



STUDY ON INFLUENCE OF ADDITIVES ON REFOLDING AND DIALYSIS OF RECOMBINANT HUMAN INTERFERON GAMMA

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Abstract

Recombinant protein accumulated intracellularly is frequently laid down in the form of inclusion bodies. So, dialysis and refolding condition optimization can be advantageous to standardize purification protocol with minimum protein yield loss. Recombinant Human interferon gamma (rhIFN- γ) IB was solubilized at alkaline pH (8.0) in 6 M Guanidinium HCl solution. The solubilized proteins were checked for protein loss when refolded and dialyzed at different buffer pH and in presence of different additives like β ME (β -Mercapto Ethanol), Tween-20, Mannitol and their combination. Phosphate buffer at 50mM concentration (pH 6.5) minimized the protein loss. Tween- 20 (0.01% W/V) increased recovery of protein from inclusion bodies.

Key words; rhIFN- γ , protein expression, refolding, dialysis, additives.

1. INTRODUCTION

Human interferon gamma is a cytokine controlling many immunologically relevant genes. The recombinant rhIFN- γ produced in *Escherichia Coli* has molecular mass of 16.7 kD and is not glycosylated, but physiologically active. *E. Coli* is advantageous as expression system providing rapid growth, cheap cultivation, well studied genetic background, and high expression level in large scale cultures [1, 2]. 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. Structurally, rhIFN- γ monomers have highly hydrophobic domains and contain no disulfide bonds. To achieve an active configuration, these monomers must be refolded and dimerized. Complex aggregate formation and misfolding is to be avoided in the isolation and purification procedures. [2] Inclusion bodies produced in *Escherichia Coli* are composed of densely packed denatured protein molecules in the form of particles. Refolding of inclusion body proteins into bioactive forms is cumbersome, results in poor recovery and accounts for the major cost in production of recombinant proteins from *E. coli*. With new information available on the structure and function of protein aggregates in bacterial inclusion bodies, it has been possible to develop improved solubilization and refolding procedures for higher recovery of bioactive protein [3,12]. Inclusion bodies are formed from partially folded protein intermediates and are composed of aggregates of mostly single types of polypeptide. This helps to isolate and purify the protein aggregates to homogeneity before solubilization and refolding [6,7, 8]. Proteins inside inclusion body aggregates have native-like secondary structures. It is assumed that restoration of this native-like

secondary structure using mild solubilization, refolding and dialysis conditions will help in improved recovery of bioactive protein in comparison to solubilization using a high concentration of chaotropic agent. Analysis of the dominant forces causing aggregation during inclusion body formation provides information to develop suitable mild purification procedures for inclusion body proteins. Refolding from such solubilized protein will be very high due to restoration of native-like secondary structure [6,7,8,9]. Typically, purified rhIFN- γ is refolded in vitro with or without arginine as an aggregation suppressor [9,10,11]. Recombinant Human interferon gamma (rhIFN- γ) inclusion bodies were homogenized from *E. coli* cells before solubilization and refolding. Pure inclusion bodies were solubilized at alkaline pH (8.0) in the presence of 6M Guanidinium HCl solution. The solubilized proteins were checked for protein loss when refolded and dialyzed at different buffer pH and in presence of different additives. Dialysis of recombinant human interferon gamma (rhIFN- γ) in 50mM Phosphate buffer at pH 6.5 along with the additives (0.01% Tween-20) help to prevent precipitation of protein during dialysis and refolding. Additive helps to increase protein yield up to 6% than the yield that can be achieved without additive.

II. MATERIALS AND METHODS

A. Plasmid and Bacterial strain

Competent *E. coli* BL21 cells were transformed with pET23 expression vector containing the rhIFN- γ DNA. Kanamycin was used for selection of transformant cells. For all experiments pre inocula were cultured overnight at 250 r.p.m. at 37 °C.

B. Culture media

Liquid broth containing 50 μ g/ mL kanamycin was routinely used to grow *E. Coli* BL21. The broth contained (per liter): 4g glucose, 12 g tryptone, 24 g yeast extract, 0.4 g MgSO₄ and 50 mg kanamycin. The pH was adjusted to 7.0 prior autoclaving for 15 minutes at 121 °C. Glucose and MgSO₄ solutions were autoclaved separately.

