

## Study on the Effect of Solution Conditions on Heat Induced-Aggregation of Human Alpha Interferon

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### Abstract

A major problem in the formulation of therapeutic proteins is the irreversible protein aggregation. Recombinant human interferon alpha2b (rhIFN $\alpha$ 2b) has poor stability and undergoes physical degradation. The aim of this study was to investigate the effect of solution conditions on the heat-induced aggregation of rhIFN $\alpha$ 2b. The protein was incubated for 1 h at 40–70 °C and for up to 240 h at 50 °C and its aggregation tendency was then studied using optical density (at 350 nm), SE-HPLC, dynamic light scattering and SDS-PAGE methods. The effect of various pH (5, 6 and 7) and buffer concentrations (10, 55 and 100 mM) on the aggregation of protein following incubation at 50 °C for 72 h was also evaluated. The results obtained for samples incubated at 50 °C for up to 240 h showed that OD<sub>350</sub> and the amount of higher molecular weight aggregates (HMW) increased and the monomer content decreased significantly ( $p < 0.05$ ) as the incubation time increased. Following incubation at various temperatures, a significant increase in OD<sub>350</sub>, drop in monomer content and increase in the amount of HMW aggregates were observed ( $p < 0.05$ ). Data obtained from incubation of samples at 50 °C for 72 h confirmed that regardless of the buffer concentration, the percentage of monomer at pH 6 was significantly higher than that at pH 7 and pH 5 ( $p < 0.05$ ). At constant pH, although not significant, the same trend was observed when the buffer concentration increased to 100 mM. In conclusion, the change in solution conditions can influence the aggregation extent of rhIFN $\alpha$ 2b.

**Keywords:** Interferon alpha2b; Thermal aggregation; SE-HPLC, Optical density; SDS-PAGE.

### Introduction

During the past three decades, many therapeutic proteins have been developed for a wide range of human disorders (1). However, the main impediment for their commercialization has been to design and develop a formulation with an acceptable long shelf life. The problem

arises from the inherent instability and molecular complexity of proteins (2, 3). Thus, a major challenge for a biopharmaceutical product formulator is to stabilize the molecule and postpone its degradation procedures. It is particularly difficult to achieve this goal, due to the protein susceptibility to chemical and physical degradation. In general, degradation causes a loss of protein product quality and, critically, may lead to a decrease of safety and efficacy and occurrence of adverse effects. Aggregation is

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one of the main physical instabilities of proteins which is recognized as a critical degradation pathway with clear influences on the efficacy and safety of a biological product and may be a potent inducer for immune responses with varying manifestations (4, 5). Aggregates are typically defined as assemblies of protein molecules, the level of which increase with time of storage and/or in response to stress.

Several types of stresses (*e.g.*, thermal, acidic, mechanical, and interface adsorption) that proteins may face during the various production and manufacturing processes (*i.e.*, expression, purification and formulation) have been identified and reported in the literature. In these processes, proteins are exposed to changes of the properties of the solution environment such as pH, temperature, salt concentration, buffer composition, surfactants, preservatives, shear rate and surfaces (6-9), which may influence the protein stability and facilitate their aggregations.

Interferons (IFNs) are cytokines with immunomodulatory, anti-proliferative and antiviral properties (10, 11). Specifically, 23 species of structurally similar proteins with molecular masses varying from 17 to 28 kDa have been identified in the human alpha interferon family (12). Among these subtypes, recombinant human IFN alpha2a (rhIFN $\alpha$ 2a) and IFN alpha2b (rhIFN $\alpha$ 2b) are the most successfully commercialized drugs (13) and used for the treatment of a variety of diseases such as hairy cell leukemia, multiple myeloma, basal cell carcinoma, and hepatitis B and C (14, 15). However, development of stable IFN $\alpha$  formulations still remains a great challenge (16). There are some reports on aggregation of IFN $\alpha$  products that could subsequently enhance their immunogenicity (17, 18). Sharma *et al.* have reported that IFN $\alpha$ 2 is likely to form partially unfolded intermediates with conformations which are sensitive to solution pH and temperature. They showed that these unfolded states could play important roles in the aggregation of IFN $\alpha$ 2 and put long-term stability of the protein at risk (6). The aim of this study was to investigate the influences of solution conditions on the aggregation behavior of rhIFN $\alpha$ 2b. For this purpose, the aggregation tendency of rhIFN $\alpha$ 2b was studied and evaluated

at different temperatures, pH values and buffer concentrations, using various analytical methods.

