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European Journal of Pharmaceutical Sciences 24 (2005) 545–552
EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES
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Microcalorimetric studies of the effects on the interactions of human recombinant interferon-α2a

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Received 6 October 2004; received in revised form 10 January 2005; accepted 16 January 2005

Abstract

The applicability of the physical stability of protein solution monitored by isothermal titration calorimetry (ITC) was evaluated. The second virial coefficient, b_2, derived from the dilution enthalpies of protein solution measured by ITC under various experimental conditions was studied. The protein applied in this work is human recombinant interferon-α2a (hrIFN-α2a), which is a commercial drug applied for the treatment of virus-infected diseases. The results obtained were used to predict the possibility of hrIFN-α2a aggregation, and the prediction can be further confirmed by size-exclusion chromatography (SEC). Various factors affecting the stability of protein solution were investigated, for example, temperature, salts, surfactants, and mechanical stress. Specifically, the results show that the dilution enthalpy of hrIFN-α2a increased with increasing temperature and NaCl concentration, while b_2 decreased, indicating that the attraction between hrIFN-α2a molecules was enhanced under these conditions. On studying the effect of mechanical stress, the data obtained reveals that the introduction of centrifugal or vortex force strengthened the attractive forces between hrIFN-α2a molecules. These implications were supported by SEC data, demonstrating that the amount of aggregated hrIFN-α2a was increased. As a consequence, the methodologies presented in this investigation offer a possibility of monitoring the physical stability of protein solution at various stages of recovery, purification as well as the development of appropriate drug storage formulations.

Keywords: Protein; Interferon; Stability; Aggregation; Isothermal titration calorimetry; ITC; Dilution enthalpy; Second virial coefficient; Size-exclusion chromatography; SEC

1. Introduction

Protein stability is an important issue in the academy as well as the pharmaceutical industry. Intensive efforts have been made to find ways to stabilize proteins using various approaches (Bam et al., 1998; Zlateva et al., 1999; Braun and Alsenz, 1997; Kline, 2001; Gross, 1998; Yuen and Kline, 1999; Kwan, 1989). However, so far, not many studies have been able to determine the conditions required for stability with any certainty. Specifically, protein aggregation is a major problem to physical stability of protein solution. In view of this, establishing a methodology that is able to rapidly and effectively monitor the factors affect the interactions between protein molecules is essential.

In this study, isothermal titration calorimetry (ITC) was utilized to monitor the physical stability of protein solution by measuring dilution heat of protein solution. ITC has been widely applied to probe the interactions between protein–solid surfaces (adsorption) as well as the protein–protein interactions (Frazier et al., 2003; Chen et al., 1999; Lin et al., 2000, 2001a, 2001b, 2001c, 2002; Lin and Chen, 2002). For example, protein–protein interactions can be explored by the dilution heat of protein solution measured under various solution conditions, and the endothermic dilution enthalpy of protein solution indicates the at-

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doi:10.1016/j.ejps.2005.01.003

tractions between the protein molecules (Lin et al., 2000, 2001c).

Furthermore, the second virial coefficient, b_2, is the parameter commonly used to probe the interactions between colloids, and it has been reported that can be derived from the dilution enthalpy of liposome and microemulsion droplets measured by ITC (Chen et al., 2000; Liu and Chen, 2000). It is accepted that a negative value of b_2 indicates a global attraction between the molecules, implying a high possibility of aggregation. In addition, b_2, can be derived from osmotic measurements as well. The osmotic b_2 can provide a thermodynamic link between molecular structure and the potential of attractions at various solution conditions, similar to the one measured by calorimetry. However, the amount of sample required for measuring this coefficient by membrane osmometry, or light scattering is much larger than by calorimetry (Haynes et al., 1992; Velev et al., 1998; Bajaj et al., 2004). This drawback limits the application of this coefficient for those pharmaceutical proteins with low concentrations and limited amounts.

