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ORIGINAL PAPER

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Process development for production of recombinant human interferon-γ expressed in *Escherichia coli*

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Abstract A simple fed-batch process was carried out using constant and variable specific growth rates for high-cell-density cultivation of *Escherichia coli* BL21 (DE3) expressing human interferon-γ(hIFN-γ). The feeding rate was adjusted to achieve an appropriate specific growth rate. The dissolved oxygen level was maintained at 20–30% of air saturation by control of airflow and stirrer speed and, where necessary, by enrichment of inlet air with pure oxygen. Glucose was the sole source of carbon and energy and was provided by following a simple exponential feeding rate. The final cell density in the fed-batch fermentation with constant and variable specific growth rate feeding strategies was $\sim$100 g dry cell wt l^{-1} after 36 and 20 h, respectively. The final specific yield and overall productivity of recombinant hIFN-γ in the variable specific growth rate strategy were 0.35 g rHu-IFN-γ g^{-1} dry cell wt and 0.9 g rHu-IFN-γ l^{-1} h^{-1}, respectively. A new chromatographic purification procedure involving anion exchange and cation exchange chromatographies was developed for purification of rHu-IFN-γ from inclusion bodies. The established purification process is reproducible and the total recovery of rHu-IFN-γ was $\sim$30% (100 mg rHu-IFN-γ g^{-1} dry cell wt). The purity of the rHu-IFN-γ was determined using HPLC. Sterility, pyrogenicity, and DNA content tests were conducted to assure the absence of toxic materials and other components of *E. coli* in the final product. The final purified rHu-IFN-γ has a specific antiviral activity of $\sim 2\times10^7$ IU/mg protein, as determined by viral cytopathic effect assay. These results certify the product for clinical purposes.

Keywords Fed-batch fermentation · High-cell-density cultivation · Purification · Recombinant human interferon-γ · Recombinant *Escherichia coli*

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Introduction

Interferon (IFN) was discovered in 1957 as a biological agent interfering with virus replication [7]. IFN-γ is secreted by lymphocytes stimulated by mitogen and is involved in the differentiation, maturation, and proliferation of hematopoietic cells. It also enhances nonspecific immunity to tumors, as well as to microbial, viral, and parasitic organisms [12, 24]. Natural human interferon-γ(hIFN-γ) is composed of 143 amino acid residues with a total molecular mass of 20–25 kDa. It is glycosylated and does not contain cysteine residues [6, 20]. In 1986, hIFN-γ cDNA was successfully cloned and expressed in *Escherichia coli,* which made possible the production of recombinant hIFN-γ (rHu-IFN-γ) in relatively large amounts [34]. rHu-IFN-γ produced in *E. coli* is not glycosylated and has methionine as its N-terminal residue instead of pyroglutamic acid. The total molecular mass of rHu-IFN-γ is less (17 kDa) than that of hIFN-γ, but nonetheless it is physiologically active. Clinical trials indicate that rHu-IFN-γ has therapeutic efficacy on kidney cell carcinoma, colon cancer, and rheumatoid arthritis [34].

E. coli is the most commonly used host for heterologous protein production [10, 19, 33]. Using expression vectors in batch and fed-batch cultivations,

a variety of therapeutic proteins have been successfully expressed in recombinant *E. coli*. However, many of these proteins accumulated in the form of insoluble biologically inactive inclusion bodies [10, 11, 33]. Thus, the volumetric productivity of a recombinant protein is proportional not only to the final cell density but also to the specific yield (the amount of product formed per unit cell mass). High-cell-density culture (HCDC) techniques have been developed for use in *E. coli* in order to improve the productivity of recombinant proteins. In HCDC, maximum cell concentrations are most often achieved by using fed-batch processes and various feeding strategies [2, 8, 10, 18].

In order to produce recombinant proteins in *E. coli* with high yield, over-expression of the recombinant protein in a fermentation process and a purification procedure allowing efficient recovery of the protein from the resultant biomass are necessary. Several methods based on multiple chromatographic steps have been reported for the purification of rHu-IFN-γ [1, 9, 14, 34]. Such multi-step procedures are cumbersome and the overall yields are low. For example, a purification scheme involving polyetylenimine precipitation, quaternary aminoethyl (QAE) column chromatography, phenyl Sepharose column chromatography, ammonium sulfate precipitation, Sephadex G-100 column chromatography, and dialysis was described for purification of rHu-IFN-γ [14]. Zhang et al. [34] described a method based on S-Sepharose chromatography, immobilized metal ion affinity chromatography, and size-exclusion chromatography with Superdex-75. A purification process based on immunosorption by highly specific monoclonal antibodies was reported by Kung et al. [9]. Arora and Khanna [1] developed a method based on S-Sepharose chromatography and S-100 size-exclusion chromatography; the final yield of recombinant protein was 14 mg/g dry cell mass.

