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**Original Research Article**

# Improved Refolding Efficacy of Recombinant Human Interferon α-2b via pH Modulation

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## Abstract

***Purpose:*** *To increase the refolding yield of Recombinant Human Interferon α-2b in order to achieve a highly potent product.*
***Methods:*** *Interferon α-2b inclusion body was dissolved in tris-HCl buffer containing 6 M guanidine-HCl and $CuSO_4$. Different refolding buffers were employed for refolding the target protein. The refolded proteins were then purified by affinity and gel filtration chromatography. The purified proteins were subjected to circular dichroism (CD) spectropolarimetry and assayed for biological activity in vitro.*
***Results:*** *Increment of pH to 8.5 improved refolding efficacies from 42.28 % to 71.22 %. However, the relative potency significantly increased up to pH 8.0 (from 19353546 to 28633902, $p < 0.05$) and then decreased to 21081305.00 at pH 8.5. The CD spectra demonstrated that by increasing pH to 8.5, the secondary structure of the protein was altered, probably due to increase in alpha-helix from 23.7 % at pH 7.0 to 28.1 %.*
***Conclusion:*** *Employing a low-cost and simple method, such as alteration of refolding buffer pH, results in higher refolding yield in downstream processing of rhIFN α-2b.*

***Keywords:*** *Recombinant human interferon α-2b, Refolding, Circular dichroism, Spectropolarimetry, Recombinant protein, pH effect*

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## INTRODUCTION

Large scale production of bio-pharmaceuticals has been made possible by recombinant DNA technology. Biomolecules produced via biotechnology are increasing, posing challenges and obstacles [1-3]. One of the recombinant proteins produced by such technology is interferon, with anti-viral, anti-proliferative, and immunomodulatory properties [4].

Recombinant human interferon α-2b (rhIFN α-2b) is currently produced as insoluble aggregate forms through recombinant technology employing *Escherichia coli*. The aggregated proteins are further processed in order to obtain functional forms of target protein *in vitro* [2-5]. Thus such proteins are outcomes of high-level expression of eukaryotic proteins deposited in cytoplasm of *E. coli* referred to as inclusion bodies (IBs) [3,4,6-8]. Protein properties such as net charge, turn-forming residual fraction, cysteine and proline fractions, and hydrophilicity correlate with inclusion body formation. Other factors controlling the partitioning of recombinant proteins are temperature, pH, and nutrient

constituents of culture/fermentation medium [4,8].

It has long been reported that IBs are formed with unfolded or misfolded polypeptides that are devoid of biological activity [9,10]. Hence, the occurrence of IBs is deemed to be problematic in a biotechnological context, which results in major economic impacts particularly when protein refolding from inclusion bodies becomes necessary [3,8,9,11]. The formation of IBs is usually the consequence of the rapid accumulation of newly synthesized polypeptides [4,12]. This occurs when rates of protein synthesis overwhelm the folding machinery or the cell's capacity for post-translational modification [13]. However, IBs cause obstacles in downstream processing in biotechnology industries. This obstacle can be solved by addition of solubilizing agents, initial protein recovery, and protein renaturation [8,14]. In this study we attempted to increase the refolding yield of rhIFN α-2b through pH alteration by optimizing the refolding buffer pHs.

## EXPERIMENTAL

### Solubilization of inclusion body

The IB in buffer (50 mM Tris-HCl and 50 mM NaCl, pH 7.0) was supplied by the Department of rhIFN α-2b production, Research and Production Complex, Pasteur Institute of Iran. It was thawed and then precipitated by centrifugation at 3000 g and at 4 °C for 15 min. The supernatant was decanted and the pellet was weighed. Subsequently, 25 mL of denaturing buffer (50 mM Tris-HCl, 50 mM NaCl and 8 M guanidine HCl pH 7.0) containing 115 mg dithiothreitol (DTT, Merck, Germany) was added to each 0.3 g of pellet and slowly stirred for 2 h at 4 °C to solubilize the protein. The insoluble fraction was then separated by centrifugation at 5000 g, and at 4 °C for 15 min. The soluble protein was filtered through 0.2 µM filter and evaluated by Bradford protein assay using bovine serum albumin (Sigma Aldrich A4737) as standard [15]. Finally, the protein concentration was adjusted to 5 mg/mL by adding denaturing buffer to minimize the concentration effect on refolding yield. The protein solution was employed in carrying out the refolding process at different pH conditions.

