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Purification of recombinant human granulocyte–macrophage colony-stimulating factor from the inclusion bodies produced by transformed *Escherichia coli* cells

M. Belew[a,*], Y. Zhou[b], S. Wang[c], L.-E. Nyström[a], J.-C. Janson[a]

[a]*Pharmacia Bioprocess Technology, S-751 82 Uppsala, Sweden*

[b]*National Laboratory of Molecular Biology and Genetic Engineering, Institute of Virology, Chinese Academy of Preventive Medicine, 100 Yingxin Street, Beijing 100052, China*

[c]*Antibiotic Institute, North China Pharmaceutical Corporation, 4 Heping Road, Shijiazhuang, Hebei, China*

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Abstract

Recombinant human granulocyte–macrophage colony-stimulating factor (rhGM-CSF), produced as inclusion bodies in genetically transformed *Escherichia coli* cells was purified to homogeneity by a three-step chromatographic procedure involving hydrophobic interaction, ion exchange and gel filtration. Each purification step is reproducible and well suited for process-scale operations. The purification process also leads to a significant decrease in DNA and endotoxin levels in the final product. Of the three gel media used, Phenyl Sepharose 6 FF (high sub) was most effective in reducing the DNA content (by a factor of ca. 2000) while Superdex 75 prep grade was more effective for removing endotoxins (reduction factor ca. 15). The recovery of purified rhGM-CSF was 35% by enzyme-linked immunosorbent assay and 70% by a biological assay method. The overall purification factor obtained was about 4.6, which is in the range of those reported for recombinant proteins produced in *E. coli* as inclusion bodies. The purified rhGM-CSF is an acidic protein ($pI = 5.4$) and has a specific activity of ca. $3.3 \cdot 10^7$ units/mg, which is in excellent agreement with that reported for its natural counterpart. Its monomer molecular mass of 14 605, as determined by electrospray mass spectrometry, corresponds exactly to the mass calculated from its cDNA sequence. Its amino acid composition and partial NH_2-terminal sequence (up to seventeen residues) are also identical with those reported for this protein. These and other results confirm the identity of the purified rhGM-CSF with its natural counterpart. However, the results also showed that it is apparently heterogeneous from its NH_2-terminal side as it is composed of three polypeptides having Met, Ala and Pro as the NH_2-terminal residues in which the intact Met analogue accounts for 60% for the mixture. This heterogeneity does not seem to have any biological significance since the specific activity of the purified rhGM-CSF is identical with that of its natural counterpart.

1. Introduction

The colony-stimulating factors (CSFs) are a group of four glycoproteins which regulate the proliferation and differentiation of granulocytes, monocyte–macrophages and certain related haemopoietic cells [1]. Their classification is based on the stimulatory effects which they exert

* Corresponding author.

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on various bone marrow progenitor cell lineages [2–6]. The granulocyte–macrophage CSF (GM-CSF) specifically stimulates the proliferation of cells of both the macrophage and granulocyte lineages [5,6]. The biological effects attributed to GM-CSF include (i) stimulation of the initial divisions of the erythroid and megakaryocyte progenitor cells [7], but mature cells in these lineages are produced only in the presence of either erythropoietin or a megakaryote stimulating factor [8]; (ii) prolonging the life span of mature granulocytes, macrophages and eosinophils [9–11]; (iii) inducing the accumulation of neutrophils at sites of infection or blood vessel damage [12]; (iv) inducing the secretion of interleukin-1 by GM-CSF-treated neutrophils, thereby involving other cells capable of fighting infections [13]; (v) stimulation of antibody-dependent T-cell-mediated cytotoxicity by neutrophils and macrophages [14,15]; and (vi) inducing macrophages to secrete a number of biological response modifiers including tumour necrosis factor [13].

Its important medical applications are (i) to restore haematopoietic dysfunction by raising cell counts from suppressed to normal levels; (ii) to stimulate the hyper-production of functionally primed effector cells [16]; and (iii) to augment host defence against infection and, possibly, malignant disease. It can thus help cancer patients to resist secondary infections developing either because of diminished resistance associated with some forms of cancer or in patients with suppressed bone marrow function after the use of myelotoxic chemotherapy or in those undergoing bone marrow transplantation following intense chemotherapy [1].

The natural human GM-CSF is composed of 127 amino acids including four cysteine residues that form two disulphide linkages [17,18]. It is a compact globular protein containing both α-helical and β-sheet structures [19]. It is also an acidic glycoprotein with a molecular mass of 18 000–30 000 and an isoelectric point ranging from 4.0 to 5.2, depending on the type of cells from which it is derived [20–22]. The molecular mass of the non-glycosylated protein is 14 700.

The cDNA of human GM-CSF has successfully been cloned and expressed in both mammalian (monkey COS cells) [23,24] and bacterial (*Escherichia coli*) cells [25]. Characterization of the purified product showed that, despite the lack of glycosylation, the bacterially synthesized human GM-CSF is the same as its natural counterpart in both conformation and biological activity [25]. In order to improve its expression level in *E. coli*, polymerase chain reaction (PCR) technology and synthesized oligonucleotide primers have been used to modify its 5′-terminal cDNA to fit the *E. coli* system. Recently, the high yield expression plasmid (pBV_{220}/GM-CSF) has been obtained [26] which will allow the production of recombinant human (rh) GM-CSF in relatively large quantities. This in turn will make it possible to perform some detailed structure–function analysis on the purified protein and its use in diverse clinical trials.

Methods for the purification of rhGM-CSF are scanty and the few that are published are based either on immunoaffinity chromatography [27] or a combination of gel filtration, ion-exchange (IEC) and reversed-phase liquid chromatography (RPLC) [24,25,28]. This paper describes an optimized downstream purification procedure for rhGM-CSF expressed in *E. coli* as inclusion bodies. The adopted procedure is reproducible and well suited for process-scale operations. The purified rhGM-CSF is homogeneous when examined by several criteria of purity and its biological activity is apparently identical with that of its natural counterpart. It has also been characterized with respect to its amino acid composition, partial amino terminal sequence analysis and its molecular mass.

2. Experimental

Unless stated otherwise, all experiments were performed at room temperature (20°C). Chromatographic columns, gel media, recorders, detectors, fraction collectors, PhastSystem electrophoresis apparatus and the BioPilot chromatographic system were products of Pharmacia Bio-Process Technology (Uppsala, Sweden). During the developmental phase of the downstream

purification procedure and for establishing the reproducibility of the adopted purification process, XK16 and XK26 laboratory columns were used. For pilot-scale applications, XK50/30 and BioProcess glass columns (BPG 100/950) were used. Relevant details will be outlined in appropriate sections.