## Experimental

### Materials

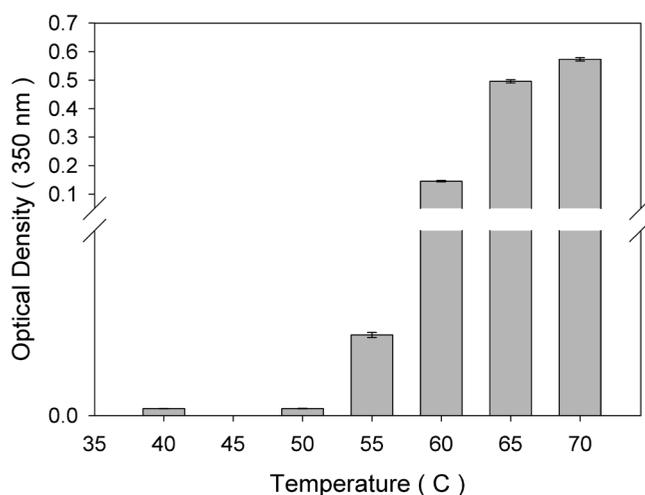
rhIFN $\alpha$ 2b was kindly gifted by Pasteur Institute of Iran (Tehran, Iran). Acrylamide/bis acrylamide solution, ammonium peroxodisulfate, bromophenol blue sodium salt, Coomassie brilliant blue, 1,4-dithiothreitol (DTT), glycerol, glycine, iso-butanol, 2-mercaptoethanol, sodium chloride, sodium dodecyl sulfate (SDS), disodium hydrogen phosphate anhydrous, sodium dihydrogen phosphate 1-hydrate, tetramethylethylenediamine (TEMED), and tris (hydroxyl methyl) amino methane (Tris) were purchased from Merck (Darmstadt, Germany). All solutions were prepared with sterile deionized water (Millipore Company, USA). Buffers were filtered through 0.2  $\mu$ m membranes prior to use.

### Stress studies

The thermal stability of rhIFN $\alpha$ 2b was investigated following heating the protein samples. Protein samples composed of 100  $\mu$ g/mL in 100 mM sodium phosphate buffer solution (pH 7.0) were incubated (IFE 500, Memmert, Germany) for 1 h at various temperatures (40, 50, 55, 60, 65 and 70 °C) and then cooled to room temperature. The level of heat-induced aggregation and monomer content were monitored by both turbidity measurement (optical density determination) at 350 nm and size exclusion high performance liquid chromatography (SE-HPLC). In another experiment, the samples were incubated at 50 °C (up to 240 h) and analyzed by SE-HPLC, optical density determination, dynamic light scattering and electrophoresis techniques. The relationship between thermal stability of protein with pH of sodium phosphate buffer solution (5, 6, and 7) and buffer concentrations (10, 55, and 100 mM) were also studied through heating the protein samples (100  $\mu$ g/mL) at 50 °C for 72 h by SE-HPLC.

### Turbidity measurements

Optical density of the protein sample was determined by ScanDrop<sup>®</sup> 250 (Analytik Jena



**Figure 1.** Optical density of rhIFN $\alpha$ 2b solutions (100  $\mu$ g/mL) following incubation at various temperatures for 1 h (mean  $\pm$  SD; n = 3).

AG, Jena, Germany) at 350 nm against sodium phosphate buffer as the blank solution to evaluate the aggregate production.