In this study, a commercial protein drug, human recombinant IFN-α2a (hrIFN-α2a) was applied. Interferons (IFNs) are a group of proteins, which can be produced by various cells upon infection by viruses and double strain RNAs (Sen, 2001). Specifically, IFNs can be divided into two groups in accordance with their various cell surface receptors and unrelated structural properties: IFN-α, β, ω, δ and τ belong to type I IFN (among them, IFN-α has many subtypes such as IFN-α2a, IFN-α2b), while only IFN-γ is a type II IFN (Stepwart et al., 1980). IFNs have great potential as drugs for the treatment of various virus-infected diseases such as hepatitis C (Lake-Bakaar, 2001) and severe acute respiratory syndrome (SARS) (Cinatl et al., 2003), but they are very unstable, and their activities can be reduced during bioprocessing, storage, freezing as well as lyophilizing. Thus, while IFNs are suitable molecules for clinical application, it is necessary and important that factors affect physical stability of IFN solution should be explored prior to develop the stable IFN formulations.

Various effects on the interactions between human recombinant IFN-α2a molecules were studied in this work. These factors include temperature (30, 40, and 50 °C), pH values (pH 6, 7, and 8), salt species (NaCl and $NaNO_3$), NaCl concentrations (0, 0.1, and 0.5 M), surfactants (Tween 20) as well as the mechanical stress (centrifugal and vortex force).

Consequently, this work presents a novel approach utilizing ITC to rapidly monitor the physical stability of protein solution, particularly for those pharmaceutical proteins with limited concentration and sample amount.

2. Materials and methods

2.1. Materials

hrIFN-α2a (Roferon®-A) was obtained from Hoffmann-La Roche Inc. Polyoxyethylene 20 sorbitan monolaurate (Tween 20) was bought from J.T. Baker (Phillipsburg, NJ). Sodium chloride, sodium nitrate, and potassium phosphate were of analytical grade and purchased from Merck (Darmstadt, Germany).

2.2. Purification (desalting) of hrIFN-α2a

Prior to perform each experiment, the pure hrIFN-α2a was extracted from the Roferon®-A vial, which contains hrIFN-α2a, benzyl alcohol, Tween 80, NaCl and ammonium acetate. Size-exclusion chromatography was performed by using Superdex 75 HR 10/30 column (from Amersham, UK) by ÄCTA purifier (Amersham, UK) at room temperature. The operational conditions were: 20 mM potassium phosphate buffer, pH 7.0, at a flow rate of 0.5 ml/min. The protein absorbance was monitored by a UV detector at a wave length of 280 nm.

2.3. Dilution heat measured by isothermal titration calorimetry (ITC)

The ITC system used in this experiment was obtained from Microcal Inc. (Northampton, MA) and employed an 'insertion' injector to fill into a reaction cell. When thermal equilibrium between the cell and the injector was reached, the protein solution in the injector was titrated into the cell, which contained phosphate buffer solution. The output signal was collected as power versus time and was integrated and quantified against the amount of protein added, to give the enthalpy change of dilution as shown in Fig. 1. The dilution enthalpy of the protein solution, $\Delta H_{\text{dilution}}$ was calculated by the following equation:

$$Q_{\text{dilution}} = C \times \Delta H_{\text{dilution}} \quad (1)$$

where Q_{dilution} is the heat attributed to the dilution of protein solution, and C is the molar concentration of protein injected into the cell. Note that the dilution heat of the protein solution measured in this study was corrected by the dilution heat of the same buffer, under the same condition.

In this study, 1 μl of hrIFN-α2a solution (0.01 mg/ml) was prepared in a 20 mM potassium phosphate buffer solution under various conditions and titrated into the cell which contains 1.4 ml, 20 mM potassium phosphate buffer under the same condition. A typical ITC thermogram and the corresponding dilution enthalpy, $\Delta H_{\text{dilution}}$, against the hrIFN-α2a molar concentration are shown in Fig. 1.

2.4. Calorimetric second virial coefficient

On the basis of the measured dilution heat of the protein under varying concentrations, the corresponding calorimetric second virial coefficient values thus can be obtained by virial equation analysis under a hard-sphere interaction potential assumption (Chen et al., 2000; Liu and Chen, 2000). Generally, the dilution heat of the protein solution can be fitted to

