Research Article

Optimizing Primary Recovery and Refolding of Human Interferon-*β* from *Escherichia coli* Inclusion Bodies

Farzaneh Ashnagar [1], Mahvash Khodabandeh [1,*], Ayyoob Arpanaei [1], Zohreh Azita Sadigh [2], Fatemeh Rahimi [1], Parvin Shariati [1]

[1]Faculty of Industrial and Environmental Biotechnology, National Institute of Genetic Engineering and Biotechnology, P.O. Box: 14965/161, Tehran, I.R. IRAN
[2]Razi Vaccine and Serum Research Institute, Tehran, I.R. IRAN

*Corresponding author: Mahvash Khodabandeh, Faculty of Industrial and Environmental Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, I.R. IRAN, Tel: +98-2144787355, Fax: +98-2144787399, E-mail: saba@nigeb.ac.ir

Received: October 15, 2013; **Revised:** September 22, 2014; **Accepted:** October 19, 2014

Background: The refolding of proteins from inclusion bodies is affected by several factors, including solubilization of inclusion bodies by denaturants, removal of the denaturant, and assistance of refolding by small molecule additives.
Objectives: The purpose of this study was optimization of recombinant human interferon-β purification in order to achieve higher efficiency, yield, and a product with a better and more suitable biological activity.
Materials and Methods: Triton X-100 and sodium deoxycholate were used to wash the recombinant human interferon-β inclusion bodies prior to solubilization. The inclusion bodies solubilization process was performed by denaturants and reducing agents; guanidine-hydrochloride, urea, β-mercaptoethanol and dithiothritol.
Results: The best recovery was obtained in the presence of 0.5% TritonX-100 (v/v). Low concentrations of urea only gave a marginal improvement on the refolding of recombinant human interferon-β. Successful refolding was achieved by gradient elution (decreasing the guanidine-hydrochloride concentration) in the presence of L-arginine. Partial purification was also achieved continuously, and recombinant human interferon-β was recovered with 93.5% purity. The interferon prepared in this project was biologically active and inhibited the replication of vesicular stomatitis virus in Hela cells, when compared to the standard interferon.
Conclusions: In this research, the best recovery of inclusion bodies was found at a concentration 0.5 M of Triton X-100, the maximum efficiency of solubility was found in pH 10.5 and the maximum efficiency of refolding was achieved by final buffer containing 2M urea and 0.6 M L-Arg.
Keywords: *Beta* interferon; Inclusion bodies; L-arginine

1. Background

Interferons (IFNs) are divided into three types: I, Π, and III, all belonging to the cytokines family. These proteins are all structurally and functionally related, demonstrating antiviral and antiproliferative effects on various cell types, and possessing a variety of potent immunomodulatory effects (1-5). Beta interferon (IFN-β), a member of type I IFN family, has been greatly utilized as an effective treatment for numbers of diseases such as multiple sclerosis and several other diseases such as cancers, and cold sores. Therefore, its large-scale production via recombinant DNA technology and obtaining a pure product is of great importance especially in pharmaceutical industries.

IFN-β is encoded by a single gene in fibroblasts. Since large-cale production of this interferon from fibroblasts is difficult, molecular cloning technology has now paved the way for massive production using varieties of host cells (6-11).

IFN-β-1b is a type of recombinant human IFN-β in which the cysteine at position 17 is replaced with serine via a T to A transition in the first base of the codon. This mutant IFN is a non-glycosylated protein with an approximate molecular weight of 18.500 kDa and 165 amino acids (12).

Heterologous expression of foreign genes in prokaryotic expression systems, especially *Escherichia coli* often leads to the production of the

expressed proteins as insoluble inclusion bodies (IBs) (13). Inclusion bodies enclose not only recombinant protein but also host proteins such as RNA polymerase, outer membrane proteins, enzymes, and other molecules, like 16S and 23S rRNA, and plasmids. These impurities can affect the protein refolding yield during the purification process (14). Therefore, purification of IBs from all such adhering impurities is of great interest and importance. The first step in the separation of IBs, is washing them with appropriate solutions (15). To recover an active protein after the washing step, two important steps must be conducted; solubilization and refolding.

Solubilization inevitably leads to monomolecular dispersion and minimum intra- or inter-chain interactions. The choice of solublizing agents, such as urea and guanidine hydrochloride plays a key role in the efficiency of this step, and in subsequent refolding process (16, 17). Protein refolding is a difficult task, needing a trial and error process to find the right conditions. Since protein refolding competes with reactions, such as misfolding and aggregation, the composition of the refolding buffer is crucial in preventing the formation of inactive proteins. In this regard, many additives to the refolding buffer has been tested. Polyethylene glycol (18), cyclodextrin (19), L-arginine (20, 21), and proline (22) have been reported to suppress aggregation (23, 24). Among these additives, arginine has been widely used to assist refolding of various recombinant proteins (25-28). In fact, L-arginine is known as a refolding enhancer and can improve the renaturation yield (25, 29-33).

This paper describes the optimization of the initial steps to recover rh-IFN-β-1b from *Escherichia coli*. The washing and solubilization steps were enhanced using effective solutions. Renaturation was investigated using refolding buffer in the presence and absence of L-arginine.

2. Objectives

The aim of this research was process optimization, focusing on the refolding of a recombinant interferon-beta.

