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Production, purification and characterization of recombinant human interferon γ

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ABSTRACT

An essentially three-step chromatographic purification procedure, *i.e.*, ion-exchange, immobilized metal ion affinity and size-exclusion chromatography, is described for the purification to homogeneity of recombinant human interferon-γ (rhIFN-γ) from the inclusion bodies produced in genetically transformed *Escherichia coli* cells. Batchwise adsorption of the cloudy solution of renatured rhIFN-γ obviated the need for high-speed centrifugation to clarify the suspension. This step effectively removed about 70% of extraneous protein impurities. The established purification process is reproducible and leads to a total recovery of 32%. Pilot-scale processing of *E. coli* cells grown in a 30-l fermentor gave about 70 mg of a homogeneous preparation of rhIFN-γ. The specific biological activity of purified rhIFN-γ is *ca.* $3.4 \cdot 10^7$ I.U./mg protein, which is comparable to that of its natural counterpart. It is basic protein (p*I* > pH 9) with a monomer relative molecular mass of 15 000. It behaves, however, as a dimer on size-exclusion chromatography. Its partial NH_2-terminal sequence is identical with that established for the rhIFN-γ. However, its amino acid composition and its relative molecular mass (15 067 as determined by electrospray mass spectrometry) indicate that the purified protein is a truncated form lacking fifteen amino acid residues from its carboxyl-terminal side. This modification does not seem to have any adverse effect on its biological potency. The levels of DNA, bacterial endotoxins and Ni(II) ions in the final product were determined.

INTRODUCTION

The human interferons (hIFNs) are a family of proteins the initial discovery of which was based on their ability to inhibit viral growth in target cells [1]. Their biological effects *in vivo* include antiviral activity, cell growth inhibition and immunomodulatory activity [2]. Based on major antigenic differences, they are grouped into three main classes, *viz.*, hIFN-α (leucocyte), hIFN-β (fibroblast) and hIFN-γ (immune) [3–5]. Few, if any, of the natural hIFN-α sub-classes are glycosylated whereas hIFN-β and hIFN-γ are produced in their glycosylated form. hIFN-α and hIFN-β are otherwise similar in many respects, *e.g.*, both maintain their activity after exposure to sodium dodecylsulphate (SDS) or pH 2 and have relative molecular masses (M_r) in the range 20 000–26 000 [6,7]; each is composed of a single polypeptide chain of about 166 amino acid residues cross-linked by one or more disulphide bridges; they also share about 30% amino acid sequence homology [8].

hIFN-γ is different from hIFN-α or -β in several

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respects. It is inactivated on exposure to pH 2 or SDS [9]. Its cell surface receptors are different from those of hIFN-α or hIFN-β [10]. Its virus and cell specificities and the antiviral mechanism it induces are also distinct [11]. It is more potent than the other two hIFNs in its immunomodulatory activities and the antiproliferative effects it elicits in transformed cells [11,12], making it a potentially useful drug against cancer. Some clinical trials indicate that recombinant hIFN-γ (rhIFN-γ) has therapeutic efficacy on kidney cell carcinoma, leukaemia, melanoma, pulmonary cell carcinoma, colon cancer and rheumatoid arthritis [13–18].

Natural hIFN-γ is composed of 143 amino acid residues with a calculated formula mass of 16 775 [19]. It is glycosylated and does not contain any Cys residues [19]. It is a basic protein with an isoelectric point above pH 9. Its reported relative molecular mass is variable ranging from 15 500 to as much as 70 000 [9,12,19–22], which is apparently due to its tendency to exist in several aggregated forms [9,22–23]. Its cDNA has been successfully cloned and expressed at high levels in *Escherichia coli* [24,25], which made possible the production of rhIFN-γ in relatively large amounts. Several methods have subsequently been published describing its purification to apparent homogeneity [23,26–28]. Its availability in large amounts has made possible some detailed structure–function analysis on the purified protein [29] and its use in diverse clinical trials.

The rhIFN-γ (produced in *E. coli*) is not glycosylated and has Met as its NH_2-terminal residue instead of pyroglytamic acid. Its amino acid sequence is otherwise identical with that of its natural counterpart without any internal insertions or deletions [19]. The observed differences in its primary structure do not seem, however, to have any adverse effect on its biological activities. It is also worth mentioning that the soluble form of hIFN-γ receptor, expressed in *E. coli*, has recently been purified and characterized [30], which is expected to simplify the rational search for agonists and antagonists of hIFN-γ activities and thereby the development of newer and more effective drugs.

This paper describes an optimized downstream purification procedure for rhIFN-γ expressed intracellularly in *E. coli* as inclusion bodies. The adopted procedure is reproducible and well suited for process-scale operations. Sufficient data are presented to show the purity of the final product and its identity with its natural counterpart. Structural analyses of the purified rhIFN-γ indicate that it is in its truncated form, lacking fifteen amino acid residues from its carboxyl-terminal end relative to its natural counterpart.

EXPERIMENTAL

Unless stated otherwise, all experiments were performed at room temperature (20°C). Chromatographic columns, gel media, electrophoretic apparatus and the BioPilot chromatographic system were products of Pharmacia LKB Biotechnology (Uppsala, Sweden). Guanidine hydrochloride (Gu · HCl) (95%) was obtained from Aldrich Chemie. During the developmental phase of the downstream purification procedure, XK16, XK26 and K50 columns were used. For large-scale applications, BioProcess glass columns (BPG 100/500 and BPG 100/950) were used. Relevant details will be outlined in appropriate sections.

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

(A) 20 m*M* sodium phosphate buffer–0.125 *M* NaCl–5 m*M* EDTA (disodium salt) (pH 7.2).