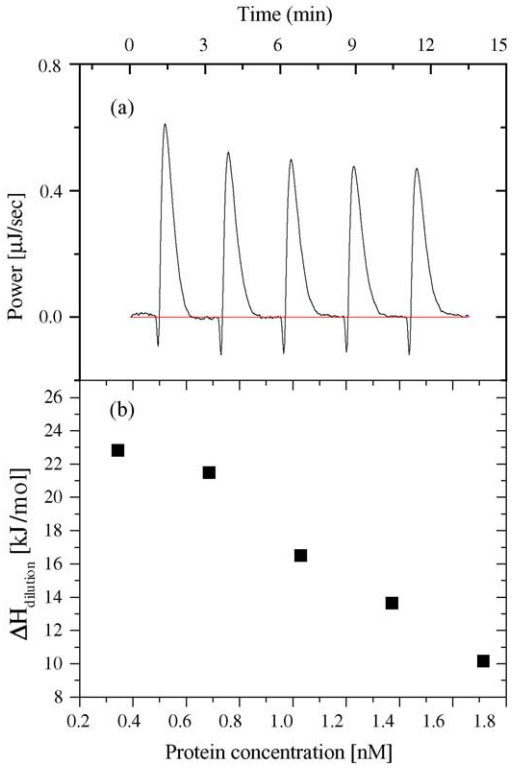

Fig. 1. (a) A typical ITC thermogram (endothermic) and (b) the corresponding dilution enthalpy, $\Delta H_{\text{dilution}}$ against the injected protein molar concentration.

a second-order polynomial function as:

$$\frac{\mathrm{d}(Q_{\text{dilution}}/NK_{\text{B}}T)}{\mathrm{d}\rho} \cong \frac{\mathrm{d}(E/NK_{\text{B}}T)}{\mathrm{d}\rho} = b_2 + b_3\rho + b_4\rho^2 \tag{2}$$

where E is the internal energy (J), ρ is the solute number density, which equals to N/V, where V is the volume of the solution (ml) and N is the number of particles, i.e., number of protein molecules in the solution, K_{B} is the Boltzmann constant (J/K), T is the absolute temperature (K), and b_2, b_3, b_4 are the calorimetric virial coefficients. Specifically, b_2 is the second calorimetric virial coefficient (ml/particle).

For an open liquid system, without any change in pressure, the dilution volume can be neglected as compared to the total solution volume of the system. The dilution heat thus measured should equal to the internal energy change of the system. Moreover, the internal energy change of the system with the number density of the protein molecules in the solution can be expressed by the virial equation as:

$$\frac{E}{NK_{\text{B}}T} = \frac{3}{2} - T\sum_{i=1}^{\infty}\frac{1}{i}\frac{\mathrm{d}B_{i+1}}{\mathrm{d}T}\rho^i \tag{3}$$

where B_i is the virial coefficient. Comparison of Eq. (2) with Eq. (3) reveals that the calorimetric coefficients obtained by fitting of the polynomial can be affiliated with the virial coefficients of Eq. (3) by the following equation:

$$b_2 = -T\left(\frac{\mathrm{d}B_2}{\mathrm{d}T}\right) \tag{4}$$

Furthermore, the second virial coefficient can be represented by the interaction potential energy function $U(r)$, on the basis of the statistic thermodynamics as:

$$B_2(T) = -2\pi\int_0^{\infty}\left[\exp\left(-\frac{U(r)}{K_{\text{B}}T}\right) - 1\right]r^2\,\mathrm{d}r \tag{5}$$

If a square-well attractive potential energy function $U(r)$ is applied and plugged into Eq. (5), therefore, Eq. (4) can be represented as follows:

$$b_2 = -T\left(\frac{\mathrm{d}B_2}{\mathrm{d}T}\right) = -B_0(\lambda^3 - 1)\,\mathrm{e}^{\varepsilon/K_BT}\left(\frac{\varepsilon}{K_{\text{B}}T}\right) < 0 \tag{6}$$

where

$$B_0 = \left(\frac{16}{3}\right)\pi R_{\text{HS}}^3$$

$$\lambda = \frac{(2R_{\text{HS}} + \sigma)}{2R_{\text{HS}}}$$

σ and ε denote the width and depth of the square-well attractive potential function, respectively, and R_{HS} is the radius of a hard-sphere.

When qualitatively analyzed, Eq. (6) demonstrates that a negative b_2 value indicates the attractive forces between the protein molecules, and a greater negative value of b_2 (higher values of σ and ε), implies protein aggregation is likely.

2.5. Probing of aggregation of hrIFN-α2a by size-exclusion chromatography

The distribution of the molecular weights of hrIFN-α2a was determined by size-exclusion chromatography (SEC), using the same Superdex 75 column as mentioned above. The experiment was performed by loading 500 μl of sample at a flow rate of 0.5 ml/min, using the same buffer and the same condition as the corresponding ITC experiment.

