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Research paper

Elimination of the free sulfhydryl group in the human serum albumin (HSA) moiety of human interferon-α2b and HSA fusion protein increases its stability against mechanical and thermal stresses

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ABSTRACT

Interferon-α2b (IFN-α2b) and human serum albumin (HSA) fusion protein (IFN-α2b–HSA) is a promising long acting formulation of IFN-α2b for the treatment of hepatitis C. However, accelerated mechanical and thermal stress tests revealed that IFN-α2b–HSA was prone to disulfide-linked aggregation. The formation of aggregates was associated with an increase in immunogenicity in mice. The addition of non-ionic surfactant Tween 80 increased the stability of IFN-α2b–HSA against agitation, but its thermal stability was not improved. Moreover, Tween 80 prompted the aggregation of IFN-α2b–HSA during quiescent storage. To increase the stability of IFN-α2b–HSA, the unpaired cysteine residue in this fusion protein was substituted with serine by site-directed mutagenesis. The resultant fusion protein was designated as IFN-α2b–HSA(C34S). IFN-α2b–HSA(C34S) had significant higher stability over IFN-α2b–HSA, which was evidenced by the facts that after agitation for 72 h or incubation at 60 °C for 2 h, more than 90% of IFN-α2b–HSA(C34S) remained monomeric. Consistent with its improved stability, the immunogenicity of IFN-α2b–HSA(C34S) increased less significantly after agitation. Pharmacokinetics studies in rats revealed that both fusion proteins had similar pharmacokinetic behavior, both with a half-life of about 50 h.

1. Introduction

The advent of protein pharmaceuticals revolutionized the treatment of many diseases, including cancer, infectious diseases, autoimmune diseases and AIDS/HIV [1]. However, the large size and complex structure of protein molecules cause chemical and/or physical instabilities, which make stable aqueous pharmaceutical protein formulation a challenging task [2]. Mechanical stress generated by shaking or stirring and thermal stress originated from intentional heat treatment or accidental exposure to high temperature environment are frequently encountered for protein pharmaceuticals, especially for liquid formulations [3]. Protein aggregation, which may be soluble or insoluble, reversible or non-reversible, covalent or non-covalent, is one of the prevalent physical instability reactions in liquid protein formulation [4]. Aggregation can compromise biological activity and increase the immunogenicity of protein pharmaceuticals [5–7]. Thus, the formulation must be designed to protect the protein from aggregation. To facilitate the formulation process, especially in early development, accelerated thermal or mechanical stress tests are frequently performed.

Human serum albumin (HSA) fusion technology has evolved as a general strategy to create long acting protein pharmaceuticals [8]. This technology had been successfully employed to create long acting form of human interferon-α2b [9], human interleukin-2 [10], human granulocyte-colony stimulating factor [11], human growth hormone [12], human insulin [13] and human glucagon-like peptide-1 [14]. All the above mentioned HSA fusion proteins are developed by Human Genome Sciences, Inc. (HGSI).

Formulation studies have demonstrated that some HSA fusion proteins such as HSA and human growth hormone fusion protein (HSA–hGH) were prone to aggregation during agitation [15], but their accurate molecular mechanism remained to be elucidated.

IFN-α2b–HSA is a novel fusion protein of human interferon-α2b and HSA created by our laboratory. IFN-α2b–HSA differs from HGSI's version of HSA and IFN-α2b fusion protein in its orientation. The alteration of the orientation alleviated the structural perturbation between IFN-α2b and HSA, and restored disulfide pairing of IFN-α2b. IFN-α2b–HSA had higher homogeneity and stability during quiescent storage [16]. However, accelerated mechanical and thermal stress tests showed that IFN-α2b–HSA was also susceptible to aggregation. The instability of interferon-α2b and HSA fusion protein necessitated lyophilized formulation, as had been reported

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by HGSI [17]. Such formulation was undesirable for protein pharmaceuticals, since lyophilization is a time-consuming and cost-expensive process [18].

In this study, we have attempted to elucidate the molecular mechanism for the instability of IFN-α2b–HSA during agitation and heat treatment. Two different strategies: addition of excipients and protein engineering methodology, were employed to enable a liquid formulation for this promising therapeutic protein candidate. Their efficacy and utility were compared in detail.

