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Cloning, High Expression and Purification of Recombinant Human Intereferon-β-1b in *Escherichia coli*

Dasari Venkata Krishna Rao · Chatadi Tulasi Ramu · Joginapally Venkateswara Rao · Mangamoori Lakshmi Narasu · Adibhatla Kali Satya Bhujanga Rao

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Abstract Sequential evaluation and process control strategy were employed for impurity profile and high recovery with quality of rhIFN-β-1b expressed in *Escherichia coli*. The high-level expression was achieved by using codon substitution (AT content of 52.6% at N-terminal region) and optimization of culture conditions. The addition of rifampicin at a concentration of 200 μg/ml has increased the specific product yield of 66 mg optical density^{-1} l^{-1} (43.5% of total cellular protein). Eighty-three percent of lipopolysaccharides, 32% of host deoxyribonucleic acid (DNA), and 78% of host cell proteins were removed by 0.75% Triton X-100 and 2 M urea wash. Eleven percent of lipopolysaccharides, 39% of host DNA, and 12% of host cell proteins were removed at the solubilization step. Ninety-two percent of protein refolding was achieved by high-pressure diafiltration method. Refolding by high-pressure diafiltration, bed height, and height equivalent to the theoretical plate value in chromatography column were identified as key parameters for high recovery with purity. Finally, the established process yielded 34% of purified protein with greater than 99% purity and is acceptable for preclinical toxicological studies. The purified rhIFN-β-1b obtained in this study is the highest that has been reported so far.

Keywords High-level expression · Recombinant human IFN-β-1b · Site directed mutagenesis · Specific product yield · High purity

Introduction

Interferons (IFNs) are a group of cytokine proteins, which possess antiviral, antiproliferative, and immunomodulatory properties [1, 2]. Two types of IFNs are recognized on

D. V. Krishna Rao (✉) · C. Tulasi Ramu · A. K. S. Bhujanga Rao
Research and Development, Biotechnology Division, Natco Research Centre, Hyderabad 500018, India
e-mail: krishnaraodv@yahoo.co.in

J. Venkateswara Rao · M. Lakshmi Narasu
Institute of Science and Technology, Department of Biotechnology,
Jawaharlal Nehru Technological University, Hyderabad 500072, India

the basis of their physical and biological properties. IFN-β is belongs to a family of IFN type 1 and used for effective treatment in multiple sclerosis [3]. IFN-β is encoded by a single gene and produced by fibroblasts. It is N-glycosylated at only a single site and produced in culture by human fibroblasts stimulated with double-standard ribonucleic acid (RNA) or poly (I), poly(C) in the presence of metabolic inhibitors [4]. Since a large quantity of IFN production from fibroblasts is difficult, it can be produced by molecular cloning technology using different host cells [5–9]. One type of recombinant IFN-β, which reduces the exacerbation rate in mass spectrometry is a modified, genetically engineered protein, in which an amino acid of cysteine at position 17 of human IFN-β protein is replaced with serine [10]. Muteins are mutationally altered biologically active proteins by site-directed mutagenesis. Hence, IFN-β-1b is a mutein of IFN-β in which cysteine^{-17} is changed to serine via a T to A transition in the first base of codon, which prevent incorrect disulfide bond formation. IFN-β-1b is a nonglycosylated protein that has 165 amino acids with an approximate molecular weight of 18,500 Da. It does not include the carbohydrate side chains as found in the native protein.

Escherichia coli is a popular host for the effective expression of nonglycosylated recombinant proteins [11–13]. Due to the sequence bias of genes in nature, messenger RNA (mRNA) stability, translational efficacy of foreign gene in native *E. coli*, and the potential toxicity of foreign protein to the host, many eukaryotic genes cannot be expressed effectively in the prokaryotic organism [14]. Major differences were found in codon usage in prokaryotes and eukaryotes for the expression of proteins by encoding genes [15, 16].

Some methods were explained for production and purification of IFN-β [17–19]. The end product obtained by adopting the methods cited in the available literature was not directly suitable as an active pharmaceutical ingredient for formulation. Conditions for high-level expression, impurity profile study, and refolding by pressurized diafiltration were not reported for rhIFN-β-1b. Since rhIFN-β-1b is a therapeutically useful protein, which is hydrophobic in nature, highly controlled process parameters are essential to get high recovery with quality.

In the present study, an attempt was made to develop a processing method by establishing a high yielding clone, culture conditions, impurity profile, refolding by pressurized diafiltration, bed height, and height equivalent to the theoretical plate (HETP) value in the chromatography column. Size exclusion and desalting were performed in a single-step operation. The experimental techniques and methodology described here results in high-level expression, aggregate free product (rhIFN-β-1b) of improved recovery, and better quality. The yield of purified protein obtained was two times higher than the results reported in earlier literature.

Materials and Methods

Materials

Plasmid vectors pET-3a and pT7 blue and *E. coli* strain BL21 (DE3) PLysS used were from our research laboratory. Trizol was purchased from Sigma, USA; complementary deoxyribonucleic acid (cDNA) synthesis kit, gel extraction kit, and polymerase chain reaction (PCR) cloning and purification kits were obtained from Qiagen, USA. Restriction endonucleases, T4 DNA ligase, and chemical reagents were procured from Invitrogen,

USA. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was supplied from Bangalore Genei, Bangalore, India. Chromatographic columns and resins were imported from GE Healthcare Uppsala, Sweden. Tangential flow filtration (TFF) system and cassettes were purchased from Pall Life Science. All the other chemicals were procured from local companies and are of molecular biology grade.