3. Results and discussion

3.1. Purification (desalting) of hrIFN-α2a by size-exclusion chromatography

The chromatogram is shown in Fig. 2. The elution volume of the monomeric hrIFN-α2a peak is 12.0 ml; and the corresponding molecular weight (obtained by the calibration curve inserted) is about 20.4 kDa. The concentration of the pure, monomeric form of hrIFN-α2a at 0.01 mg/ml was determined by Bradford method (Bradford, 1976) using bovine serum albumin as a standard and this was used for all further studies.

3.2. Effects of temperature

To simply discuss the temperature effects on the interactions between hrIFN-α2a molecules thermodynamically, the averaged value of dilution enthalpy, $\Delta H_{\text{dilution}}$, calculated from the average of five measured enthalpy data, and the derived calorimetric second virial coefficient, b_2 are shown in Figs. 3 and 4, respectively. The corresponding statistic data were summarized in Table 1. Since the heat of dilution indicates the interactions between protein molecules and/or other small molecules, such as salts, the dilution enthalpy change of a protein solution should be an index of the interactions between protein molecules. For instance, a positive (endothermic) value for dilution enthalpy implies that there are attractive forces between the protein molecules, that is, the protein unfavorably dissolves into the buffer solution. In this study, the results reveal that the interactions between the hrIFN-α2a molecules were attractive and increased with temperature. This is mainly attributed to enhanced hydrophobic interactions, resulting from the structural changes to the protein under the heating process. Since the compact conformation of the protein became loosely at a higher temperature and gradually exposed its hydrophobic regions to the solvent, increased hydrophobic attractions thus can be expected. The study of Sharma and Kalonia (2003) on temperature effect of hrIFN-α2a structure by circular dichroism also reveals that a significant loss in the tertiary structure of hrIFN-α2a by increasing temperature from 15 to 50 °C.

In addition, as can also be seen in Figs. 3 and 4, the $\Delta H_{\text{dilution}}$ and b_2 values at 30 °C are quite different when compared to the same after cooling from 40 or 50 °C. This implies that the structural changes in hrIFN-α2a induced by heating are irreversible. Consequently, based on the ITC and b_2 data presented, the aggregation of hrIFN-α2a would seem to be increased by increasing the temperature. In conclusion,

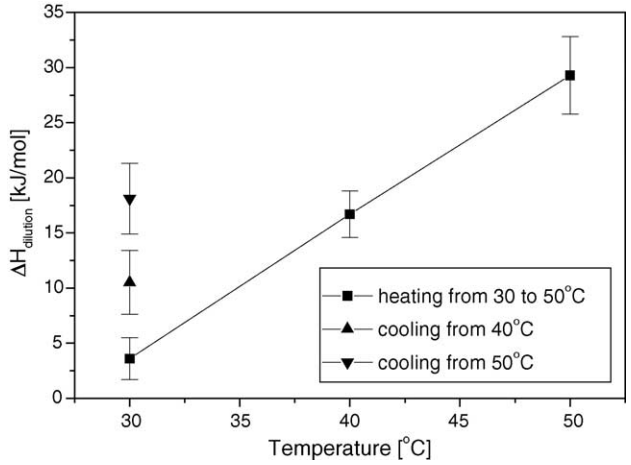

Fig. 3. The effect of temperature on the dilution enthalpy, $\Delta H_{\text{dilution}}$, of hrIFN-α2a solution.

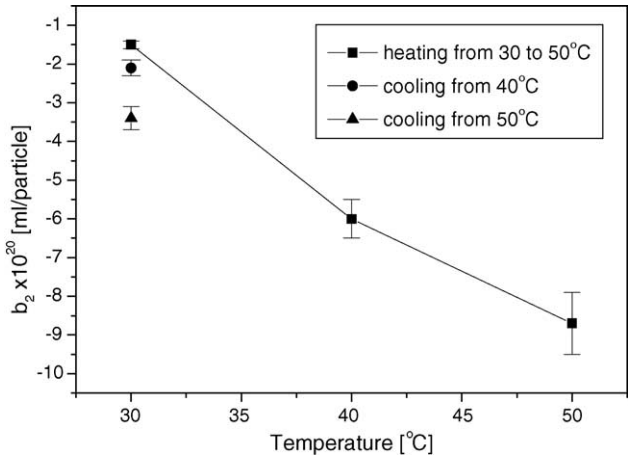

Fig. 4. The effect of temperature on the calorimetric second virial coefficient, b_2, of hrIFN-α2a solution.