#### *Size exclusion high performance liquid chromatography (SE-HPLC)*

Size exclusion separation was carried out on a TSKgel G3000 SWXL column (7.8 mm ID $\times$ 300 mm, 5- $\mu$ m particles, 250 $\text{\AA}$  pore size, Tosoh Bioscience, Japan). Analysis was performed at 25  $^{\circ}$ C under isocratic elution at a rate of 0.5 mL/min with a mobile phase consisting of 100 mM sodium phosphate (pH 7.0), 150 mM NaCl and 0.05% (w/v) sodium dodecyl sulfate (SDS). The column was calibrated by protein standard markers.

#### *Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)*

Gels were composed of a separating gel containing 12.5% (w/v) acrylamide and 0.1% (w/v) SDS, and a stacking gel containing 3% (w/v) acrylamide and 0.1% (w/v) SDS. Gels were run under reducing (sample buffer containing 5% v/v  $\beta$ -mercaptoethanol) and non-reducing (sample buffer without  $\beta$ -mercaptoethanol) conditions at 200 V at room temperature, using a MP300 electrophoresis instrument (Clever Scientific, UK).

#### *Dynamic light scattering (DLS)*

A Malvern Zetasizer Nano ZS

(Worcestershire, UK) laser light scattering system, equipped with a Nano ZS<sup>®</sup> software for data acquisition and analysis, was used for particle size measurements.

#### *Statistical analysis*

Results are reported as mean  $\pm$  SD. Data obtained was compared using one-way ANOVA. Differences between the treatments were assumed to be significant at  $p < 0.05$ . Statistical analysis of all data was performed using IBM<sup>®</sup> SPSS<sup>®</sup> Statistics, v19, 2010.

## **Results and Discussion**

#### *Heating stress studies*

An increase in optical density signal in the non-absorbing region of a protein UV spectrum (320 nm) is typically considered as an indicator of larger species of aggregate forms (19, 20). In this study, the optical density of protein samples incubated at various temperatures for 1 h was monitored (Figure 1). As depicted, compared with non-incubated rhIFN $\alpha$ 2b, no significant change in optical density was observed when incubation was performed at 40 and 50  $^{\circ}$ C, whereas at temperatures higher than 50  $^{\circ}$ C, optical density increased significantly.

SE-HPLC is one of the few analytical techniques suitable for the quantitative assessment of proteins in their native state

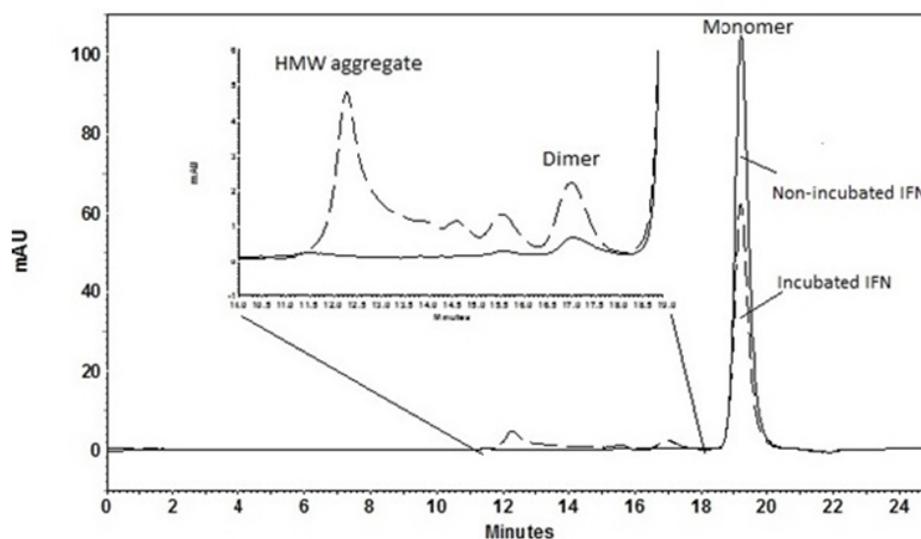


Figure 2. SE-HPLC chromatograms of non-incubated rhIFN $\alpha$ 2b and a sample incubated at 50 °C for 240 h.