In this report we describe the production of large quantities of biologically active rHu-IFN-γ using recombinant *E. coli* BL21 (DE3), which over-expressed rHu-IFN-γ in the form of insoluble inclusion bodies. The fed-batch culture was optimized to obtain maximum overall productivity of rHu-IFN-γ through HCDC. A purification process was also developed to obtain biologically active rHu-IFN-γ of therapeutic grade from inclusion bodies.

Materials and methods

Microorganism and vector system

Escherichia coli strain BL21 (DE3) (Novagen, UK) was used as the host for rHu-IFN-γ expression. This strain was transformed with a commercially available plasmid, pET3a inducible expression vector (Novagen), in which the hIFN-γ gene (Noor Research and Educational Institute, Tehran, I.R. Iran) was inserted into the *Not*I and *Nde*I sites. Host cells were transformed with the plasmid using the calcium chloride procedure [13]. The transformed cells were spread on several LB agar plates containing 100 mg ampicillin l^{-1} .

Media and solutions

LB (Luria-Bertani) agar medium was used for plate cultivation of *E. coli* strain. M9 modified medium consisted of: 10 g glucose, 12.8 g $Na_2HPO_4{\cdot}7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 0.24 g $MgSO_4$; 1 ml trace element solution per liter was used for preparation of seed culture, batch and fed-batch fermentations. The trace element solution consisted of [g $(l\ 1\ M\ HCl)^{-1}$]: 2.8 $FeSO_4{\cdot}7H_2O$, 2 $MnCl_2{\cdot}4H_2O$, 2.8 $CoSO_4{\cdot}7H_2O$, 1.5 $CaCl_2{\cdot}2H_2O$, 0.2 $CuCl_2{\cdot}2H_2O$, and 0.3 $ZnSO_4{\cdot}7H_2O$ [22].

The glucose and $MgSO_4$ solutions were sterilized separately to prepare the feeding solution, which consisted of 750 g glucose l^{-1}, 20 g $MgSO_4\ {\cdot}7H_2O\ l^{-1}$ and 5 ml trace element solution l^{-1}. This solution was used for feeding during fed-batch culture.

The following buffers were used for cell disruption, preparation and solubilization of inclusion bodies, renaturation and purification of rHu-IFN-γ. They are referred to in abbreviated form throughout.

A_1: 50 mM sodium phosphate buffer, 2.0 mM EDTA (di-sodium salt), 0.001% (w/v) phenyl methane sulfonyl fluoride (PMSF), pH 7.5

A_2: 3 M urea, 50 mM sodium phosphate buffer, 2.0 mM EDTA (di-sodium salt), 0.001% (w/v) PMSF, pH 7.5

B: 8 M urea, 40 mM sodium phosphate buffer, 2.0 mM EDTA (di-sodium salt), pH 9.0

C: 6 M urea, 40 mM sodium phosphate buffer, 2.0 mM EDTA (di-sodium salt), pH 7.5

D_1: 6 M urea, 50 mM ammonium acetate, 2.0 mM EDTA (di-sodium salt), pH 7.2

D_2: 6 M urea, 0.2 mM ammonium acetate, 2.0 mM EDTA (di-sodium salt), pH 7.2

D_3: 6 M urea, 0.5 mM ammonium acetate, 2.0 mM EDTA (di-sodium salt), pH 7.2

E_1: 20 mM Tris-HCl, 2.0 mM EDTA (di-sodium salt), pH 7.7

E_2: 20 mM Tris-HCl, 0.2 M NaCl, 2.0 mM EDTA (di-sodium salt), pH 7.7

E_3: 20 mM Tris-HCl, 0.8 M NaCl, 2.0 mM EDTA (di-sodium salt), pH 7.7

Fermentation conditions

Fed-batch fermentation was carried out in a 2-l bench top bioreactor (INFORS, Bottmingen, Switzerland) with a working volume of 1 l. The initial batch culture was started by adding 100 ml of an overnight-seed culture (0.4–0.6 g dry cell wt l^{-1}) and 1 l of defined medium into the bioreactor. The pH was controlled at 7 ± 0.05 by addition of 25% (w/w) aqueous ammonia or 1 M H_3PO_4 solutions. The ammonium concentration was maintained in the range of 0.1–1.5 g l^{-1}.