(B) 20 m*M* sodium phosphate buffer–0.125 *M* NaCl–0.5 *M* urea–1 m*M* EDTA (disodium salt) (pH 7.0).

(C) 7 *M* Gu · HCl dissolved in 50 m*M* boric acid and the pH adjusted to 7.2 with 0.1 *M* NaOH.

(D) 20 m*M* sodium phosphate buffer–0.125 *M* NaCl–1 m*M* EDTA (disodium salt)–5% (w/v) sucrose (pH 7.2).

(E) 20 m*M* sodium phosphate buffer–0.125 *M* NaCl–1 m*M* EDTA (disodium salt)–5% (w/v) sucrose–2 *M* Gu · HCl (pH 7.2).

(F) 20 m*M* sodium phosphate buffer (pH 7.0) (conductivity = 3 mS/cm at 21°C).

(G) 20 m*M* sodium phosphate buffer–0.3 *M* NaCl (pH 7.0) (conductivity = 25 mS/cm at 21°C).

(H) 20 m*M* sodium phosphate buffer–0.6 *M* NaCl (pH 7.0) (conductivity = 45 mS/cm at 21°C).

(I) 20 m*M* sodium phosphate buffer–0.3 *M* NaCl (pH 7.5).

(J) 20 m*M* sodium phosphate buffer–9 m*M* imidazole–0.5 *M* NaCl (pH 7.5).

(K) 50 m*M* Na_2HPO_4–0.5 *M* NaCl, pH adjusted to 4.0 with dilute phosphoric acid solution.

Production of rhIFN-γ

E. coli strain DH5α was transformed by plasmid pBV220/IFN-γ, which contains hIFN-γ cDNA inserted downstream of P_RP_L promoter and CIts857 regulator gene. The expression of hIFN-γ by the cultured *E. coli* cells was induced by raising the temperature of the cell culture from 30 to 42°C within about 15 min [31,32]. About 600 ml of an overnight cell culture in LB medium was seeded in a 30-l fermenter containing 25 l of modified M 9 medium and the fermentation was performed essentially as described by Song and Tong [33]. After about 14 h of continuous culturing, the temperature was raised to 42°C and the fermentation allowed to continue for a further 4 h. The cells were then harvested by centrifugation at 3000 *g* at 4°C for 10 min using a Heraeus Cryofuge 1000 fitted with a model 6606 rotor.

Preparation of crude rhIFN-γ extract

One part by weight of *E. coli* cells were suspended in ten volumes of buffer A at 4°C and dispersed using an Ultra Turrax (IKA-Werk, Germany). The homogenized suspension was passed through an APV Gaulin press for a total of three passages, maintaining the pressure at 500 bar during the milling process. The press was cooled by a circulating water bath maintained at 4°C throughout the entire operation. The suspension of disintegrated cells was centrifuged at 1000 *g* for 30 min at 4°C. The pellet was resuspended in buffer A, homogenized using the Ultra Turrax and centrifuged as above. The resulting pellet was resuspended in buffer B followed by homogenization and centrifugation.

The washed pellet, containing highly purified inclusion bodies, was suspended in 3 ml of buffer C per gram of wet pellet and solubilized by homogenization for about 5 min using the Ultra Turrax. This was followed by centrifugation at 17 000 *g* and 4°C to remove insoluble material and residual cell debris. The supernatant, containing solubilized but denatured proteins of the inclusion bodies, was stored at 4°C until further use. It will be referred to as the "crude extract" throughout.

The crude extract was renatured by diluting it about 70-fold with buffer D followed by buffer E to obtain a final protein concentration of 0.1–0.2 mg/ml in 0.2 *M* Gu · HCl. This was stored overnight at 4°C for optimum renaturation of the proteins. The product obtained is a cloudy suspension, probably owing to the presence of partially renatured and/or aggregated proteins.

Ion-exchange chromatography (IEC)

The renatured rhIFN-γ suspension (1.4 l, corresponding to 20 ml of crude extract) was clarified by centrifugation at 17 000 *g* for 30 min at 4°C. The supernatant was applied to a K50 column (bed height = 5 cm; bed volume = 98 ml) of S-Sepharose FF equilibrated with buffer F followed by washing with 2 bed volumes of the equilibration buffer to elute unbound proteins. The column was then washed with 4.5–5 bed volumes of buffer G to elute weakly bound impurities and finally with 2.5–3 bed volumes of buffer H to elute the strongly bound rhIFN-γ fraction. The column was regenerated by washing it with 2–3 bed volumes of 1 *M* NaCl dissolved in buffer F and equilibrated for the next run by washing it with about 3 bed volumes of buffer F. This procedure was used during the developmental stages of the downstream purification process.

For large-scale operations, batch adsorption was adopted. To 1.4 l of the cloudy suspension of renatured rhIFN-γ were added 80 g of suction-dried S-Sepharose FF (corresponding to 100 ml of packed gel) that had previously been equilibrated with buffer F. The mixture was stirred intermittently for 1 h at 20°C and then filtered through a No. G2 glass filter, which allows the passage of the fine precipitates through its pores. The suctioned gel was then washed with 200-ml portions of buffer F until the filtrate was no longer cloudy (a total of about 600 ml of the buffer was required). The gel was then packed in a column and the bound proteins were eluted according to the procedure outlined above.