2. Materials and methods

2.1. Strains, plasmid and culture media

Pichia pastoris strain GS115 and plasmid pPIC9 were from Invitrogen Life Sciences. Minimal Dextrose (MD) plate containing the following components (per liter): 13.4 g yeast nitrogen base (YNB) with ammonium sulfate without amino acids (Difco), 20 g dextrose, 0.4 mg biotin, 10 g agar, was used for selection of transformants. Yeast Extract Peptone Dextrose (YPD) medium containing the following components (per liter): 10 g yeast extract (Oxiod), 20 g peptone (Oxiod) and 20 g dextrose, was used as growth medium. Buffered Glycerol-complex Medium (BMGY) containing the following components (per liter): 10 g yeast extract, 20 g peptone, 100 mM potassium phosphate, pH 6.0, 13.4 g YNB, 0.4 mg biotin, 10 g glycerol and Buffered Methanol-complex Medium (BMMY) containing the following components (per liter): 10 g yeast extract, 20 g peptone, 100 mM potassium phosphate, pH 6.0, 13.4 g YNB, 0.4 mg biotin, 5 ml methanol, were used for protein expression in shake flask.

2.2. Cloning and plasmid construction

The genes encoding HSA and IFN-α2b were both artificial genes synthesized according to the *P. pastoris* codon usage preference [19]. IFN-α2b–HSA is a seamless fusion protein of IFN-α2b and HSA. To prompt the disulfide pairing between Cys1 and Cys98 of IFN-α2b, its orientation is with N-terminal IFN-α2b followed by C-terminal HSA. The gene encoding IFN-α2b–HSA fusion protein was amplified using a two-step PCR strategy as described previously [16].

2.3. Site-directed mutagenesis

Cysteine residue at position 34 of HSA was substituted with serine by PCR-mediated mutagenesis. The introduction of this mutation was performed using a two-step PCR strategy with following forward primers, F: 5′-CCCTCGAGAAAAGATGTGACTTGCCACAAACC CACTCC-3′ and C34SF: 5′-TACTTGCAACAATCTCCATTCGAAGAC-3′ and reverse primers, R: 5′-GGGAATTCCTATTACAAACCCAAAGCAG CTTGAGAAGC-3′ and C34SR: 5′-GTCTTCGAATGGAGATTGTTGC AAGTA-3′. In each mutagenesis reaction, a forward or reverse mutagenic primer was used that flanked the nucleotide(s) to be changed. One set of PCR employed forward primer F and a reverse mutagenic primer C34SR while the other set of PCR used a forward mutagenic primer C34SF and reverse primer R. These two reactions amplify the 5′ and 3′ portions of the fusion protein, respectively. The products from the above mentioned reaction were mixed together in equimolar amounts and a second round of PCR was performed with primers F and R, amplifying the full length fusion protein containing the desired mutation. The PCR product from each of these reactions was digested with XhoI–EcoRI and cloned into the XhoI–EcoRI sites of plasmid pPIC9. The sequence of the PCR product in each plasmid was confirmed by DNA sequence analysis and shown to contain only the desired changes.

2.4. Protein expression and purification

IFN-α2b and HSA fusion proteins were expressed from *P. pastoris* and purified to homogeneity as described previously [16].

2.5. Protein aggregation induced by mechanical stress

A sample of 10 ml containing 1 mg/ml fusion protein was placed into a 30 ml borosilicate glass test tube (diameter = 16 mm), which provided sufficient head space for bubble entrainment into the solution from the air–liquid interface during agitation. Samples were orientated horizontally and agitated at 200RPM on an orbital shaker at 30 °C for 72 h. Sample tubes were removed for testing at 0, 24, 48 and 72 h. The total soluble aggregates were detected by size-exclusion chromatography (SEC), the covalent aggregates were detected by non-reducing and reducing SDS–PAGE. To study the effects of Tween 80 on the stability of fusion protein, Tween 80 (Farco Chemical Supplies, Hong Kong, Lot No. 0160Fc0099A28) was added to a final concentration of 0.05%.