Cloning and Expression of rhIFN-β-1b

T lymphocytes were isolated from normal human peripheral blood by ficoll-hypaque gradient method. RNA isolation and poly (A) mRNA preparation from T lymphocytes was performed by oligo (dT) chromatography [20]. Human IFN-β cDNA was synthesized from mRNA using the following gene specific primers, including *Nde*1 and *Bam*H1 restriction sites (indicated by italics): Forward primer 5′-GCGAATT*CATATG*AGCTACAACTTGC TTG-3′ and reverse primer 5′-GATA*GGATCC*TCAGTTTCGGAGGTAACCTG-3′. The PCR amplified gene was subjected to site directed mutagenesis for alteration of an amino acid cysteine by serine at 17th position and subcloned into pT7 blue vector and sequenced.

The native gene sequence of human IFN-β-1b (IFN-$\beta^{ser\text{-}17}$) was altered by site-directed mutagenesis at immediate downstream of the initiation codon to the eighth codon. The forward primer 5′-GT*CATATG*AGTTATAACCTGCTTGGCTTTCTAC-3′ and reverse primer 5′-GATA*GGATCC*TCAGTTTCGGAGGTAACCTG -3′ were used. The PCR-amplified gene was cloned into pET-3a vector between the *Nde*1 and *Bam*H1 restriction sites. The *E. coli* BL21 (DE3) PLysS-competent cells were transformed with the recombinant vector (pET-3a+IFN-β-1b) and screened by using antibiotic-resistant markers (ampicillin and chloramphenicol). The recombinant clone was then subjected to protein expression by using Luria–Bertani medium for seed development and modified terrific broth (TB) medium (tryptone 12 g/l, yeast extract 24 g/l, glucose 7.5 g/l, KH_2PO_4 2.2 g/l, and K_2HPO_4 9.4 g/l) for expression. The protein expression was analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) [21] and silver-stained [22]. The yield was quantified by gel densitometry method using Imagequant gel documentation system (GE).

Evaluation of Some Culture Conditions and Fermentation

The effect of culture medium, concentration of inducer, and rifampicin were evaluated using the above high-yielding clone. The fermentation was performed in shake flasks at 2.0-l level using optimized culture conditions.

Harvesting and Isolation of IBs

The fermented broth (from shake flask; 1,000 ml) was harvested by centrifugation (Kubota, Japan) at 8,000 rpm for 30 min at 4 °C. The cell pellet was washed twice with 50 mM phosphate buffer pH 7.4. The wet pellet (16 g) was suspended in 65 ml of lysis buffer (100 mM Tris–HCl pH 8.0 containing 3 mM ethylenediamine tetraacetic acid [EDTA] and 1 mM phenylmethylsulfonyl fluoride [PMSF]), and the cells were lysed by sonication (Vibra cell, Sonics, USA) at 40% amplitude of power for 6 min using different sonication pulses such as 2, 5, 10, and 15 s, respectively. The inclusion bodies (IBs) were separated from lysate by centrifugation at 8,000 rpm for 30 min at 4 °C.

The IB pellet was sequentially washed with 0.75% Triton X-100 and 2 mM urea in each 65 ml of wash buffer (50 mM Tris–HCl pH 8.0, 2 mM EDTA, and 1 mM PMSF). The

pellet obtained was washed with 65 ml of pyrogen-free water. The concentration of Triton X-100 and urea were optimized.

IB Solubilization and Refolding

The washed IB was solubilized by using different denaturants such as 10% SDS, 2% sarkosyl, 6 M Guanidine HCl, and 8 M urea in 25 mM Tris–HCl pH 8.0 containing 20 mM dithiothreitol (DTT) and 1 mM EDTA. The protein complete dissolution was made by adding 1 N NaOH, and the concentration was adjusted 5.0 to 6.0 mg/ml. The solution was incubated at 25 to 28 °C for 45 min and spun down at 10,000 rpm for 30 min. The supernatant pH was adjusted to 8.2, and refolding was performed by diafiltration against 1.0% glycerol in water (pH 8.2) using the TFF system containing 5,000 Da molecular weight cutoff-regenerated cellulose membrane. The efficiency of refolding was monitored by reverse-phase liquid chromatography (RP-LC) analysis through change in the retention time of peak (reduced form to oxidized form). After refolding, the protein pH was adjusted to 6.5 with 0.5 M acetic acid and clarified by centrifugation at 10,000 rpm for 20 min at 4 °C.

Desalting

XK 26/1,000 mm column was packed with 370 ml of Sephadex G-75 fine matrix. The column temperature and flow rate were maintained at 20 °C and 45 cm/h, respectively, throughout the process. The column was serially equilibrated with five bed volumes of 0.2 M sodium acetate buffer pH 6.5 and five bed volumes of rhIFN-β-1b storage buffer (50 mM sodium acetate buffer, 0.1% SDS, 1.0% glycerol, pH 6.5). The refolded protein was loaded on the column and eluted with rhIFN-β-1b storage buffer. The single peak fraction containing rhIFN-β-1b was collected and characterized.

Analytical Methods for Impurity Quantitation

The cell lysis efficiency was measured by colony counting, residual endotoxin levels by LAL kit [23], host DNA by A_{260}, and hybridization technique and host cell proteins (HCPs) by enzyme-linked immunosorbent assay (ELISA) [24]. Protein aggregates during refolding and chromatography were measured photometrically at 340 nm [25].

