic shock could lead to a better protein extraction along with lesser contamination with cytoplasmic proteins, DNA or other compounds.

Several factors play role in ion exchange chromatography among which pH and salt concentration are the two key factors. As a general rule and depending on the type of exchanger (anion or cation exchanger), proteins bind to the exchanger at one pH unit above or below their p*I* [38]. In order to optimize buffer condition, by applying standard rhGM-CSF, p*I* of 5.4 was determined similar to other former reports (data not shown) [13]. Thus, in subsequent purification steps, the pH of buffers were adjusted to 6.5 which favors anion exchange chromatography and optimal rhGM-CSF binding to the column. This pH was chosen for enhancing removal of proteins with p*I*s higher than rhGM-CSF in the process of chromatography. Additionally, applying a pH around the p*I* of the protein of interest also protects it from the possible denaturation [38].

In order to achieve a simplified purification procedure for rhGM-CSF, we started with a two-step purification protocol applied on osmotic shock protein product prepared from shaken flask culture including ion exchange chromatography and gel filtration (Figs. 1 and 3). Practicing many times of purifications, it was found that addition of 15 mM NaCl to dialysis and washing buffers with pH of 6.5 could be effective in removing lots of proteins which seem to be weakly adsorbed to the exchanger (Fig. 2 lane E) without affecting binding of rhGM-CSF. This effect of salt seems to be due to the perturbation of interaction of such proteins with the exchanger and thus removal during column washing. Fig. 1 shows the purification profile of rhGM-CSF by anion exchange chromatography and Fig. 2 SDS-PAGE analyses of eluted fractions. Lane F corresponds to the partially purified rhGM-CSF following to elution with 50 mM Tris–HCl, pH 6.5 containing 100 mM NaCl. As shown, applying

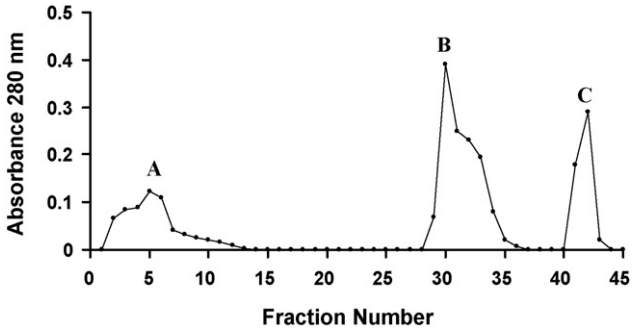

Fig. 1. The anion exchange chromatogram of rhGM-CSF. Peak A, washed fractions; peak B, 100 mM NaCl eluted fractions containing rhGM-CSF and peak C, 1 M washed fractions.

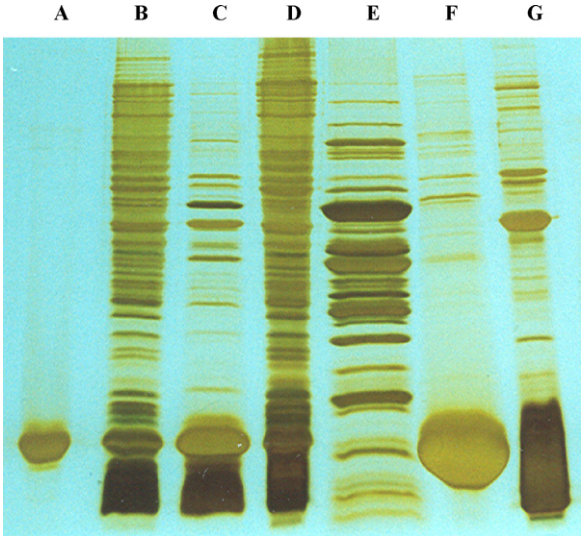

Fig. 2. SDS polyacrylamide gel electrophoresis of different fractions obtained from anion exchange chromatography. Lane A; standard rhGM-CSF, lane B; total recombinant *E. coli* proteins, lane C; periplasmic proteins obtained by osmotic shock, lane D; total insoluble proteins after osmotic shock, lane E; column washed fraction (peak A, Fig. 1), lane F; washed fraction with100 mM NaCl (peak B, Fig. 1), lane G; 1 M NaCl eluted proteins (peak C, Fig. 1).