2.6. Protein aggregation induced by heat treatment

A sample of 10 ml containing 1 mg/ml fusion protein was placed into a 30 ml borosilicate glass test tube (diameter = 16 mm), and was placed in water bath of 60 °C for 2 h. The total soluble aggregates were detected by size-exclusion chromatography (SEC), the covalent aggregates were detected by non-reducing and reducing SDS–PAGE. To study the effect of Tween 80 on the stability of fusion protein, Tween 80 (Farco Chemical Supplies, Hong Kong, Lot No. 0160Fc0099A28) was added to a final concentration of 0.05%.

2.7. Size-exclusion chromatography (SEC)

SEC was utilized to quantify levels of monomeric protein and soluble aggregates. A Superdex 200 prep grade (26 × 600) gel filtration column connected to AKTAprime chromatography system was used. The mobile phase was 20 mM sodium phosphate, 100 mM NaCl, pH 7.0, and the flow rate was 2.4 ml/min. The amounts of native monomer, non-native dimer, and large soluble aggregates were quantified using UV absorbance at a detection wavelength of 280 nm.

2.8. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE)

The gels consisted of a separating gel containing 10% (w/v) acrylamide and 0.1% (w/v) SDS, and a stacking gel containing 4.5% (w/v) acrylamide and 0.1% (w/v) SDS. Gels were run under reducing (sample buffer containing 5% (v/v) β-mercaptoethanol) and non-reducing conditions at 180 V at room temperature. The electrophoresis buffer was 25 mM Tris(hydroxymethyl) aminomethane, 192 mM glycine and 0.1% (w/v) SDS. Gel electrophoresis was performed with a mini VE system (Amersham Pharmacia Biotech). Samples analyzed under reducing conditions were first boiled for 5 min. Protein bands were visualized by coomassie blue staining.

2.9. Immunogenicity studies

The immunogenicity of different protein preparations (IFN-α2b–HSA or IFN-α2b–HSA(C34S), agitated or unagitated) was assessed in Kunming (KM) mice (20 ± 2 g in weight, 8–9 weeks in age) (Experimental Animal Center, Academy of Military Medical Sciences of China, Beijing, China). Eight groups of KM mice, three male mice and three female mice per group, received 100 μg of

different protein preparations subcutaneously twice a week for 4 weeks. Bleeds were taken from the mice at 7 days subsequent to the final administration.

Sera were analyzed by enzyme-linked immunosorbent assay (ELISA) for antibodies against unagitated fusion proteins. Unagitated IFN-α2b–HSA or IFN-α2b–HSA(C34S) were diluted to a concentration of 2 μg/ml in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.5). Ninety-six well plates (Biousing, Wuxi, China) were coated for 16 h at 4 °C with 100 μl/well of the above antigen solution. The plates were blocked with 2% (w/v) non-fat milk powder in PBS (blocking buffer) for 1 h at room temperature. The plates were washed extensively with PBS containing 0.05% Tween 20. Serial dilutions of the sera were prepared in blocking buffer, added to the wells, and incubated for 2 h at room temperature. After repeating the washing steps, the plates were incubated with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG diluted 1 in 1000 in blocking buffer. Washing was repeated and detection was performed using 100 μl of 1,2-phenylenediamine (Merck) solution. After 15 min of incubation, the absorbance was read at 492 nm with a ZS-3 ELISA plate reader (Xinhua Electrical and Mechanical Technology Company, Beijing, China). The absorbance values were plotted against log dilution. Curves were fitted with a sigmoidal curve (version 3.0; GraphPad Software, Inc., San Diego, CA). The dilution needed to obtain 50% of the maximum absorbance was taken as the titer of the serum.

2.10. Pharmacokinetics studies

IFN-α2b–HSA and IFN-α2b–HSA(C34S) fusion proteins were labeled with ^{125}I by iodogen methods [20], and single dose pharmacokinetics studies were conducted in Sprague–Dawley rats (Experimental Animal Center, Academy of Military Medical Sciences of China, Beijing, China). Two groups of Sprague–Dawley rats (250 ± 20 g), three male rates and two female rats per group, were administered a single subcutaneous (s.c.) injection of 30 μg kg^{-1} of IFN-α2b–HSA or IFN-α2b–HSA(C34S). Blood samples were collected at 0, 30 min, 2 h, 12 h, 24 h, 48 h, 72 h, 120 h and 144 h. Plasma concentrations were determined with gamma counter.

