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Increased Yield of High Purity Recombinant Human Interferon- γ Utilizing Reversed Phase Column Chromatography

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Abstract

Increasing therapeutic applications for recombinant human interferon- γ (rhIFN- γ), an antiviral proinflammatory cytokine, has broadened interest in optimizing methods for its production and purification. We describe a reversed phase chromatography (RPC) procedure using Source-30TM matrix in the purification of rhIFN- γ from *Escherichia coli* that results in a higher yield than previously reported. The purified rhIFN- γ monomer from the RPC column is refolded in Tris buffer. Optimal refolding occurs at protein concentrations between 50–100 μ g/ml. This method yields greater than 90% of the dimer form with a yield of 40 mg g⁻¹ cell mass. Greater than 99% purity is achieved with further purification over a Superdex G-75 column to obtain specific activities of from 2 to 4 $\times 10^7$ IU/mg protein as determined via cytopathic antiviral assay. The improved yield of rhIFN- γ in a simple chromatographic purification procedure promises to enhance the development and therapeutic application of this biologically potent molecule.

Keywords

recombinant human interferon-gamma; protein expression; reversed phase chromatography; protein purification; antiviral assay

Introduction

A member of the antiviral interferons, interferon- γ (IFN- γ) is a proinflammatory immunomodulatory cytokine involved in a broad range of biological activities [1–5]. As a result of its numerous functions, recombinant human IFN- γ (rhIFN- γ) is finding increasing therapeutic applications. In the treatment of chronic granulomatous disease [6–9], and in severe malignant osteopetrosis [10,11], rhIFN- γ has clinically accepted beneficial effectiveness. Additionally, in the almost uniformly fatal progressive fibrosing lung disease, idiopathic pulmonary fibrosis (IPF), a recent metaanalysis has concluded that rhIFN- γ treatment is associated with reduced mortality [12]. Interest is also being expressed in the use of rhIFN- γ in the treatment of drug resistant tuberculosis [13], atopic dermatitis [14], and hepatitis B infections [15]. In the food and animal industries, IFN- γ is effective against a variety of

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economically important animal viral diseases [16,17]. Hence, the commercial interests in efficiently optimizing the production yields and specific activities of IFN- γ preparations are tremendous [18–22].

Most commonly, rhIFN- γ is prepared via cloning and gene expression in *Escherichia coli* (*E. coli*). The peptide accumulates in inactive aggregates, inclusion bodies (IB), that must be isolated and then solubilized [23]. Structurally, rhIFN- γ monomers have highly hydrophobic domains and contain no disulfide bonds. To achieve an active configuration, these monomers must be refolded [24,25] and dimerized [26]. Complex aggregate formation and misfolding is to be avoided in the isolation and purification procedures. Solubilized rhIFN- γ has been purified using a variety of chromatographic and affinity techniques, including size exclusion gel-filtration [25], CM-Sepharose [27], MonoS [28], *S*-Sepharose [29], and cation exchange [30] chromatography, sometimes incorporating monoclonal antibodies [31], and affinity tags [32].

Typically, purified rhIFN- γ is refolded *in vitro* with or without arginine as an aggregation suppressor [29,33,34]. While original techniques used solution phase refolding procedures, more recent attempts have utilized hydrophobic chromatographic column matrices as templates or stabilizing surfaces for the refolding process in order to help avoid inactive aggregate formation seen in the solution phase [35–37]. The problem of aggregate clogging of chromatographic columns can be reduced or eliminated through the use of expanded bed adsorption chromatography [24]. Molecular chaperones that play an essential role in the correct folding of proteins *in vivo* have been explored for *in vitro* refolding of rhIFN- γ [38–40]. Attempts to enhance rhIFN- γ stability and specific activity are being made through the exploration of potential mutant analogues [41]. Purified product yields from all procedures range from 0.3–14 mg g⁻¹ cell mass with specific activities of 10⁷–10⁸ IU/mg.

We present a technique whereby rhIFN- γ solubilized from genetically engineered *E. coli* inclusion bodies is chromatographically purified utilizing rigid, monosized, polystyrene/divinyl benzene reversed phase chromatography columns in a process that enhances the yield after refolding nearly 3-fold to 40 mg g⁻¹ cell mass while retaining specific activities of from 2×10^7 to 4×10^7 IU/mg protein. Additionally, the DNA and endotoxin content per mg of protein is considerably less than that allowed for therapeutic purposes.