3. Materials and Methods

3.1. Culture and Growth Conditions

Escherichia coli BL21 (K12) was provided by the Fermentation Group at the National Institute of Genetic Engineering and Biotechnology (NIGEB), Iran. Expression vector PYG87 of IFN-β has been described thoroughly by Ghane *et al*. (34).

To cultivate higher cell densities, a 3-1 bioreactor (New Brunswick, Biofilo 3000, USA) was used, and the fermentation process was carried out according to the method by Ghane *et al*. (34).

3.2. Harvesting and Isolation of Inclusion Bodies

The culture medium was harvested by centrifugation at 1789g, for 30 min at 4ºC (Sigma, 3K30, USA). The collected cells were suspended in 1500 ml of lysis buffer (Table 1). The cells were disrupted by a homogenizer (Niro Sovani, Italy) at 600 bar for two cycles in an ice bath. The lysate was centrifuged at 18894g for 30 min and 4ºC, and the resulting inclusion bodies (IBs) were obtained in pellet form.

3.3. Washing of Recombinant Human Interferon βIBs

The IB pellet was resuspended in different detergent buffers such as 2% and 0.5% (v/v) Triton-X100, and sodium deoxycholate in 50 mM Tris–base pH 8.0 containing 100 mM NaCl and 1 mM EDTA. The resulting suspensions were washed by stirring with a magnetic agitator for 1 h. The washing process was conducted at room temperature using 1 g IBs/15 ml of detergent buffer. After centrifugation at 18407g for 1 hour and 4ºC, an impurity profile was established for the inclusion bodies by applying SDS-PAGE electrophoresis and subsequent densitometric analysis using Alpha Ease FC software (Genetic Technologies, Inc., USA). The recovery yield after each washing step was defined as below:

Recovery yield (%) =

$$\frac{\text{Dry weight of washed IB pellet (g)}}{\text{Dry weight of unwashed IB pellet (g)}} \times 100$$

3.4. Solubilization

Two denaturing agents, Guanidine HCl (4-8 M) and urea (6 and 8 M) were investigated. The most effective reducing agents, *i.e*., β-mercaptoethanol and dithiothreitol were also examined. Washed IBs (1 g) were dissolved in 100 ml of various denaturing buffers (Table 1). Solubilization was conducted at room temperature with continuous stirring for 1 h, until complete denaturation and reduction of IBs were achieved. The insoluble particles were removed by centrifugation at 18407g for 30 min and 4ºC. The resultant supernatant, containing denatured rhIFN-β was diluted in. The total protein concentration for all supernatants was measured by the Bradford assay (35). Solubilization yield was defined as mentioned below:

Solubilization yield (%) =

$$\frac{\text{Denatured rhIFN-}\beta\text{ in supernatant (mg)}}{\text{IB pellet weight before solubilization (mg)}} \times 100$$

3.5. Refolding by Dilution

Two sets of experiments were carried out. One experiment involved diluting the denatured protein 20-fold in refolding buffers, A_1-A_4 (Table 1). However, in the second set, 40-fold dilution of the denatured protein was carried out with the same buffers. Accordingly, refolding was initiated by adding the denatured protein slowly to a glass container filled with the refolding buffers, A_1-A_4, in a drop-wise fashion. For the first few minutes, a fast and efficient mixing was implemented, after which the solution was incubated at 4°C overnight without further agitation. Finally the solution was centrifuged at 18407 g for 20 min at 4°C, to remove precipitated materials and the resulting supernatant was used in the subsequent analyses. Turbidity was measured at 340 nm using a UV visible spectrophotometer (BECKMAN DU 530, USA), to determine aggregation during refolding.

Refolding by dilution in the presence of L-arginine

Table 1. List of buffers used in this work

Buffers		Compositions
Lysis buffer		1% Triton X-100, 120 mM EDTA, 150 mM NaCl and 8 $\mu g.g^{-1}$ PMSF , 50 mM Tris-base pH 8.5-9
Washing buffer	A	0.5% Triton X-100, 1 mM EDTA, 100 mM NaCl, 50 mM Tris-base pH 8.0.
	B	0.5% Triton X-100, 1 mM EDTA, 100 mM NaCl, 0.02 M Sodium deoxycholate, 50 mM Tris-base pH 8.0
	C	2% Triton X-100, 1 mM EDTA, 100 mM NaCl, 0.02 M Sodium deoxycholate, 50 mM Tris-base pH 8.0
Denaturing buffer	A_1	4 M Guanidine HCl, 100 mM β-ME, 10 mM Tris-base pH 10
	A_2	6 M Guanidine HCl, 100 mM β-ME, 10 mM Tris-base pH 10
	A_3	8 M Guanidine HCl, 100 mM β-ME, 10 mM Tris-base pH 10
	B_1	4 M Guanidine HCl, 20 mM DDT, 10 mM Tris-base pH 10
	B_2	6 M Guanidine HCl, 20 mM DTT, 10 mM Tris-base pH 10
	B_3	8 M Guanidine HCl, 20 mM DTT, 10 mM Tris-base pH 10
	C_1	6 M Urea, 100 mM β-ME, 10 mM Tris-base pH 10
	C_2	8 M Urea, 100 mM β-ME, 10 mM Tris-base pH 10
	D_1	6 M Urea, 20 mM DTT, 10 mM Tris-base pH 10
	D_2	8 M Urea, 20 mM DTT, 10 mM Tris-base pH 10
Refolding buffer	A_1	0.5 mM EDTA, 10 mM Tris-base pH 9.5
	A_2	0.5 M Urea, 0.5 mM EDTA, 10 mM Tris-base pH 9.5
	A_3	1 M Urea, 0.5 mM EDTA, 10 mM Tris-base pH 9.5
	A_4	2 M Urea, mM EDTA, 10 mM Tris-base pH 9.5
	B_1	0.5 M L-Arg, 0.5 mM EDTA, 10 mM Tris-base pH 9.5
	B_2	0.5 M L-Arg, 0.5 M Urea, 0.5 mM EDTA, 10 mM Tris-base pH 9.5
	B_3	0.5 M L-Arg, 1 M Urea, 0.5 mM EDTA, 10 mM Tris-base pH 9.5
	B_4	0.5 M L-Arg, 2 M Urea, mM EDTA, 10 mM Tris-base pH 9.5
Affinity Chromatography		
Buffer	A	10 mM Tris-base pH 7.2
Binding buffer	B	0.15 M NaCl, 0.02 M phosphate buffer pH 7.2
Elution buffer	C	2 M NaCl, 0.02 M phosphate buffer pH 7.2
Elution buffer	D	2 M NaCl, 50% ethylene glycol, 0.02 M phosphate pH 7.2