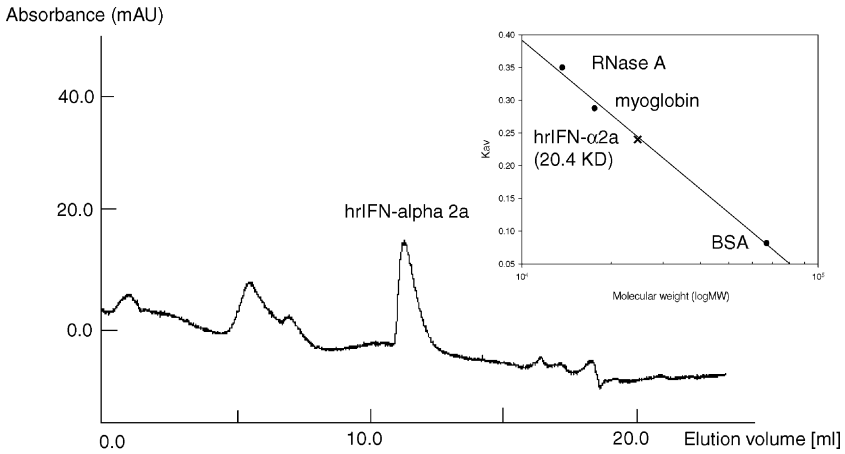

Fig. 2. A gel filtration chromatogram of purification (desalting) of hrIFN-α2a from Roferon®-A; inserted is the corresponding calibration curve, in which ribonuclease A (RNase A), myoglobin, and bovine serum albumin (BSA) are used and their molecular weights are 13.7, 17.6, and 67 kDa, respectively.

Table 1
The values of dilution enthalpy, $\Delta H_{dilution}$ and calorimetric second virial coefficient, b_2 of hrIFN-α2a solution obtained under various conditions

Experimental conditions[a]		$\Delta H_{dilution}$ (kJ/mol)[b]	$b_2 \times 10^{20}$ (ml/particle)[c]
Blank		3.6 ± 1.9	−1.5 ± 0.1
Temperature	Heating from 30 to 40 °C	16.7 ± 2.1	−6.0 ± 0.5
	Heating from 30 to 50 °C	29.3 ± 3.5	−8.7 ± 0.8
	Cooling from 40 to 30 °C	10.5 ± 2.7	−2.1 ± 0.2
	Cooling from 50 to 30 °C	18.8 ± 3.2	−3.4 ± 0.3
Salts	0.1 M NaCl	10.5 ± 2.0	−3.0 ± 0.2
	0.5 M NaCl	25.1 ± 4.3	−9.6 ± 0.8
	0.1 M $NaNO_3$	7.5 ± 1.2	−2.5 ± 0.2
pH	pH 6.0	16.7 ± 2.6	−3.3 ± 0.3
	pH 8.0	3.0 ± 1.5	−1.3 ± 0.1
Tween 20	0.01% (w/v)	2.2 ± 0.2	−1.2 ± 0.1
	0.02% (w/v)	1.9 ± 0.2	−1.0 ± 0.1
Mechanical stress	Centrifugation	9.2 ± 1.9	−2.6 ± 0.2
	Vortex	23.8 ± 5.5	−9.7 ± 0.9

[a] The experimental condition of blank is 20 mM potassium phosphate buffer, pH 7.0, without NaCl or $NaNO_3$, Tween 20 and mechanical stress, at 30 °C.
[b] $N = 5$.
[c] $N = 3$.

for bioprocessing, purification or storage of IFN-related protein, a low operation temperature is recommended.

3.3. Effects of salts and their concentrations

The values of $\Delta H_{dilution}$ as well as b_2 of hrIFN-α2a in different salts and at different concentrations are shown in Figs. 5 and 6, respectively. The results demonstrate that the attractions between hrIFN-α2a molecules increased with NaCl concentration. This is because in the presence of salts, the native structure of hrIFN-α2a shifts towards being less compact and the solvent-exposed hydrophobic surfaces increased, leading to stronger hydrophobic interactions between protein molecules. Furthermore, the strength of electro-repulsion between hrIFN-α2a molecules would seem to be decreased with increasing salt concentrations. Consequently, protein aggregation can be caused either by enhancing hydrophobic interactions or by decreasing electro-repulsive forces between hrIFN-α2a molecules. The SEC chromatogram also confirms this. In Fig. 7, the chromatogram shows that an additional peak with a molecular weight of about 44.7 kDa mainly due to aggregation of hrIFN-α2a appeared in the 0.5 M NaCl condition as compared to blank condition. These observations were similar to reported data (Melander and Horvath, 1977), which has shown that proteins can be easily aggregated by adding salts.