Materials and Methods

Materials

Plasmid (pET21a) and *E. coli* host cells (Rosetta) were obtained from Novagen (Darmstadt, Germany). Source-30TM reversed phase chromatography matrix and Superdex-75TM gel filtration columns were purchased from Amersham Biosciences (Buckinghamshire, England). Ultrafiltration membrane cassettes were purchased from Sartorius (Goettingen, Germany). The BioLogic DuoFlow chromatography system used for column purification was purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A.). HEP2C cells were obtained from the American Type Culture Collection (Manassas, Virginia, U.S.A.), and the encephalomyocarditis virus (EMCV) was from the National Institute of Virology (Pune, India). rhIFN- γ standard was procured from Chemicon International (Temecula, CA, U.S.A.).

cDNA Synthesis, Cloning and Expression of rhIFN- γ

Normal human peripheral blood was centrifuged over Ficoll-Hypaque gradients to isolate T-lymphocytes. T-lymphocytes were then incubated at a density of 10⁶ cells/ml in serum-free RPMI 1640 medium containing phytohemagglutinin at 5 μ g/ml for 24 h at 37° C in a humidified CO₂ atmosphere. Cells were centrifuged, resuspended in lysis buffer [5 M guanidine thiocyanate, 50 mM Tris pH 7.6, 8% (vol/vol) 2-mercaptoethanol], and disrupted for 1 min

with a Polytron tissue homogenizer. RNA was isolated, and poly(A) mRNA was prepared by oligo(dT) chromatography as described [42].

IFN- γ cDNA was synthesized from mRNA using the following gene specific primers:

forward primer: 5'-ATGCAGGACCCATACGTAAAAGAAGCA-3'

reverse primer: 5'-TCGACCTCGAAACAGCATCTGAC-3'.

The PCR amplified human IFN- γ gene was cloned into pET21a as an NdeI and EcoRI fragment. The internal NdeI restriction site was altered while doing PCR amplification using specific primers:

forward primer: 5'-GGCCATATGCAGGACCCATACGTAAAAGAAGCA-3'

reverse primer: 5'-GGCGAATTCTCATCGACCTCGAAACAGCATCTGAC-3'.

E. coli strain Rosetta(DE3)pLysS was transformed with recombinant plasmid containing the hIFN- γ gene. The expression of the hIFN- γ gene was driven by a Lac promoter, which can be regulated by inducing the culture with isopropyl β -D-thiogalactopyranoside (IPTG) thereby allowing high level of expression of the hIFN- γ gene.

Cell Growth and Harvesting

Luria-Bertani (LB) medium (300 ml containing 100 μ g/ml ampicillin) was inoculated with 500 μ l of glycerol stock of *E. coli* host cells and cultured overnight at 37° C, 200 rpm.

The overnight culture was inoculated into 3 L of LB with 100 μ g/ml ampicillin and distributed aseptically into 1 L flasks (300 ml per flask). The cells were cultured at 37° C at 200 rpm. The culture was then induced after reaching an optical density of 0.6 at 600 nm by the addition of IPTG to a final concentration of 0.5 mM and cultured at 37° C, 200 rpm for 4 h.

The culture was centrifuged at 6,600g for 10 min, the supernatant carefully removed, and the cell pellet washed by gently suspending in Buffer A (100 mM Tris pH 8.0, 10 mM EDTA, 100 mM NaCl). The washed cell mass (5.8 g) was collected by centrifuging at 14,900g, 4° C for 15 min and stored at -20° C.

Cell Lysis and Inclusion Bodies Purification

The cell pellet was suspended in 58 ml of Buffer A in a beaker maintained on ice. The cells were disrupted with a sonicator programmed for 5 cycles of 5 min on and 5 min off. The sonicate was then centrifuged at 14,900g at 4° C for 15 min. The supernatant was discarded. The crude inclusion body pellet was suspended in Buffer A containing 4 M urea and incubated while being magnetically stirred for 45 min at 4° C. The sample was then centrifuged as before and the supernatant discarded.

The pellet was suspended in Buffer B (100 mM Tris pH 8.0, 1 mM EDTA, 1 M NaCl, 0.5% Triton X-100) and centrifuged at 14,900g at 4° C for 15 min. The pellet was washed with Buffer A and suspended in Buffer C (10 mM Tris pH 8.0, 0.25 M Sucrose, 1 mM EDTA). The sample was centrifuged at 3,700g, 4° C for 15 min.

The pellet was then washed with Buffer A and the inclusion bodies pellet was collected by centrifugation at 14,900g for 30 min. The inclusion bodies pellet (2.8 g) was stored at -70° C.