3. Results

3.1. Instability of IFN-α2b–HSA under mechanical stress

After 72 h of agitation, the solution containing 1 mg/ml IFN-α2b–HSA remained clear and turbidity measurement at 405 nm did not increase (data not shown), which indicated that no

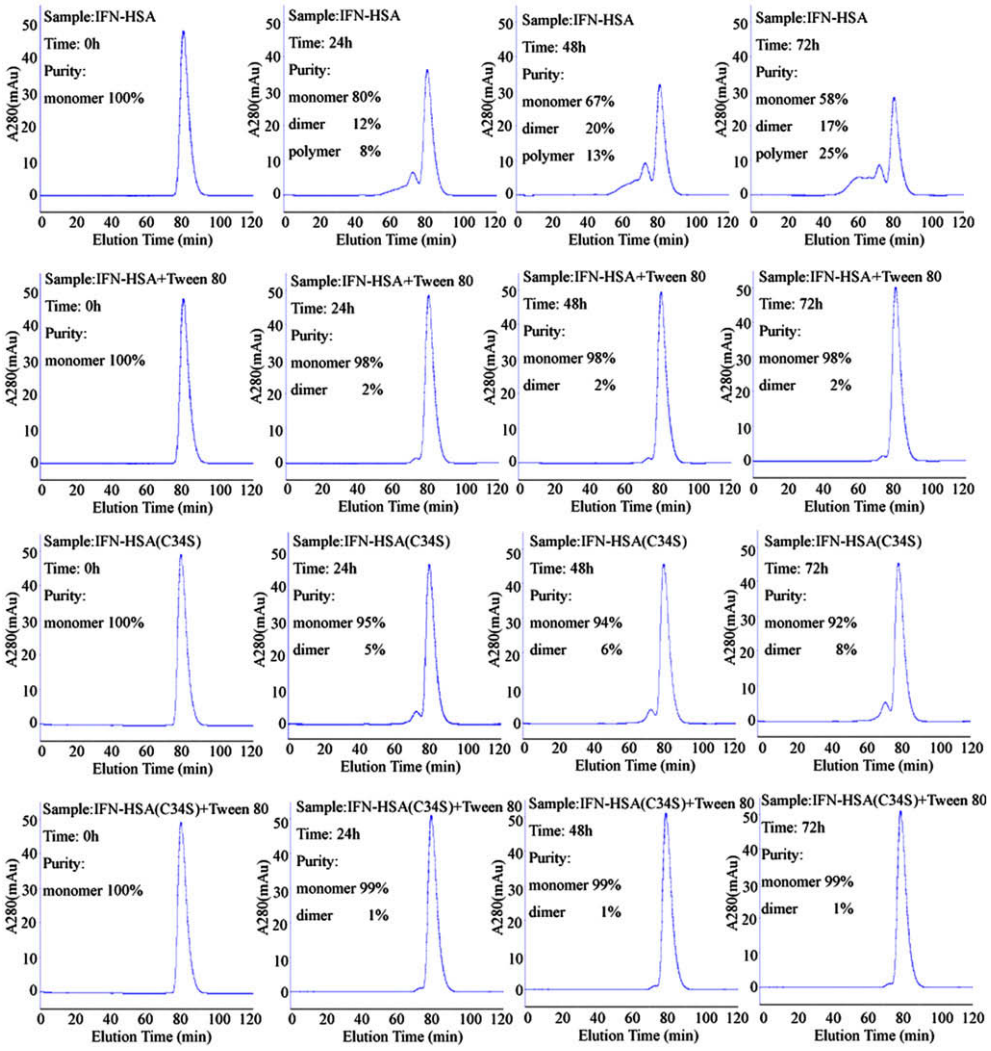

Fig. 1. Size-exclusion chromatography (SEC) chromatogram of IFN-α2b–HSA and IFN-α2b–HSA(C34S) agitated for 0, 24, 48 and 72 h. The purity of each sample represents an average of three separate experiments.

insoluble aggregates were formed [21]. However, size-exclusion chromatography (SEC) revealed that after being agitated for 24 h, 48 h, and 72 h, the level of monomeric protein decreased from 100% to 80%, 67%, 58%, respectively (Fig. 1). The decrease in the level of the monomeric protein was associated with a concurrent increase in the amount of dimer and polymer (Fig. 1). These dimers and polymers of IFN-α2b–HSA could be detected by non-reducing SDS–PAGE (Fig. 2A), but reducing SDS–PAGE failed to do so (Fig. 2B).