Dissolved oxygen was analyzed using a polarographic electrode (Ingold, Mettler Toledo, Germany) and was controlled at 20–30% of air saturation by control of both airflow and stirrer speed. During fed-batch phase, the inlet air was enriched with pure oxygen, and foam was controlled by the addition of silicon-antifoaming reagent.

After depletion of the initial glucose in the batch medium, as indicated by an increase in the dissolved oxygen concentration, feeding was initiated and the flow rate was increased stepwise based on an exponential feeding strategy. The exponential feeding rate was determined by a simple mass-balance equation of the cell and substrate [30].

$$(d/dt)(VX) = V\ \mu\ X \quad \text{(biomass)} \tag{1}$$

$$(d/dt)(VS) = FS_0 + (V\ \mu\ X)/Y_{X/S} \quad \text{(substrate)} \tag{2}$$

where V is the medium volume in the bioreactor (L), X is the biomass concentration in the bioreactor (g dry cell wt l^{-1}), t is the time (h), μ is the specific growth rate (h^{-1}), S is the glucose concentration in the bioreactor (g l^{-1}), S_0 is the glucose concentration in the feeding solution (g l^{-1}), F is the feeding rate

($l\ h^{-1}$), and $Y_{x/s}$ is the glucose yield coefficient [g dry cell weight (g glucose)$^{-1}$].

Equation 1 can be integrated as:

$$XV = X_0V_0\exp.\left(\int_0^t \mu\,(t)dt\right) \quad (3)$$

where X_0 (g dry cell wt l^{-1}) is the biomass concentration at the start of feeding, and V_0 (L) is the medium volume in the bioreactor at the start of feeding. Assuming that a quasi-steady state exists for the substrate concentration, and constant volume fed-batch fermentation $(d/dt)(V\,S) = 0$, if $Y_{x/s}$ is constant, then by substituting Eq. 3 into Eq. 2, Eq. 4 will be:

$$Ms(t) = Fs(t)S_0 = \left(\mu\,(t)X_0V_0/Y_{x/s}\right)\exp.\left(\int_0^t \mu\,(t)dt\right) \quad (4)$$

where $M_s(t)$ is the mass flow rate of glucose(g glucose h^{-1}). Then, at constant specific growth rate, Eq. 5 will be:

$$Ms(t) = Fs(t)S_0 = \left(\mu\,X_0V_0/Y_{x/s}\right)\exp(\mu\,t) \quad (5)$$

Cell disruption and preparation of inclusion bodies

The fermentation broth was centrifuged at 5,000 *g* for 5 min at 4 °C and the supernatant was discarded. The biomass was resuspended in distilled water and centrifuged at 5,000 *g* for 5 min at 4 °C to remove residual salts. The washed pellet was resuspended in buffer A_1 (1:20, w/v) at 4 °C and cells were disrupted by passing them twice through a high-pressure homogenizer at 1,000 bar. The suspension of disrupted cells was immediately cooled to 4 °C and centrifuged at 10,000 *g* for 20 min at 4 °C. The pellet was washed separately with buffers A_1 and A_2 and centrifuged at 10,000 *g* for 20 min at 4 °C, respectively. This washed pellet containing inclusion bodies was used for purification of rHu-IFN-γ.

Purification and refolding of rHu-IFN-γ

Inclusion bodies were solubilized in buffer B (1:40, w/v) at 4 °C using a mechanical homogenizer for about 5 min. This suspension was stored at 25 °C for 30 min then centrifuged at 15,000 *g* for 20 min at 4 °C to remove insoluble material and residual cell debris. The supernatant containing solubilized and denatured rHu-IFN-γ was stored at 4 °C for further use. Denatured rHu-IFN-γ solution was loaded on the first chromatography column containing 350 ml Q-Sepharose-FF packed gel which was pre-equilibrated with buffer C. The column was eluted with 1,000 ml buffer C and the effluent was collected. This solution was loaded on the second chromatography column containing 200 ml SP-Sepharose-FF packed gel which was pre-equilibrated with buffer D_1. Then the column was washed with 1,000 ml buffer D_2 and rHu-IFN-γ was eluted with 300 ml buffer D_3. The fraction obtained from Sp-Sepharose-FF was renatured with ten volumes of refolding solution (40 mM sodium phosphate buffer, 2.0 mM EDTA, pH 7.0). The refolded solution of rHu-IFN-γ was loaded on SP-Sepharose-FF column pre equilibrated with buffer E_1. The column was then washed with 1,000 ml buffer E_2, and purified rHu-IFN-γ was eluted with 300 ml buffer E_3.