Moreover, as also shown in Figs. 5 and 6, for the same salt concentration (0.1 M), NaCl has a greater ability to increase the attractions between hrIFN-α2a molecules than does $NaNO_3$. This is because NaCl has a relatively larger value for its molal surface tension increment compared $NaNO_3$ (the values of molal surface tension increment for NaCl and $NaNO_3$ are 1.64 and 1.06, respectively (Melander and Horvath, 1977)). The study of Beldarrain et al. (2001) also demonstrates that the stabilization of hrIFN-α2a is highly dependent on the salt species and ionic strength. As a consequence, the aggregation of hrIFN-α2a can be increased by adding salts and increasing the salt concentra-

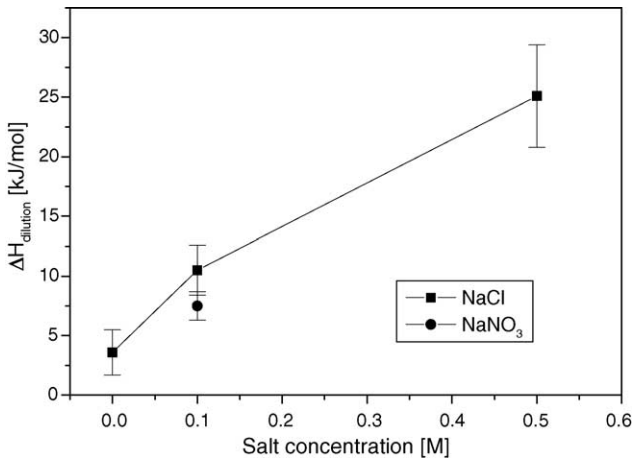

Fig. 5. The effects of salt species and their concentrations on the dilution enthalpy, $\Delta H_{dilution}$, of hrIFN-α2a solution.

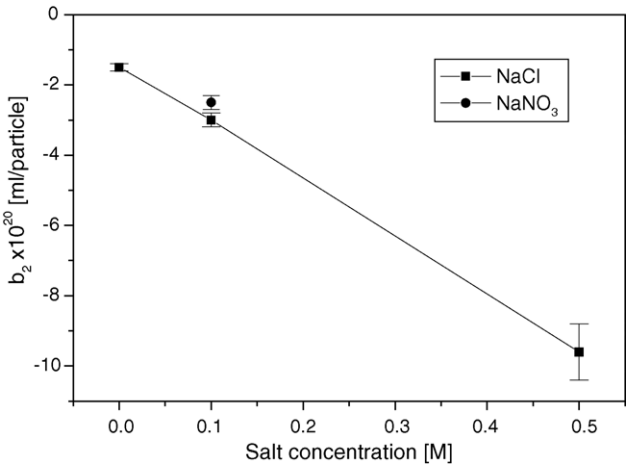

Fig. 6. The effect of salt species and their concentrations on the calorimetric second virial coefficient, b_2, of hrIFN-α2a solution.