Solubilization of Inclusion Bodies

The inclusion bodies pellet was suspended in 28 ml of solubilization buffer (6 M guanidine hydrochloride, 100 mM Tris pH 8.0, 0.2 mM EDTA) and dissolved by stirring overnight at 4°

C with a magnetic bar. The solution was centrifuged at 14,900g at 4° C for 30 min. The supernatant was then collected and stored at 4° C.

Reversed Phase Chromatography

The solubilized sample was loaded onto a column (1.2 cm × 10 cm) packed with Source-30 RPC matrix equilibrated with 0.1% trifluoroacetic acid (TFA) in water. The column was then washed with 0.1% TFA and the protein was eluted with a linear gradient of acetonitrile. The eluted fractions were analyzed by SDS-PAGE. Pure fractions were pooled for renaturation.

Renaturation

The pooled fractions (50 ml) from the column were renatured by dilution in refolding buffer (1100 ml; 100 mM Tris pH 7.2, 0.2 mM EDTA). The protein concentration (2.3 mg/ml) of the pooled fraction was determined by the Bradford method and diluted for optimal refolding. This buffer was stirred while diluting the protein pool and the solution was incubated for 24–36 h at 4° C for refolding.

After refolding the solution was concentrated by ultrafiltration to a protein concentration of approximately 2–5 mg/ml.

Gel Filtration Chromatography

The concentrated rhIFN- γ was purified on a Superdex-75 column equilibrated with PBS buffer. Ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and ribonuclease (13.7 kDa) were also loaded onto the column as calibration standards. The profile of rhIFN- γ was then compared with the standards for molecular weight confirmation and determination of dimer formation.

Dialysis

The refolded protein was dialyzed against 50 mM sodium acetate at pH 5.0. The dialysis was carried out at 4° C with frequent buffer changes. The dialyzed sample was then stored at 4° C.

SDS-PAGE and Western Blotting

Electrophoresis of the expression samples, purified protein and the crosslinked rhIFN- γ was carried out on SDS-PAGE gels, stained with Coomassie Brilliant Blue (CBB) G-250. For western blots, after electrophoresis the samples and molecular weight standards were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (0.2 μ m pore size) from Invitrogen (Carlsbad, CA, U.S.A.). After transfer the PVDF membrane was incubated in blocking buffer (Pierce, Rockford, IL, U.S.A.) for 30 min at room temperature. The transferred rhIFN- γ were probed with a mouse monoclonal anti-INF- γ antibody (#ab9801; Abcam, Cambridge, U.K.) for 16 h at 4° C followed by incubation with specific secondary antibodies in blocking buffer for 1 h at room temperature. Three washes with a buffer containing 10 mM Tris, 100 mM NaCl and 0.1% Tween 20 were then performed. SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, U.S.A.) was used for detection and results were recorded using chemiluminescent-sensitive Kodak X-OMAT AR film (Kodak, Rochester, NY, U.S.A.).

Cross-linking Analysis

Interchain cross-linking of rhIFN- γ was performed using disuccinimidyl suberate (DSS) (Pierce, Rockford, IL, U.S.A.). For reaction, 40 μ l of rhIFN- γ (0.6 mg/ml in PBS) and 10 μ l DSS (20 mM in DMSO) were mixed and incubated for 5 min at room temperature. The reaction was stopped by the addition of 25 μ l 0.5 M Tris pH 8.7.

Antiviral Assay

An antiviral assay of rhIFN- γ activity was performed as previously described by Lewis [43] by challenging the human lung carcinoma cell line (HEP2C) with EMCV in the presence and absence of rhIFN- γ . 20,000 cells in 100 μ l were seeded in each well of 96 well microtiter plates along with various concentrations of rhIFN- γ or the reference standard. Cells were seeded in DMEM containing 10% FBS and incubated for 24 h. 100 μ l of 10^3 diluted virus was then added to all but the control wells. The plate was then developed by tapping off the media and adding amido black. Colorimetric analysis was performed at 450 nm after adding 0.38% NaOH. The antiviral activity was calculated in comparison with the standard. Absorbance values were calculated for each well and plotted against the concentration of rhIFN- γ using a semi-log plot. The standard and sample curves were fitted using nonlinear sigmoidal (4 parameter) regression (SigmaPlotTM software, Systat Software Inc., Point Richmond, CA, U.S.A.). The concentration yielding a 50% increase in absorbance between baseline and maximum plateau absorbance values (marking a 50% reduction in cytopathic effect) defined the assay endpoint. The potency of the experimental sample was calculated by comparing the endpoint concentration of the rhIFN- γ preparation against that of the standard.