3.2. Instability of IFN-α2b–HSA under thermal stress

The accelerated thermal stress tests were performed at 60 °C for 2 h because (1) such condition has been employed to inactivate protease when recovering recombinant human serum albumin secreted from yeast [22]; (2) at 60 °C, the formation of soluble covalent aggregates remained to be the main degradation pathway. It was reasonable to predict the stability of IFN-α2b–HSA at lower temperature based on the results of accelerated thermal stress tests.

As shown in Fig. 3, IFN-α2b–HSA was prone to heat-induced aggregation. Size-exclusion chromatography revealed that after being incubated at 60 °C for 2 h, the level of monomeric protein decreased from 100% to 69%, with a concurrent increase in the amount of dimer and polymer. Similar levels of dimer and polymer could be detected on non-reducing SDS–PAGE (Fig. 4A), but not on reducing SDS–PAGE (Fig. 4B).

3.3. The effects of Tween 80 on the stability of IFN-α2b–HSA

Previous studies had demonstrated that the addition of non-ionic surfactant Tween 80 increased the stability of Albutropin (HSA and human growth hormone fusion protein) against agitation [15]. As shown in Figs. 1 and 2, the addition of 0.05% Tween 80 also inhibited the aggregation of IFN-α2b–HSA during agitation. However, the addition of 0.05% Tween 80 had marginal effect on the aggregation of IFN-α2b–HSA during heat treatment (Figs. 3 and 4). We also assessed the effects of Tween 80 on the stability of IFN-α2b–HSA during quiescent storage. As shown in Fig. 5, non-reducing SDS–PAGE revealed that the addition of 0.05% Tween 80 prompted the aggregation of IFN-α2b–HSA during motionless storage.

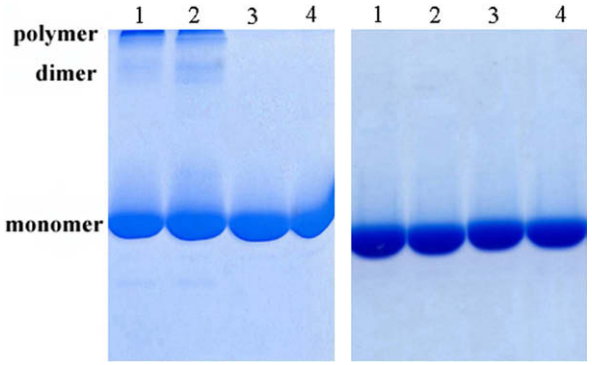

Fig. 4. Heat-induced protein aggregation detected by non-reducing SDS–PAGE (A) and reducing SDS–PAGE (B). Lane 1: IFN-α2b–HSA incubated at 60 °C for 2 h; lane 2: IFN-α2b–HSA incubated at 60 °C for 2 h in the presence of 0.05% Tween 80; lane 3: IFN-α2b–HSA(C34S) incubated at 60 °C for 2 h; lane 4: IFN-α2b–HSA(C34S) incubated at 60 °C for 2 h in the presence of 0.05% Tween 80.