Guanidine hydrochloride (Gu·HCl) (95%) was obtained from Aldrich Chemie, urea from Prolabo, Berol 185 from Berol Kemi (Stockholm, Sweden), EDTA (disodium salt, dihydrate) and 2-mercaptoethanol (2-ME) from Fluka, reduced glutathione (GSH) and oxidized glutathione (GSSG) from Sigma, RPMI 1640 medium and foetal calf serum from Gibco and a reference standard for rhGM-CSF (specific activity = $6 \cdot 10^7$ units/mg) from Boehringer (Mannheim, Germany).

The following buffers were used for extraction, solubilization and renaturation of the inclusion bodies and for the chromatographic experiments; they will be referred to in abbreviated form throughout:

(A) 20 m*M* sodium phosphate (93 parts of 0.2 *M* Na_2HPO_4 solution + 7 parts of 0.2 *M* NaH_2PO_4 solution diluted 10-fold)–0.125 *M* NaCl–5 m*M* EDTA (pH 7.6).
(B) 0.1% (v/v) Berol 185 in buffer A (pH 7.5).
(C) 0.5 *M* urea in buffer A (pH 8.1).
(D) 7 *M* guanidine hydrochloride–100 m*M* 2-mercaptoethanol–50 m*M* Tris–HCl–50 m*M* NaCl–1 m*M* EDTA (pH 7.5).
(E) 20 m*M* Tris–HCl–1 m*M* reduced glutathione–0.1 m*M* oxidized glutathione–0.1% Berol 185 (pH 8.4).
(F) 50 m*M* sodium phosphate (210 ml of 0.2 *M* Na_2HPO_4 + 40 ml of 0.2 *M* NaH_2PO_4 solutions diluted to 1 l)–10% (w/v) ammonium sulphate (pH 6.9).
(G) 20 m*M* sodium phosphate (61 ml of 0.2 *M* Na_2HPO_4 + 39 ml of 0.2 *M* NaH_2PO_4 solutions diluted to 1 l) (pH 7.0).
(H) 30% 2-propanol in buffer G.
(I) 20 m*M* sodium phosphate buffer (31 ml of 0.2 *M* Na_2HPO_4 + 69 ml of 0.2 *M* NaH_2PO_4 solutions diluted to 1 l) (pH 6.5).
(J) 20 m*M* sodium phosphate buffer (40 ml of 0.2 *M* Na_2HPO_4 + 60 ml of 0.2 *M* NaH_2PO_4 solutions diluted to 1 l)–0.15 *M* NaCl (pH 6.5).
(K) 20 m*M* sodium phosphate buffer (58 ml of 0.2 *M* Na_2HPO_4 + 42 ml of 0.2 *M* NaH_2PO_4 solutions diluted to 1 l)–1.0 *M* NaCl (pH 6.4).

2.1. Production of rhGM-CSF

The procedure described here is essentially identical with that used in ref. [29]. *E. coli* strain DH5α was transformed by plasmid pBV220/GM-CSF, which contains rhGM-CSF cDNA inserted downstream of P_RP_L promoter and CIts857 regulator gene. Fermentation of the transformed cells was performed essentially as described by Song and Tong [30]. About 1000 ml of an overnight cell culture in LB medium was seeded into a 40-l fermenter containing 25 l of modified M 9 medium. The cells were allowed to grow at 30°C, maintaining the pH at 7.0 and the glucose concentration at 0.2–0.4% (w/v) until the absorbance of the cell suspension at 600 nm (A_{600}) was about 3. This was reached after about 9 h of continuous culturing. The temperature of the fermenter was then raised to 42°C to induce the expression of the rhGM-CSF [31,32]. The fermentation was allowed to continue for a further 4 h and the cells were then harvested by centrifugation at 10 000 rpm at 4°C in a Beckman J2-21 centrifuge fitted with a continuous rotor.

2.2. Extraction, solubilization and renaturation of the inclusion bodies

About 120 g of the *E. coli* cells were suspended in 1200 ml of buffer A followed by dispersion using an Ultra Turrax (IKA-Werk) run at moderate speed for about 5 min. The homogenized suspension was cooled to 15°C and passed through an APV Gaulin Press maintained at a pressure of 500 bar (50 MPa) throughout the milling operation. The temperature of the partially disrupted cell suspension rose to 30°C during this first passage. It was cooled to 20°C in an ice-bath and the milling operation was re-

peated twice more. At the third and last passage, the temperature of the disrupted cell suspension rose to 27°C. Microscopic examination of the milled suspension showed no intact *E. coli* cells, indicating that at least 98% of the cells were disrupted.

The suspension was centrifuged at 5000 *g* for 30 min and at 4°C. The supernatant was discarded and the pellet was resuspended in buffer B (600 ml) followed by homogenization for about 5 min using the Ultra Turrax and centrifugation as above. The resulting pellet was further washed by resuspension in buffer C (600 ml) followed by homogenization and centrifugation as above. The supernatant was discarded and the pellet was resuspended in 300 ml of buffer A followed by homogenization and centrifugation at 10 000 *g* for 30 min at 4°C. The supernatant was discarded and the pellet (ca. 14 g wet mass), containing highly purified inclusion bodies, was dissolved in 56 ml of buffer D by continuously stirring it for 3 h at 4°C. The resulting cloudy solution was centrifuged at 40 000 *g* for 30 min at 4°C to remove insoluble material and residual cell debris.

The clarified supernatant, containing denatured and solubilized proteins of the inclusion bodies, was renatured by stepwise dilution with buffer E (a total of seven steps were used). This procedure resulted in a significant decrease in the amount of precipitate formed during renaturation compared with a one-step dilution procedure we had previously used [29]. To 1 part of the supernatant was added sufficient amount of buffer E (1.17 parts) to decrease the concentration of Gu · HCl from 7 to 6 *M*. The solution was then stirred continuously for 10 min at 4°C and a further amount of buffer E was added to decrease the concentration of Gu · HCl to 5 *M*. The solution was stirred as above for 10 min and this process was repeated until the final concentration of Gu · HCl was decreased to 1 *M*. At this point, the solution turned cloudy. To this was added sufficient buffer E to decrease the concentration of Gu · HCl to 0.1 *M* (total dilution factor = 75) and the resulting cloudy suspension was stirred for 10 min and allowed to stand at 4°C overnight for optimum renaturation of the proteins. The product obtained was distinctly cloudy, probably owing to the presence of partially renatured and/or aggregated proteins. It was clarified by centrifugation at 17 000 *g* for 30 min at 4°C and the clear supernatant, containing renatured rhGM-CSF, was used as the starting material for purifying the active protein according to the optimized procedure described below.