Results and Discussion

Cloning and Expression

The first reported recombinant interferon- γ [44] consisted of 146 amino acids with the N-terminal portion of the molecule commencing with the sequence Cys-Tyr-Cys. Later, however, it was shown that the native human interferon- γ lacks the Cys-Tyr-Cys sequence at the N-terminal. Our cloned rhIFN- γ protein is identical with the native IFN- γ except for having an N-terminal methionine residue (an artifact encoded by the mRNA translational “start” signal AUG for *E. coli* expression, which is not removed by this bacterial host system) (Fig. 1A). The rhIFN- γ expressed in transformed and induced *E. coli* cells demonstrated to be ~55% of the total cell proteins and was harvested as inclusion bodies (Fig. 1B). These inclusion bodies were then purified and renatured to yield biologically active rhIFN- γ .

IB Purification

The crude IB preparation was washed with chaotropic agent (urea) and detergent (Triton X-100) to remove *E. coli* proteins and other soluble components. The procedure also removes significant amounts of endotoxins (to less than 5 EU/mg protein) and DNA from the host cells (to less than 100 pg/mg of protein). The IB preparation was then washed with sucrose and centrifuged at low speed to remove low-density protein contaminants. This final IB isolate was 80% pure.

Reversed Phase Chromatography

Solubilized IB of rhIFN- γ was loaded onto a Source-30 column and eluted with a linear gradient of acetonitrile. The elution profile showed three peaks (Fig. 2). The first peak contained contaminants (lane 1 of Fig. 2 inset). The major peak was collected in fractions. The fractions were then analyzed by SDS-PAGE (lanes 2 to 6, inset of Fig. 2). These fractions proved to be uncontaminated and were pooled for renaturation of the protein.

Renaturation and Gel Filtration

The pure fractions from the Source-30 RPC column were renatured by dilution and incubated in refolding buffer. Optimal refolding occurred at a final protein concentration between 50–100 μ g/ml. Concentrations above 100 μ g/ml resulted in excessive protein aggregation, whereas below 50 μ g/ml, suboptimal dimer formation was observed (data not shown). The refolded protein was concentrated and then dialyzed several times to remove traces of acetonitrile.

The refolded rhIFN- γ was then purified on Superdex-75 to yield greater than 90% of the dimer form (in some batches even 95%). Properly assembled dimers were assessed against ovalbumin, chymotrypsinogen and ribonuclease standards using gel filtration chromatography on a Superdex-75 fast protein liquid chromatography column. The rhIFN- γ eluted between ovalbumin (43 kDa) and chymotrypsinogen (25 kDa) indicating that it was a dimer of ~38 kDa (Fig. 3).

The eluted dimer peak was collected and then both rechromatographed on a Superdex-75 column (Fig. 4) and assessed by SDS-PAGE (Fig. 5) to analyze purity followed by western blotting to determine immunoreactivity (Fig. 6). The host cell protein and DNA content were measured in the concentrated sample and were found to be absent. The protein yield obtained in the present method was ~40 mg of purified rhIFN- γ per gram of cell mass (Table 1). Purified product yields from the published procedures previously discussed ranged from 0.3–14 mg per gram cell mass. Interestingly, one study [20] reported a batch process yield of 100 mg per gram of *dry* cell mass. Since *E. coli* is from 70–80% water, this still converts to a value less than what we obtained per gram of *wet* cell mass (although an exact conversion cannot be calculated). Therefore, our yields represent a significant improvement over previously published reports.

Cross-linking Analysis

Dimer formation was further confirmed by interchain crosslinking of the monomeric rhIFN- γ using DSS as a cross-linker [37]. The crosslinked dimer was then analyzed by SDS-PAGE. A single band corresponding to ~33 kDa was confirmed (Fig. 6). This experiment verifies that the most stable configuration of the purified product is, in fact, a homodimer and not the monomeric form.

Antiviral Assay

Antiviral assay of interferon- γ was evaluated as described by Lewis [43] by challenging the human lung carcinoma cell line (HEP2C) with EMCV in the presence and absence of rhIFN- γ . In three determinations, the calculated specific activities of rhIFN- γ were found to range from 2×10^7 to 4×10^7 IU/mg of protein as assessed against the standard (Fig. 7). The specific activities obtained from our recombinant protein were similar to those obtained with the standard (2×10^7 IU/mg protein) and previously published results.