Immobilized metal ion affinity chromatography (IMAC) on Ni(II)–chelating Sepharose Fast Flow

The chelating Sepharose Fast Flow gel (CS FF) was packed in an XK 16 column (bed height = 15 cm; bed volume = 30 ml) and washed with about 2 bed volumes of distilled water. It was then charged with Ni(II) ions (8 ml of 0.2 *M* $NiSO_4$ solution, pH 4.7) followed by washing with about 2 bed volumes of deionized water to elute the excess of metal ions.

The column was then washed with 2 bed volumes of buffer K, to elute loosely bound metal ions, followed by 6 bed volumes of buffer I for equilibration.

The partially purified rhIFN-γ fraction obtained from the ion-exchange step was applied to the column followed by washing with about 3 bed volumes of buffer I to elute unbound impurities. The column was then washed with about 7 bed volumes of buffer J to elute the adsorbed rhIFN-γ fraction and finally with about 3 bed volumes of buffer K to elute the strongly bound impurities and also to regenerate the column. The column was then re-equilibrated with buffer I and can be used again for at least ten consecutive runs without any detectable deterioration in its capacity or selectivity.

Size-exclusion chromatography (SEC)

The active fraction obtained from the Ni(II)–CS FF column was applied to a prepacked Superdex 75 column (60 × 6 cm I.D.; bed volume = 1696 ml) equilibrated with buffer I. The volume of sample applied corresponded to *ca.* 6% of the total bed volume. The flow-rate was maintained at 15–20 cm/h. For laboratory-scale operations, fractions corresponding to 1% of the total bed volume were collected. For large-scale operations, fractions were pooled directly on the basis of the continuous chart recording of the effluent.

From the various pooled fractions obtained after each chromatographic step, aliquots of 1 ml were saved for electrophoretic analysis and for determining their biological activity and protein content. Samples used for biological assay contained 1% (w/v) human serum albumin (HSA) to prevent the loss of interferon by adsorption to surfaces.

Analytical methods

The distribution of proteins in the column effluents was determined by continuous on-line measurement of the absorbance at 280 nm (A_{280}) and direct recording. Proteins were determined according to the Lowry procedure using a Sigma protein assay kit. The concentration of purified rhIFN-γ in solution was determined using the factor $A_{280}^{1\%}$ = 8.62. This was calculated from the quantitative amino acid composition analysis of the purified rhIFN-γ and its UV absorption spectrum. Its amino acid composition was determined after hydrolysis in 6 *M* HCl for 24 and 72 h at 110°C. The hydrolysates were analysed on a Model 4151 Alpha Plus amino acid analyser (Pharmacia LKB Biotechnology).

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

Determination of biological activity

This was a standard microtitre assay [34] based on the reduction of the cytopathic effect (CPE). The assay was performed by incubating a fixed count of WISH cells with a serially diluted rhIFN-γ sample followed by challenging the cells with difined plaque-forming units of vesicular stomatitis virus (VSV), all according to a standardized procedure [34]. The interferon activity was calculated as the reciprocal of the dilution in the well of the titre plate where 50% of the WISH cell monolayer is protected from the CPE of the challenging virus.

SDS–polyacrylamide gel electrophoresis (SDS-PAGE)

This was performed routinely to assess the progress achieved at each stage of the downstream purification process and to examine the electrophoretic homogeneity of purified rhIFN-γ preparations. The Pharmacia LKB PhastSystem electrophoresis apparatus and 20% homogeneous or 8–25% gradient PhastGel media were used following the recommended procedures of the manufacturer. Molecular mass marker proteins were also run simultaneously.

Approximately 20–30 μg of each sample were applied to the gel for electrophoresis. The separated protein bands were stained using either the Coomassie Brilliant Blue or silver staining techniques according to the detailed procedure outlined in the PhastSystem manual. The molecular mass of the purified rhIFN-γ was calculated relative to the migration distance of standard calibration proteins run simultaneously. This was performed accurately and conveniently using the Pharmacia LKB PhastImage gel scanner.

Reversed-phase high-performance liquid chromatography (RP-HPLC)

RP-HPLC was used to check the homogeneity of the purified rhIFN-γ preparation. The column (250 × 4 mm I.D. packed with Pep-S, C_2–C_{18} gel of 5-μm average particle diameter) was equilibrated with 5% acetonitrile in 0.05% trifluoroacetic acid (TFA) and developed with a linear gradient of acetonitrile (from 5 to 80%) in 0.05% TFA at a flow-rate of 1 ml/min in a total gradient time of 66 min. The effluent was monitored continuously at 226 nm.

Analytical ultracentrifugation

Analytical equilibrium sedimentation analysis was performed in an MSA Centriscan ultracentrifuge fitted with a six-place analytical rotor and a photoelectric UV scanner. The sample was centrifuged at 26 000 *g* for 48 h at 20°C. The concentration of the purified rhIFN-γ was 0.49 mg/ml dissolved in buffer G.

Mass spectrometry

The purified rhIFN-γ was subjected to electrospray mass spectrometric (ES-MS) analysis [35,36] to determine its molecular mass accurately and also to characterize the size of the fragments generated on digesting it with a *Staphylococcus aureus* V8 protease (Glu-C endoproteinase, obtained from Boehringer, Mannheim, Germany).

Quality control

Contaminating residual DNA was determined using the Threshold total DNA detection system [37]. The content and concentration of bacterial endotoxin were determined using a standard Limulus test [38]. Trace amounts of Ni(II) ions were determined by atomic emission spectrometry using a JY 70 Plus inductively coupled plasma atomic emission spectrometer (Jobin Yvon, Longjumeau, France).