2.3. Hydrophobic interaction chromatography (HIC)

Phenyl Sepharose 6 Fast Flow (high sub) was packed in an XK50 column (bed height = 20 cm; bed volume = 400 ml) and washed with two bed volumes of deionized water followed by three bed volumes of equilibration buffer F. In the sample of renatured and clarified rhGM-CSF (4.3 l) was dissolved ammonium sulphate to a final concentration of 0.76 *M* (10%, w/v) before applying it to the equilibrated column at a flow-rate of 100 cm/h. After sample application, the column was washed with three bed volumes of the equilibration buffer F to elute the unbound fraction. The bound fraction, containing rhGM-CSF, was eluted by washing the column with 2.5 bed volumes of buffer G followed by about four bed volumes of buffer H. This last washing also serves to regenerate the column. For subsequent use, the column was washed with at least three bed volumes of deionized water to elute the 2-propanol prior to equilibrating it with buffer F. Fractions were pooled as they eluted from the column on the basis of their A_{280} recorder tracing.

After using the column for 3–5 consecutive runs, it was regenerated by washing with 2–3 bed volumes of 0.5–1 *M* NaOH. The column was allowed to stand in this solution overnight and then washed with deionized water (two bed volumes) followed by buffer F for re-equilibration (see above). This procedure serves to remove strongly bound proteins and lipids and thereby restore the function of the adsorbent.

2.4. Ion-exchange chromatography

The active fractions (B and C) obtained from

the HIC step were pooled (total volume 880 ml) and applied to an XK50 column (bed height = 22.5 cm; bed volume = 450 ml) packed with Q Sepharose Fast Flow which was equilibrated with buffer I. After sample application, the column was washed with the equilibration buffer I until all the unbound fraction was eluted, followed by 2.5–3 bed volumes of buffer J to elute the fraction containing active rhGM-CSF. The column was finally washed with three bed volumes of buffer K to elute the most strongly bound proteins and also to regenerate it.

2.5. Gel filtration

This was performed on a BioPilot glass column (BPG 100/950) packed with Superdex 75 prep grade (bed volume = 4.9 l). The column was equilibrated with buffer J and the flow-rate was maintained at 15 cm/h. The active fraction obtained from the IEC step (total volume = 620 ml) was applied to the column in portions, or after concentrating it over a PM 10 membrane fitted to an Amicon concentrating cell, such that the sample volume corresponded to approximately 5% of the total bed volume of the column. The eluted fractions were pooled directly on the basis of the continuous chart recording of the A_{280} of the effluent.

2.6. Analytical methods

The distribution of proteins in column effluents was determined by continuous on-line measurement of the absorbance at 280 nm and direct recording. Quantitative determinations were made according to a modified method of Lowry using a Sigma protein assay kit. The concentration of protein in solutions of purified rhGM-CSF was determined using a factor $A^{1\%}$ at 280 nm of 10.1 which we have established (see Results). Its amino acid composition was determined according to standard procedures on a purified sample which has been hydrolysed in 6 *M* HCl for 24 and 72 h at 110°C in evacuated and sealed glass tubes. Cysteine was determined as cysteic acid on an oxidized sample that was hydrolysed for 24 h. Tryptophan was determined on a sample which was hydrolysed for 24 h in 3 *M* mercaptoethanesulphonic acid (MESA) to avoid its destruction by air oxidation. The hydrolysates were analysed on a Model 4151 Alpha Plus amino acid analyser (Pharmacia LKB Biotechnology).

The NH_2-terminal sequence of purified rhGM-CSF was determined using the Edman degradation method on an Applied Biosystems Model 477A sequencer. The resulting phenylhydantoin (PTH)-amino acid derivatives were identified using an Applied Biosystems Model 120A PTH analyser.

2.7. Determination of biological activity

This is based on determining the survival of TF-1 cells cultured in the presence of rhGM-CSF relative to those cultured in its absence. TF-1 (tri-factor dependent) is a unique cell line established from the bone marrow cells of a patient with erythroleukaemia that is dependent for its growth and proliferation on each of three haematopoietic factors, i.e. erythropoietin (EPO), granulocyte–macrophage colony-stimulating factor (CM-CSF) and interleukin 3 (IL-3) [33]. The procedure we employed routinely is an adaptation of the method described by Kitamura et al. [34]. The activity of rhGM-CSF was also determined by an indirect enzyme-linked immunosorbent assay (ELISA) using horseradish peroxidase conjugated with sheep anti-mouse (BALB/c) IgG.

2.8. Native or sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

This was performed routinely according to the procedure outlined in Ref. [29] to follow the progress achieved at each stage of the downstream purification process and to examine the electrophoretic homogeneity of purified rhGM-CSF preparations. The dilute protein solutions were concentrated by lyophilization after desalting of each sample on a PD 10 (Sephadex G-25) column equilibrated with 50 m*M* ammonium acetate (pH 6.8). The dried samples were then dissolved in a suitable volume of distilled water

to obtain an A_{280} of ca. 10 (i.e. 10 μg/μl). Low-molecular-mass marker proteins were also run simultaneously. Isoelectric focusing of the purified rhGM-CSF was performed on a PhastGel IEF 3–9 medium according to the standard procedure described in the PhastSystem manual.

Approximately 20 μg of each sample were applied to the gel for electrophoresis. The separated protein bands were stained using either the Coomassie Brilliant Blue (PhastGel Blue R) or silver staining techniques according to the detailed procedure outlined in the PhastSystem manual. The relative molecular mass of the purified rhGM-CSF was calculated by comparing its migration distance with that of standard calibration proteins run simultaneously.

2.9. Analytical gel filtration and ion-exchange chromatography

High-performance gel filtration was performed on a Superdex 75 HR 16 column (48.5 × 1.6 cm I.D.; bed volume = 97 ml) equilibrated and eluted with buffer J. The sample of purified rhGM-CSF (2 ml) was applied to the column and eluted at a linear flow-rate of 15 cm/h. The effluent was monitored by continuous on-line detection at 280 nm. Analytical IEC was performed on a Mono Q HR 5/5 column fitted to a Pharmacia-LKB HPLC system. Various buffers (ranging in pH from 6.5 to 7.8) and salt gradient profiles were used to separate any minor component(s) that might be present in the purified sample of rhGM-CSF. The linear flow-rate was maintained at 150 cm/h in most routine analyses.

2.10. Mass spectrometry

The purified rhGM-CSF was analysed by electrospray (ES-MS; see [35] and [36]) or laser desorption (LS-MS) mass spectrometry to determine accurately its molecular mass. The results obtained, in conjunction with the published sequence of rhGM-CSF and its partial amino terminal sequence as determined by us, also formed the basis for further characterization of the purified molecule.

2.11. Quality control

These were performed in accordance with the detailed guidelines outlined in the WHO Expert Committee's Report on Biological Standardisation [37], whose aim is to ensure the safety of biological products which are to be used as therapeutic agents in human patients. Contaminating residual DNA in chromatographic fractions containing rhGM-CSF activity was determined using the Threshold total DNA detection system [38] after pretreating the samples with proteinase K. The content and concentration of endotoxins in the same samples were determined using a standard Limulus test [39].