Analytical procedures

Cell growth was monitored by measuring culture turbidity and dry cell weight. Optical density (OD) was measured at 600 nm. Samples were diluted with NaCl solution (9 g l^{-1}) to obtain an OD_{600} between 0.2 and 0.7. In order to determinate dry cell weight, 5 ml broth was centrifuged at 5,000 *g* for 10 min, washed twice with deionized water, and dried at 105 °C to constant weight. Glucose and ammonia were analyzed enzymatically with glucose and ammonia kits (ChemEnzyme, I.R. Iran), and acetate was analyzed using an enzymatic analysis kit (Boehringer Mannheim/R-Biopharm, Germany) according to the procedures suggested by the suppliers. The expression level of rHu-IFN-γ was determined by SDS-PAGE using polyacrylamide gel (12.5%). Gels were stained with Coomassie brilliant blue R250, silver stained, and quantified by densitometry. Total soluble protein was analyzed by the Bradford method and rHu-IFN-γ was measured by ELISA assay. The stability of the plasmid in the recombinant *E. coli* strain was determined by aseptically sampling from the bioreactor at different cell densities. The sample was diluted with sterile NaCl (9 g l^{-1}) to yield 100–300 colonies per plate on LB-agar medium and incubated at 37 °C for 16 h. All colonies on three plates were transferred by replica plating to selective LB-agar plates supplemented with 100 mg ampicillin l^{-1} . Plasmid stability was calculated by taking the ratio between the average number of colonies from three selective LB-agar plates and the average from three nonselective LB-agar plates [15]. Host-cell and vector-derived DNA were assayed in finally purified rHu-IFN-γ using the threshold method [23, 25]. Bacterial endotoxin contamination was tested in the final product by using a limulus amebocyte lysate (LAL) chromogenic kit [3, 5, 21]. Covalent dimers and oligomers, monomers, and aggregate forms of rHu-IFN-γ were analyzed by HPLC. Covalent dimer and oligomer analysis was done by size-exclusion chromatography using an Ultropak TSK G3000SW LKB column (10,000–300,000 Dalton, 7.5×300 mm) and 0.1% SDS in 20 mM phosphate buffer (pH 6.8) as mobile phase at a flow rate of 1.0 ml min^{-1} and a spectrophotometer detector set at 214 nm [4]. Monomer and aggregates were also analyzed by size-exclusion chromatography using an Ultropak TSK G3000SW LKB column (10,000–300,000 Dalton, 7.5×300 mm), a potassium chloride R solution (1.2 M) as the mobile phase at a flow rate of 0.8 ml min^{-1}, and a spectrophotometer detector set at 214 nm [4]. Deamidated, oxidized, and heterodimer forms of rHu-IFN-γ in final purified product were analyzed by cation-exchange chromatography. A cation-exchange column with a stainless steel column (Altex Sphero-Gel TSK CM-35 W LKB, 7.5×75 mm) was used in an HPLC system with the spectrophotometer detector set at 214 nm to separate the different ionic isoforms of rHu-IFN-γ . The elution buffers were 0.05 and 1.2 M ammonium acetate (pH 6.0) as buffer A and B, respectively [4]. A standard biological assay based on reduction of the cytopathic effect (CPE) of vesicular stomatitis virus (VSV) on Vero cells was carried out using serially diluted rHu-IFN-γ. The results were compared with those obtained from commercial rHu-IFN-γ (Imukin, Boehringer, Germany) [32].