was performed in the same way as mentioned above, except that the buffer in this case contained 0.5 M L-arginine.

3.6. Purification of Recombinant Human Interferon β by Affinity Chromatography

Purification was performed using a 1×10 cm column packed with 7 ml of the Blue-Sepharose Fast Flow gel (Pharmacia, USA). Three buffers were used to carry out the purification process. Prior to sample application, the column was equilibrated with buffer A (Table 1). The refolded protein was loaded on the column, five bed volumes of Buffer B (Table 1) and 10 bed volumes of buffer C (Table 1) were used consecutively. Buffer D (Table 1) was applied for elution of target protein from the column. The column temperature and flow rate were maintained at 20°C and 1 $ml.min^{-1}$, respectively, throughout the process. A chromatogram was generated as a result of measuring the absorbance of the collected fractions at 280 nm.

3.7.Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In order to study the protein pattern, electrophoresis was carried out under denaturing conditions (35). Protein bands were developed by the Coomassie blue staining method. Two standards were applied during the reducing SDS-PAGE procedure: a primary standard of rhIFN-β (pharmaceutical interferon β from Shering, Germany) and a molecular weight marker composed of several denatured proteins (MW between 14.4 and 94 kDa, Roche, Germany). Loading buffer (0.15 M Tris-base, 15% v/v glycerol, 0.05% v/v bromophenol blue, 10% v/v β-mercaptoethanol) was added to samples and standards, prior to boiling them for 3 min. They were loaded onto the gel and ran at a constant voltage of 150 v for 4 h (36).

After staining and destaining, the gels were photographed in a scanner. This purity was defined as the ratio of the density of the rhIFN-β band to the total density of all bands in the gel.

3.9. Biological Activity of Refolded Protein

To evaluate the biological activity of the interferon, the most common assay based on the antiviral property of this cytokine was used (37). Hela cells and the vesicular stomatitis virus (VSV) as the lytic virus were used in assay. Hela cells were grown in a 96-well microplate, at 37°C in a CO_2 incubator. After Hela cells were reached confluency, refolded interferon and the purchased standard were added to the cells, separately. Two rows of wells (sixteen) were considered for the cell and virus control samples. Cells (with the exception of the control cells) were inoculated with VSV at 10^2 $CCID_{50}.ml^{-1}$ (cell culture infective dose) following 24 h. The cells were monitored for the cytopathic effect induced by viruses, using an inverted microscope (Axiovert 25, zeiss, Germany).

3.10. Western Blotting

Proteins in the samples were electrophoretically separated by SDS-PAGE and transferred to a PVDF membrane. Western blot was performed as described previously with rabbit anti human interferon-β (prepared by NIGEB, Iran) and horseradish peroxidase-conjugated sheep anti-rabbit immunoglobulin (Serotec, UK) (38). The developed blots were photographed by the Eagle Eye system (cell phone C6_01, Nokia).

4. Results

4.1.Washing of rhIFN-β inclusion bodies

The yield resulting from the washing process was detected by SDS-PAGE and the dry weight of the pellet remaining after each wash was measured.

The three washing buffers did not have any significant effects on the removal of impurities. However, the best protein recovery was achieved with detergent buffer A (76.3%), which was higher than buffer B (62%) and C (21%) (Table 2). The presence of sodium deoxycholate and an increased concentration of Triton X-100 in buffers B and C, made the solution more viscose and improper for solubilization of IBs. After washing with buffer A, the IBs were dissolved in denaturing buffer, followed by centrifugation, yielding a supernatant containing the denatured rhIFN-β. To find the optimal combination of denaturant/reducing agent in the denaturing buffer, a set of experiments was car-

Table 2. Impurity profile and recovery of rhIFN-β inclusion bodies during Triton X- 100 washing

Detergent buffer	Inclusion body	
	Purity (%)	Recovery (%)
A	41.3	76.3 ± 2.5
B	41.2	62 ± 1.8
C	38.7	21± 1.4

Data are means of three measurements. Multiple comparisons were carried out using one-way analysis of variance (ANOVA) followed by Duncan Test. *P* values less than 0.05 were regarded significant

ried out. Yield of recovery and purity, measured via Bradford assay and SDS-PAGE, differed significantly when urea was replaced with Guanidine HCl. Solubilization yields in guanidine hydrochloride were higher than urea (Figure 1).The use of β-mercaptoethanolas a reducing agent instead of DTT, further improved the recovery of protein during the solubilization step. The highest recovery value (9.1%) was obtained when the highest concentration of Guanidine HCl (8 M) was used (Figure 1). A lower concentration of Guanidine HCl significantly decreased the recovery of the protein (data not shown). Purity of solubilized rhIFN-β (up to 48%) was achieved at 8 M Guanidine HCl+ β-mercaptoethanol (β-ME) (Figure 1C).