In order to perform these analyses, all chromatographic experiments were carried out under asceptic conditions in an isolated room equipped with a continuously lit UV lamp. All buffers and vessels used for collecting fractions were sterilized by autoclaving for 1 h at 121°C. Sterile-filtered air was also circulated out of the room throughout the duration of the experiment. Each packed column was cleaned in place (CIP) with 2–3 bed volumes of 0.5 *M* NaOH which, after about a 15-h contact time, was washed out with 3–5 bed volumes of sterilized water. Each column was then equilibrated with the appropriate starting buffer and the chromatographic procedure described above was followed to purify the rhGM-CSF.

3. Results and discussion

During the initial stages of this investigation, the extraction, washing and renaturation of the inclusion bodies were performed following the procedure outlined in Ref. [29]. The results were unsatisfactory as relatively large amounts of protein impurities were co-extracted with the inclusion bodies and the activity of the renatured rhGM-CSF was low. The entire procedure was thus modified to circumvent this problem. A neutral detergent (0.1% Berol 185) was included in the washing buffer (buffer B) to remove impurities bound to inclusion bodies by hydrophobic forces [40,41]. Unlike other detergents

used for such purposes, e.g., sodium deoxycholate, Triton X-100 [40,41], any Berol 185 bound to the inclusion bodies (IBs) can be removed by washing with aqueous buffers. We believe that this washing procedure had resulted in highly purified inclusion bodies.

As concerns the renaturation of reduced proteins, a variety of recommendations have been published [41] whose basic aim was to obtain a high yield of correctly formed disulphide bonds and thereby of the active protein. Common to all the published procedures is the use of air oxidation or a variety of redox systems to obtain Cys–Cys linkages in the diluted protein solution. Of the various redox systems that are recommended [41], a 10:1 mixture of reduced to oxidized glutathione (introduced by Saxena and Wetlaufer [42]) was found to be an efficient system for generating disulphide bonds from reduced proteins. However, the ratio of reduced to oxidized glutathione is variable depending upon the number of disulphide bonds to be formed [41]. We have used this and other redox systems to optimize the yield of the renatured rhGM-CSF. We also included 2-mercaptoethanol in the solubilizing buffer (buffer D) to stabilize the Cys residues in the denatured protein by forming mixed disulphides [41]. The results we obtained are summarized in Table 1 which indicate that the highest yield of active rhGM-CSF was obtained by using 7 *M* Gu · HCl (containing 100 mmol/l of 2-ME) for solubilizing the IBs followed by a 75-fold stepwise dilution of the solution in the presence of a 10:1 ratio of GSH–GSSG and a 0.1% (w/v) solution of Berol 185. Replacing Gu · HCl with urea markedly decreased the formation of active rhGM-CSF. The inclusion of PEG in the renaturation buffer, as suggested by Cleland et al. [43], had a marginal effect on the overall recovery of active protein.

Each of the chromatographic steps described below was optimized in order to obtain maximum selectivity and capacity of the media for the rhGM-CSF. The reproducibility of each step was checked with satisfactory results. The recovery, specific activity and homogeneity of the purified

Table 1
Effect of varying the conditions for solubilization and renaturation of the rhGM-CSF on the activity of the renatured product

Solubilization conditions			Renaturation conditions							
Denaturant	2-ME (mmol/l)	Berol (%, v/v)	GSH/GSSG (mmol/l)	PEG (μmol/l)	Berol (%, v/v)	Sucrose (%, w/v)	Gu · HCl (mol/l)	Urea (mol/l)	Cys/Cys–Cys (mmol/l)	ELISA activity
Gu · HCl						5	0.2			2.4
Gu · HCl						5	0.2		3/0.3	2.3
Urea			2/0.2					4		5.9
Urea			2/0.2	70				4		7.5
Urea			2/0.2		0.1			4		8.6
Gu · HCl			2/0.2			5	0.1			4.5
Gu · HCl		7.5	2/0.2		0.1	5	0.1			8.4
Gu · HCl	100		1/0.1		0.1		1.0			8.6
Gu · HCl[a]	*100*		*1/0.1*		*0.1*		*0.1*			*9.6*
Urea	100		1/0.1		0.1			1		5.1
Urea	100		1/0.1		0.1			0.2		5.0

The concentration of urea for solubilizing the IBs was 8 *M* (pH 8.5) while that of Gu · HCl was 7.0 *M* (pH 7.5). About 1 g (wet mass) of inclusion bodies was used for each of the conditions listed. For details of the solubilization and renaturation procedure and the method for determining activity by indirect ELISA, see text. The unit for ELISA is mg of rhGM-CSF/g of inclusion bodies. GSH = reduced glutathione; GSSG = oxidized glutathione; PEG = polyethylene glycol (average molecular mass 3350); 2-ME = 2-mercaptoethanol.

[a] Values in italics are optimal renaturation conditions that result in the highest yield of renaturated protein.

rhGM-CSF was also found to be consistently high and reproducible in all the pilot experiments we performed.

3.1. Step 1: hydrophobic interaction chromatography

A typical elution profile obtained after chromatography of ca. 4.3 l of the renatured rhGM-CSF on a 400-ml column of Phenyl Sepharose 6 FF (high sub) is shown in Fig. 1. Fraction A was inactive and apparently contained very little protein as virtually no bands could be detected after PAGE of the concentrated sample followed by PhastGel Blue R staining (see Fig. 5). Pooled fractions B and C together account for approximately 40% of the A_{280} (60% of the proteins) and 92% of the rhGM-CSF activity applied to the column. This step is thus very effective in removing most of the impurities and also the buffer constituents (Gu · HCl, Berol 185, glutathione, etc.) found in the renatured sample. The overall degree of purification obtained is apparently low (twofold; see Table 2) and might be due to the fact that the highly purified inclusion bodies contain very little extraneous protein contaminants.

In general, many proteins lose part or most of their activity on exposure to aqueous organic solvents. However, the rhGM-CSF was found to be stable when eluted with 30% 2-propanol from the Phenyl Sepharose 6 FF column. The possible denaturing effect of this solvent might be time dependent and it is advisable to avoid storage of the eluted fraction C for longer periods than necessary.