C. Recombinant human interferon gamma expression

Cultures were generated in 500 mL flasks containing 100 mL medium and shaken at 250 r.p.m. Each flask was inoculated with 5 mL of pre inocula to provide an initial optical density at 600 nm (OD_{600nm}) of 2.0. Cells were grown at 37 °C. Cells were induced with 0.5 mM IPTG (Isopropyl-beta-thio galactopyranoside) concentration. Before induction (BI) and after induction (AI) samples were analyzed by SDS-PAGE for rhIFN- γ protein expression. After 90 minutes, cells were harvested by centrifugation at 5000 r.p.m. for 10 minutes. These pellets were processed for inclusion body (IB) isolation [13].

D. Isolation of inclusion bodies (IBs)

Pelleted cells were homogenized in 50 mM Tris, 20mM EDTA (Ethidium diamine tetra acetate), pH 8.0 (T₅₀E₂₀) buffer at 11,000 r.p.m. for 2 minutes. Cells were lysed at 0.3 mg/mL lysozyme concentration on rotary shaker for 1 hour at room temperature. Cells were further lysed at 0.5 M NaCl and 3% Triton X-100 concentration, under shaking condition at 350 r.p.m. for 1 hour. Solution was centrifuged at 8000 r.p.m. for 90 minutes. Pellet was washed with 25% Triton X-100 followed by two washes with T₅₀E₂₀. During washing steps homogenization was done at 19,000 r.p.m for two minutes followed by centrifugation at 8000 r.p.m. for 90 minutes [14].

E. Solubilization of Inclusion Bodies

In each experiment, 25 mg of protein was solubilized in 2.5 ml of Solubilization buffer (0.1 mM Tris, pH 8.0, 2 mM EDTA, 6 M Guanidinium HCl) [14].

F. Dialysis pH optimization

Solubilized protein was added to 250 ml of refolding buffer containing 0.1 M Tris pH- 8.0, 2 mM

EDTA, 0.5 M L-Arginine. (Final concentration of protein 0.1 mg/ml). The protein was refolded for 48 hrs at 60 °C using a pulsatile renaturation process. From refolded protein, 100 ml was dialyzed against 500 ml of 50mM Phosphate buffer at different pH (6.5, 7.0, and 7.5) for 48h. Three changes of fresh buffer were given at 16hrs, 24 hrs and 48 hrs. Samples were collected at 48 h for protein analysis and SDS-PAGE analysis [15].

G. Refolding and dialysis in Presence of different additive

Solubilized protein was added to 250 ml of refolding buffer (Final concentration of protein 0.1 mg/ml). The protein was refolded for 48 hrs at 60 °C using a pulsatile renaturation process. From refolded protein 100 ml was dialyzed against 500 ml of respective dialysis buffers for 48 h. Three changes of fresh buffer were given at 16hrs, 24 h and 48 h. Samples were collected at 48 h for protein analysis and SDS-PAGE analysis [15].

Refolding Buffers used:

- 0.1 M Tris pH- 8.0, 2 mM EDTA, 0.5M L-Arginine
- Buffer a + 0.1% (V/V) βME (β-Mercapto Ethanol)
- Buffer a + 0.01 % (W/V) Tween-20
- Buffer a + 4 % (W/V) Mannitol
- Buffer a + 0.01% (W/V) Tween-20 and 4 % (W/V) Mannitol.

Dialysis Buffers used:

- 50mM Phosphate Buffer, pH- 6.5,
- Buffer a + 0.1% (V/V) βME (β-Mercapto Ethanol)
- Buffer a + 0.01 % (W/V) Tween-20
- Buffer a + 4 % (W/V) Mannitol
- Buffer a + 0.01% (W/V) Tween-20 and 4% (W/V) Mannitol.

H. SDS-PAGE Analysis

500 µl of dialyzed protein sample was taken and centrifuged at 12,000 r.p.m. for 30 minutes at 4 °C. To the pellet, 500 µl of 1X non reducing (NR) dye was added. To 150 µl of supernatant, 350 µl of 1X NR dye was added. Rest of the supernatant was filtered through 0.45 µm syringe filter and 150 µl of filtered supernatant taken and 350 µl of 1X NR was added. The 1x NR dye contained per 100 mL: 12.5 mL stacking gel buffer, 10mL glycerol, 2 g SDS and 0.5 mg bromo phenol blue. From all the three samples 20 µl was loaded in the well for SDS-PAGE analysis. The Bio-Rad Protean gel-electrophoresis system was used in the present study. SDS-PAGE analysis was performed using 15% (W/V) polyacrylamide gels. The electrophoresed gels were stained with Coomassie Brilliant Blue (CBB) G-250 [15].