an optimized concentration of salt and appropriate pH determined based on p*I* of rhGM-CSF could lead to the reduction of most unwanted proteins and elution of those with column washing buffer (compare lane E with lane G). Fig. 3 shows fur-

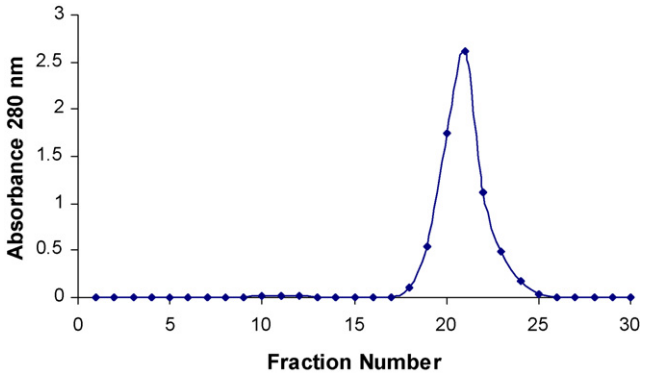

Fig. 3. Gel filtration (Sephacryl S-100) chromatogram of rhGM-CSF.

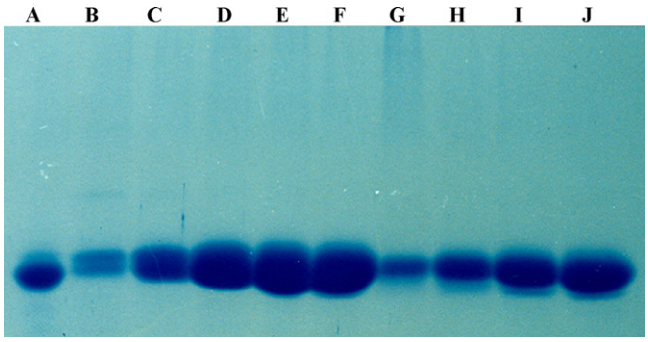

Fig. 4. SDS-PAGE analysis of rhGM-CSF containing fractions obtained by gel filtration. Lane A, standard rhGM-CSF; lanes B–F, fraction numbers 18–22, and lane G–J, fraction numbers 26, 25, 24 and 23, respectively.

ther purification by applying Sephacryl S100 gel filtration. The appearance of homogeneous bell shaped peak is an indication for the proper chromatography process and the presence of only a single protein in the eluted fractions. Analysis of the fractions with SDS/PAGE (Fig. 4) additionally confirmed that all of the obtained fractions contain rhGM-CSF without contamination with other proteins. Combining fractions and carrying out immunoblotting (Fig. 5) by applying anti-rhGM-CSF antibody further supported the successful purification of rhGM-CSF.

As noted in Section 1, the available commercial ion exchangers due to their highly activated surface groups might encumber purification of some proteins among which large proteins due to their multipoint interactions with exchanger. Thus it is required that high ionic strength buffers to be applied for protein desorption. Achieving a selective adsorption of proteins to ion

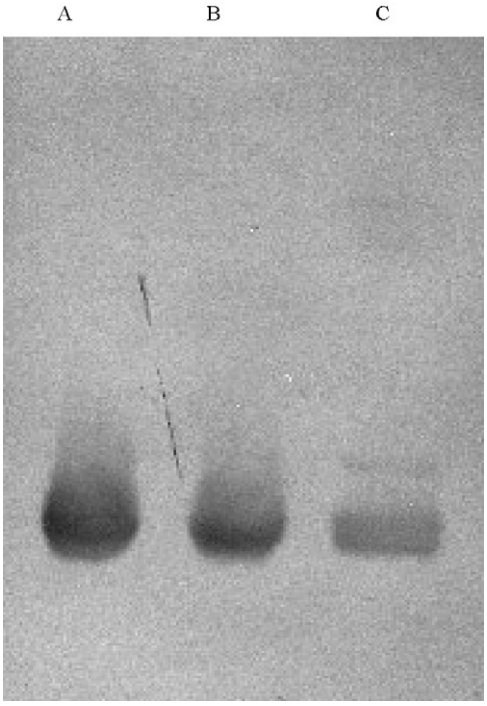

Fig. 5. Immunoblotting of rhGM-CSF following to purification with gel filtration. Lanes A and B, the purified rhGM-CSF and lane C, total proteins of *E. coli* expressing recombinant rhGM-CSF. The antibody specifically reacts only with rhGM-CSF and not any other *E. coli* specific proteins.