The capacity of Phenyl Sepharose 6 FF (high sub) for rhGM-CSF is low (about 1 mg/ml gel) and might be due to the presence of chaotropic ions (Gu · HCl), detergents and other competing solutes present in the renatured rhGM-CSF. However, this is compensated for by the fact that the partially purified rhGM-CSF so obtained is virtually free from buffer salts and DNA which can interfere with the subsequent IEC step. Another important advantage is that the active fractions 1B and 1C are eluted in a volume corresponding to approximately one fifth of the

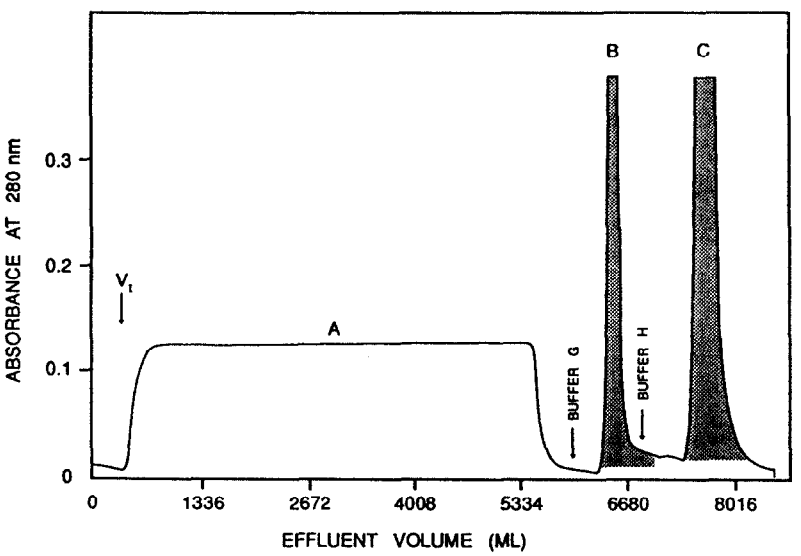

Fig. 1. Hydrophobic interaction chromatography (HIC) of 4340 ml of renatured crude extract (A_{280} = 0.21; total protein ≈ 580 mg) on an XK50/30 column packed with Phenyl Sepharose FF (bed height = 20 cm; bed volume = 400 ml). The renatured sample was centrifuged at 17 000 *g* to remove the suspension of finely divided precipitate prior to its application on the column that was equilibrated with buffer F. The flow-rate was 100 cm/h and fractions were pooled as they were eluted from the column. Fraction A was virtually inactive while pooled fractions B and C (shaded) contained approximately 96% of the activity (ELISA) and 40% of the A_{280} (ca. 60% of the protein) applied to the column.

Table 2
Recovery of rhGM–CSF activity after each chromatographic step used for its purification from the inclusion bodies of transformed *E. coli* cells

Step	Sample	Total protein (mg)	Total activity [biological] ($\times 10^7$ units)	Specific activity ($\times 10^7$ units/mg)	Recovery (%) (biological activity)	Total activity (ELISA) (mg)	Recovery (%) (ELISA)	Purification factor (-fold)
–	Renatured rhGM-CSF	578.6	416.6	0.72	100	181.6	100	1
I (HIC)	Pool 1 (B + C)	315	439.1	1.39	105 (68–125)	167.4	92.1 (85–97)	1.9
II (IEC)	Pool 2B	116	204.1	1.76	49 (33–72)	75.8	41.7 (35–47)	2.5
III (GF)	Pool 3B	88.4	289.8	3.28	70 (39–86)	64.1	35.3 (31–36)	4.6

The tabulated values are averages of three independent experiments covering the entire procedure adopted for its purification. The recovery in biological or ELISA activity is calculated relative to the corresponding total activity in the renatured sample. The purification factor is calculated on the basis of the specific activity obtained from the biological assay. Figures in parentheses indicate the range of values obtained. For identification of the samples referred to here, see Figs. 1–3. GF = Gel filtration.

volume of the renatured sample applied to the column. These considerations are especially important in reducing the processing time for large-scale applications. Finally, attempts have been made to elute the bound fraction (1B + 1C) by washing the column directly with buffer H but the recovery was found to be low. This might be due to the residual ammonium sulphate present in the column which must be washed out with buffer G prior to elution with buffer H.

3.2. Step 2: ion-exchange chromatography

The elution profile obtained after chromatography of 880 ml of pooled fractions 1B + 1C on a 450-ml column of Q Sepharose FF is shown in Fig. 2. The volume of media used is the minimum required to bind all of the rhGM-CSF activity in the pooled fractions. Neither the pH nor the salt concentration of the pooled sample was adjusted because the applied activity was bound satisfactorily to the column under the equilibration conditions used here.

Various salt gradient elution strategies have been tried but the activity was spread over several separated fractions and the total recovery in activity was not satisfactory. This also led to a considerable dilution of the eluted fractions. Changing the pH of the equilibration buffer from 6.5 to 7.5 or 8.0 resulted in a progressive strong binding of the active protein but did not result in

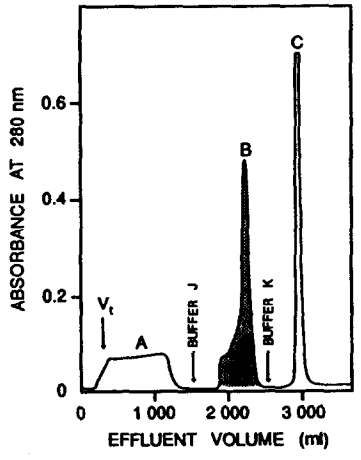

Fig. 2. Ion-exchange chromatography of 880 ml (ca. 300 mg of protein) of pooled fractions B + C (see Fig. 1) on an XK 50/30 column packed with Q Sepharose FF (bed height = 22.5 cm; bed volume = 450 ml). The column was equilibrated with buffer I and a flow-rate of 100 cm/h was used throughout. The bound fractions 2B and 2C were eluted by a stepwise change of the eluent buffers J and K and were pooled directly as they were eluted. Most of the applied activity (ca. 70%) was found in the shaded fraction 2B.

any improvement of the resolution obtained. It is therefore apparent that the procedure adopted here is simple, results in a high recovery of activity and leads to a concentration of the active protein in fraction 2B by a factor of about 1.4 relative to the volume applied to the column. All these considerations make this procedure well suited for large-scale operations.

Of the total ELISA activity applied to the column, less than 5% was found in the unbound fraction A, about 45–50% in fraction B and about 25–30% in fraction C. Electrophoretic analysis of these fractions (see Fig. 5) showed that the band corresponding to M_r 14 000 (i.e. the approximate molecular mass of rhGM-CSF) was predominantly found in fraction 2B. It is virtually absent in fraction 2A and is only a minor component of the many bands (most of which are of high molecular mass) detected in fraction 2C. The majority of the electrophoretic bands in fraction 2C might thus represent insufficiently renatured rhGM-CSF or its aggregation products.

3.3. Step 3: gel filtration

This step effectively removes the last traces of impurities (both high- and low-molecular mass proteins) present in the highly purified fraction 2B (see Fig. 3). The active fraction from the IEC step (pool 2B) can be applied as such or after concentration over an Amicon PM 10 membrane concentration cell such that the volume of sample applied to the Superdex 75 column corresponds to ca. 4–6% of the total bed volume. The active fraction 3B is diluted about 1.5-fold after elution from the column. Fractions 3A and 3C are inactive and together contain about 15% of the A_{280} applied to the column. The active fraction contains about 80% of the A_{280} and 90% of the rhGM-CSF activity applied to the column.