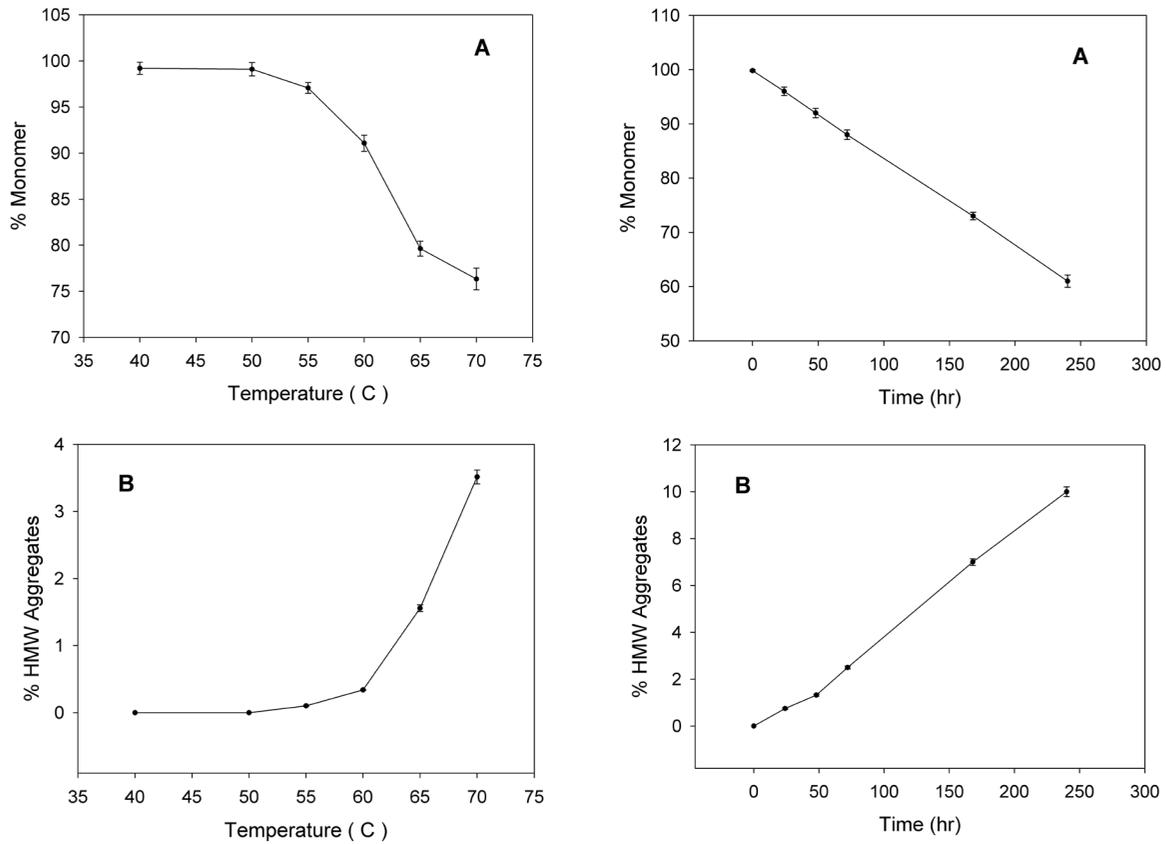
and for the determination of their oligomers and aggregates (21, 22). Non-incubated rhIFN $\alpha$ 2b used in this investigation, contained 99.8% monomer and 0.2% dimer. However, incubation was shown to increase the content of high molecular weight (HMW) aggregates and dimers. Figure 2 indicates SE-HPLC chromatograms of non-incubated rhIFN $\alpha$ 2b and a sample incubated at 50 °C for 240 h. Plots of the percent of remained rhIFN $\alpha$ 2b monomer and HMW aggregates as a function of incubation temperature are shown in Figure 3A and Figure 3B, respectively. Compared with non-incubated rhIFN $\alpha$ 2b, incubation at temperatures greater than 50 °C for 1 h resulted in significant changes in the contents of monomer and HMW aggregates. Results obtained from optical density measurements and SE-HPLC for incubated rhIFN $\alpha$ 2b at 50 °C for 24, 48, 72, 168 and 240 h, also revealed a significant drop in the monomer content and a significant rise in the amount of HMW aggregate and OD<sub>350</sub>, as the incubation time increased (Figure 4).