Results and discussion

High-cell-density culture of recombinant *E. coli*

The effects of constant and variable specific growth rate feeding strategies on rHu-IFN-γ production level and plasmid stability were compared in fed-batch cultures of *E. coli* BL21 (DE3) harboring pET3a-*hifn*-γ vector. By using experimental data from various fed-batch cultures, a specific growth rate of 0.12 h^{-1} was selected as the set point of the specific growth rate in Eq. 5 in order to avoid formation of growth-inhibitory metabolites, particularly acetate. The glucose yield coefficient ($Y_{x/s}$) was assumed to be 0.33 [g dry cell wt (g glucose)$^{-1}$], which was chosen based in previous fed-batch experiments in which the total amount of cells produced from known amounts of glucose consumed was determined (data not shown). Changes in the feeding rate were made at 1-h

intervals and were adequate to control the specific growth rate at the selected level. The specific growth rate was controlled at the set point, and the calculated cell density agreed well with the experimental data. The glucose concentration was easily maintained at zero throughout the fermentation, and acetate was controlled below 2 g l^{-1}. The final cell density was approximately 100 g dry cell wt l^{-1} after 36 h cultivation, which is equal to or higher than that reported by other researchers for recombinant plasmid-containing *E. coli* expressing foreign proteins [8, 10, 12, 15, 18, 19, 22, 29, 30, 31, 33]. Plasmid stability decreased continuously throughout the fermentation to ~50% in the late fermentations, and the rHu-IFN-γ concentration increased slightly to 3.4 g rHu-IFN-γ l^{-1} (Fig. 1).

A problem often encountered in high-level expression systems, such as T7, is plasmid instability. Plasmid stability was not affected significantly by the addition of 5 g ampicillin l^{-1} in the feeding solution (data not shown). The expression of foreign proteins usually causes a significant reduction of specific growth rate of plasmid-containing cells, while that of plasmid-free cells remains high. This behavior will amplify the effect of cultural instability, resulting in a rapid decrease in the fraction of recombinant cells [13, 15]. In the T7 expression system, such instability is difficult to overcome by adding ampicillin, because the cells excrete β-lactamase which degrades ampicillin [13].

For this reason, fed-batch culture of recombinant *E. coli* BL21 (DE3)[pET3a-*hifn*-γ] was carried out under glucose-unlimited conditions. Based on data obtained from these experiments, an appropriate equation was used to calculate a decrease in the specific growth rate (Eq. 6) such that the formation of growth inhibitory metabolites could be avoided:

$$\mu = -0.004(t - t_0)^2 - 0.03(t - t_0) + 0.52 \quad 0.1 < \mu < 0.52 \tag{6}$$

where t is the time of fermentation and t_0 is the time when feeding started.

By substituting Eq. 6 into Eq. 4, the glucose-feeding rate was determined for a variable specific growth rate feeding strategy. The final cell density of ~100 g dry cell wt l^{-1} was reached after 20 h. Plasmid stability remained approximately constant throughout the fermentation, and rHu-IFN-γ increased slightly, to 1.2 g rHu-IFN-γ l^{-1}, by the end of the fermentation. These results showed that fermentation time was decreased and plasmid stability increased compared to the fed-batch process at constant specific growth rate (Fig. 1). Variations in plasmid stability with changes in specific growth rate have been reported [15, 29, 31]. However, these results indicate that the use of a variable specific growth rate is both more convenient and more efficient than the constant specific growth rate in a fed-batch process for HCDC of recombinant *E. coli* BL21 (DE3)[pET3a-*hifn*-γ].

It is well established that various *E. coli* strains accumulate acetate during growth on glucose. Acetate accumulation occurs when the carbon flux exceeds the