Based on the specific activity of the highly purified fraction 3B, the adopted downstream purification procedure leads to a 4.6-fold purification of the renatured rhGM-CSF (see Table 3). Such a low figure seems to be characteristic for recombinant proteins produced as inclusion bodies (cf., [29]) and might reflect the much lower level of extraneous protein contaminants present in highly purified inclusion bodies.

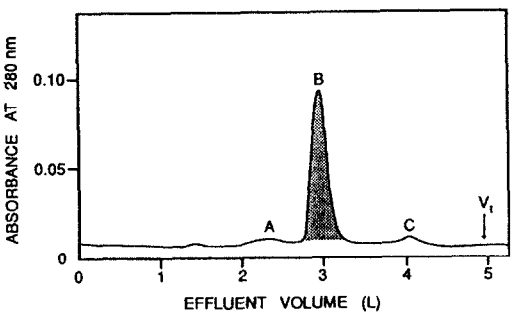

Fig. 3. Gel filtration of pooled fraction 2B (see Fig. 2) on Superdex 75 Prep Grade packed in a BioPilot glass column (BPG 100/950; bed volume = 4.9 l). The column was equilibrated and eluted with buffer J at a flow-rate of 15 cm h. The volume of sample applied was approximately 5% of the total bed volume of the column and fractions were pooled directly as they eluted from the column. Approximately 80% of the applied A_{280} and 90% of the rhGM-CSF activity was found in pooled fraction 3B.

The entire purification scheme is presented in Fig. 4.

3.4. Criteria of purity

SDS-PAGE of the purified rhGM-CSF (fraction 3B) gave a single but diffuse band with an apparent molecular mass of 14 000 (see Fig. 5, lane 7 of each gel). Silver staining of an overloaded gel (not shown here) revealed the presence of a faint second band with a molecular mass of ca. 30 000. With all probability, this band represents a dimer of the single chain molecule.

The electrophoresis gels shown in Fig. 5 were overloaded with the purified sample (see lane 7 of each gel) in order to detect any minor impurities that might be present in the purified protein. This resulted in diffuse bands, especially with the SDS-treated samples, most likely due to the higher dye uptake of SDS-denatured proteins whose structure is in an extended, random coil state.

Isoelectric focusing of fraction 3B on PhastGel IEF 3–9 gave a single band with an isoelectric point near pH 5.2. Even when the gel was overloaded to the extent that the focused protein

Table 3
Stability of purified rhGM-CSF at pH 4.2 or 7.0 during storage for 45 days at 4 or at −20°C

Storage time (days)	pH	Temperature (°C)	Activity (ELISA) (μg/ml)	Biological activity ($\times 10^7$ units/ml)
1	4.2	4	157	
	7.0	4	180	
15	4.2	4	152	
	4.2	−20	144	2.6
	7.0	4	170	
	7.0	−20	185	3.7
25	4.2	4	122	
	4.2	−20	137	2.4
	7.0	4	158	
	7.0	−20	183	1.0
35	4.2	4	81	
	4.2	−20	135	
	7.0	4	156	
	7.0	−20	167	
45	4.2	4	84	
	4.2	−20	121	1.3
	7.0	4	155	
	7.0	−20	178	1.4

About 12 ml of a purified sample of rhGM-CSF in 20 m*M* sodium phosphate buffer (pH 7.0) (protein concentration = 0.34 mg/ml) was used in this study. From this sample, 0.5-ml aliquots were transferred to ten plastic tubes of which five were stored at 4°C and the other five were stored at −20°C. To the remaining solution (7 ml) was added 0.7 ml of 1 *M* acetic acid dropwise until the pH decreased to 4.2. From this solution, 0.5-ml aliquots were transferred to ten plastic tubes of which five were stored at 4°C and the remaining five were stored at −20°C. One tube from each of the four categories was then taken at intervals of several days (see tabulated values) and the activity in each was determined by ELISA or by using cultured cells as described in detail in the text. The tabulated values are averages of triplicate analyses performed on the same sample.

could be seen as a distinct opalescent band without staining, no further bands were seen after staining the gel. The results indicate that the purified protein is homogeneous with respect to surface charge and any variants or deamidation products could not be detected by this method. This leads us to conclude that deamidation of the four asparagine or eight glutamine residues in this protein [18,24,26] has not occurred during the extraction and purification process.

High-performance gel filtration of fraction 3B on an analytical column of Superdex 75 HR 16 showed a single symmetrical peak eluting at a position corresponding to an apparent molecular mass of ca. 28 000. This result seems to suggest that the rhGM-CSF exists as a dimer in its native state in a neutral pH milieu. However, this is contrary to the report by Wingfield et al. [19], who found that the rhGM-CSF is a monomeric protein with a molecular mass of 14.7 000, as determined by sedimentation equilibrium analysis. The reason for this anomaly is difficult to explain. It is worth mentioning, however, that we obtained a similar result for rhIFN-γ [29] when run under the same experimental conditions as used here.

High-performance IEC of fraction 3B on a Mono Q HR 5/5 column run at different pH values (6.5–7.8) and salt gradient elution profiles gave a single symmetrical peak. Some of our earlier preparations showed a minor peak (ca. 2–5%) eluting slightly earlier than the major peak, but this was not seen on later preparations carried out according to the standardised protocol described in this paper.