SDS-PAGE results of samples incubated at 50 °C for 240 h were in consistent with those obtained from other analyses. Under non-reducing condition, the monomer band of incubated rhIFN $\alpha$ 2b sample was weaker than the non-incubated one (Figure 5A), whereas at reducing condition, this band showed higher

intensity (Figure 5B) which may be possibly due to the formation of intermolecular disulfide bonds in thermally induced aggregates. DLS analysis also revealed the presence of aggregates (heterogeneous in size) in the sample incubated at 50 °C for 240 h. Figure 6 depicts that the size distribution peak shifted to the large particles for incubated samples.

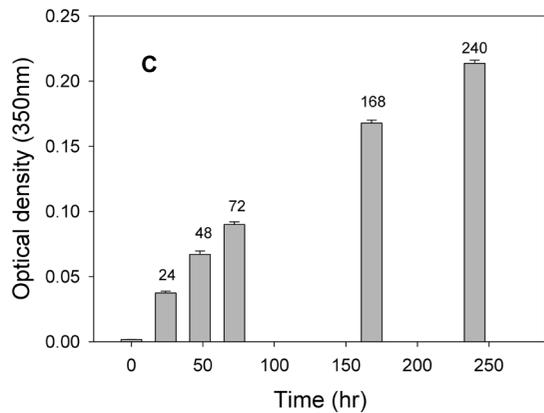
#### *Effect of pH and buffer concentration*

Theoretically, a protein has minimum solubility at its pI due to the minimum protein charge-charge repulsions. Nevertheless, the rate and extent of protein aggregation were expected to be maximal around the pI and negatively correlated with the absolute value of charge (23, 24). Militello *et al.* found that the aggregation of bovine serum albumin increased as the pH was adjusted closer to its pI (about 5) at 58 °C (25). Giger *et al.* studied the aggregation of insulin (with pI of 5.5) in 10 mM NaCl solution at room temperature in the pH range of 3-9 by turbidity measurement and found that this process was fastest at pH 5.6 (26). However, this is certainly not the case for all proteins. Some proteins actually aggregate at a lower rate around their pI values. Majhi *et al.* (27) found that the initial aggregation rate of  $\beta$ -lactoglobulin at the concentration of 1 mg/mL in 4.5 mM NaCl is bell shaped at ambient temperature with a maximum



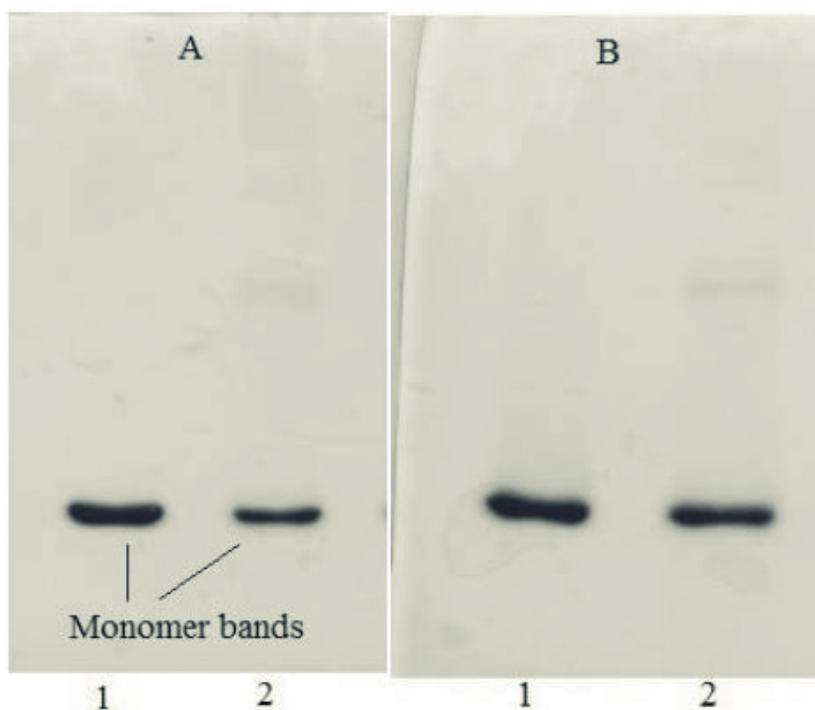
**Figure 3.** A) Plots of percent of monomer and B) percent of high molecular weight aggregates, following incubation of rhIFN $\alpha$ 2b solutions (100  $\mu$ g/mL) for 1 h at various temperatures (mean  $\pm$  SD; n = 3).