exchangers was the subject of several recent studies [22,23,39]. For example Pessela et al. [22] and Fuentes et al. [23] have shown that purification of large proteins such as β-galactosidase is favored with tailor made lowly activated surface ion exchanger. With such ion exchangers, due to large surface of interactions in large proteins and multipoint interactions with exchanger such proteins are selectively adsorbed while small proteins are excluded due to lower surface charges. The extent of adsorption of large proteins is such that renders them resistant to high ionic strength and thus requirement of high salt concentration for elution [40]. Also, it was shown that such tailor made exchangers not only are useful for isolation of large proteins but also for isolation of protein–protein complexes and for studying the very weak protein–protein interactions [23]. Furthermore, dissociating such protein–protein complexes into individual components comes with the loss of interaction of each individual resultant protein with ion exchanger [39]. It seems that application of salt in our study may play a role for selective binding of proteins to the exchangers when commercially exchangers are used. In fact, application of salt favors desorption of most unwanted proteins by changing ionic strength. However, for different proteins with different surface charges, salt concentration should be optimized. Application of salt increases the ionic strength of the medium and the resultant Na^+ and Cl^- ions can affect the specificity of proteins for interaction with the exchanger especially weakly interacting proteins. While Cl^- competes with proteins for positively charged exchanger, the counter ion, Na^+ interact with the negative surface charges of proteins and thus lead to the reduction of binding to the exchanger. As a result, proteins with higher surface negative charge such as rhGM-CSF will be able to bind and remain stably bound to the exchanger during binding and washing steps till the final elution with high salt concentration. Supporting our suggestion is the recent study of Fuentes et al. [41] that have used divalent (Mn^{2+}) and polyvalent (Cr^{3+}) cations to enhance adsorption of enzymes such as β-galactosidase and chymotrypsin to dextran sulfate coated supports. This group observed a higher absorption of proteins in presence of di- and trivalent cations. In fact, application of salt would be effective for isolation of proteins with high surface charge such as rhGM-CSF which could be deduced from their p*I*s.

In order to achieve an improved purification protocol for rhGM-CSF, additional efforts were done to make the process simple, speedy and cost effective. This goal was accomplished by further attempts that finally led us to the reduction of chromatography from two steps into a single step anion exchange chromatography. Considering advantages associated with the presence of salt, more works on the optimization of salt concentration led us to increase salt concentration up to 20 mM in dialysis and column washing buffers and up to 120 mM in elution buffer of anion exchange chromatography. Instead of applying shaken flask for culturing *E. coli*, fermenter was used which provides large amounts of starting bacterial culture and thus more protein for purification. Because of obtaining a large amount of bacterial culture following to cultivation in fermenter it was necessary to apply larger volumes of osmotic shock buffer and thus it was required to concentrate extracted

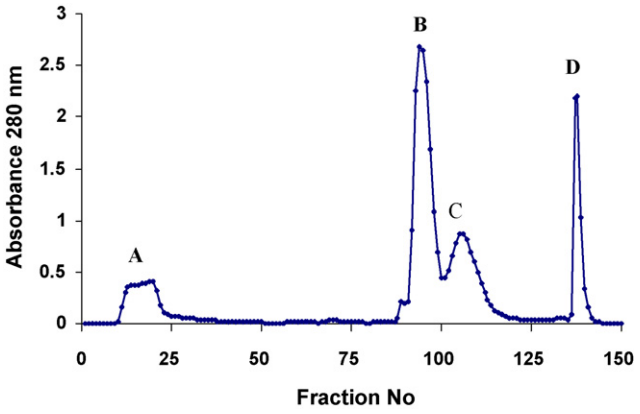

Fig. 6. The single step anion-exchange chromatogram of the purified rhGM-CSF. Peak A, washed fractions of the column; peaks B and C, fractions obtained following to washing the column with 120 mM NaCl; peak D, fractions eluted with 1 M NaCl.