Protein Characterization

Total protein was quantified by Lowry method using bovine serum albumin as the reference standard [26]; rhIFN-β-1b purity was measured by circular dichorism (CD), RP-LC [27], size exclusion liquid chromatography (SE-LC), and SDS-PAGE (15% gel) and quantified by Bradford [28]. Molecular weight was determined using Applied Biosystems 4800 matrix-assisted laser desorption/ionization (MALDI) time of flight instrument with alpha-hydroxy cinnamic acid as the matrix.

Antiproliferate Activity

A549 cells were seeded in 96-well plates, 5,000 cells per milliliter, in 1,000 ml complete Ham's F12K medium containing 1 mM L-glutamine, 1.5 g/l sodium bicarbonate, and 10% fetal bovine serum. rhIFN-β-1b was added to the cells (in triplicate) in serial dilutions, initiating at 125 ng/ml; cells were allowed to grow for 4 days, and the cell proliferation

assay was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Results and Discussion

Construction of Recombinant Clone and Expression of rhIFN-β-1b

The cDNA encoding the human IFN-β-1b gene was cloned as described under "Materials and Methods." The nucleotide sequence of the cloned gene was identical to that of native sequence (except at amino acid cysteine[17]) deposited in the Genbank (gi50593016). The rhIFN-β-1b expression was studied according to standard laboratory protocol (Sambrook et al., a laboratory manual) and was low (12 mg optical density $[OD]^{-1}$ l^{-1}). Therefore, further studies were carried out to improve the rhIFN-β-1b expression by employing codon substitution at the N-terminal region of the gene and optimization of culture conditions.

Effect of Codon Substitution on Expression of rhIFN-β-1b

The native gene codon sequence was altered at immediate down stream of the initiation codon to the eighth codon by incorporating six silent mutations. Upon the codon substitution, the protein expression was increased to 29 mg OD^{-1} l^{-1}. Though the variation in AT content (eight codons at N-terminal region) was not in a considerable level between altered (AT content 52.6%) and native genes (AT content 48.2%), the efficiency of expression was 2.4-fold higher in the altered gene over the native gene. The high-level expression in the altered gene may be due to codon replacement and has the potential to cause significant changes in stability of secondary structure of mRNA, immediately downstream of the initiation codon. The sequence with increased AT content (52.6%) at the N-terminal region may facilitate the entry of ribosome to enhance translation initiation and subsequent high-level protein expression. A similar effect with the AT-rich sequence was reported by other researchers [29]. Other studies also reported that the stable secondary structure of mRNA inhibits the translational efficiency [30, 31]. Other researchers have not described about the specific product yield of rhIFN-β-1b in *E. coli* by altering the AT content at the N-terminal region [10, 17, 19]. The high specific product yield has a significant impact in high recovery with acceptable quality [32].

Effect of Media Composition on the Expression of rhIFN-β-1b

The TB medium with 0.8% glucose was used as culture medium to maintain neutral pH and plasmid stability and prevent expression instability [33]. The cultures growing in certain complex medium induced the target protein expressions to high levels, and even the T7 promoter was used [34]. Therefore, in the present study, we have investigated the effect of TB serial media and defined serial media on cell growth, plasmid stability, and efficiency of rhIFN-β-1b expression. The cell growth reached a plateau after 6 h of induction in all samples, but lower OD values were observed in TB serial media samples over defined serial media.

The results reveal that the cell growth in the defined medium supplemented with 1% glucose was 1.75-, 1.30-, and 1.12-fold higher than normal TB medium, TB supplemented with glucose, and the defined medium supplemented with glycerol, respectively. The plasmid stability was maintained at the highest level till the end of process in all samples,

but a slight decrease was noticed in the postinduction period of samples cultured in the medium containing glucose as a carbon source (data not shown). The specific product yield in the defined medium supplemented with glucose was higher than other samples. Target protein expression was lower in the TB medium supplemented with glucose, among the media tested, probably because of the acid generated by glucose metabolism. This was clearly noticed by the fact that both cell growth and rhIFN-β-1b expression level in defined serial media were higher than TB serial media. After 6 h of induction, the pH of the culture in the defined medium supplemented with glucose was 6.7, whereas in the TB medium supplemented with glucose, it was 6.1.

The above studies clearly indicate that the metabolic changes are controlled by nutrient salts and thus lead to high-level expression. Generally, glycerol supports the cell growth by suppressing the metabolism of other carbon sources. It could balance the acid produced as a result of glucose metabolism and allow high-density cell growth but significantly less than that of glucose [35]. It was supported by our studies where the pH of the medium with glycerol was higher than the medium with glucose but with the cell density of the culture in medium with glycerol being lower than in the medium with glucose. The level of rhIFN-β-1b expression in the defined medium supplemented with glycerol was also higher than the TB medium supplemented with glucose.

Volumetric yield depends on both cell densities and specific product yield in cultures. In our study, to obtain higher cell density, cells grown in different media were induced at 3 h. The volumetric product yields obtained in the defined medium with glucose was 2.41-, 1.65-, and 1.29-fold higher in comparison to TB, TB supplemented with glucose, and defined medium supplemented with glycerol, respectively.

Effect of Inducer Concentration on Expression of rhIFN-β-1b

The available literature suggests that the optimal concentration of inducer for expression of heterologous genes is product specific [36]. In this study, the culture induced with 3 mM IPTG has shown specific product yield of 1.21-, 1.12-, and 1.25-fold higher and volumetric product yield of 1.14-, 1.08-, and 1.36-fold higher when compared with 1, 2, and 4 mM IPTG induction, respectively (Fig. 1). The high concentration of nonmetabolizable IPTG present in the cytoplasm probably might have caused toxicity to host cells [37], leading to lower yields when 4 mM IPTG was used. This clearly indicates the impact of inducer concentration on protein expression.