Multiple chromatography steps were followed to remove remaining contaminations within both denatured and solubilized rhIFN-β proteins (0.820 mg.ml^{-1}).

4.2. Investigation of Factors Affecting on Refolding

Figure 2 shows the effect of dilution in the final concentrations of protein and recovery. In the case of the 20-fold dilution, protein concentration reached 40 μg.ml^{-1}, and precipitation happened in the first few minutes after the addition of the denatured protein to the refolding buffers, A_1-A_4. In fact, maximum turbidity (0.15) occurred in the presence of buffer A_1 (without any urea; Figure 2A). The presence of urea in the other refolding buffers, A_2-A_4, significantly decreased the negative effects of high protein concentration; a minimum turbidity of 0.05 was obtained in the presence of the buffer A_4, which contained 2 M urea (Figure 2A). SDS-PAGE results of the supernatant sample showed all the soluble proteins had aggregated during refolding and there was no band of interferon on the gel (Figure 3A, lanes 1-4). Moreover, no

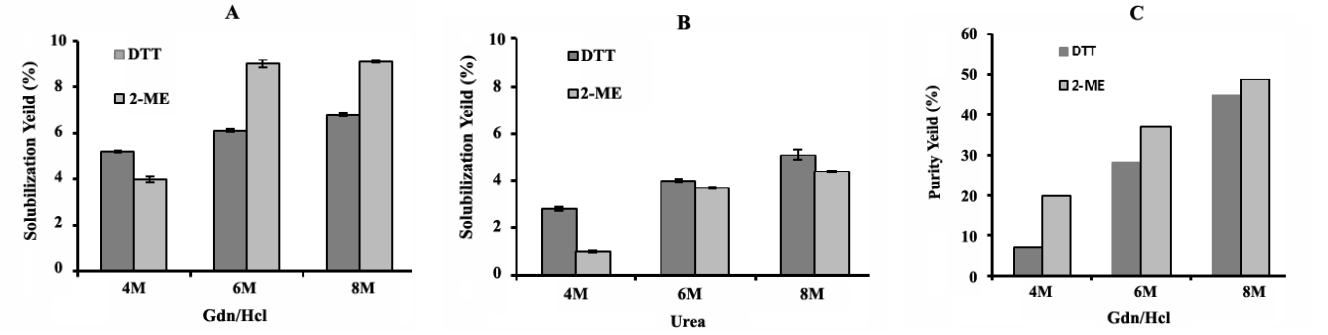

Figure 1. Solubilization of rhIFN-β inclusion bodies in denaturing buffers. A: Solubilization of inclusion bodies using Guanidine HCl buffer and DTT, β-ME as reducing agents has already been used. B: Solubilization of inclusion bodies using urea buffer and DTT, β-ME as reducing agents has already been used. C: Purity yield of solubilized inclusion bodies in Guanidine HCl buffers

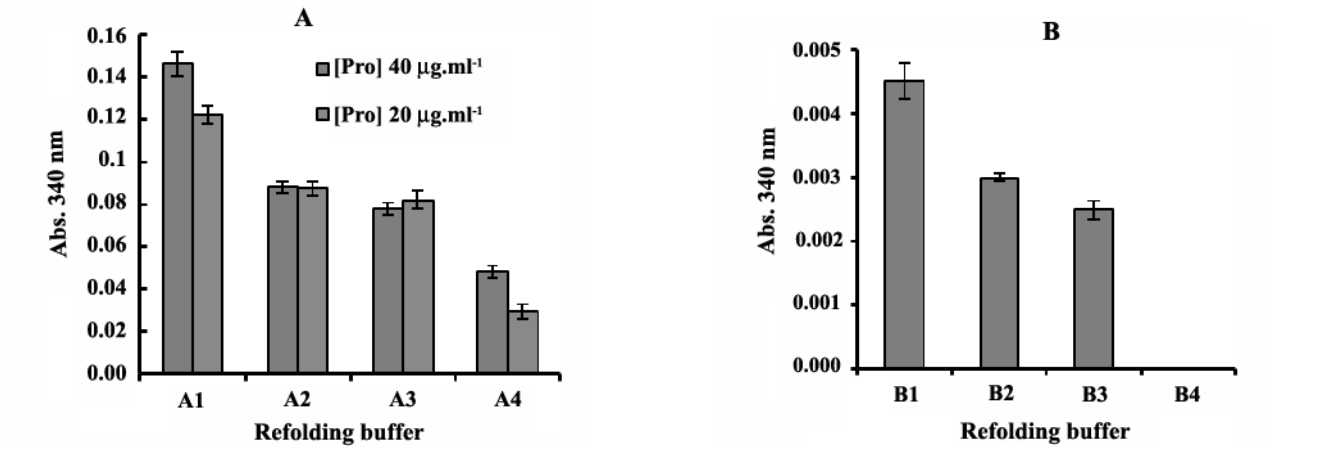

Figure 2. Effect of final protein concentration, urea concentration, and L-Arginine on renaturation of rhIFN-β: A: Reduced and denatured inclusion bodies were diluted (20- and 40-fold) by refolding buffer A_1-A_4, containing different concentrations of urea as described in the text. B: Refolding was performed by refolding buffers B_1-B_2, containing L-Arg. The protein concentration was 0.8 mg.ml^{-1}. Arginine concentration was 0.5 M