I. Protein analysis

Protein samples withdrawn were analyzed by standard Bradford's protein assay method [16].

II. RESULT AND DISCUSSION

A. Cloning and expression

The rhIFN-γ expressed in transformed and induced *E. Coli* BL21 cells demonstrated to be approximately 51% of the total cell proteins and was harvested as inclusion bodies (Fig.1). Homogeneity of the transformed gene expression in all four different flasks was as shown in Fig.1. These inclusion bodies were then denatured and dialyzed using different pH buffers and additives.

B. Cell lysis and IB purification

The crude IB preparation was washed with Triton X-100 to remove *E. Coli* proteins and other soluble components. The procedure also removes some amount of rhINF-α while T50E20 buffer wash did not showed any protein loss as shown in Fig.1.

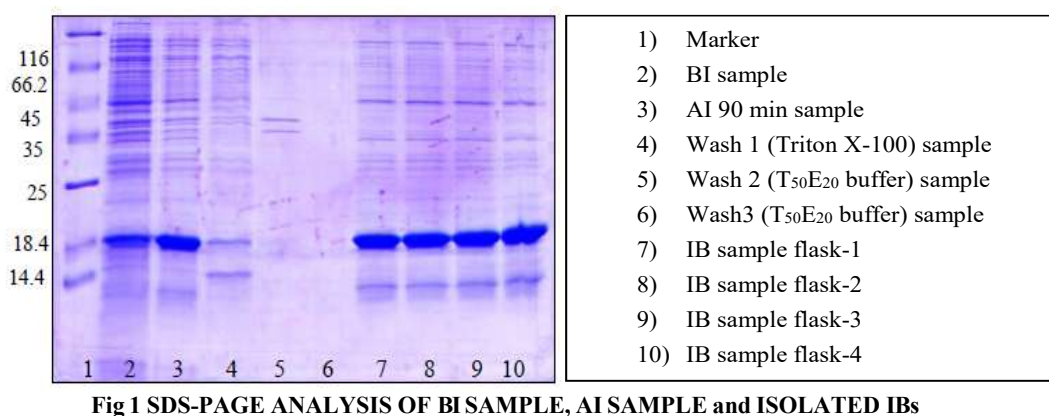


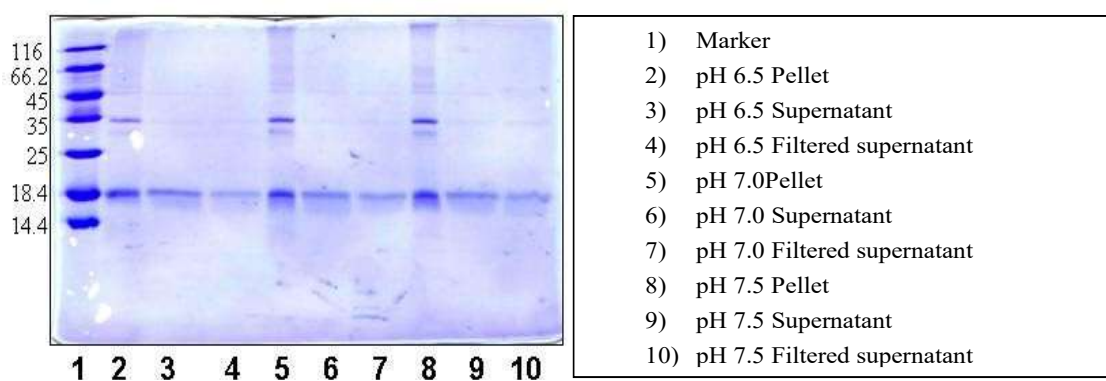
Fig 1 SDS-PAGE ANALYSIS OF BI SAMPLE, AI SAMPLE and ISOLATED IBs

C. Dialysis pH optimization

Solubilized *rhIFN-γ* was tested for dialysis in 50mM phosphate buffer at pH 6.5, 7.0 and 7.5 as shown in Fig.2. Phosphate buffer (50mM) at pH 6.5 minimized protein loss during dialysis as shown in Table I.