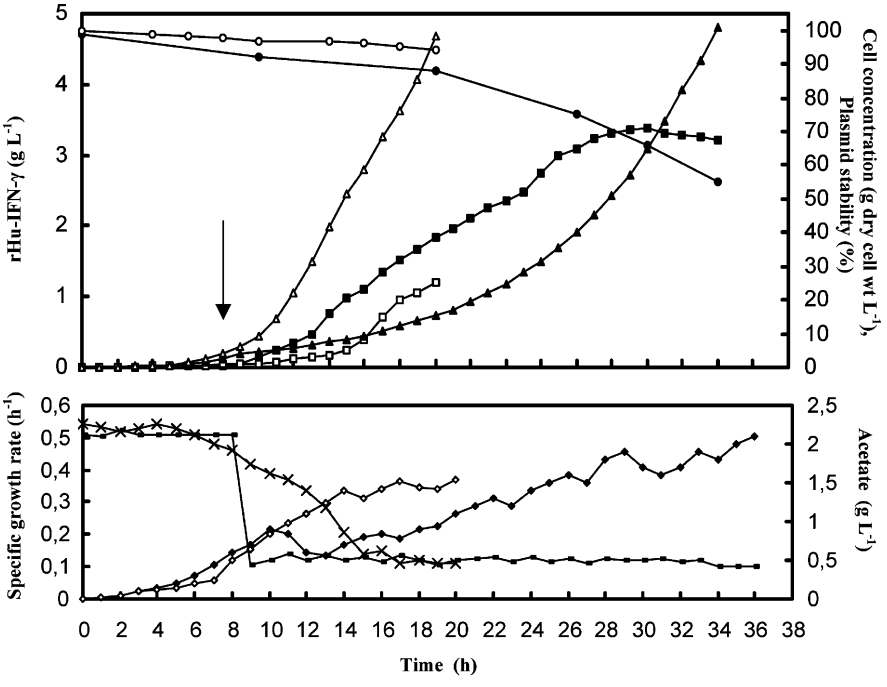


Fig. 1 Growth kinetics of recombinant *Escherichia coli* BL21 (DE3) harboring pET3a-*hifn*-γ in a 2-l bench top bioreactor containing 1 l of defined M9 modified medium using a fed-batch fermentation process. *Filled symbols* Exponential feeding rate with a constant specific growth rate (0.12 h^{-1}) according to Eq. 5. *Empty symbols* Exponential feeding rate with a variable specific growth rate according to Eqs. 4 and 6. ▲, △ Cell concentration, ■, □ rHu-IFN- γ concentration, ■, □ acetate concentration, ●, ○ plasmid stability, —, × specific growth rate. *Arrow* Start time of feeding

capacity of the Krebs cycle (Crabtree effect). Accumulation of acetate depends on the medium composition and on the strain and is connected to the growth and carbon source uptake rates. *E. coli* BL21 was derived from an *E. coli* B strain reported to be a low acetate producer compared to the *E. coli* K12 strain [26, 27]. For all fed-batch cultures, acetate concentration in the culture medium was below 2.5 g l^{-1}. This level of acetate concentration is much lower than the reported growth-inhibitory concentration of acetate [8, 26].

In all of the HCDC techniques, because of mass-transfer limitation, growth was limited by the dissolved oxygen concentration. In this research, HCDC was successfully obtained by controlling only the specific growth rate using exponential feeding. This approach is simple and efficient and does not need any special equipment, advanced computer controller, or special feedback control system.

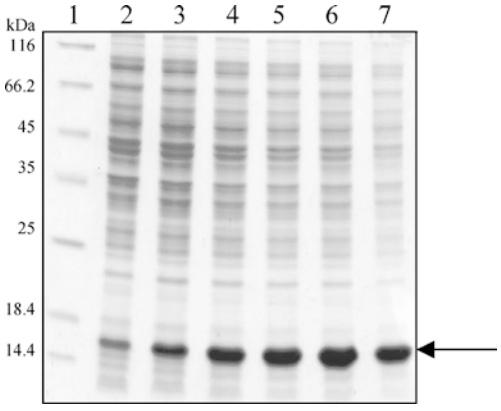


Fig. 3 SDS-PAGE analysis of the total cell lysate of *E. coli* BL21 (DE3) harboring pET3a-*hIFN-γ* from a variable specific growth rate fed-batch process. At 50 g dry cell wt l^{-1} the culture was induced with 3 mM IPTG. *Lane 1* Molecular mass marker, *lane 2* cell lysate before induction, *lanes 3–7* cell lysate at 1–5 h after induction. *Arrow* Position of rHu-IFN-γ