proteins in the subsequent step. For concentrating proteins, precipitation with ammonium sulfate was used and subsequently dialysis with buffer containing 20 mM NaCl was carried out for removing ammonium sulfate. Consequently, anion exchange chromatography was carried out using elution buffer containing 120 mM NaCl as noted above. Implementing these concentrations was found to improve purification of rhGM-CSF into a single step anion exchange chromatography (Fig. 6 peaks B and C and Fig. 7 lanes F and G). As the chromatogram in Fig. 6 shows the fractions containing rhGM-SF appear as homogeneous peak which further validates the purity of protein (see also Figs. 8 and 9). Consequently, two antibodies were used for further assessment of purification process. The first antibody was raised against total *E. coli* proteins and the second antibody against pure rhGM-CSF. Application of antibody against total *E. coli* proteins in blotting (part II Fig. 7) showed that the antibody interacts with *E. coli* proteins (lanes B–E and lane H), however it does not interact with any protein in lane A which belongs to pure rhGM-CSF that was used as positive control and lanes F and G which correspond to the purified rhGM-CSF. This observation suggests that the purified rhGM-CSF is free from other *E. coli* specific proteins. We also applied anti rhGM-CSF antibody in immunoblotting. It could be inferred from part III of Fig. 7 that despite the fact that rhGM-CSF was expressed in *E. coli*, however the absence of interaction of this protein with antibody against total *E. coli* proteins (compare with part II lanes A, F and G) was in fact due to the lack of purified protein from any *E. coli* specific proteins. In the other word, the condition for preparation and purification of rhGM-CSF was suitable. It could also be deduced from parts II and III of this figure that both antibodies show no cross reactivity or non-specific reactions with other proteins. Fig. 7 also indicates that rhGM-CSF was efficiently bound to the column during initial washing step containing 20 mM NaCl (lane E of the parts I–III) and almost completely eluted with elution buffer (lanes F and G of Fig. 7 parts I and III). Also the absence of this protein in lane H (or washing with 1 M NaCl) additionally supports the efficacy of elution with 120 mM of salt. Further validation on the purity of the rhGM-CSF was done by reverse phase HPLC (Fig. 9). Elut-