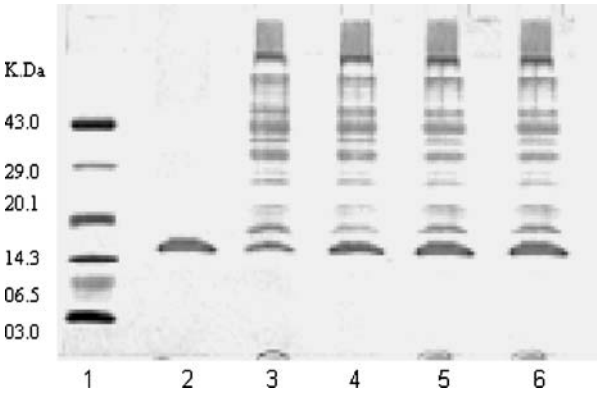

Fig. 1 Effect of inducer concentration on expression of rhIFN-β-1b in *E. coli* BL21 (DE3) PLysE. The cell pellet 25 μg from culture harvest equivalent was electrophoresed on 15% gel. *Lane* 1, molecular weight markers; *lane* 2, IFN-β-1b standard (Betaseron); *lanes* 3 to 6: Concentration of inducer from 1 to 4 mM

Similarly, the efficiency of expression was different in all samples in terms of soluble protein fraction. The variation observed was between 15% and 45% of total rhIFN-β-1b expression. The induction with 4 mM IPTG led to formation of IBs within 2 h after induction, while at other concentrations, the IBs were formed in 3 h (data not shown). The early IB formation may be attributed to the metabolic changes or burdens that probably lead to inhibition of protein expression. Previous studies have not described IPTG-related induction in detail for the expression of rhIFN-β-1b [5, 6, 10, 17, 19]. Hence, the present study becomes important for the large-scale production of recombinant protein production using *E. coli* as the host system.

Effect of Rifampicin on the Expression of rhIFN-β-1b

The initiation of RNA synthesis is inhibited by binding the rifampicin to the β-subunit of prokaryotic RNA polymerase [38]. The addition of rifampicin at 15–20 min of postinduction with IPTG leads to inhibition of *E. coli* RNA polymerase, which in turn affects the production of the HCPs [39]. To each of the culture, rifampicin was added separately at the concentration of 50, 100, 150, 200, and 250 μg/ml after 15 min of IPTG (3 mM) induction. After 6 h of induction, OD_{600} of the cultures were 7.8, 7.2, 6.5, 6.0, and 6.0, respectively, whereas the OD_{600} of culture untreated with rifampicin was 9.6 (Table 1). The rhIFN-β-1b-specific product yields were 52, 58, 62, and 66 mg OD^{-1} l^{-1} (34.3%, 38.3%, 40.9%, and 43.5% of the total cellular protein), respectively, leading to suppression of background proteins, with no further improvement at 250 μg/ml of rifampicin. While, untreated cells accounted for 45 mg OD^{-1} l^{-1} of rhIFN-β-1b (about 30% on total cellular basis) and background proteins were not suppressed. The results illustrate that the efficiency of protein expression was proportional, and the cell growth was inverse to the concentration of rifampicin used. Contrary to higher protein expression level, the increase in cell density of the rifampicin-treated culture was only 10% after induction, while cell density accounted for 70% in the untreated culture.

The soluble fraction in the cells treated with rifampicin was 14% of total rhIFN-β-1b, while in untreated cells, it was about 27%. The increase in concentration of rifampicin addition increases the suppression of RNA synthesis; resultantly, the molecular chaperons content are decreased in the cells. But, there are enough HCPs for the cells to survive, and rifampicin-resistant T7 RNA polymerase triggers the transcribing target gene. The

Table 1 Effect of rifampicin concentration on expression of rhIFN-β-1b that the cells were induced with 3 mM IPTG.

Sample number	Rifampicin (μg/l)	Growth (OD_{600})	SPY[a] (mg OD^{-1} l^{-1})	VPY[b] (mg/l)	Expression (%)	Avarage error (%)
1	Control	9.6	45	432.0	29.7	2.5
1	50	7.8	52	405.6	34.3	3.2
2	100	7.2	58	417.6	38.3	3.8
3	150	6.5	62	403.0	40.9	3.6
4	200	6.0	66	396.0	43.5	2.8
5	250	6.0	66	396.0	43.5	3.2

The above values are the mean of four experiments.

[a] *SPY* Specific product yield

[b] *VPY* Volumetric product yield

molecular chaperons are limited in the cytoplasm, and as such, the target protein was not subjected to complete refolding to the native form resulting them to form IBs [40, 41].

Purification of Human rhIFN-β-1b

Cell lysis and IBs isolation

The rhIFN-β-1b IBs was isolated by lysing the bacterial cells, and the efficiency of cell lysis was observed at different sonication pulses of 2, 5, 10, and 15 s. Although the cell lysis efficiency (by plating technique) was almost similar at all sonication pulses, the protein IB recovery (94%) was high at a sonication pulse of 5 s (data not shown). The low efficiency of IB recovery with higher pulsing could be due to fragmentation of IBs and its subsequent passing off into the supernatant. No clarity was evident in the previous reports for rhIFN-β-1b [17, 19]. Thus, optimization of the initial step of the downstream process will have an impact on overall product recovery with purity.