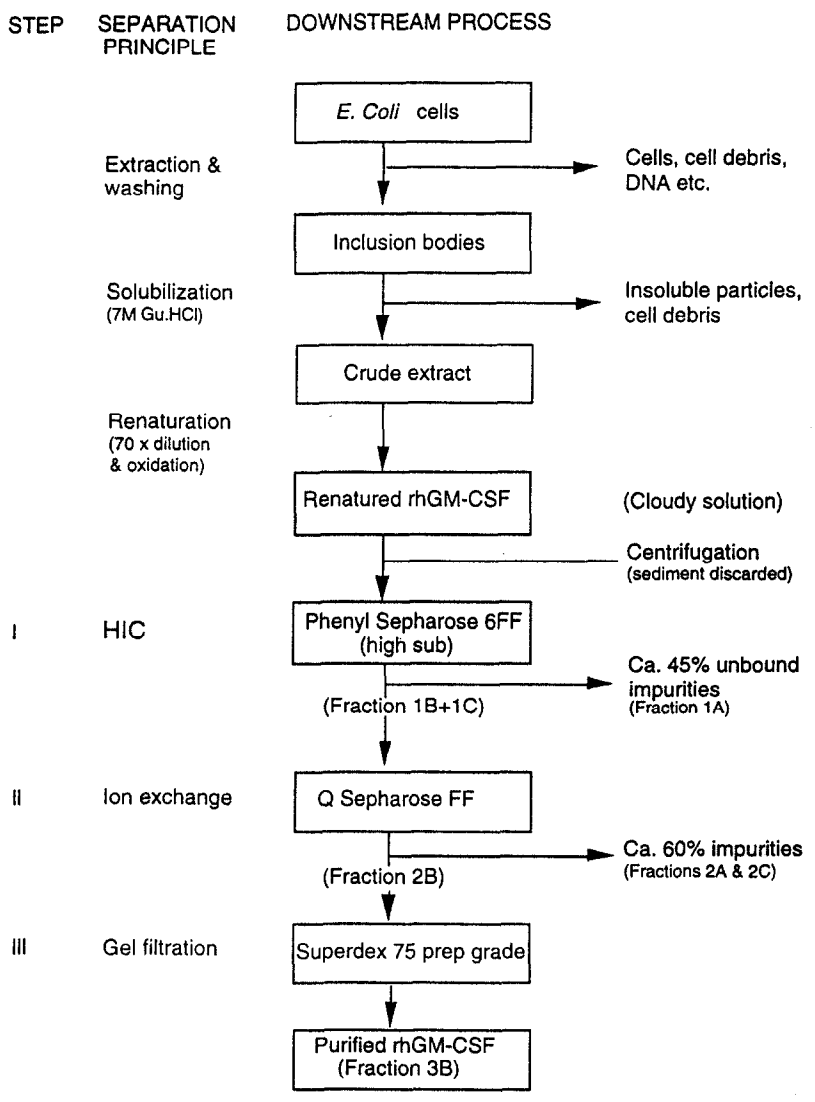

Fig. 4. Pilot-scale purification scheme for rhGM-CSF.

High-performance RPLC of fraction 3B on a column of Pep-S, C_2–C_{18} (5-μm silica particles) showed one major peak (representing ca. 96% of the eluted material) and two very minor peaks eluting just in front of it. This indicates that the otherwise homogeneous preparation of rhGM-CSF can be further purified by RPLC, if required, to remove the last traces of impurities it might contain.

3.5. Biological potency and stability

The specific biological activity of the purified rhGM-CSF is about $3.3 \cdot 10^7$ units/mg (Table 2), which is comparable to the values reported for this protein derived from mammalian cell cultures [24] or *E. coli* cells [25]. The results in Table 2 also show that the specific biological activity of fraction 3B (Fig. 3) is almost double

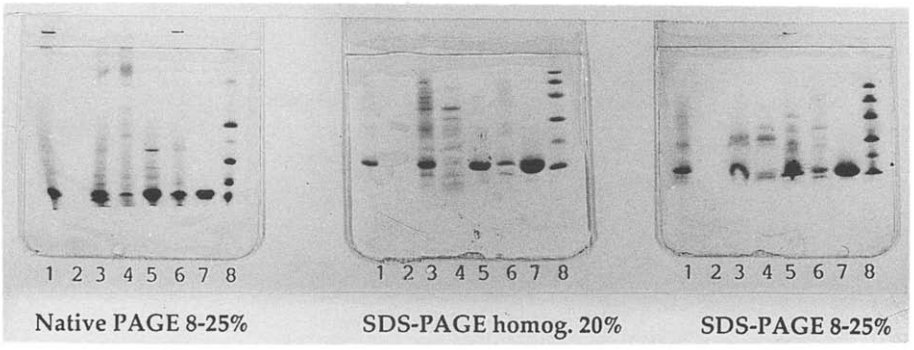

Fig. 5. PhastGel PAGE patterns of the various chromatographic fractions obtained during the three-step purification process for rhGM-CSF. About 20 μg of each sample (native or reduced) were applied to the gels and analysed by three PAGE techniques as indicated under each photograph. The gels were stained with PhastGel Blue R according to a standardized procedure. Lanes: 1 = renatured rhGM-CSF; 2, 3 = unbound (inactive) and buffers G- and H-desorbed and combined fractions, respectively, from a Phenyl Sepharose 6 FF (high sub) column; 4–6 = unbound (inactive); buffer J- and K-desorbed fractions, respectively, from Q Sepharose FF column; 7 = the main fraction 3B (see Fig. 3) that was eluted from a Superdex 75 prep grade column (purified rhGM-CSF); 8 = molecular mass marker proteins (top to bottom: 94 000, 67 000, 43 000, 30 000, 20 000, 14 000). The variations in the densities of the stained bands of the native and SDS-treated samples might be due to the higher dye uptake of the latter.

that of fraction 2B (Fig. 2), indicating that the gel filtration step is very effective in removing some crucial contaminants present in fraction 2B.

Purified rhGM-CSF showed no significant loss in its ELISA activity when stored for 4 weeks at −20°C and pH 7.0, but lost about 60% of its biological activity (Table 3). Storing it for 4 weeks at −20°C and pH 4.2 decreased its ELISA activity by ca. 15% and its biological activity by ca. 50%. The results thus indicate that, irrespective of the pH at which it is stored, the biological activity of purified rhGM-CSF progressively decreases with time of storage. Moreover, although there is an initial drop of its biological activity by 30% on lowering the pH from 7.0 to 4.2, the long-term effects on its biological activity are negligible if stored at −20°C. However, there is a significant loss in ELISA activity (45%) when it was stored for 4 weeks at 4°C and pH 4.2 compared with a loss of only 10% at pH 7.0 during the same period.

3.6. Molecular size and structure

The amino acid composition of the purified rhGM-CSF is shown in Table 4. The total number of residues found in the purified protein is 127, which is less by one residue of glutamic acid compared with the composition calculated from the cDNA sequence coding for this protein [26]. This discrepancy might be due to the limits of accuracy of this analytical method when a single amino acid residue (glutamic acid + glutamine) represents 16% of the total amino acids in this protein. With this exception, the number of residues for the other amino acids is identical with those calculated from its cDNA sequence data [26]. The data also show that, of the charged amino acids, 60% are acidic (Asp + Glu) and 40% are basic (Arg + Lys), which accounts for the low isoelectric point which we have determined for the purified protein (approximately pH 5.2). Based on the quantitative amino acid composition data and the absorption spectrum of purified rhGM-CSF, we have established that its $A^{1\%}$ at 280 nm (10 mm light path) is 10.1, which is in good agreement with the reported value of 9.58 [19].