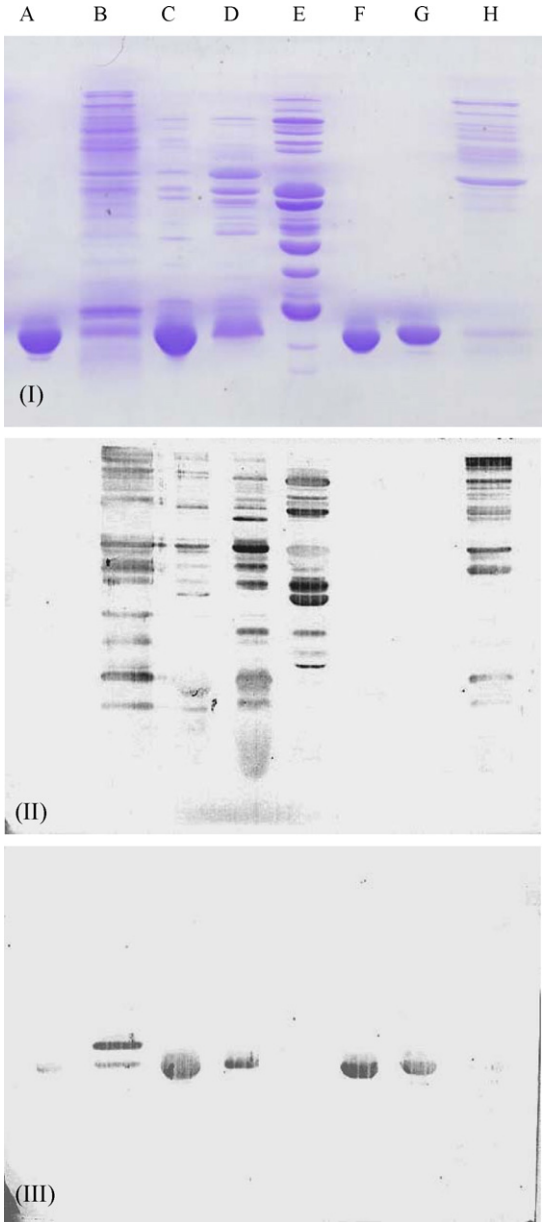

Fig. 7. SDS/PAGE analysis and immunoblotting of the purified rhGM-CSF by application of antibodies raised against total *E. coli* proteins and pure rhGM-CSF. Part I, SDS/PAGE: Lane A, rhGM-CSF; lane B, total recombinant *E. coli* proteins; lanes C and D, soluble and insoluble proteins obtained after ammonium sulfate precipitation; lane E, column washed fraction (peak A, Fig. 6); lanes F and G, fractions eluted with 120 mM NaCl (peaks B and C, Fig. 6); lane H, fraction eluted with 1 M NaCl (peak D, Fig. 6). Part II, immunoblotting carried out against part I by applying antibody against total *E. coli* proteins, and part III; the same as part II but anti rhGM-CSF specific antibody was used.

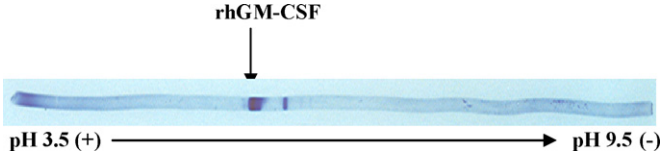

Fig. 8. Capillary isoelectric focusing analysis of the mixed purified rhGM-CSF of peaks B and C of the Fig. 6.

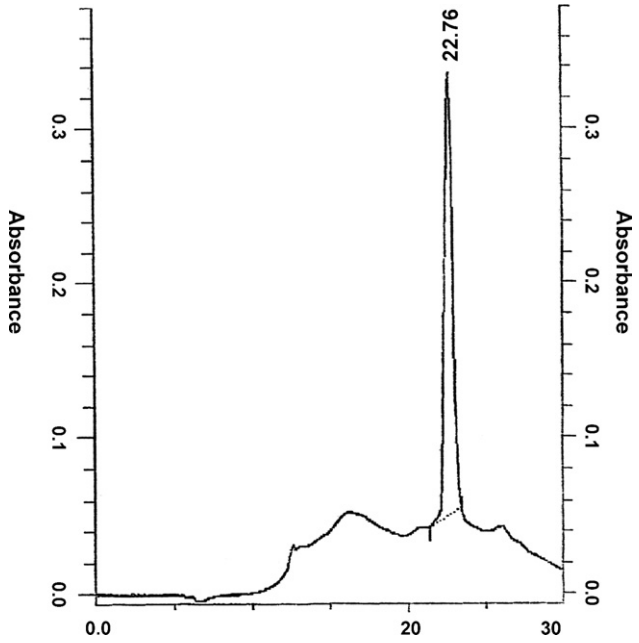

Fig. 9. Reverse-phase HPLC chromatogram of the purified rhGM-CSF. The protein elutes as a single peak which points out its purity.

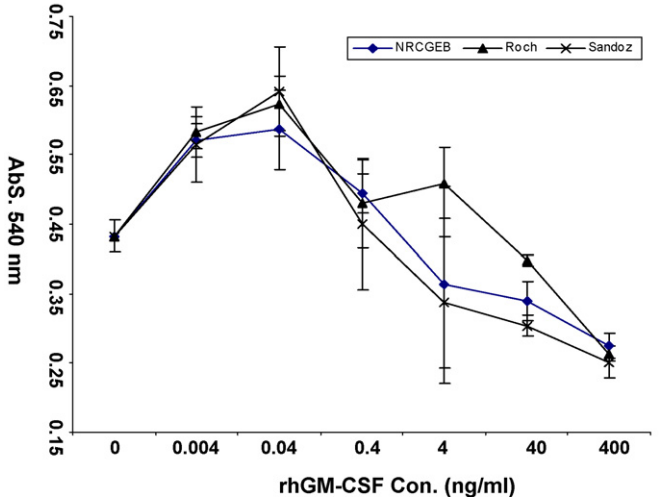

Fig. 10. TF-1 leukemia cell proliferation assessment of the purified rhGM-CSF. TF-1 cells were cultured and exposed to the increasing concentrations of rhGM-CSF (two commercially available rhGM-CSFs and the purified rhGM-CSF from periplasmic space) for 96 h and MTT cell proliferation assay. The purified rhGM-CSF exhibits a comparable biological activity with that of two other commonly used rhGM-CSF for therapeutic purposes.

ing as a single polypeptide peak additionally indicates the purity of rhGM-CSF.