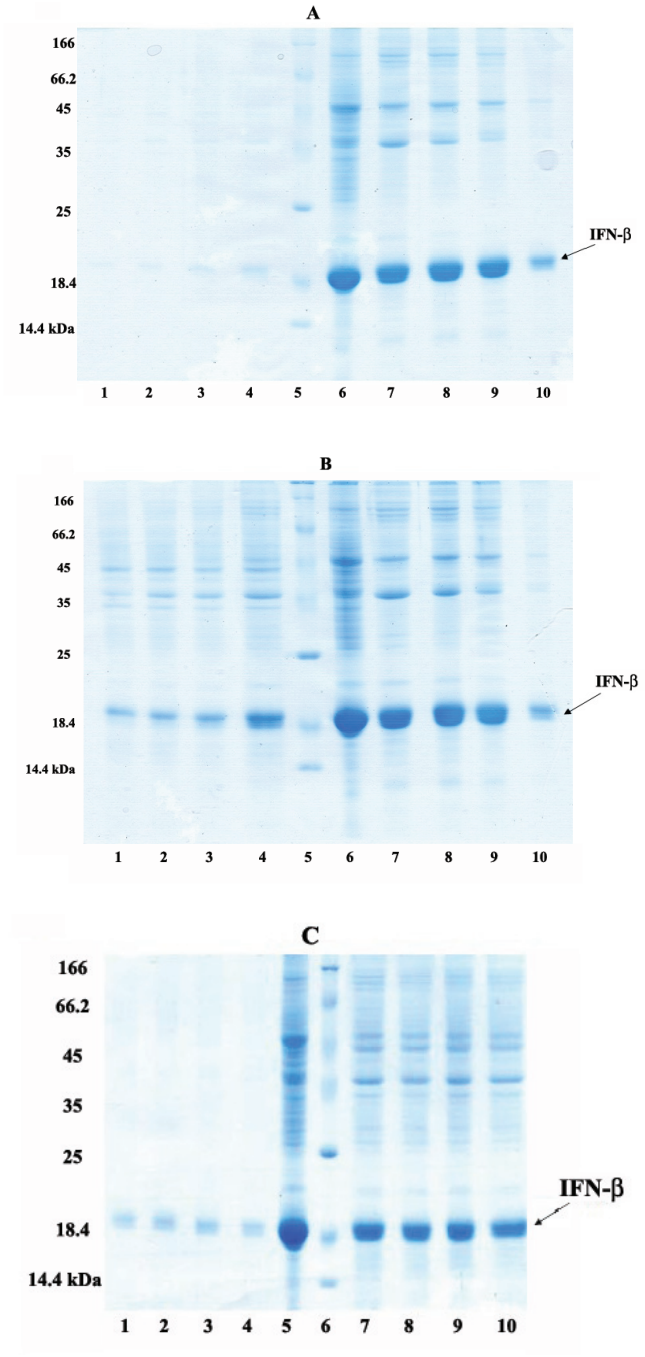

Figure 3. SDS-PAGE analysis of refolded protein samples**.** Samples were analyzed after centrifugation. A: Protein concentration was 40 µg.ml^{-1}: Lanes 1-4: samples (supernatants) in the presence of 0, 0.5, 1 and 2 M urea; lane 5: molecular weight standards; lane 6: rhIFN-β; lanes 7-10: samples (pellets) in the presence of 0, 0.5, 1 and 2 M urea. B: Protein concentration was 20 µg.ml^{-1}: Lanes 1-4: samples (supernatants) in the presence of 0, 0.5, 1 and 2 M urea; lane 5: molecular weight standards; lane 6: rhIFN-β; lanes 7-10: samples (pellets) in the presence of 0, 0.5, 1 and 2 M Urea. C: Refolding buffers B_1-B_4 containing arginine: Lanes 1-4: samples (pellets) in the presence of 2, 1, 0.5 and 0 M urea; lane 5: rhIFN-β; lane 6: molecular weight standards; lanes 7-10: samples (supernatants) in the presence of 2, 1, 0.5 and 0 M urea

remarkable suppressive impact on aggregate formation was observed after adding different concentrations of urea to the refolding buffer. Meanwhile, the amount of precipitation decreased to some extent in the presence of 2 M urea (buffer A_4; Figure 3A, lane 10).

Recent reports have demonstrated that arginine can suppress formation of insoluble aggregates during the refolding of rhIFN-γ. In this study, the effects of arginine on the aggregation process during the refolding of rhIFN-β were also investigated. Accordingly, in order to increase the refolding yield and minimize aggregation during the refolding process, L-arginine was applied to the refolding buffers, B_1-B_4 (Table 1). The absorbance of protein solution was significantly reduced and reached zero at 340 nm, when buffer B_4 was used (Figure 2B). Refolding yield was remarkably enhanced following arginine application. Additionally, sharp bands associated with soluble interferon were observed in the supernatant samples (Figure 3C, lanes 7-10), along with a noticeable decrease in precipitate (Figure 3C, lanes 1-4).