The possible presence of viruses was assayed by determining any CPE produced in MDBK cells (from bovine kidney) co-cultured with a sample of purified rhIFN-γ at 37°C for 7 days in a 5% carbon dioxide atmosphere. The cells were frozen and thawed three times to release any viruses and the supernatant was poured off to a new MDBK cell culture and incubated as above. This procedure was repeated one more time and the cells were examined for any CPE produced during the above process.

RESULTS AND DISCUSSION

During the explorative stages of this investigation, a variety of hydrophobic and metal chelate adsorbents were tried for the initial purification of the renatured rhIFN-γ. Their selectivities and adsorption capacities were low, however. The most promising results were obtained using S-Sepharose FF as the initial adsorbent for the renatured rhIFN-γ from the cloudy suspension without the necessity for dialysis or high-speed centrifugation. The results so obtained and the subsequent purification steps used for obtaining a homogeneous rhIFN-γ preparation are described below. The reproducibility of the adopted downstream purification procedure shown in Talbe I was checked by at least fifteen independent runs with satisfactory results.

Step 1: ion-exchange chromatography

A typical elution profile obtained after this initial purification step is shown in Fig. 1. In batch adsorption experiments, fraction 1A is not seen on the elution diagram as it is washed out prior to packing of the gel to elute the bound fractions 1B and 1C. Fraction 1A contains about 60–70% of the proteins and 5–20% of the activity in the renatured rhIFN-γ sample applied to the column. The corresponding figures for fraction 1B are 1–2% of the proteins and 2–7% of the activity and for fraction 1C 25–35% of the proteins and about 85% of the activity (see Table II). This step is therefore very effective in removing most of the impurities and also leads to the simultaneous concentration of the active fraction 1C, which is eluted in a volume that corresponds to approximately one tenth of the volume of the renatured sample applied to the column.

Batch adsorption is adopted as the preferred method as it is simple, fast and can easily be scaled up for large-scale operations. The cloudy solution of renatured rhIFN-γ need not be clarified by centrifugation prior to the adsorption step. The recovery of activity was, in most instances, more than 100%. This is probably due to the further solubilization of active rhIFN-γ from the precipitate found in the renatured sample as it comes into contact with the S-Sepharose FF gel beads [39].

The apparent capacity of the S-Sepharose FF adsorbent was low, amounting to *ca.* 2 mg of proteins

TABLE I

PILOT-SCALE PURIFICATION SCHEME FOR rhIFN-γ

Step	Separation principle	Downstream process
		E. Coli cells
	Extraction	→ Cells, cell debris, DNA etc.
		Inclusion bodies
	Solubilization (7 *M* Gu · HCl)	→ Insoluble particles, cell debris
		Crude extract
	Renaturation (70 × dilution)	
		Renatured rhIFN-γ (Cloudy solution)
I	Ion exchange	S-Sepharose FF
		→ Ca. 70% unbound impurities (Fractions 1A & 1B)
		(Fraction 1C)
II	IMAC	Ni(II) - CS FF
		→ Ca. 20% impurities (Fractions 2A & 2C)
		(Fraction 2B)
III	Size exclusion	Superdex 75 prep grade (Sephacryl S-100)
		Purified rhIFN-γ (Fraction 3A)

(in 14 ml of the renatured rhIFN-γ) per millilitre of the ion exchanger. This is due to the presence of high concentrations of competing ions (0.2 *M* Gu · HCl, 0.125 *M* NaCl) in the renatured sample. The removal of these ions in order to increase the adsorption capacity of the ion exchanger implies the use of time-consuming procedures such as desalting or dialysis, which are inconvenient for use in large-scale operations.

Step 2: IMAC on Ni(II)–Chelating Sepharose FF

Fig. 2 shows the elution profile obtained after chromatography of pooled fraction 1C on a column of Ni(II)–CS FF. The pH or salt concentration of the sample was not adjusted prior to its chromatography, a consideration which is very advantageous in large-scale operations. The unbound fraction 2A contains about 6–12% of the proteins and at most 4–5% of the activity, while fraction 2B contains about 70–80% of the proteins and more than 70% of the activity applied to the column (see Table II). Fraction 2C contains about 6–10% of the proteins and is virtually inactive.

It takes about 7 column volumes of the desorption buffer J to elute fraction 2B completely in a total of about 2–3 bed volumes. Usually, this fraction was eluted without dilution relative to the volume of sample applied to the column. The results

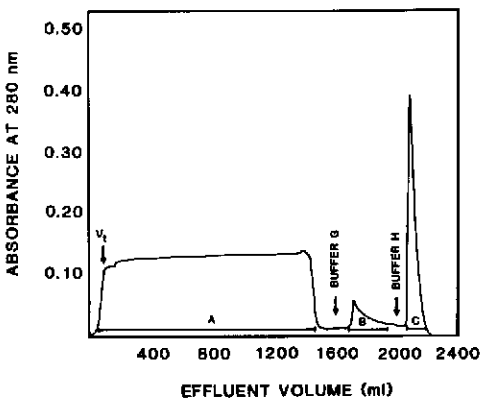

Fig. 1. IEC of 1400 ml of renatured crude extract on a K50/20 laboratory column (5 × 5 cm I.D.; bed volume = 98 ml) of S-Sepharose FF. The sample was centrifuged at 17 000 *g* to remove the suspension of finely divided precipitate prior to its application to the column. The flow-rate was maintained at 60 cm/h throughout and fractions were pooled as they were eluted from the column on the basis of the continuous chart tracing. For further experimental details, see text. Pooled fractions are indicated by the horizontal bars. Approximately 85% of the eluted IFN-γ activity was found in the strongly bound fraction 1C.