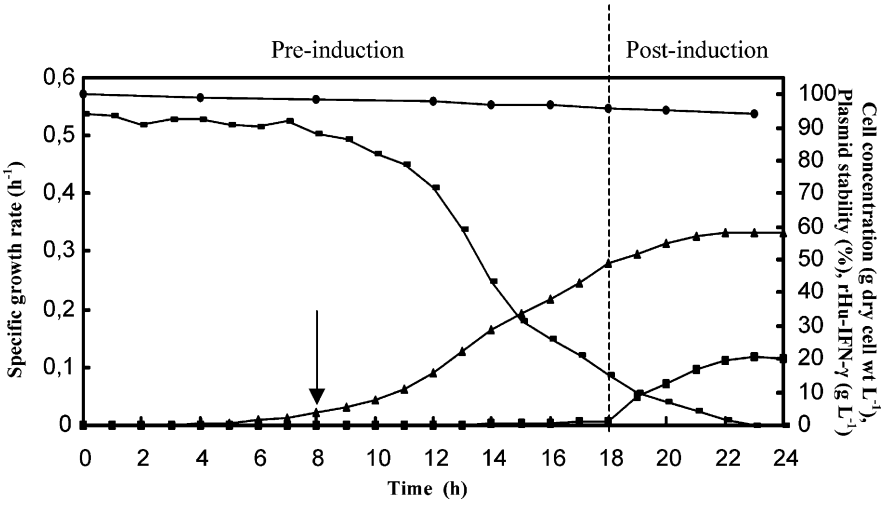
Production of rHu-IFN-γ in HCDC

In the fed-batch culture of recombinant *E. coli* BL21 (DE3)[pET3a-*hifn-γ*] at variable specific growth rate feeding strategy, rHu-IFN-γ expression was induced at a cell density of ~50 g dry cell wt l^{-1} by adding 3.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration) (Fig. 2). Overproduction of rHu-IFN-γ leads to a decrease in growth rate and inhibition of cell mass production, as reported by others [15, 31]. The maximum amount of rHu-IFN-γ was attained after 5 h post-induction (Fig. 3). The final cell density of about 58 g dry cell wt l^{-1} was reached at 23 h. The final specific yield and overall productivity of rHu-IFN-γ were as 0.35 g rHu-IFN-γ (g dry cell wt)$^{-1}$ and 0.9 g rHu-IFN-γ l^{-1} h^{-1}, respectively. These are higher than the results reported for recombinant proteins in HCDC [2, 10, 11, 15,17].

Fig. 2 The effect of IPTG-induction in high-cell-density culture of *E. coli* BL21 (DE3) harboring pET3a-*hifn-γ*, and production of rHu-IFN-γ using a variable specific growth rate feeding strategy according to Eqs. 4 and 6 in a 2-l bench top bioreactor containing 1 l of defined M9 modified medium. ▲ Cell concentration, ■ rHu-IFN- γ concentration, ● plasmid stability, — specific growth rate. *Arrow* Start time of feeding

Purification and quality control of rHu-IFN-γ

After solubilization of inclusion bodies, the solution was loaded on a Q-Sepharose-FF column and washed with buffer C. On this column, rHu-IFN-γ is not adsorbed but impurities such as proteases and other proteins are adsorbed on the bed. The fractions collected from Q-Sepharose-FF were loaded on a SP-Sepharose-FF column and washed with D_2 buffer to remove impurities. rHu-IFN-γ was then eluted with D_3 buffer. The fraction obtained from Sp-Sepharose-FF was diluted eight- to ten-fold with refolding solution and stored at 4 °C for 24 h. The refolded solution was loaded on a SP-



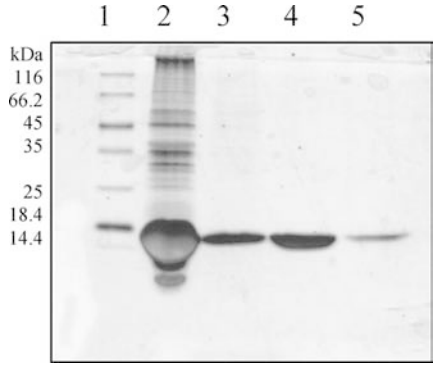


Fig. 4 Silver-stained SDS-polyacrylamide gel of fractions obtained from different steps of pilot-scale purification of rHu-IFN-γ. *Lane 1* Molecular mass marker, *lane 2* inclusion body, *lane3* Q-Sepharose-FF fraction, *lane 4* SP-Sepharose-FF fractions before refolding, *lane 5* SP-Sepharose-FF fractions after refolding

Sepharose-FF column pre-equilibrated with buffer E_1, the column was washed with buffer E_2, and rHu-IFN-γ was eluted with buffer E_3 (as described in Materials and methods). Figure 4 shows a silver-stained SDS-polyacrylamide gel of the various stages of rHu-IFN-γ production. The purity of rHu-IFN-γ after Q-Sepharose-FF and secondary SP- Sepharose-FF was about 85% and 99.9%, respectively.