4.3.Purification of interferon

Purification of rhIFN-β was carried out by a simple step of Blue Sepharose CL-6B chromatography, achieving a protein purity of 93.5%. Purified sample was separated on SDS-PAGE (data not shown) and transferred onto a membrane and analyzed via specific antibody (Figure 4). The blot was indicative of the presence of purified rhIFN-β.

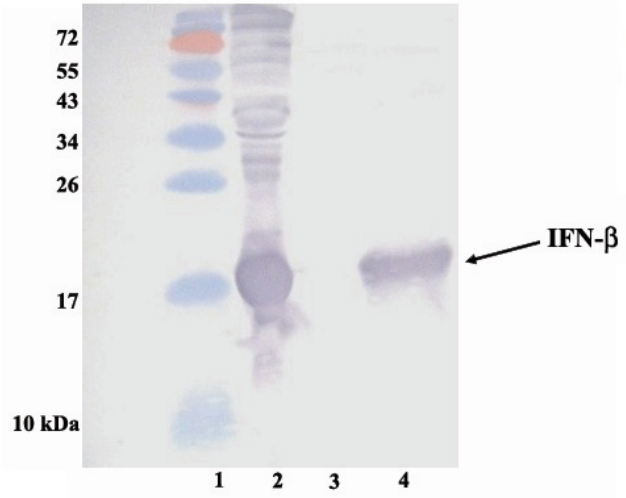

Figure 4. Western blot analysis of the expressed and partially purified human IFN-β in *E. coli* using the rabbit anti-human IFN-β antibody. Lane 1, molecular weight marker; lane 2, expressed human IFN-β in *E. coli;* lane 3, negative control; lane 4, partial purified human IFN-β

4.4.Biological Activity of rhIFN-β

The biological assay of the interferon on HeLa cells showed that rhIFN could inactivate the replication cycle of the virus. However the inactivation effect was smaller once compared with the standard rhIFN-β. Control cells showed no change in size and shape (Figure 5A), while HeLa cells treated with virus (VSV) demonstrated cytopathic effect (Figure 5B) on the contrary, no cytopathic effect was observed for the inoculated cells with VSV after treatment with standard IFN (Figure 5C). HeLa cells inoculated with VSV after exposure to rhIFN (40 $ng.ml^{-1}$) had no detectable cytopathic effect (Figure 5D).

5. Discussion

Eukaryotic proteins expressed in bacteria in large quantities, are insoluble products. One of the most important steps in purification is separation of insoluble bodies. The most difficult contaminants to remove from the crude inclusion body protein were membrane-associated proteins that were released upon cell disruption. In order to remove such contaminants, washing step was performed. The use of the different buffers demonstrated that the presence of sodium deoxycholate and Triton X-100 is essential to achieve maximum purity (28). Already has used sodium deoxycholate and Triton X-100 for removing membrane proteases (39). Dasari also showed that the increase in the concentration of Triton X-100 causes IBs to precipitate via increasing buffer viscosity (40). In this research, the best efficiency of IBs recovery was found at a concentration 0.5 M of Triton X-100 (without the use of Sodium DeoxyCholate in the second phase of the washing stage).

Optimum buffering conditions were defined for protein resolvation similar to Valent *et al*. (2005) (38). Buffer solution contained 8 M Guanidine HCl and 100 mM β-2ME. The dissolving phase is usually performed in the neutral pH (pH 7-8) and in a buffering system that is compatible with the protein of interest.

In this research pH 10.5 showed most effective, once pH ranges of 6.5-10.5 were applied to find out the best buffering system to bring rhIFN-β into solution. For the refolding of dissolved IBs, it is necessary to remove reducing agents such as Urea and Guanidine HCl. Several variables have an important role in this process, including components of refolding buffer, final concentration of desired protein, and refolding procedure (41). Normally low concentrations (0.1-2 M) of chaotropic agents such as urea next to some other additives like L-Arg are being used in refolding buffer (41). It has proven that L-Arg is capable to inhibit of precipitation during the refolding, although the echanism has remained elusive. Dialysis or dilution method is typically used to remove denaturants. Here, refolding buffer contained 2 M Urea +0.6 M L-Arg.

It is obvious that a high final protein concentration would inevitably produce aggregates. Increasing the protein concentration, leaves an insufficient volume of buffer, thus leading to increased local protein concentration, whereby some protein molecules come into contact with one another in the process of refolding. The denatured rhIFN-β has many hydrophobic amino acid residues, which are liable to aggregation by hydrophobic interactions.
Under refolding conditions, unfolded proteins or intermediate components are extremely prone to aggregation.