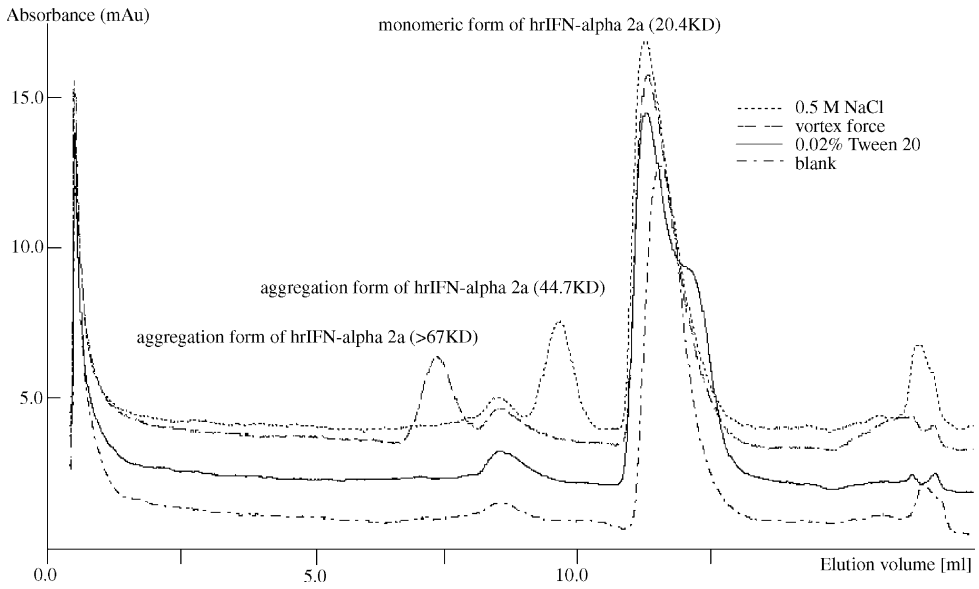

Fig. 7. The SEC chromatograms of hrIFN-α2a under various experimental conditions. Blank means hrIFN-α2a was at 20 mM potassium phosphate buffer, at pH 7.0, without salts, Tween 20, and mechanical stress.

tion, although salts usually serve as an isotonizing agent in commercial IFN formulations (Gross, 1998; Yuen and Kline, 1999).

3.4. Effects of pH values

The $\Delta H_{\text{dilution}}$ and b_2 values of hrIFN-α2a at different pH values are presented in Figs. 8 and 9, respectively. The data show that the attractive forces between hrIFN-α2a molecules changed at the pH values studied. Specifically, at pH 6, the attraction between hrIFN-α2a is much stronger than that at pH 7 or 8. This is because at pH 6, which is near the p*I* of hrIFN-α2a (5.7), the protein becomes electro-neutralized, the repulsions between the protein molecules are minimized and the hydrophobic attractions enhanced. This is also supported by the literature (Yuen and Kline, 1999), where the formation of hrIFN-α2a aggregation as well as the adsorption of hrIFN-α2a onto the glass surface, reached a maximum at pH 5–6. Note that selection of an optimal pH value for an IFN solution needs careful consideration. For example, in addition to physical stability as discussed above, the degradation of hrIFN-α2a can be minimized at a pH near p*I* (Yuen and Kline, 1999). Therefore, an optimal pH value for stabilizing the IFN solution should be determined by analysis of a variety of factors.

3.5. Effects of mechanical stress

The effects of mechanical stress on the stability of hrIFN-α2a were tested by centrifugal forces at 3000 × *g* for 30 min

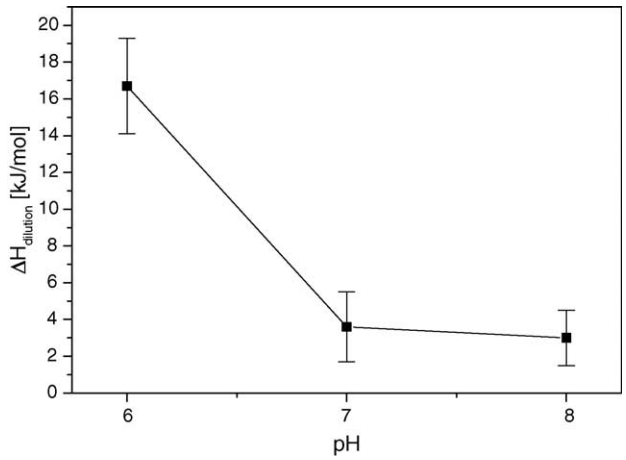

Fig. 8. The effect of pH value of potassium phosphate buffer on the dilution enthalpy, $\Delta H_{\text{dilution}}$, of hrIFN-α2a solution.

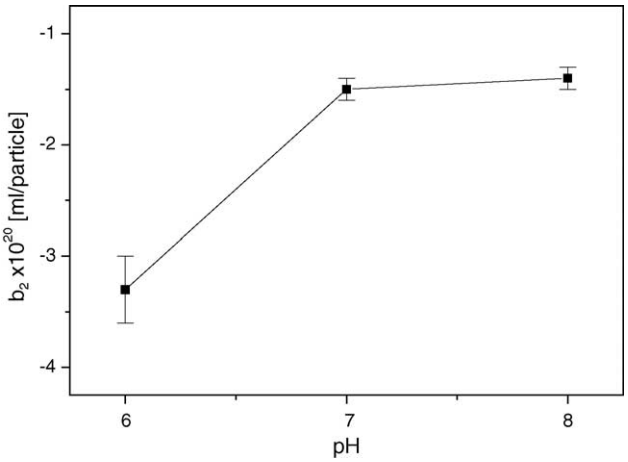

Fig. 9. The effect of pH value of potassium phosphate buffer on the calorimetric second virial coefficient, b_2, of hrIFN-α2a solution.