IBs Washing

The IBs isolated were found to be contaminated with endotoxins, host DNA, and HCPs. These impurities interfere with refolding and significantly affect the process yield and purity [42]. Therefore, the rhIFN-β-1b IBs were washed with detergents and impurity profile (endotoxins, HCP, and host DNA) was established by measuring the supernatant of Triton X-100 and urea wash using LAL kit, ELISA, and by A_{260}. The removal of impurities did not vary much significantly with different concentrations of Triton-X100 ranging from 0.25% to 1.0%. However, the recovery of protein IB was best with 0.75% of Triton-X100 (Table 2). The increased concentration of Triton-X100 leads to an increase in viscosity of wash buffer causing improper sedimentation of IBs.

In this step, the effect of urea on removal of host impurities was evaluated. Urea wash (2 M) showed 41±4% of lipopolysaccharides [LPS] and 48±4% of HCP and 22% on nucleic acid removal. The purity of rhIFN-β-1b was increased from 79% to 89%, but step

Table 2 Summary of impurity profile and recovery of rhIFN-β-1b inclusion bodies during Triton X-100 wash.

Triton X-100 (%)	LPS[a] (EU)	DNA (pg/ml)	HCP[b] (%)	Inclusion body[c]		Avarage error (%)
				Purity (%)	Recovery (%)	
IB pellet (before wash)	1,400	1,300	50	50	94	1.7
0.25 (supenatant)	880	175	24	74	90	2.3
0.50 (supenatant)	940	175	28	78	88	3.8
0.75 (supenatant)	990	175	29	79	88	3.0
1.00 (supenatant)	992	175	32	82	78	3.9

Data are means of four measurements.

[a] *LPS* Lipopolysaccharides

[b] *HCP* Host cell protein

[c] The inclusion bodies were suspended in Tris–HCl buffer (pH 8.0) and washed with Triton-X100 at 0.25%, 0.50%, 0.75%, and 1.0% concentrations.

Table 3 Summary of impurity profile and recovery of rhIFN-β-1b inclusion bodies during urea wash.

urea concentration (M)	LPS[a] (EU)	DNA (pg/ml)	HCP[b] (%)	Inclusion body[c]		Avarage error (%)
				Purity (%)	Recovery (%)	
IB from Triton wash	410	1,125	21	79	–	2.9
2.0	170	250	10	89	85	3.3
3.0	195	375	12	91	66	1.7
4.0	230	520	13	92	48	3.7
5.0	260	710	15	94	35	2.5

Data are means of four measurements.

[a] *LPS* Lipopolysaccharides

[b] *HCP* Host cell protein

[c] The Triton washed inclusion bodies were suspended in Tris–HCl buffer (pH 8.0) and washed with urea at 2.0, 3.0, 4.0, and 5.0 M concentrations.

recovery was 85% only (Table 3). The use of 3, 4, and 5 M urea had a positive impact on removal of host impurities such as 47%, 56%, and 64% of LPS, and 33%, 46%, and 63% of host DNA, respectively. The purity was improved from 79% to 94% but strong negative impact on the recovery front, which dropped to 66%, 48%, and 35% respectively (Table 3). Available literature indicates the use of urea for washing of some other recombinant protein IBs [42–45]. There is no information available in the literature about the evaluation of impurity profile of rhIFN-β-1b [10, 17, 19]. At best, the use of twin detergents like Triton-X100 at 0.75% concentration and urea 2 M concentration have shown a significant impact on impurity removal and increased the IB recovery.

IB Solubilization

The washed rhIFN-β-1b IBs was biologically inactive and still contaminated with some host impurities. To attain it's biologically activeness, the IBs are to be solubilized and refolded. During this, the cell debris is also co-solubilized and interferes in the refolding process, which in turn affects the process yield and quality [42]. Therefore, a set of initial experiments were performed by solubilizing the IBs in 50 mM Tris–HCl pH 8.0 containing 20 mM DTT with 10% SDS, 2% Sarkosyl, 8 M urea, and 6 M Guanidine HCl. The protein concentration was adjusted to 5.0 to 6.0 mg/ml, and the IBs were completely solubilized at pH ~12.0, where the host impurities/cell debris was also completely dissolved. Therefore, the pH was gradually decreased to separate the host impurities. During this, protein aggregation was observed at pH 8.5 in all samples, whereas partial aggregation was noticed at the 10% SDS-used sample. This may be due to the association of hydrophobicity, conductivity, concentration of protein, and denaturant agents, etc.

Based on the above findings, solubilization experiments were performed at different concentrations of protein such as 1.0, 2.0, 3.0, and 4.0 mg/ml using 10% SDS/20 mM DTT in 25 mM Tris–HCl. High rate of protein aggregation was observed at 3.0 and 4.0 mg/ml of concentration at pH of 8.5, while no protein aggregation was observed at 1.0 and 2.0 mg/ml. But, host impurities were not precipitated. Therefore, the pH was decreased to acidic condition (pH 6.0), where the host impurities were precipitated without protein aggregation. The results incorporated in Table 4 clearly illustrates that 64% of LPS and 57% of host DNA were precipitated with 95% protein recovery at concentration of 2.0 mg/ml at pH 6.0.

Table 4 Summary of IBs solubilization at different pH.