Conclusions

The methods described here for the preparation of rhIFN- γ are simple and easily reproducible. The chromatographic purification step utilizing the Source-30 RPC column followed by solution-phase refolding and dimerization provided overall yields that are higher than those obtained by previously described methods. Our methods should prove useful for not only making rhIFN- γ , but potentially for other recombinant proteins as well. rhIFN- γ and other biologically important recombinant proteins are being studied and used extensively in a variety of diseases and thus efficient production of these molecules is of significant importance.

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Abbreviations

EMCV	encephalomyocarditis virus
rhIFN-γ	recombinant human interferon- γ
IB	inclusion bodies
RPC	reversed phase chromatography
DSS	disuccinimidyl suberate
TFA	trifluoroacetic acid
EU	endotoxin unit
IU	International Units

Fig 1. Expression of rhIFN- γ

(A) The *E. coli* expression vector. The IFN- γ gene was spliced into pET21a as an NdeI and EcoRI fragment. Expression was driven by the Lac promoter regulated with IPTG. (B) SDS-PAGE of the expressed gene product. Lane 1: uninduced; lanes 2–5: induced samples after 1, 2, 3 and 4 h, respectively. IB denotes inclusion bodies prepared as described in Materials and Methods.

Fig 2. Reversed phase HPLC purification of the rhIFN- γ monomer

The solubilized inclusion body was fractionated on a Source-30TM column (1.2 cm $\times$ 10 cm) equilibrated with 0.1% TFA and eluted with a linear gradient of acetonitrile. The pure monomer peak (fractions of peak 2), which eluted at 43–48% acetonitrile, free from other *E. coli* proteins (peaks 1 and 3), was pooled and subsequently refolded. The figure inset shows the SDS-PAGE of peak fractions. Lane 1: peak 1; lane 2–6: various fractions of peak 2.

Fig 3. Confirmation of dimerization of rhIFN- γ on Superdex-75

The refolded fraction of the RP column was concentrated to 2–5 mg/ml and purified on a FPLC Superdex-75 column. Peaks 1, 3 and 4 represent the standards: ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and ribonuclease A (13.7 kDa), respectively. Peak 2 is the pure rhIFN- γ dimer with an apparent mass of approximately 38 kDa.

Fig 4. Pure rhIFN- γ dimer on Superdex-75

Final highly purified rhIFN- γ showing a single peak of the dimer form on Superdex-75.

Fig 5. SDS-PAGE analysis of monomeric and cross-linked rhINF- γ

Purified monomeric rhINF- γ was cross-linked with DSS. The dimeric form of the rhINF- γ was then visualized on a 4–20% SDS-PAGE gel. Lane 1: monomeric form of rhINF- γ ; lane 2–3: different concentrations of cross-linked protein. M, molecular weight marker.

Fig 6. Western blot analysis of purified and cross-linked rhINF- γ

Lane 1: Purified rhINF- γ migrating at ~18 kDa as a monomer; lane 2: cross-linked rhINF- γ migrating at ~33 kDa as a dimer. Western blots were developed using a mouse monoclonal anti-INF- γ antibody.

Fig 7. Antiviral assay

Purified rhIFN- γ activity was assessed against a commercially available standard obtained from Chemicon. The assay involved challenging the human lung carcinoma cell line (HEP2C) with EMCV in the presence and absence of rhIFN- γ . The concentration yielding a 50% increase in absorbance between baseline and maximum plateau absorbance values (marking a 50% reduction in cytopathic effect) defined the assay endpoint. The calculated specific activities of the prepared rhIFN- γ were found to range from 2×10^7 to 4×10^7 IU/mg of protein after three determinations. The graph is representative of one experiment. Squares: Chemicon standard (specific activity 2×10^7 IU/mg protein); circles: purified rhIFN- γ (specific activity 3.1×10^7 IU/mg protein).

Table 1
Yields and purities achieved at each purification step

Step Number	Purification Step	Total protein (mg)	IFN- γ (mg)	Step yield (%)	Purity (%)
1	Whole Cells *	1740	957	100	55
2	Cell Lysis	1040	728	60	70
3	Purified Inclusion Bodies	850	680	82	80
4	IB Solubilization	657	558	77	85
5	Source-30 Chromatography **	115	109	70	95
6	Refolding	76	72	66	95
7	Superdex-75 Chromatography	58	58	76	99.99

* Initial wet cell mass: 5.8 gm

** One fourth of solubilized protein (164 mg) was loaded onto the Source-30 column.