The NH_2-terminal sequence of the first seventeen residues of the purified protein is shown in Table 5. The results are based on two independent analyses of two different preparations of purified rhGM-CSF and were found to be identi-

Table 4
Amino acid composition of purified rhGM-CSF

Amino acid	Notation	Relative No. of residues	Residues per mole
Lysine	K	6.1	6
Histidine	H	2.9	3
Arginine	R	6.2	6
Half-cystine	C	4.1	4
Aspartic acid	D	8.2	8
Threonine	T	11.0	11
Serine	S	9.4	9
Glutamic acid	E	20.0	20 (21)
Proline	P	10.9	11
Glycine	G	3.3	3
Alanine	A	7.8	8
Valine	V	5.1	5
Methionine	M	4.9	5
Isoleucine	I	4.9	5
Leucine	L	14.0	14
Tyrosine	Y	2.1	2
Phenylalanine	F	5.2	5
Tryptophan	W	2.0	2
Total		128.1	127

The numbers of amino acid residues per molecule are averages of the values calculated from the analyses performed on 24- and 72-h hydrolysed samples. They represent the nearest integers obtained from the normalized values for each amino acid so obtained. Tryptophan was determined on a sample hydrolysed for 24 h in the presence of 3 *M* mercaptoethanesulphonic acid. The value obtained for Glu (20) is less by one residue compared with that expected from its sequence, probably owing to limits of accuracy of this type of analysis. Glu and Gln account for 16% of the total number of residues in this protein.

cal. In both instances, three NH_2-terminal residues (i.e., Met, Ala and Pro) were found, indicating that the purified protein is apparently heterogeneous from its amino-terminal end. This heterogeneity is not due to extraneous protein impurities or other artefacts in the purified rhGM-CSF since the sequence for all three variants is identical from the third amino acid up to fifteen stages of the Edman degradation cycle. The sequence we obtained corresponds exactly to that reported for this protein [18,24,26], suggesting that the purified rhGM-CSF is identical with its natural counterpart. Based on the area of the peaks for each amino acid after stage

Table 5
Amino-terminal sequence of residues 1–17 of purified rhGM-CSF

	1 5 10 15
I	H_2N · Met–Ala–Pro–Ala–Arg–Ser–Pro–Ser–Pro–Ser–Thr–Gln–Pro–Trp–Glu · · · · · · · · · COOH (128)
	1 5 10 15
II	H_2N · Ala–Pro–Ala–Arg–Ser–Pro–Ser–Pro–Ser–Thr–Gln–Pro–Trp–Glu–His · · · · · COOH (127)
	1 5 10 15
III	H_2N · Pro–Ala–Arg–Ser–Pro–Ser–Pro–Ser–Thr–Gln–Pro–Trp–Glu–His–Val · · · COOH (126)

The sequence was generated from data obtained after analysis of a purified sample of rhGM-CSF on an Applied Biosystems Model 477A automatic micro-sequencing apparatus according to standardized procedures. The relative occurrence of the three variants of rhGM-CSF is 58% for variant I (i.e., the full length sequence of rhGM-CSF), 29% for II and 12% for III.

1 of the Edman degradation cycle, the relative occurrence of each amino acid was calculated and the values obtained were Met = 58%, Ala = 29% and Pro = 12%. This indicates that the majority (58%) of the molecules are in their intact form with Met as the NH_2-terminal residue.

The occurrence of the above three variants of rhGM-CSF is further supported by data obtained from LS-MS and ES-MS analyses which showed that the purified protein is composed of three distinct components with molecular masses of 14 605, 14 474 and 14 402. The difference in their molecular masses can be accounted for by the lack of Met (residue $M_r = 131$) and Met + Ala (combined residue $M_r = 202$) from the main component whose molecular mass is 14 605. This value is in excellent agreement with that determined by SDS-PAGE (14 000, see Fig. 5) or that determined from the gene sequence (14 650, see ref. [24]) or from ultracentrifugal analysis (14 700, see ref. [19]). The spectrum also shows that each of these variants contains at least 1 mol of Na per mole of protein and that the main component accounts for 55% of the total area of the three peaks seen in the spectrum.

We can only speculate at this stage about the occurrence of the two modified variants of the parent Met (1) analogue of rhGM-CSF. Despite this apparent molecular heterogeneity, the mixture of the three variants showed a specific biological activity (Table 2) that is comparable to that reported for the recombinant protein produced in mammalian or bacterial cells [24,25]. This might indicate that the occurrence of this protein as three variants may not have any biological significance.

3.7. Quality control

Recombinant proteins intended for use as biopharmaceuticals must not only fulfil the rigorous requirements of purity, identity and biological efficacy vis-à-vis their natural counterparts, but must also fulfil the safety requirements related to their content of DNA, endotoxins and chromatographic artefacts [37]. The first set of these requirements have been satisfactorily fulfilled by the results we have presented above. The results in Table 6 relate to the reduction in DNA and endotoxin levels obtained at each stage of the downstream purification process. According to a WHO Study Group on Biologicals, the probability of risk associated with heterologous contaminating DNA is negligible when the amount of such DNA is 100 pg or less

Table 6
Levels of DNA and endotoxins in renatured rhGM-CSF and the active fractions obtained after each chromatographic purification step

Step	Sample	Total volume (ml)	Total protein (mg)	Endotoxin (EU/ml)[a]	Total endotoxin (EU)[a]	DNA (ng/ml)	Total DNA (ng)
–	Renatured rhGM-CSF	4340	490	421	1 827 140	180	781 200
I (HIC)	Pool 1 (B + C)	880	220	243	213 840	0.46	405
II (IEC)	Pool 2B	620	88	251	155 620	0.14	87
III (GF)	Pool 3B	1045	62	9	9405	0.08	84

The results serve as guidelines in assessing the efficiency of removal of these impurities by the gel media used for chromatography. For identification of the samples referred to here, see Figs. 1–3.
[a] EU = Endotoxin units.

in a single dose administered parenterally [37]. The DNA content in the purified rhGM-CSF is 80 pg/ml, which corresponds to 1.4 pg of DNA per μg of protein. For comparison, the level of DNA in the sterilized buffer used for the final gel filtration step is approximately 10 pg/ml. The results thus indicate that a single dose of ca. 70 μg of the purified protein contains a level of DNA that is within the acceptable upper limit recommended by the WHO study group.

The results also show that Phenyl Sepharose 6 FF (high sub) decreases the DNA content effectively (by a factor of ca. 2000), probably because most of the DNA does not bind to it. The anion-exchange step further reduces the level about fivefold. The reduction obtained here is less than expected but might be due to the fact that some of the DNA bound by Q Sepharose FF co-elutes with the rhGM-CSF at pH 6.5. Taken as a whole, the combination of gel media used here for removing protein impurities from the rhGM-CSF is also effective in removing minor amounts of DNA present in the crude extract.

The total decrease in the level of endotoxins in the purified product is about 190-fold relative to the renatured rhGM-CSF. After chromatography on Phenyl Sepharose 6FF, an eightfold decrease was obtained, indicating that most of the endotoxins are not bound under the experimental conditions employed. An even higher reduction (seventeenfold) was obtained after chromatography on Superdex 75, probably owing to large differences in the molecular size of the rhGM-CSF and the contaminating endotoxins. On the other hand, the reduction obtained after the anion-exchange step on Q Sepharose FF (ca. twofold) is much less than expected considering that endotoxins are negatively charged at pH 6.5 and should have been bound strongly. This result seems to suggest that some of the endotoxins and the rhGM-CSF have similar surface charge characteristics and therefore co-elute from the column at pH 6.5. If such is the case, it may be possible to bind the endotoxins more strongly than the rhGM-CSF at a higher pH (e.g., pH 8.0), thereby facilitating their efficient removal.

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