### Refolding of rhIFN α-2b

All refolding processes were performed batchwise at 4 °C and the refolding efficiency was determined as the ratio of solubilized protein concentration to the total denatured protein [16,17] at pH 7.0, 7.5, 8.0 and 8.5. The refolding buffer (50 mM Tris-HCl and 50 mM NaCl) was prepared at various pHs (pH 7.0, 7.5, 8.0 and 8.5). The buffer of pH 7.0 was considered as control for refolding. Effects of different pH values on refolding of rhIFN α-2b were studied. by addition of 22.5 mL of each refolding buffer at different pH containing 2.5 µL of 80 mM $CuSO_4$ to 2.5 mL of denatured protein (5 mg/mL) in separate 50 mL beakers using a peristaltic pump (Bio-Rad, USA) at the flow rate of 0.06 ml/min for 6 h with gentle stirring using magnetite stirrers with similar size and shape (PLT Scientific, Thailand). Each experiment was performed in triplicate. The speed of stirrers was adjusted to 150 rpm such a way there was no foam formation. Finally, refolding efficacy was calculated for each pH.

### Purification of refolded rhIFN α-2b

The purification procedures were as similar for all proteins obtained at different pH values to minimize data variation affected by purification steps. The purification steps were composed of two sequential column chromatography including immobilized metal affinity chromatography (IMAC) and size exclusion chromatography (SEC). All solutions and buffers were HPLC grade and were filtered, de-gassed prior to use and applied to the column at the flow rate of 1 ml/min. In IMAC, 15 mL chelating Sepharose Fast Flow gel (GE Healthcare, USA) was packed into the column (1.5 × 12 cm, Econo-Pac®) with column adaptor (Bio-Rad, USA). It was washed by water for injection (WFI) till the inlet and outlet pH and conductivity were the same. The column was sanitized by washing 2 column volumes with 0.5 M NaOH solution. It was then neutralized by 0.03 % (V/V) phosphoric acid and washed again with WFI. The column was equilibrated with phosphate buffered saline (PBS; pH 6.6 and 14 ± 2 µS conductivity). Thereafter, 6 mM $CuSO_4$ solution was applied to the column till the absorption at 280 nm was above 0.2. Again the column was washed with washing buffer (7.5 g/L glycine, 7.8 g/L NaCl pH 2.2 and conductivity of 33 ± 4 µS) till the absorption at 280 nm was below 0.2.

After equilibration of the column, the refolded protein at each pH was loaded and washed first with 2 column volumes of PBS and then with sodium acetate solution (pH adjusted to 5 with glacial acetic acid). Finally, the protein was eluted with elution buffer (7.5 g/L glycine, 7.8 g/L NaCl pH 2.2 and conductivity of 33 ± 4 µS) and the eluted protein solution under the peak area was pooled for the next purification step. In the second step, Sephadex G-75 gel (15 mL of

swollen Sephadex) was packed in the column (1.5 × 12 cm, Econo-Pac®) using a column adaptor (Bio-Rad, USA). After packing, the column was sanitized with 0.5 M NaOH and washed with 2 column volumes of PBS (pH 6.6 and 14 ± 2 µS conductivity). All packed columns qualities were controlled according to the company's instruction by loading 1 % V/V of acetone to the packed columns. Only columns with acceptable asymmetry factor range (0.8-1.5) was used.

The equilibrated columns were loaded with 0.5 ml of protein from the previous step and eluted with PBS (pH 6.6 and 14 ± 2 µS conductivity). The protein fractions were detected and collected at 280 nm based on standard rhIFN α-2b (Institute Pasteur, Iran) using a fraction collector unit equipped with ultraviolet detector (Bio-Rad, USA). The recovery of interferon from each purification step was calculated as: dividing the concentration of eluted interferon by concentration of applied interferon and multiplied by 100 to express it as percent recovery. The protein concentration of interferon α-2b was determined densitometrically by SDS-PAGE and GS-800™ densitometer (Bio-Rad, USA).