TABLE I. PROTEIN ANALYSIS AT DIFFERENT DIALYSIS pH

Dialysis Buffer pH	Protein Under Treatment (mg)	Protein After Refolding (mg)	Protein After Dialysis (mg)
6.5	25	17	15.9
7	25	16.9	14.1
7.5	25	16.8	13.2



D. Effect of additives on protein yield

Refolding and dialysis of solubilized protein in presence of additives like β ME, Tween 20 and Mannitol, helped to prevent protein loss, as shown in Fig.3(A) and Fig. 3(B). Protein yield increase of 2.4% (with 0.1% v/v β ME), 6% (with 0.01% W/V Tween 20), 3.2% (with 4% W/V Mannitol) and 5.8% (with combination of 0.01% W/V Tween 20 and 4% W/V Mannitol) were observed as shown in Table II. The results indicate use of 0.01% W/V Tween 20 is advantageous during refolding and dialysis of *rhIFN-γ* to have higher protein yields.

TABLE II. PROTEIN ANALYSIS OF SAMPLES, DIALYZED IN PRESENCE OF ADDITIVES

Additive Combination in Buffer	Concentration of Protein Under Treatment	Protein Concentration After Refolding (mg)	Protein Concentration After Dialysis (mg)	Yield Increase (%)
a	25	17	15.9	0
b	25	17.3	16.5	2.4
c	25	18.4	17.4	6
d	25	17.5	16.7	3.2
e	25	18.2	17.35	5.8

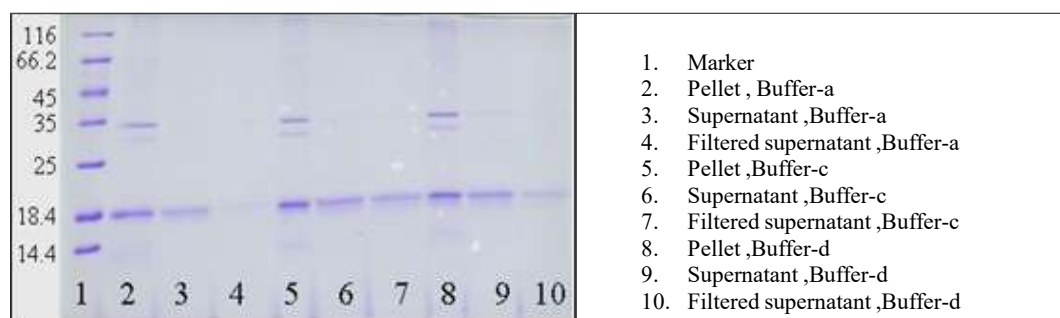


FIG 3(A) SDS-PAGE ANALYSIS OF SAMPLES DIALYZED WITH DIFFERENT ADDITIVES

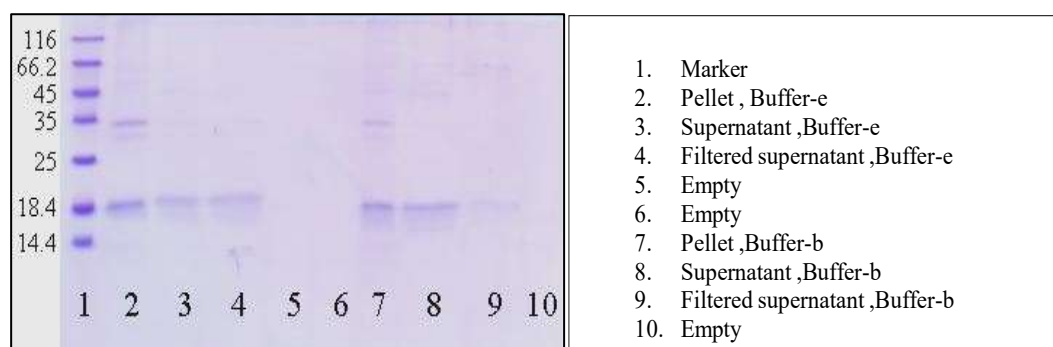


FIG 3 (B) SDS-PAGE ANALYSIS OF SAMPLES DIALYZED WITH DIFFERENT ADDITIVES

III. CONCLUSION

Recombinant clone of *E. coli* tested shows high level (approximately 51% of total cell protein) expression of *rhIFN-γ* within 90 minutes after induction. Homogeneity of expression of gene is observed. Most of the impurities are being removed during the step of isolation of IB and analysis of IB indicates negligible loss of protein of interest. Phosphate buffer (50mM) at pH 6.5 helps to lower protein precipitation during dialysis. The additive 0.01% T-20 helps to prevent precipitation of protein during refolding and dialysis. The additive helps in 6% yield increase of the protein.

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