In this study, with regard to the 40-fold dilution of protein solution, when the final protein concentration decreased, aggregation also declined. So the minimum value of OD_{340} (0.032) was obtained when buffer A_4 was used (Figure 3A). SDS-PAGE data was illustrative of lower protein concentration favors protein refolding (Figure 3B, lanes 1-4). The effect of the urea concen-

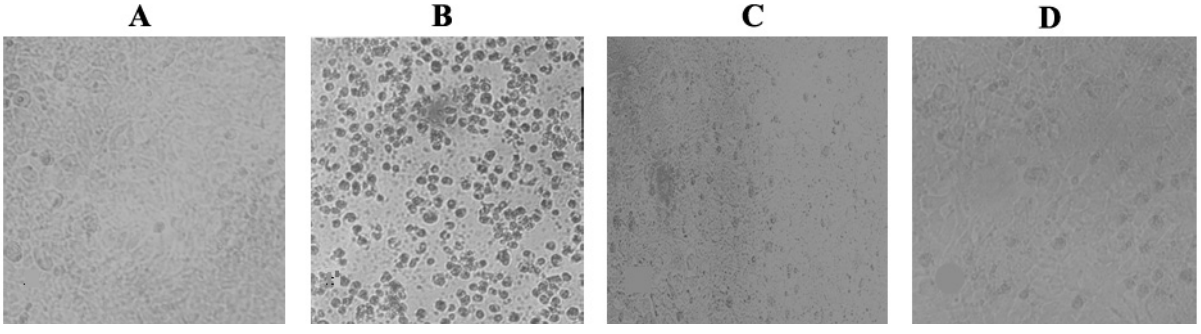

Figure 5. Biological activity of recombinant IFN: A: Control cells**,** B: Infected cells by Virus (VSV) control, C: Standard IFN in the infected cells (40 $ng.ml^{-1}$), D: Recombinant IFN in the infected cells (40 $ng.ml^{-1}$)

tration on rhIFN-β refolding is shown in Figure 3B. The weak interferon band associated with the pellet sample (Figure 3B, lane 10) indicates that 2 M urea causes protein refolding and lowers aggregation. Urea concentrations lower than 2 M would not restrain the aggregation of proteins, efficiently (Figure 3B, lanes 7-9).

This study determined the capability of arginine to suppress the formation of insoluble precipitants throughout the rhIFN-β refolding process. The ability of arginine has also been proved for other proteins (21). Here, the use of refolding buffer B_2 containing a low urea concentration can be considered as an optimized protocol for refolding of IFN-β.

Acknowledgments

The authors would like to acknowledge Mr. Habib Ghomi for helping with the fermentation.

Funding

Results presented in this paper were extracted from the data of project 298 supported by National Institute of Genetic Engineering and Biotechnology (NIGEB) of Islamic Republic of Iran.

References

1. Issacs A, Lindenmann J. *Virus interferenceI*. The interferon: Proc. Roy. Soc, London Ser. B; 1957.
2. Pfeffer LM, Dinarello CA, Herberman RB, Williams BRG, Borden ECR, Bordens W, M.R Biological properties of recombinant alpha-interferons: 40th anniversary of the discovery of interferons. *Cancer Research*. 1998;**58**(12):2489-2499.
3. Baron S, Tyring SK, Fleishmann WR, Niesel DH, Klimpel GR, Stanton J. The IFNs.mechanisms, *JAMA*. 1991;**266**:1375-1383.
4. Nagabhushan TL, Giaquinto A. *Zinc mediated dimer of human interferon α 2 b revealed by x-ray crystallography*. New York: John Wiley & Sons; 1995.
5. Jaeckel E, Cornberg M, Wedemeyer H, Santantonio T, Mayer J, Zankel M, Pastore G, Dietrich M, Trautwein C, Manns MP. Treatment of acute hepatitis C with interferon alfa-2b. *New England J Med*. 2001;**345**(20):1452. doi: 10.1056/NEJMoa011232.
6. Taniguchi T, Guarente L, Roberts T, Kimelman M, Douhan DJ, Ptashne M. Expression of the human fibroblast interferon gene in Escherchia coli. Proceedings of the National Academy of Sciences of the United States of America. 1980;**77**:5230-5233.
7. Derynck R. Expression of human fibroblast interferon gene in Escherichia coli. *Nature* 1980;**287**:193-197. doi:10.1038/287193a0
8. Goeddel DV, Shepard HM, Yelverton E, Leung D, Crea R, Sloma A. Synthesis of human fibroblast interferon by *E. coli*. *Nucleic Acids Research*. 1980;**8**:4057-4074. doi: 10.1093/nar/8.18.4057
9. Ohno S, Taniguchi T. Inducer-responsive expression of the cloned human interferon β1 gene introduced into cultured mouse cells. *Nucleic Acids Research*. 1982;**10**:967-977. doi: 10.1093/nar/10.3.967
10. Cormick M, Trahey M, Innis M, Dieckmann B, Ringold G. Inducible expression of amplified human beta interferon genes in CHO cells. *Mol Cell Biol*. 1984;**4**(1):166-172.
11. Smith GE, Summers MD, Fraser MJ. Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Mol Cell Biol*. 1983;**3**(12):2156-2165.
12. Mark DF, Lu SD, Creasey AA, Yamamoto R, Lin LS. Site-specific mutagenesis of the human fibroblast interferon gene. Proceedings of the National Academy of Sciences of the United States of America. 1984;**81**(18):5662-5666.
13. Thatcher D. Recovery of therapeutic proteins from inclusion bodies: problems and process strategies. *Biochem Soc Trans*. 1990;**18**:234-235.
14. Clark EB. Refolding of recombinant proteins. *Curr Opin Biotechnol*. 1998;**9**:157-163.
15. Fischer B, Sumner I, Good enough P. Isolation, renaturation, and formation of disulfide bonds of eukaryotic proteins expressed in *Escherichia coli* as inclusion bodies. *Biotechnol Bio Eng*. 1993;**41**:3-13. doi: 10.1002/bit.260410103
16. Yamaguchi S, Yamamoto E, Mannen T, Nagamune T, Nagamune T. Protein refolding using chemical refolding additives. *Biotechnol J*. 2013;**8**(1):17-31. doi: 10.1002/biot.201200025
17. Yamaguchi H, Miyazaki M. Refolding Techniques for Recovering Biologically Active Recombinant Proteins from Inclusion Bodies. *Biomolecules* 2014;**4**(1):235-251. doi:10.3390/biom4010235
18. Cleland JL, Hedgepeth CD, Wang IC. Effect of additives on protein aggregation. *J Biol Chem*. 1992;**267**:1327-1334.
19. Karuppiah N, Sharma A. Cyclodextrins as protein folding aids. *Biochem Biophys Res Commun*. 1995;**211**:60-66. doi:10.1006/bbrc.1995.1778
20. Asano R, Kudo T, Makabe K, Tsumoto K, Kumagai I. Antitumor activity of interleukin-21 prepared by novel refolding procedure from inclusion bodies expressed in *E. Coli*. *FEBS Lett*. 2002;**528**:70-76. doi:10.1016/S0014-5793(02)03254-4
21. Liu YD. A newly proposed mechanism for argenin associated protein. *Protein Expr Puri*. 2006;**51**:235-242.
22. Samuel D, Kumar TK, Ganesh G, Jayaraman G, Yang PW, Chang MM. proline inhibits aggregations during protein refolding. *Protein Sci*. 2000;**9**:344-352. doi: 10.1110/ps.9.2.344
23. Cleland JL, Wang DIC. Cosolvent assisted protein refolding. *Biotechnology* 1990;**8**:1274-1278. doi:10.1038/nbt1290-1274
24. Tsumoto K, Umetsu M, Kumagai I, Ejima D, Arakaw T. Aggregation of therapeutic proteins. *Biochem Biophys Res Commun*. 2003;**312**:1383-1386.
25. Buchner J, Pasta I, Brinkmann U. A method for increasing the yield of properly folded recombinant fusion proteins,single-chain immunotoxins from renaturation of bacterial inclusion bodies. *Anal Biochem*. 1992;**205**(2):263-270. doi:10.1016/0003-2697(92)90433-8