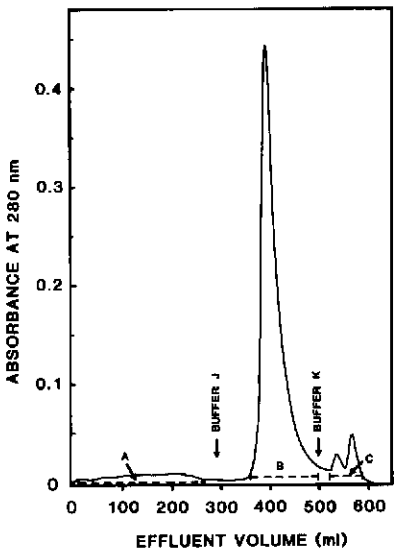

Fig. 2. IMAC of pooled fraction 1C (see Fig. 1) on an XK26/20 laboratory column (6 × 2.6 cm I.D.; bed volume = 32 ml) of Ni(II)–Chelating Sepharose FF. A flow-rate of 60 cm/h was used throughout. Bound fractions were desorbed by stepwise change of the elution conditions and were pooled directly as shown by the horizontal bars. For further experimental details, see text. The IFN-γ activity was localized in fraction 2B.

indicate that the column needs to be saturated with the competing ion (*i.e.*, imidazole) before this fraction is eluted. The process could be speeded up by using a higher concentration of imidazole in the eluent buffer, but this led to the co-elution of some strongly bound contaminants. Lowering the pH of the eluent buffer to 6.8 paradoxically led to a broadening of the peak. In normal IMAC procedures

TABLE II

RECOVERY OF rhIFN-γ ACTIVITY AFTER EACH CHROMATOGRAPHIC SEPARATION STEP USED FOR ITS PURIFICATION FROM THE INCLUSION BODIES OF TRANSFORMED *E. COLI* CELLS

The values obtained are averages of four independent experiments performed on 1400 ml of renatured rhIFN-γ samples prepared from 20 ml of the same batch of crude extract (7 *M* Gu · HCl-solubilized inclusion bodies). The recovery and purification factors are calculated relative to the total activity in 1400 ml of the renatured sample. Details of the pooled fractions referred to here are shown in Figs. 1–3. The numbers in parentheses are ranges of values obtained for the recovery of activity.

Step	Sample	Total protein (mg)	Total activity (× 10^7 I.U.)	Specific activity (× 10^7 I.U./mg)	Recovery (%)	Purification factor (-fold)
–	Renatured rhIFN-γ	118.9	146.7	1.2	100	1
I (IEC)	Pool 1C	31.7	126.0	4.0	85.8 (54–115)	3.2
II (IMAC)	Pool 2B	27.2	94.5	3.5	64.4 (37–73)	2.8
III (SEC)	Pool 3A	13.7	46.8	3.4	31.9 (21–47)	2.8

[40], a lower pH should have facilitated the desorption of the bound proteins. We have also observed that the concentration of NaCl in the equilibration buffer, ranging from 0.1 to 1.0 *M*, did not have any influence on the adsorption–desorption kinetics, contrary to what one would expect for such a basic protein [41].

The bound rhIFN-γ fraction could also be eluted by decreasing the pH of the desorption buffer to 4.5 or lower [26]. However, there was a marked decrease in activity of the eluted fraction, apparently due to the instability of this rhIFN-γ in an acidic medium.

IMAC was found to be a necessary step for the efficient purification and high recovery of the rhIFN-γ activity. Some orienting experiments were performed in which this step was omitted and the partially purified fraction 1C was further fractionated on a column of Superdex 75 Prep Grade. The purified fraction so obtained still contained some protein impurities, as judged by SDS-PAGE, and its overall recovery was low. The results clearly showed that IMAC on Ni(II)–CS FF effectively removes some low-molecular-mass impurities in fraction 1C, thereby facilitating its further purification to homogeneity by a subsequent SEC step.

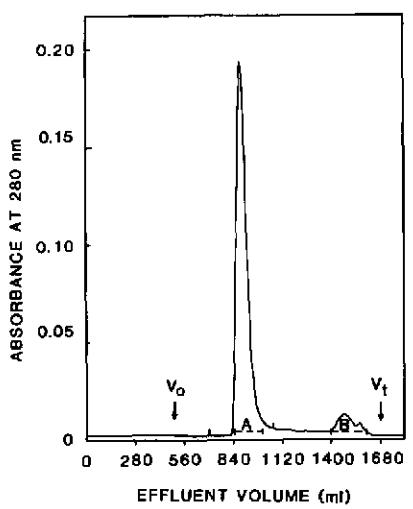

Fig. 3. SEC of pooled fraction 2B on a BioPilot 60/600 column (bed volume = 1700 ml) packed with Superdex 75 Prep Grade. Usually, the pooled fraction 2B from the previous step was applied to the column without further concentration as it is eluted from the IMAC column in a sufficiently concentrated form. The column was equilibrated and eluted with buffer I at a flow-rate of 15 cm/h. The volume of the applied sample corresponded to about 6% of the total bed volume of the packed Superdex 75 Prep Grade column. Fractions were pooled directly as shown by the horizontal bars. More than 96% of the eluted IFN-γ activity is localized in the major fraction 3A. The calculated relative elution volume (V_e/V_t) for fraction 3A was 0.54.