Solubilization pH	LPS[a] (EU)	DNA (pg/ml)	HCP[b] (%)	inclusion body recovery (%)	Avararage error (%)
IB from urea wash (pH 7.0)	240	875	11	–	3.9
4.0	45	250	6	45	3.2
6.0	85	375	5	94	3.8
8.0	160	650	2	90	3.1
10.0	215	780	10	95	3.6
12.0	240	875	11	99	2.8

Data are means of four measurements. The washed IBs were solubilized in 10% SDS in 25 mM Tris–HCl containing 20 mM DTT as a reducing agent.

[a] *LPS*: Lipopolysaccharides

[b] *HCP* Host cell protein

Whereas, 19% of LPS and 12% of host DNA were precipitated from the same concentration with a recovery of 90% at pH 8.5. The removal of host-related impurities at the solubilization step has a significant impact in shortening the purification process.

Refolding

The denatured and solubilized rhIFN-β-1b protein was still contaminated with some host impurities, and it attracts multiple chromatography steps before or after refolding [42]. In this study, the solubilized rhIFN-β-1b protein (2.0 mg/ml) pH was increased to 8.2 with 0.5 M Tris–HCl pH 8.5, and diafiltration was performed against 1.0% glycerol in water for injection pH 8.2 at 15–19 °C using feed/retentate pressure of 1.8/0.6 bar till completion of the refolding process. After 8 h of diafiltration, the efficiency of refolding was about 60%, but protein recovery was only 20%. This may be attributed to the high concentration of protein and removal of the denaturant at high pressure, which leads to misfolding and aggregation. Therefore, a set of experiments was performed at different concentrations of protein such as 0.5, 1.0, 1.5, and 2.0 mg/ml using similar conditions. These experiments revealed that 0.5 and 1.0 mg/ml concentration of protein have increased refolding and recovery efficiency over 1.5 and 2.0 mg/ml. Though the results were satisfactory, they were unexpected. Therefore, in order to increase the refolding and minimizing of the aggregate formation during diafiltration, we have set up the experiments at different pressure conditions like feed/retentate pressures of 0.9/0.3, 1.2/0.4, 1.5/0.5, and 1.8/0.6 bar. The efficiency of refolding from the reduced form to the oxidized form was monitored by RP-LC analysis through drifting of the retention time of the peak.

The results obtained showed a variation in refolding efficiency and protein recovery at all pressure conditions and had a similar effect on removal of endotoxins and HCPs as expected. However, host DNA was not at all removed. The high recovery of refolded protein (65%) was observed at a feed/retentate pressure of 1.2/0.4 bar; whereas the recovery of the refolded protein in samples diafiltered at 0.9/0.3, 1.5/0.5, and 1.8/0.6 bar was 47%, 56%, and 52%, respectively (Table 5). The permeate was collected under atmospheric pressure at 15–20 °C. The permeate fluxes for the feed/retentate pressure of 0.9/0.3, 1.2/0.4, 1.5/0.5, and 1.8/0.6 bar were 2.9, 4.8, 5.2, and 5.3 l m^{-2} h^{-1} and the transmembrane pressures (TMP) were 0.6, 0.8, 1.0, and 1.2, respectively, throughout the refolding process. The results reveal that the permeate flux was increased till the TMP value of 0.8 with a later decline. This indicates that the TMP value more than 0.8 imposes the physical stress on the

Table 5 Summary of rhIFN-β-1b refolding.

Feed/retentate pressure (Bar)	LPS (EU)	Host DNA (pg/ml)	HCP (%)	Refolding efficiency (%)	Protein yield (%)	Avarage error (%)
Solubilized protein	85	375	5	–	–	3.4
0.9/0.3	04	195	3	85	47	3.9
1.2/0.4	>1	145	3	92	65	3.0
1.5/0.5	>1	125	2	88	56	3.5
1.8/0.6	>1	120	2	85	52	3.7

Data are means of four measurements. The solubilized IBs were refolded by diafiltration at different pressure conditions using a TFF system.

protein, which in turn may lead to structure damage/misfolding/aggregation during the diafiltration. The variation in the refolding and recovery may be due to high physical stress on protein and membrane by longer exposure time and high feed/retentate pressures. Available literature indicates the use of a high-pressure system for refolding of some other recombinant proteins [43–45] but not for rhIFN-β-1b [10, 17, 19]. The results of our experiments demonstrated an inverse relationship between SDS removal and refolding.

Purification by Gel Filtration Chromatography

In this study, different types of resins like Sephacryl-200, Sephadex G-25, Sephadex G-75, and Sephadex G-100 were used, and Sephadex G-75 (fine) was chosen as suitable matrix. The protein-to-resin ratio such as 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml were studied, and 0.2 mg/ml was