### Circular dichroism spectropolarimetry

The percentage of α-helix and β-sheet in each refolded and purified recombinant human interferon α-2b at different pH was measured using a circular dichroism (CD) spectropolarimetry instrument at far UV regions (190 – 250) (JASCO 810 (Japan) based on 0.1 cm path cell and scanning speed of 200 nm/min at 22 °C. The concentration of each refolded and purified recombinant human interferon α-2b at different pH was 0.1 µg/µL.

### *In vitro* bioassay

The bioassay of rhIFN α-2b is based on the inhibitory activity of the interferon on the cytopathic effect of encephalomyocarditis virus on Hep2c cells. The potency is determined by protective effect of interferon against a cytopathic virus and comparing such with an appropriate standard interferon calibration in international unit. The standard rhIFN α-2b was obtained from National Institute of Biological Standards and Control, (NIBSC; Code 95/566). The bioassay was carried out according to British Pharmacopoeia 2012. The "relative potency" of the purified samples was determined and compared using ParLin version 5.0 software. The “comparative potency” was calculated by dividing the relative potency values of each pH by the relative potency value of pH 7.0.

### Statistical analysis

Graphs were drawn by Excel 2007 and OriginPro version 7.0. Bioassay data were analyzed by PLA version 5 using one way ANOVA test. The level of statistical significance was $p < 0.05$.

## RESULTS

### Refolding efficacy at different pH

A significant linear correlation between refolding efficacies and pH through pH increment up to pH 8.0 was recorded and compared to the control pH 7.0 ($p < 0.05$). At pH 8.5 refolding efficacy was higher than at other pH values but the biological activity was decreased (Table 1).

### Purified refolded rhIFN α-2b and interferon recovery

The electropherogram of the refolded and purified rhIFN α-2b reveals a 19 kDa band that showed 95 % purity of protein (Figure 1). Interferon recovery for each purification step was also calculated as listed in Table 2.

**Table 1:** Comparison of refolding efficiency, relative and comparative potencies of rhIFN α-2b

| pH | Refolding Efficiency (%) | Relative Potency | *Comparative potency to pH 7.0* |
|---|---|---|---|
| 7.0 | 42.28 ± 2.11 | 19353546.94 ± 1384515.09 | 1.00 |
| 7.5 | 49.48 ± 2.04 | 24641854.72 ± 478347.18 | 1.27 |
| 8.0 | 61.80 ± 2.85 | 28633902.24 ± 795729.06 | 1.48 |
| 8.5 | 71.22 ± 2.59 | 21081305.00 ± 923234.28 | 1.09 |

*All experiments were performed in triplicate and data are represented as mean ± SD. For easy comparison, comparative potency was calculated by dividing relative potency of each pH values by relative potency value of pH 7.0*

### CD-spectropolarimetric data for purified rhIFN α-2b

CD-spectropolarimetry revealed that with increasing pH of refolding medium, the percent of β-sheet present in the aforementioned protein was decreased whereas relative increment in α-helix can be observed when compared with values at pH 7.0 (Table 3 and Figure 2).

### *In vitro* biological activity of refolded rhIFN α-2b

The *in vitro* biological activities of refolded rhIFN α-2b at pH 7.5 and 8.0 were significantly higher than at pH 7.0 ($p < 0.05$). Surprisingly, the