Step 3: Size-exclusion chromatography

Some minor impurities present in fraction 2B (see Fig. 4) were effectively removed by SEC on a column of Superdex 75 Prep Grade or Sephacryl S-100 (Fig. 3). The main fraction 3A contained the interferon activity whereas the minor fraction 3B was inactive. The recovery of activity in fraction 3A was *ca.* 50% relative to the activity applied to the column and *ca.* 32% relative to the total activity in the renatured sample (Table II). The significant decrease in activity after this step is surprising and might be due to unspecific adsorption of the already highly purified fraction 2B on the gel surfaces. The addition of various "stabilizers" (*e.g.*, 5% sucrose, 0.5% SDS or 1% Dextran T40) to the elution buffer, or of 1% purified HSA to the pooled fraction 3A soon after its elution from the column, did not prevent the observed loss of interferon activity. Despite this loss, however, about 14 mg of highly purified rhIFN-γ were obtained (Table II) after processing 1400 ml of the renatured sample according to the adopted downstream purification scheme shown in Table I.

The Superdex 75 Prep Grade column was run at a flow-rate of 15 cm/h and a sample volume corresponding to 4–6% of the total bed volume. A flow-rate of 25 cm/h also gave satisfactory results. According to Kågedal *et al.* [42], Superdex 75 Prep Grade retains its high resolving power for some model proteins at flow-rates in excess of 60 cm/h. It is therefore possible to optimize further the flow-rate and sample load for use in the future that would consistently lead to a homogeneous rhIFN-γ preparation with a minimum of processing time.

As shown in Fig. 4, the purified rhIFN-γ was homogeneous by SDS-PAGE with a calculated molecular mass of 16 000. We have, on different occasions, also observed the presence of a minor band that migrated as a an *M* 31 000 protein. On desitometric analysis of two different gels using the Phast-Image gel scanner, this band represented about 1–10% of the proteins in the purified rhIFN-γ. At first, we thought that it was an impurity and several

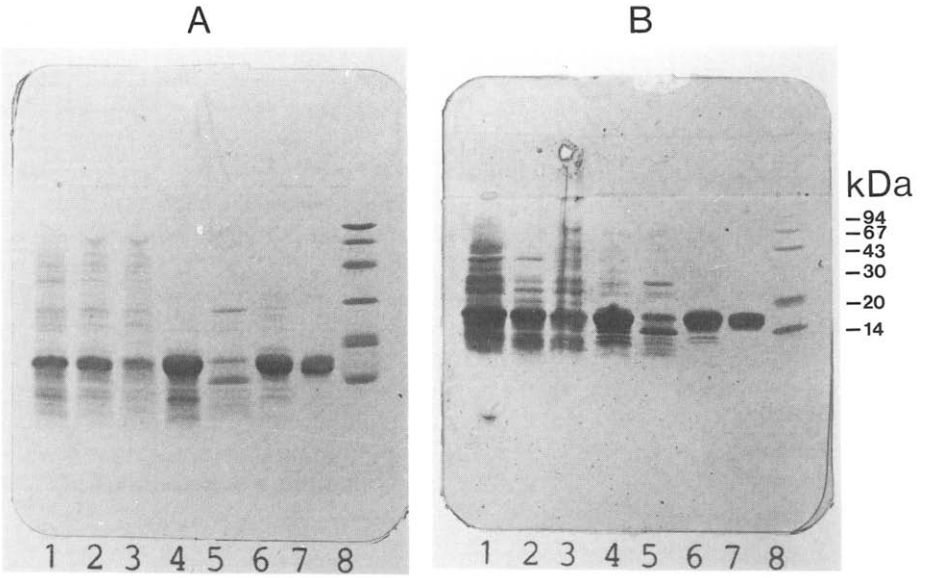

Fig. 4. PhastGel homogeneous (20%) SDS-PAGE patterns of the various fractions obtained during the three-step chromatographic purification process of rhIFN-γ. The gels were stained with PhastGel Blue R (plate A) or by the silver staining technique (plate B). About 25 μg of each sample, in 2 μl of solution, were applied to plate A and about half of this amount to plate B. 1 = Crude extract (in 7 M Gu · HCl); 2 = renatured crude extract; 3 and 4 = unbound and buffer H-desorbed fractions, respectively, from an S-Sepharose FF column; 5 and 6 = unbound and buffer J-desorbed fractions, respectively, from a Ni(II)–chelating Sepharose FF column; 7 = major fraction that was eluted from a Superdex 75 Prep Grade column (purified rhIFN-γ); 8 = molecular mass marker proteins with indicated M_r on the right (kDa = kilodalton).

separation methods (including modifications to the procedures we had adopted) were used in an attempt to remove it. None of these approaches gave the desired outcome. We later observed that the native molecular mass of the purified rhIFN-γ is about 31 000 as determined by analytical SEC on a calibrated column of Superdex 75 HR or Sephacryl S-100 HR. The results suggested that this minor electrophoretic band is the dimeric form of the purified rhIFN-γ which was not denatured by SDS. On heating the purified sample in 5% SDS at 100°C for about 2–3 times the recommended time, a single band of M_r 16 000 was obtained.

Criteria of purity

On SDS-PAGE in gradient (8–25%) or homogeneous (20%) gels, the purified rhIFN-γ showed a single band with an apparent M_r of 16 000 (Fig. 4A and B). Considering that the silver staining technique (performed according to the PhastSystem development method) can detect impurities as low as 1 ng/μl of sample, it is concluded that the level of trace impurities, if any, in 12 μg of the purified rhIFN-γ is less than 1 ng.

Analysis of the purified rhIFN-γ on an analytical SEC column gave a single symmetrical peak. Further analysis on an Ni(II)–CS FF column (25 × 10 mm I.D.) developed with a linear gradient of imidazole from 1 to 10 mM in buffer I also gave a single peak. On chromatography of the purified protein (*ca.* 1 mg) on a Mono S HR 5/5 fast protein liquid chromatography column and gradient elution (from 0–1.0 M NaCl in buffer F) of the bound protein, no material was desorbed. Further elution with 2 M NaCl in buffer F also gave the same result. The highly purified rhIFN-γ has thus been irreversibly adsorbed on the ion exchanger.