To assess the yield of purification we started with 194 mg total protein obtained following to preparation of 1 l of osmotic shock. This amount of protein was subsequently precipitated, dialyzed and subjected to anion exchange chromatography. Consequently, the yield of purification was assessed and total amount of 183.4 mg of protein was estimated. Comparing this amount with the starting total osmotic shock protein product (194 mg), it could be concluded that 95% of the total applied protein was retained following to precipitation, dialysis and anion exchange chromatography. This quantity was estimated by summing up the combined fractions of peaks B and C that contain rhGM-CSF and totally compose 131.7 mg with the amount of proteins in peaks A (washed fraction) and D (fractions eluted with 1 M NaCl) that are 31.7 and 20 mg, respectively. From these data, it could be concluded that the yield of rhGM-CSF purification was 72% at the end of final step or anion exchange chromatography. Additionally, rhGM-CSF composes 68% of total osmotic shock proteins.

Appearing as two peaks, a major (B) and a minor (C) in chromatography indicate that there should be two fractions of rhGM-CSF with minor charge differences. To assess this possibility, isoelectric focusing was carried out. Fractions obtained from peaks B and C were pooled and focused (Fig. 8). As could be expected two bands; a major which correlates to the fractions obtained from peak B and a minor band that correlates to the fractions obtained from peak C were observed. Moreover, the absence of other protein bands further indicate the purity of the rhGM-CSF. To explain the appearance of two peaks signal peptide program (http://www.cbs.dtu.dk/services/SignalP/) was used. It was found that there are two available cleavage sites for signal peptidase in the signal sequence (MKYLLPTAAAGLLLAAQPAMA▼A▼PARSPSPSTQ) of the recombinant protein, a most likely cleavage site between position 22/23 (Ala/Ala) and a less likely cleavage site between position 23/24 (Ala/Pro) as shown with arrowheads. As a result of second cleavage, a positive charge would be eliminated and thus more acidic rhGM-CSF with higher affinity for anion exchanger would be generated. However, it is required that N-terminal sequencing to be carried out for ascertaining this suggestion. There are also reports that have described formation of minor fractions of recombinant human growth hormone during preparation with modifications such as deamination, methylation of lysine residues or oxidation of methionine, which altogether might affect charge of protein. It was also observed that such changes may favor protein activity by increasing binding activity of protein to its receptor [42]. It is possible that either of the above modifications might have occurred on rhGM-CSF during preparation and purification without affecting its biological activity as comparison of its biological activity with two other commercially available rhGM-CSFs (Fig. 10) points to this proposition.

Final assessment on the purified rhGM-CSF was done by evaluating its biological activity using human TF-1 cell line in MTT cell proliferation assay. As Fig. 10 shows, the purified rhGM-CSF is biologically active and comparable with two other commercially available rhGM-CSFs which are currently used for therapeutic purposes.

Our results show that we were able to simplify the process of purification of rhGM-CSF into single step anion exchange chromatography. We are currently focused our efforts on the improvement of the process of purification of rhGM-CSF by applying batched chromatography, a procedure that reduces the time needed for purification and provides the possibility of the application of a large amount of starting material for protein purification.

4. Conclusions

By implementing minor modifications in preparation and purification conditions it became possible to purify rhGM-CSF appropriately. Applying $MgCl_2$ in osmotic shock due to its protecting action on the plasma and outer membranes of host bacterium (*E. coli*) could be effective in freedom of purified proteins from contaminants such as endotoxins and other bacterial components including nucleic acids and undesired cytoplasmic proteins. Application of appropriate pH determined based on isoelectric point of protein and optimized salt concentration due to its effect on ionic strength of the medium and thus binding of proteins to the ion exchanger are two other factors which contribute both for preparation and simplification of purification by ion exchange chromatography.

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