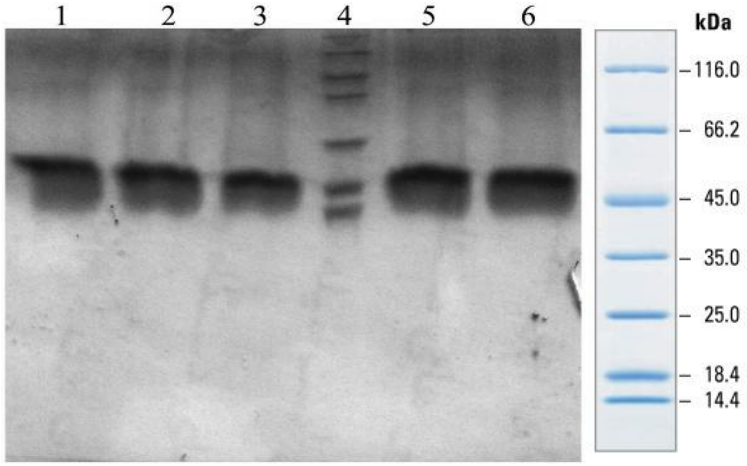


**Figure 1:** SDS-PAGE analysis of purified rhIFN α-2b on 12 % gel; Lane 1: pH 7.0, Lane 2: pH 7.5, Lane 3: rhIFN α-2b Standard (NIBSC; Code 95/566), Lane 4: Protein marker (Cat. No. 26610, Thermo Scientific, USA), Lane 5: pH 8.0 and lane 6: pH 8.5

**Table 2:** Interferon recovery in each purification step

| pH | Interferon recovery in IMAC step (%)* | Interferon recovery in SEC step (%)* |
|---|---|---|
| 7.0 | 84.5 ± 7.1 | 94.6 ± 6.7 |
| 7.5 | 82.4 ± 9.4 | 90.2 ± 7.1 |
| 8.0 | 85. ± 8.2 | 96.3 ± 5.6 |
| 8.5 | 84.6 ± 9.3 | 89.8 ± 9.4 |

**All data are presented as mean ± SD*

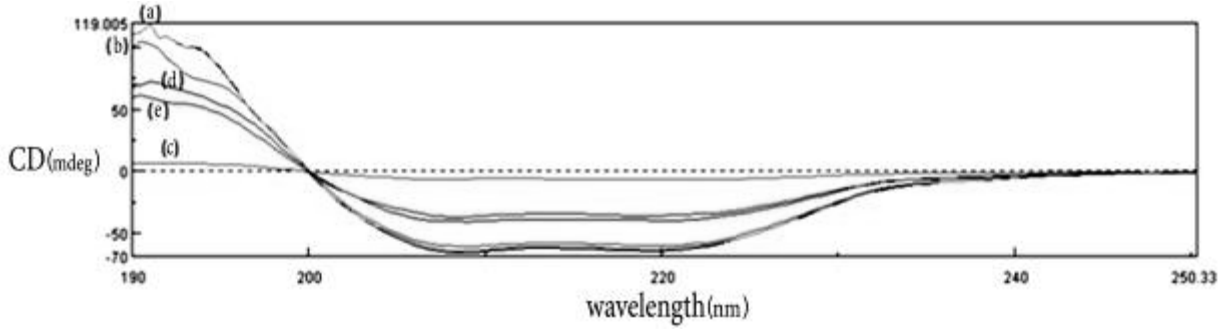


**Figure 2:** CD-polarimetric spectra showing changes in secondary structure of refolded rhIFN α-2b at different pH values; (a) pH 8.5, (b) pH 7.0, (c) blank, (d) pH 8.0 and (e) pH 7.5

**Table 3:** Secondary structure of refolded rhIFN α-2b determined by CD spectropolarimetry at different pH values

| pH | Helix (%) | Beta Sheet (%) | Turn (%) | Random Coil (%) |
|---|---|---|---|---|
| 7.0 | 23.7 | 46.0 | 0.0 | 30.3 |
| 7.5 | 24.8 | 45.1 | 0.0 | 30.1 |
| 8.0 | 27.3 | 43.1 | 0.0 | 29.6 |
| 8.5 | 28.1 | 39.8 | 0.0 | 23.1 |

activity at pH 8.5 was dramatically decreased when compared to corresponding value at pH 8.0 (Table 1).The highest potency i.e. 1.5-fold, caused by correct refolding of rhIFN α-2b, was observed at pH 8.0 (Table 1).

## DISCUSSION

The yield of correct folded protein and monomer formation depend strongly on the protein concentrations in initial step of refolding process [2,18]. The fact is that the effect of concentration variation was not minimized even by using the equal weight of pellets [4,5]. This may be due to different water contents of the harvested inclusion bodies when subjected to the refolding process. Therefore, the equal weight of IB leads to different protein concentration and refolding efficacy. In this study, concentration variation of rhIFN α-2b was eliminated by adjustment concentration before starting the refolding process.