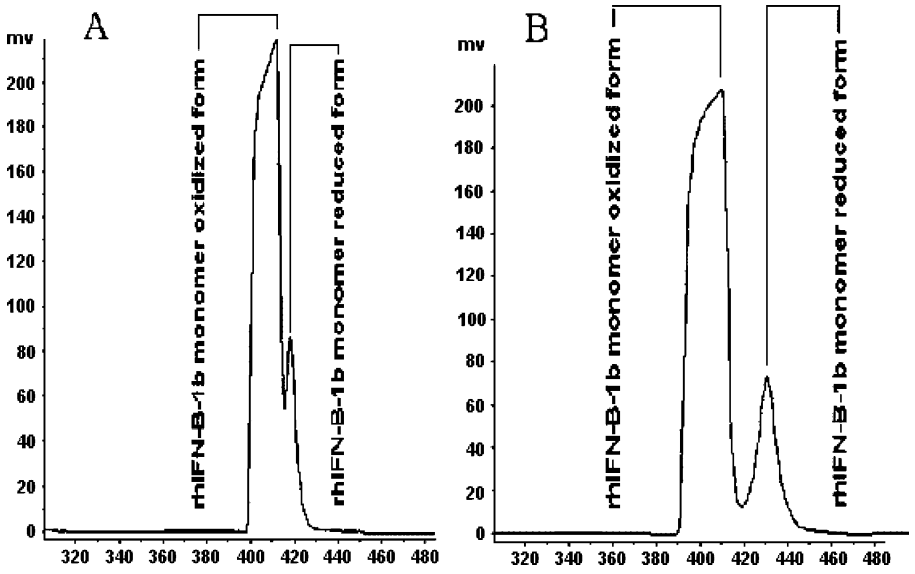

Fig. 2 **a** Desalting chromatography of rhIFN-β-1b on sephadex G-75 fine matrix. Elution was carried out with rhIFN-β-1b storage buffer (50 mM sodium acetate, 0.1% SDS and 1.0%, pH 6.5). The peaks represent the improper separation of oxidized and reduced forms of rhIFN-β-1b. **b** Desalting chromatography of rhIFN-β-1b on sephadex G-75 fine matrix. Elution was carried out with rhIFN-β-1b storage buffer (50 mM sodium acetate, 0.1% SDS and 1.0%, pH 6.5). The peaks represent the clear separation of oxidized and reduced forms of rhIFN-β-1b

found to be optimal (based on the separation of aggregates).The XK 26/400 mm column was packed with 175 ml of resin, and 35 mg of protein was loaded after equilibration with IFN-β-1b storage buffer at a linear flow rate of 45 cm/h. The protein was eluted with the same buffer and found to be free from salts of the previous step (conformed by conductivity), but it was contaminated with closely related impurity by peak merging due to improper separation (Fig. 2a). Therefore, bed height was standardized by performing the experiments at different bed heights such as 55, 60, 65, and 70 cm using the same flow rate of 45 cm/h. Maximum resolution was observed at a bed height of 70 cm. HETP values were established by packing the column at different pressure conditions (0.1 to 0.3 MPa). Clear resolution and high

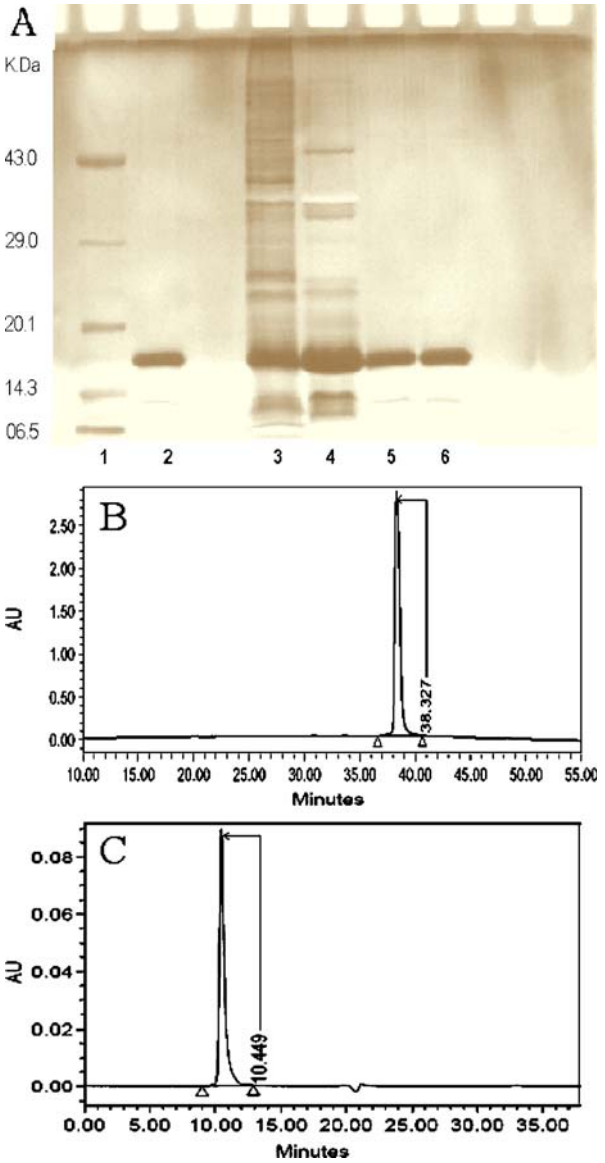

Fig. 3 **a**–**c** The purity profile of rhIFN-β-1b expressed in *E. coli*. **a** The purity of rhIFN-β-1b was observed by 15% reduced PAGE (silver-stained) gel. 20 μg of each sample was loaded for analysis. Lane 1, molecular weight marker; lane 2, reference standard (Betaseron); lane 3, fermentation sample; lane 4, washed inclusion body pellet; lane 5, refolded rhIFN-β-1b and lane 6, purified rhIFN-β-1b (desalting column). **b** RP-LC analysis of rhIFN-β-1b: The RP-LC was carried out using a ZORBAX 300SB C-18 (4.6×250 mm) column on Waters chromatography system with photodiode array (PDA) detector. The mobile phase consisted of 0.1% (v/v) trifluoroacetic acid (TFA) in water (A) and 0.1% (v/v) TFA in acetonitrile (B). Flow rate was maintained as 0.5 ml/min using a linear gradient of A to B. Analysis was carried out at a wavelength of 214 nm. **c** SE-LC analysis of rhIFN-β-1b: The SE-LC was carried out using TSK gel G 2000 SW (7.5× 300 mm, 10 μm) column on waters chromatography system with photodiode array (PDA) detector. The mobile phase consisted of 0.68% H_3PO_4 in water pH 2.5. Flow rate was maintained as 0.5 ml/min and analysis was carried out at a wavelength of 280 nm