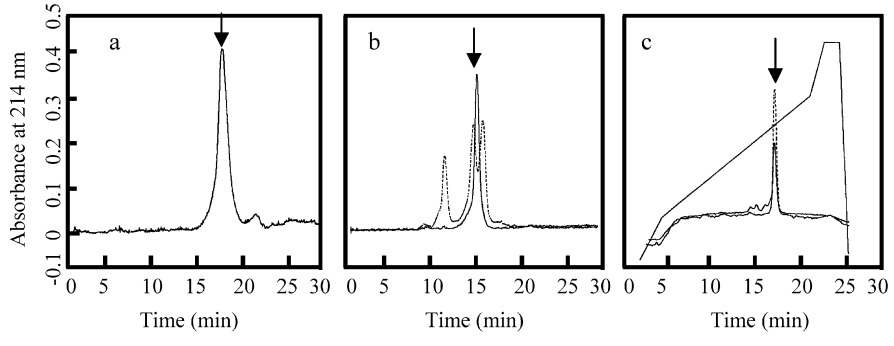
Compared to reported procedures for purification of rHu-IFN-γ, in our procedure, rHu-IFN-γ was purified in a denatured state in two chromatographic steps. The impurities were discarded during two chromatographic steps, the purified rHu-IFN-γ was refolded by reverse dilution, and then concentrated using a second cation-exchange chromatography step. This strategy increased the final yield of the purification method to approximately 100 mg rHu-IFN-γ (g dry cell mass)$^{-1}$ [$\sim$190 mg rHu-IFN-γ (g total cell protein)$^{-1}$] from inclusion bodies, which is higher than results reported for the purification of rHu-IFN-γ derived from *E. coli* [1, 9, 16, 17, 28, 34].

The quality-control results showed that the amount of host-cell and vector DNA were less than 100 pg/mg purified final rHu-IFN-γ. The bacterial endotoxin contamination level was less than 5 EU/mg purified final rHu-IFN-γ. Gel filtration chromatographic analysis of purified rHu-IFN-γ under native conditions showed that the final product did not contain considerable amounts of monomer and aggregate forms (Fig. 5a). This test, under denatured conditions in the presence of SDS, established the correct relative molecular mass ($\sim$17 kDa) of final purified rHu-IFN-γ. Its peak appeared between those of soybean trypsin inhibitor (20.1 kDa) and lysozyme (14.4 kDa) (Fig. 5b). Our final product did not contain covalent dimers or oligomeric forms, which are not as active as non-covalent dimers (Fig. 5c). Freshly prepared rHu-IFN-γ also did not contain significant amounts of ionic isoforms (deamidated, oxidized, and heterodimers) although during storage some isoforms with fewer positive charges than the main product appeared (data not shown) that were also detected in IEF gel electrophoresis. The biological activity of purified rHu-IFN-γ was determined and compared with that of commercial rHu-IFN-γ (Imukin, Boehringer). Compared to commercial rHu-IFN-γ (3×10^7 IU/mg protein), our rHu-IFN-γ had a specific antiviral activity of 2.8×10^7 IU/mg protein, which is comparable to data reported for the *E. coli*-derived rHu-IFN-γ [1, 16, 17, 34].

Conclusion

High-cell-density cultivation of recombinant *E. coli* was successfully established by controlling only the specific growth rate and by using exponential feeding. This approach is simple and efficient and does not require special equipment. The results obtained indicate that a variable specific growth rate feeding strategy is more convenient for over-expression of rHu-IFN-γ during fed-batch cultivation of recombinant *E. coli* BL21 (DE3)[pET3a-*hifn*-γ]. The purification procedure developed consisted of two steps of anion exchange

Fig. 5a–c HPLC analysis of the final purified rHu-IFN-γ. **a** Size-exclusion chromatographic analysis of final purified rHu-IFN-γ under native conditions for monomer and aggregate forms of rHu-IFN-γ. **b** Size-exclusion chromatography of final purified rHu-IFN-γ under denatured conditions in the presence of SDS in order to remove covalent dimers and oligomers of rHu-IFN-γ (*solid peak*). Molecular mass marker proteins (*dashed peaks*) are BSA (66 kDa), soybean trypsin inhibitor (20.1 kDa) and lysozyme (14.4 kDa). **c** Cation-exchange chromatography of final purified rHu-IFN-γ for removal of deamidated, oxidized, and heterodimer forms (*dashed peak*), and of standard commercial rHu-IFN-γ (*solid peak*). *Arrows* Peaks of final purified rHu-IFN-γ



(Q-Sepharose) and cation exchange (Sp-Sepharose) chromatography. These two strategies increased the final yield of rHu-IFN-γ production to approximately 100 mg rHu-IFN-γ (g dry cell mass)$^{-1}$ [$\sim$190 mg rHu-IFN-γ (g total cell protein)$^{-1}$], which is higher than the yields reported elsewhere in the literature [1, 9, 16, 17, 28, 34]. The results obtained from quality-control analyses also certify the product for clinical purposes.

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