and vortexing (at speed 8) for 1 min. The data shown on Table 1 demonstrate that the attractions between hrIFN-α2a molecules can be significantly increased by mechanical stress, partly due to change in the structural conformation of hrIFN-α2a molecule, especially under vortex conditions. This observation is further confirmed by the SEC results. As shown in Fig. 7, an additional peak with a molecular weight higher than 67 kDa (out of the calibration range) was obtained under vortex treatment compared to the untreated condition, and this is probably the aggregated form of hrIFN-α2a. This is also similar to a previous report (Bam et al., 1998), where the α-helical content of IFN-γ decreased with the introduction of mechanical stress, such as agitation, leading to the irreversible conformational changes and this favored protein aggregation. It should be noted that the significant effect of vortexing is probably more a result of the exposure to air (hydrophobic interface) than the solely changes in protein structure. Furthermore, the process of inactivation by mechanical stress during bioprocessing probably involves either the linking together of the sulfhydryl groups or disulfide interchange reactions to form inter or intra-molecular disulfide bridges, leading IFN to adopt a biologically inactive configuration. From this point of view, reducing agents such as reduced glutathione, can be applied to retain IFN sulfhydryl groups in a reduced state without affecting the disulfide linkages which are essential for IFN activity (Yuen and Kline, 1999).

3.6. Effects of surfactants

Tween 20 is useful as a stabilizer and prevents adsorption of proteins (such as recombinant human growth hormone and recombinant human factor XIII) onto stainless steel or glass surfaces or damage from the mechanical stress as well as protein aggregation (Bam et al., 1998; Kreilgaard et al., 1998). In this study, two concentrations of Tween 20, 0.01% (w/v), and 0.02% (w/v), equals to 86 and 172 μM, respectively, were studied. The results presented in Table 1 demonstrate that the attractions between hrIFN-α2a molecules can be minimized with the addition of Tween 20, probably because the hydrophobic patches on the hrIFN-α2a surface are able to bond with (or are blocked by) this non-ionic surfactant, and therefore, there is a decrease in the hydrophobic interactions. Specifically, the attractions between hrIFN-α2a decreased with the concentration of Tween 20, although the difference in b_2 values between 0.01 and 0.02% of Tween 20 is not significant. This result is also similar to the study of Kreilgaard et al. (1998), where the insoluble protein aggregates are not obviously decreased by an increasing concentration of Tween 20 from 60 to 250 μM. Furthermore, as shown in Fig. 7, the SEC chromatogram demonstrates that no aggregated form of hrIFN-α2a appeared in the presence of 0.01% Tween 20. Note that the shoulder on the monomeric hrIFN-α2a peak is probably due to the retention behavior of the protein being changed by the surfactant.

4. Conclusions

Physically, proteins can be stabilized in two ways: stabilizing the inter-molecular forces (the structural conformation) or decreasing the attractive inter-molecular forces (protein–protein interactions). The latter interaction may also be seen as an increase in the repulsive inter-molecular forces. In this study, a brief survey on the effects of interactions between hrIFN-α2a molecules was achieved by ITC measurements and SEC so as to evaluate the applicability of the calorimetric b_2. The data obtained shows that the storage of hrIFN-α2a solution at a low temperature and/or at a pH away from its p*I*, together with a limitation of mechanical stress on the protein in the presence of the correct concentration of a surfactant such as Tween 20 can really decrease attractive protein–protein interactions and thus minimizes hrIFN-α2a aggregation. However, it should be noted that the addition of salts can stabilize the structural conformation of hrIFN-α2a, but also enhances protein aggregation as well. These data demonstrate that the identification of a formulation able to stabilize protein physically will depend critically on balancing the intra- and inter-molecular forces. As a consequence, optimization of protein stability requires a systematic approach, and our results show that using ITC to carry out such a systematic study of the physical stability of protein solution is possible.

Acknowledgment

The authors would like to thank Mr. B.-Y. Song for the contribution of chromatography of hrIFN-α2a.

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