Further analysis of the purified protein on an analytical RP-HPLC column also gave a single symmetrical peak (Fig. 5). During an orienting experiment, a small peak eluted at the end of the gradient. Checking the system by running a blank experiment showed, however, that this minor peak was due to reagent artifacts and not to a contaminating protein.

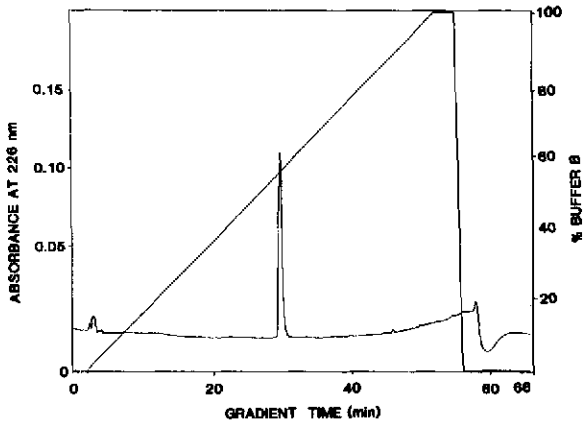

Fig. 5. RP-HPLC of purified rhIFN-γ (pool 3A in Fig. 3) on a column (250 × 4 mm I.D.) of Pep-S, C_2–C_{18} gel of 5-μm particle diameter. The chromatogram was developed with a linear gradient of acetonitrile (from 5 to 80%) in 0.05% TFA at a flow-rate of 1 ml/min in a total gradient time of 66 min.

The purified r-HuIFN-γ sedimented as a single, monodisperse protein when subjected to analytical equilibrium sedimentation analysis. Isoelectric focusing on IEF 3–9 PhastGel media also indicated that the purified rhIFN-γ migrated as an essentially homogeneous band. However, as the p*I* of this protein is above pH 9, the band seen on the gel migrated beyond the separation range of the IEF 3–9 gel.

Biological potency

The specific biological activity of the purified rhIFN-γ is about $3.4 \cdot 10^7$ I.U./mg protein (Table II), which is equal to or better than that reported by other investigators for the *E. coli*-derived rhIFN-γ or its natural counterpart [12,23,28]. It also remained constant after the IEC step indicating that the biological assay is not sensitive enough to respond to the minor impurities which are clearly revealed by SDS-PAGE.

Molecular size and structure

Data concerning the amino acid composition, partial amino-terminal sequence and molecular mass of the purified rhIFN-γ are presented in Tables III, IV and V, respectively. Its partial NH_2-terminal sequence (Table 4) is identical with that reported for the rhIFN-γ [28]. Its amino acid composition data (Table III) show, however, that it lacks fifteen amino acid residues compared with that established for the recombinant protein [19,28]. The calculated residue molecular mass of the rhIFN-γ we purified, based on its amino acid composition, is 15 046, which agrees very well with that obtained by ES-MS (15 067; see Table V). The latter method has an accuracy of 0.01% for proteins with molecular mass up to 30 000 [36]. These results suggest that the purified rhIFN-γ is in its truncated form.

According to Rinderknecht *et al.* [19], the *E. coli*-derived rhIFN-γ has the same primary structure as its natural counterpart without any internal insertions or deletions. The only difference is that the recombinant protein has Met as its amino-terminal residue instead of pyroglutamic acid [23]. The missing fifteen amino acid residues must therefore have

TABLE III

AMINO ACID COMPOSITION OF PURIFIED rhIFN-γ

The numbers of amino acid residues per molecule are averages of the values calculated from the analyses performed on a 24- and 72-h hydrolysed sample. They represent the nearest integers obtained from the normalized values for each amino acid so obtained. Its content of Trp was not determined but is taken to be 1 residue per mole based on the sequence established for its natural counterpart [19] or the recombinant protein [29]. The values obtained for Met are based on analysis of the sample hydrolysed for 24 h and are lower than expected owing to its partial oxidation during acid hydrolysis.

Amino acid	Notation	Relative No. of residues	Residues per mole
Lysine	K	18.7	19
Histidine	H	2.0	2
Arginine	R	3.3	3
Half-cystine	C		
Aspartic acid	D	19.7	20
Threonine	T	4.8	5
Serine	S	9.2	9
Glutamic acid	E	16.3	16
Proline	P	2.1	2
Glycine	G	4.0	4
Alanine	A	7.2	7
Valine	V	7.9	8
Methionine	M	3.2	(4)
Isoleucine	I	6.8	7
Leucine	L	9.3	9
Tyrosine	Y	3.9	4
Phenylalanine	F	9.0	9
Tryptophan	W	(1)	(1)
Total		128.4	129

TABLE IV

AMINO-TERMINAL SEQUENCE OF RESIDUES 1–15 OF PURIFIED rhIFN-γ

The sequence is generated from data obtained after analysis of a purified sample of rhIFN-γ on an Applied Biosystems Model 477A automatic micro-sequencing apparatus according to standardized procedures.