rate at pH 4.6-4.7. They also observed that the aggregation rate at the pI point (pH 5.2) was 30 times lower than that at pH 4.7. Similarly, non-native aggregation of recombinant human granulocyte colony stimulating factor in phosphate buffer solution upon incubation at 37  $^{\circ}$ C for 5 days was found to take place easily at pH 6.9 (with 96% loss of monomer), but only 30% is lost at pI (pH 6.1) (28). In this research, we studied the aggregate formation of rhIFN $\alpha$ 2b at its pI (about 6) and evaluated the effect of pH 5 and 7 on the protein aggregation. The results confirmed that following 72 h incubation at 50  $^{\circ}$ C, the percentage of monomer was significantly higher and the percentage of HMW aggregates was significantly lower, at pH 6 compared to pH 7 and pH 5 in all buffer concentrations (Table 1).



**Figure 4.** Plots of A) percent of monomer, B) percent of high molecular weight aggregates, and C) optical density as a function of time, following incubation of rhIFN $\alpha$ 2b solutions (100  $\mu$ g/mL) at 50  $^{\circ}$ C (mean  $\pm$  SD; n = 3).

Buffer concentrations can also influence the aggregation behavior of proteins to a great extent (29-32). It has been reported in the literature that by increasing the concentrations that by increasing the concentrations of both phosphate and citrate buffers from 0 to



**Figure 5.** SDS-PAGE results of 1) non-incubated rhIFN $\alpha$ 2b, and 2) incubated rhIFN $\alpha$ 2b at 50 °C for 240 h, under non-reducing (A) and reducing (B) conditions.

80 mM, the aggregation rate of recombinant human interleukin -1 receptor antagonist at pH 6.5 in the presence of 80 mM NaCl was reduced (33). In the present study, the effect of different concentrations of phosphate buffer on the aggregation behavior of rhIFN $\alpha$ 2b incubated for 72 h at 50 °C was evaluated. Data obtained by SE-HPLC technique revealed that irrespective of the pH applied, the lowest percentage of dimer and HMW aggregates (and in turn the highest percentage of monomer) was produced at the buffer concentration of 100 mM (Table 1). Data presented in Table 1 indicates that approximately 8% of the protein was not detected by SE-HPLC method following incubation which may be due to the surface adsorption of protein and to a

lesser extent, the production of non-detectable, very high molecular weight aggregates during incubation.

### Conclusions

Due to the poor stability, proteins are susceptible to physical degradation, specifically aggregation, affecting the efficacy and safety of the biomolecules. To stabilize the protein against aggregation, solution conditions should be carefully chosen and controlled during various processes. The results obtained in this study indicate that thermal stress mediates rhIFN $\alpha$ 2b aggregation. Our observations supported a pH and buffer concentration-dependent model for

**Table 1.** Effects of pH and buffer concentration on heat-induced aggregation of rhIFN $\alpha$ 2b after 72 h incubation at 50°C (Mean  $\pm$  SD; n = 3).