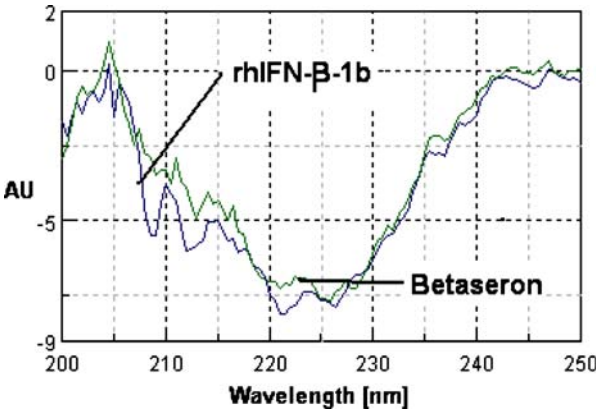

Fig. 4 The native conformation of rhIFN-β-1b was evaluated by CD. The rhIFN-β-1b was tested along side innovator product (Betaseron) by Jasco J-715 CD Spectropolarimeter. The sample concentration was 10 μg/ml

recovery of protein was observed at a HETP value of not less than 11,000 theoretical plates per meter. Therefore, the XK 26/1000 column was packed with 370 ml of Sephadex G-75 fine resin and maintained a HETP value of 14,500 theoretical plates per meter. The protein (75 mg) was loaded after equilibration and eluted with the same buffer at the same flow rate. The results clearly illustrate the significance of bed height and HETP value on the resolution of rhIFN-β-1b monomer from its related impurities (Fig. 2b).

In earlier reports, significance of bed height and HETP value in chromatography was not studied for rhIFN-β-1b [10, 17, 19]. In the present study, an attempt was made to address the significance of bed height and HETP value on chromatography. Desalting and size exclusion were simultaneously performed in a single step. The novel experimental techniques and methodology described here result in an aggregate-free product (rhIFN-β-1b) with improved recovery and better stability.

Table 6 Summary of analytical profile of rhIFN-β-1b at different stages.

Impurity profile	LPS[a] (EU)	DNA[b] (pg/ml)	HCP[c] (%)	Total[d] (mg)	IFN-β-1b[e] (mg)	Purity[f] (%)	Step yield[g] (%)	Overall yield[h] (%)	Avarage error (%)
Cell mass	1,600	1,400	70	1,440	432	30	30	100	2.0
Crude IB pellet	1,400	1,300	50	812	406	50	94	94	2.8
Washed IB pellet	240	875	11	342	304	89	75	70	3.4
Solubilization	85	375	05	301	286	95	94	66	3.0
Refolding	>1	145	02	190	186	98	65	43	3.5
Desalting	>1	95	Nill	149	149	100	80	34	2.7

Data are means of four measurements.

[a] LPS was measured by LAL test.

[b] DNA was measured by A_{260} and hybridization technique.

[c] HCP was measured by ELISA test.

[d] Total protein was measured by Lowry method.

[e] IFN-β-1b was measured by gel densitometry, RP-LC, and Bradford method.

[f] Purity was measured by RP-LC, SE-LC, and SDS-PAGE.

[g] Step yield was measured by difference between output and input of the corresponding step.

[h] Overall yield was measured by difference between output of the corresponding step and input of the initial step.

Characterization of Human rhIFN-β-1b

The protein obtained was characterized by using Betaseron® as the reference standard. The purity of the protein was more than 99% by PAGE (Fig. 3a), RP-LC (Fig. 3b), and by SE-LC (Fig. 3c).The protein was analyzed by CD along side the innovator sample, and the overlaid CD spectra reveal that the native form of rhIFN-β-1b was on par to the Betaseron (Fig. 4).The quantitative analysis reveals that the purified protein yield was 9.3 mg/g of wet cell mass (34%) by RP-LC, Bradford, and SDS-PAGE (gel densitometry). The product impurity profile reveals that endotoxins and host DNA levels were within the acceptable limits (Table 6). The molecular weight of the protein was found to be 18,508.20 Da by MALDI analysis against the theoretical (calculated) mass weight of 18,510.2 Da. The biological activity of the rhIFN-β-1b was tested as described under analytical methods and found to be comparable to innovator product (2×10^7 IU/mg). Based on the above results, the product has been found to be acceptable for preclinical toxicological studies.

Conclusion

The present work describes a high-yielding clone, culture conditions, process control, and sequential evaluation strategy for high recovery with purity of rhIFN-β-1b expressed as IBs in *E. coli*. Codon substitution at the N-terminal region (AT content increase) and evaluation of culture conditions have increased the protein expression. The process control strategy for the impurity profile and refolding by high-pressure diafiltration (TFF) has improved the protein recovery and quality. The bed height and HETP value at the gel filtration column resulted in the shortening of the purification process, and they have a key impact on product yield, stability, cost, and process time. The established and optimized process yielded 34% of rhIFN-β-1b with more than 99% purity. The cost-effective and economically feasible process developed in this study is extremely beneficial to the healthcare community and also finds an application in the development of other therapeutic proteins expressed in *E. coli*.

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