The main objective of all refolding methods in therapeutic protein production from inclusion bodies is to extract functional proteins with high yield and low cost [2]. Aggregation and aggregates are the main obstacles in preparation of functional proteins from inclusion bodies which contribute directly to the decrement in production or refolding yield of the target protein [2, 14]. This phenomenon can be avoided by using pH modulated refolding buffer. Based on previous studies, the Refolding efficacy (RE) was calculated as earlier suggested (Eq 1) [17].

$$RE = (C_2V_2/C_1V_1)100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

where $C_1$ is the concentration (mg/mL) of denatured and reduced protein inputted to the refolding process, $V_1$ is the volume in (mL) of $C_1$, $C_2$ is the concentration of solubilized protein after the oxidized and refolding process and $V_2$ is the final volume of refolding process (mL).

On the other hand, $C_2$ can be calculated as in Eq 2.

$$C_2 = C_{mf} + C_{cf} + C_{im} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

where $C_{mf}$ is the concentration of misfolded, $C_{cf}$ is the concentration of correctly folded, $C_{im}$ is the concentration of impurity. The units are in mg/mL.

Therefore, refolding efficacy of correctly folded protein cannot be directly related to soluble protein [17]. The refolding efficacy is directly dependent on corrected, soluble-misfolded and soluble-impure proteins, it can be named “mixed refolding efficacy. If $C_{mf}$ value was more considerable than $C_{cf}$ value, the "mixed refolding efficacy" was quietly different from bioassay results and had no predictable relationship. So, the term of “mixed refolding efficacy” is fairly clearer than two-sided refolding efficacy term. In this study, unlike “mixed refolding efficacy” curve, the bioassay curve (was not shown) has hyperbolic shape with an optimum pH for correct refolding. In fact, increasing pH above 8.0 dramatically decreases $C_{cf}$ value. Thus, the previous data obtained from refolding efficacy revealed that solubilization cannot be a true approach to finding the optimum correct protein refolding conditions [16,17]. Data from this study showed that “mixed refolding efficacy”, correct folding of interferon, as determined by potency assay were increased simultaneously and was predictable till pH 8.0. At a pH higher than 8.0, the relationship between increment in solubilization and increase in biological activity disappeared. In other words, the increment in solubilization causes a reduction in biological activity and the highly difference appears between refolding efficacy and mixed refolding efficacy. Thus, data resulted from solubilization are not in accordance with biological activity and are misleading for estimating biological activity. Therefore, increasing pH results in increasing solubilization but cannot induce higher biological activity of rhIFN α-2b.

In this study, purification method was applied to purify both folded and misfolded protein in order to directly investigate the effect of different pH on refolding process. By applying this procedure, the direct effect of pH on refolding of rhIFN α-2b was evaluated by *in vitro* assays. This study showed a complexity between solubilization and correct refolding of proteins.

According to secondary structure analysis through CD spectropolarimeter, the changes in secondary structures of aforementioned protein (as compared to standard interferon α-2b obtained at pH 7.0) affect the biological activity of the protein. Structural changes from β-sheet to α-helix, till pH 8.0 increased the protein potency. However, refolding of interferon α-2b at pH 8.5 led to the spatial protein structure far away from its native structure. This can be related to the S-S bound formation in the refolding process of IFN α-2b.

Our results suggest that low refolding efficacy of IFN α-2b could be improved by increasing the proteins net charge using an optimized pH refolding medium (above isoelectric point of IFN

α-2b). Our data also show that the optimal pH for higher solubilization of IBs of interferon is pH 8.5 but the optimal pH for highly functional protein recovery is pH 8.0. Since the amount of active protein recovery is more important than the amount of protein solubilization, this procedure can reduce the production cost and increase the amount of functional protein per batch of production. By changing only pH of the refolding buffer to the optimum point, the refolding efficacy and potency can be reached concurrently to the optimal yield as shown in Table 1.

## CONCLUSION

Employing a low-cost and simple method such as optimizing the pH of the refolding buffer results in higher refolding yield in downstream processing of rhIFN α-2b purification. In addition, our findings indicate that any method with high solubilization will not necessarily lead to active refolded IFN α-2b.

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