pH	5			6			7		
Buffer conc. (mM)	10	55	100	10	55	100	10	55	100
% monomer	84.6 $\pm$ 0.65	85.2 $\pm$ 0.38	87.5 $\pm$ 0.50	88.1 $\pm$ 0.55	88.7 $\pm$ 0.85	91.1 $\pm$ 1.36	85.7 $\pm$ 0.87	86.2 $\pm$ 0.72	88.6 $\pm$ 0.56
% dimer	2.5 $\pm$ 0.10	2.3 $\pm$ 0.09	1.7 $\pm$ 0.06	1.44 $\pm$ 0.22	1.2 $\pm$ 0.06	0.76 $\pm$ 0.04	1.79 $\pm$ 0.08	1.59 $\pm$ 0.08	1.23 $\pm$ 0.06
% aggregates	4.1 $\pm$ 0.10	3.9 $\pm$ 0.10	2.7 $\pm$ 0.08	2.4 $\pm$ 0.05	2.3 $\pm$ 0.05	0.95 $\pm$ 0.05	3.64 $\pm$ 0.05	3.5 $\pm$ 0.1	2.4 $\pm$ 0.1

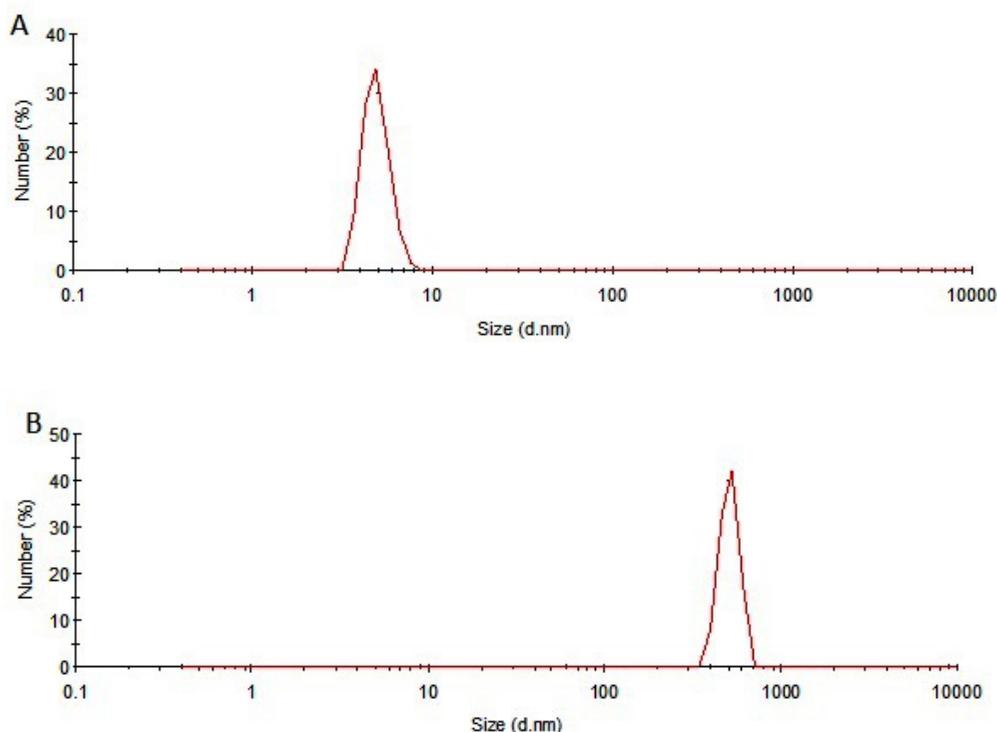


Figure 6. Size distribution of A) non-incubated and B) incubated rhIFN $\alpha$ 2b at 50 °C for 240 h.

heat-induced aggregation behavior of rhIFN $\alpha$ 2b solutions and confirmed that both factors could diminish the aggregation of the protein during thermal stress. Understanding the effects of such factors would play a key role in the protein stabilization and development of therapeutic protein formulations.

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