26. Arora D, Khanna N. Method for increasing the yield of properly folded. *J Biotechnol*. 1996;**52**:127-133. doi:10.1016/S0168-1656(96)01636-7
27. Raina K, Panda AK, Ali MM, Talwar GP. Purification, refolding, and characterization of recombinant LHRH-T multimer. *Protein Expres Purif*. 2004;**37**:8-17. doi:10.1016/j.pep.2004.03.008
28. Wright E, Serpersu EH. Isolation of aminoglycoside nucleotidyltransferase (2″)-Ia from inclusion bodies as active, monomeric enzyme. *Protein Expres Purif*. 2004;**35**:373-380. doi:10.1016/j.pep.2004.02.003
29. Buchner J, Rudolph R. Renaturation, purification and characterization of recombinant fab-fragments produced in Escherichia coli. *Biotechnology* 1991;**9**:157-162. doi:10.1038/nbt0291-157
30. Stoyan T, Michaelis U, Schooltink H, Van Dam M, Rudolph R, Heinrich PC. Recombinant soluble human interleukin-6 receptor. Expression in Escherichia coli, renaturation and purification. *Eur J Biochem*. 1993;**216**:239-245. doi: 10.1111/j.1432-1033.1993.tb18138.x
31. Hevehan DL, Clark EB. Oxidative renaturation of lysozyme at high concentrations. *Biotechnol Bioeng*. 1997;**54**:221-230. doi: 10.1002/(SICI)1097-0290(19970505)54:3<221::AID-BIT3>3.0.CO;2-H
32. Suenaga M, Ohmae H, Tsuji S, Itoh T, Nishimura O. Renaturation of recombinant human neurotrophin-3 from inclusion bodies using a suppressor agent of aggregation. *Biotechnol Appl Biochem*. 1998;**28**:119-124.
33. Swaminathan S, Khanna N. Affinity purification of recombinant interferon-α. *Protein Expression and Purification*. 1999;**15**:236-242. doi:10.1006/prep.1998.1017
34. Ghane M, Yakhchali B, Khodabandeh M. Over expression of biologically active interferon beta using synthetic gene in *E. coli*. *J Sci Islamic Republic of Iran*. 2008;**19**:203-209.
35. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *An Analytical Biochem*. 1979;**72**:248-254. doi:10.1016/0003-2697(76)90527-3
36. Laemmle UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;**227**(259):680-685. doi:10.1038/227680a0
37. Meagers A. Biological assays for interferons. *J Immunol Methods*. 2002;**261**:21-36. doi:10.1016/S0022-1759(01)00570-1
38. Valente CA, Prazeres DM, Cabral JM, Monteiro GA. Optimization of the primary recovery of the human interferon α 2b from E.coli inclusion bodies. *Appl Environ Microbiol*. 2004;**50**:5033-5036.
39. Walsh G. Proteins: Biochemistry and Biotechnology: John Wiley & Sons; 2002. doi:10.1016/S0300-9084(02)01394-9
40. Dasari Venkata RK. Cloning,high expression and purification of recombinant human interferon β-1b in *E. coli*. *Appl Biochem Biotechnol*. 2009;**158**:140-54.doi: 10.1007/s12010-008-8318-9
41. Cabrita LD. A practical guid to protein expression and refolding from inclusion bodies. *Biotechnol Annu Rev*. 2004;**10**:31-50. doi:10.1016/S1387-2656(04)10002-1