1	5	10	15 · · · · · · · · · · · · · · · 129
H_2N · Met–Gln–Asp–Pro–Tyr–Val–Lys–Glu–Ala–Glu–Asn–Leu–Lys–Lys Tyr----------COOH			

TABLE V

RELATIVE MOLECULAR MASS OF PURIFIED rhIFN-γ AS DETERMINED BY VARIOUS METHODS

Method	M_r
SDS-PAGE	16 000
Ultracentrifugation	14 000
Analytical SEC	31 000
ES-MS	15 067

been situated at its carboxyl-terminal side and are cleaved off from the parent molecule owing to post-translational processes either during or after its secretion. Identical results were obtained by Kung *et al.* [27], who also showed that the truncated rhIFN-γ has a comparable specific activity to the natural hIFN-γ, leading them to conclude that the last fifteen COOH-terminal amino acids are not essential for full biological activity. On the basis of the known primary structure of the intact rhIFN-γ [19,28] and the results obtained here, it is concluded that the truncated form arises by proteolytic cleavage between Lys_{128}–Arg_{129} of the native molecule.

The reliable molecular mass of the purified rhIFN-γ is 15 000 as determined by three independent analytical methods (Table V). It behaves, however, as a protein with M_r 31 000 on analytical SEC (Table V), indicating that, under physiological conditions, the purified protein exists as a dimer. This result is consistent with the conclusions of other investigators [12,19,23,43–45]. However, for reasons which are not apparent to us, this molecule sediments as a monomer (Table V) on anlytical equilibrium sedimentation analysis.

TABLE VI

LEVELS OF SOME EXTRANEOUS COMPONENTS IN RELEVANT CHROMATOGRAPHIC FRACTIONS OBTAINED DURING THE PURIFICATION OF rhIFN-γ

The analyses were performed on samples purified on the BioPilot system using sterilized buffers, gel media and connecting tubes for the chromatographic columns. The results serve as guidelines in assessing the efficiency of removal of these components by the gel media used during the entire purification process. For identification of the samples referred to here, see Figs. 1–3. Blank: 20 m*M* sodium phosphate–0.3 *M* NaCl (pH 7.5) (buffer I).

Sample	Protein concentration (mg/ml)	DNA[a] (ng/ml)	Endotoxin[a] (I.U./ml)	Viruses
Renatured rhIFN-γ	0.24	194	745	nd[b]
Fraction 1A	0.13	nd	45	nd
Fraction 1C	0.85	3.9	5.2	nd
Fraction 2B	0.75	2.3	3.6	nd
Fraction 3A	0.35	1.6	1.2	Negative
Buffer blank	0	< 10 pg/ml	< 0.3	nd

[a] Assuming that one dose of purified rhIFN-γ corresponds to *ca.* 29 μg of protein, the amount of DNA per injection in the final product is *ca.* 133 pg. According to the WHO Study Group on Biologicals [46], the probability of risk associated with heterogeneous contaminating DNA is negligible when the amount of such DNA is 100 pg or less in a single dose administered parenterally. The level of bacterial endotoxin in the final product is as low as 0.1 I.U. per dose.

[b] nd = Not determined.

TABLE VII

CONCENTRATION OF Ni(II) IONS IN rhIFN-γ SAMPLES OBTAINED AFTER CHROMATOGRAPHY ON AN Ni(II)–CHELATING SEPHAROSE FF COLUMN FOLLOWED BY SEC ON A SUPERDEX 75 PREP GRADE COLUMN

The samples were analysed by atomatic emission spectrometry using a JY 70 Plus inductively coupled plasma atomic emission spectrometer. The limit of detection of Ni(II) ions by this method is 2.7 μg/l. Blank = equilibration buffer (buffer I) for the Superdex 75 Prep Grade column. In the purified rhIFN-γ sample obtained from the final gel filtration step, the level of Ni(II) ions is as low as that in the column equilibration buffer. The total amount of Ni(II) ions that are stripped off the Ni(II)–CS FF column (V_t = 30 ml) is also very low. About 1000 μmol of Ni(II) ions are immobilized on the column, corresponding to *ca.* 58.7 mg of Ni(II) ions. The column is washed with *ca.* 210 ml (*i.e.*, $7 \cdot V_t$ of the column) of the desorption buffer. Hence, only 0.04 mg of Ni(II) ions were washed out during the elution of the adsorbed rhIFN-γ from the IMAC column.

Sample	Column	Protein concentration (mg/ml)	Concentration of Ni(II) ions (mg/l)
Fraction 2B	Ni(II)–CS FF	0.24	0.21
Fraction 3A	Superdex 75 Prep Grade	0.22	<0.01
Blank		0	<0.01

Quality control

Recombinant proteins expressed in genetically transformed cells must accurately represent the natural molecules that they are intended to replace or complement. They should also fulfil the minimum requirements for biological efficacy, safety and quality criteria as do other preparations intended for pharmaceutical use. The results presented here give ample evidence for the identity of the purified rhIFN-γ with its natural counterpart to the extent that it can be used in its place for clinical trails. Additional data are presented in Tables VI & VII regarding the level of biological active components and chemical substances derived from those used during its purification.

The DNA content in the final product (1.6 ng/ml; see Table VI) corresponds to about 133 pg per dose, which is about 50% higher than the recommended level for such a product (see ref. 46 and footnote to Table VI). It is possible to decrease its concentration further by using an anion-exchange column, such as Q Sepharose FF, to bind the remaining traces of DNA. The level of bacterial endotoxins is very low, indicating its efficient removal during the downstream purification process.

The same applies to the amount of Ni(II) ions (Table VII), whose concentration in the final product is reduced to that in the blank buffer. The leakage of Ni(II) ions from the IMAC column after each chromatographic run is extremely low and corresponds to about 0.07% of the total amount of Ni(II) ions immobilized on the chelating Sepharose FF column. The column can therefore be used for several cycles of adsorption–desorption experiments without regeneration.

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