3.4. The rationale for the design of IFN-α2b–HSA(C34S)

The comparison of band pattern of stressed IFN-α2b–HSA on reducing and non-reducing SDS–PAGE revealed that the majority of the aggregates were linked by disulfide bonds. The formation of disulfide-linked aggregates indicated the presence of reactive sulfhydryl group in the fusion protein. IFN-α2b contains four cys-

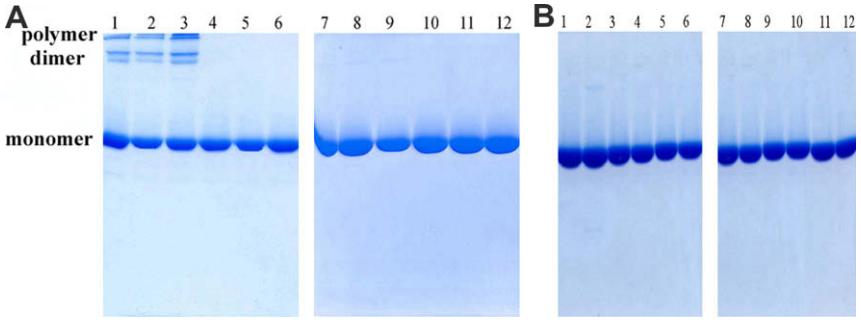

Fig. 2. Agitation-induced protein aggregation detected by non-reducing SDS–PAGE (A) and reducing SDS–PAGE (B). Lanes 1, 2, 3: IFN-α2b–HSA agitated for 24, 48, 72 h; lanes 4, 5, 6: IFN-α2b–HSA agitated for 24, 48, 72 h in the presence of 0.05% Tween 80; lanes 7, 8, 9: IFN-α2b–HSA(C34S) agitated for 24, 48, 72 h; lanes 10, 11, 12: IFN-α2b–HSA(C34S) agitated for 24, 48, 72 h in the presence of 0.05% Tween 80.

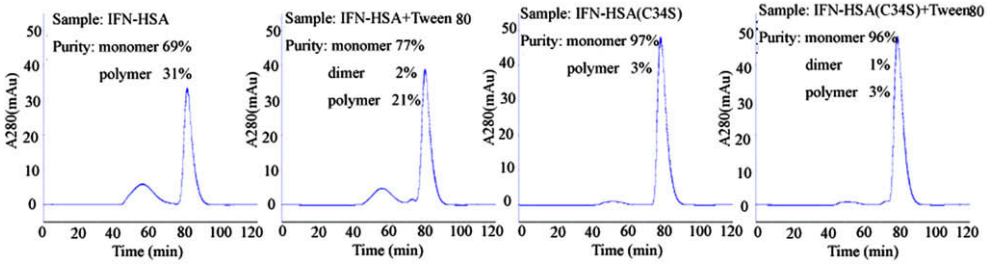

Fig. 3. Size-exclusion chromatography (SEC) chromatogram of IFN-α2b–HSA and IFN-α2b–HSA(C34S) incubated at 60 °C for 2 h. The purity of each sample represents an average of three separate experiments.

teine residues, all of them participate in the formation of disulfide bridges: Cys1-Cys98 and Cys29-Cys138. There are 35 cysteine residues in HSA, 34 of which form 17 disulfide bridges, and Cys34 is the only cysteine residue that does not participate in any disulfide bridge [23]. Cys34 was completely conserved within mammalian albumins.

In both plasma-derived human serum albumin and recombinant human serum albumin secreted from *P. pastoris*, about 30% of the Cys34 thiols are present as mixed disulfides with cysteine, homocysteine, or glutathione. Preparations of albumin usually contain 5–10% of dimeric species, with Cys34 being a possible site of dimerization [24]. Moreover, it had been demonstrated that, under stressed conditions such as ultrafiltration [25], heat treatment [26] or exposure to peroxides [27], Cys34 of albumin can trigger thiol–disulfide interchange reaction and result in the formation of complex and heterogeneous intermolecular aggregates. Thus, it had been well recognized that Cys34 was the main origin for heterogeneity and instability of mammalian albumins.

With the assumption that the free sulfhydryl group originated from Cys34 in the HSA moiety should also be responsible for the instability of IFN-α2b–HSA fusion protein, Cys34 of HSA moiety was substituted with Ser by site-directed mutagenesis, and the resultant fusion protein was designated as IFN-α2b–HSA(C34S).

3.5. Expression and purification of IFN-α2b and HSA fusion proteins

IFN-α2b–HSA(C34S) were secreted from *P. pastoris* at the same efficiency of IFN-α2b–HSA, and accumulated to about 500 mg/L in high cell density bioreactor culture. As can be seen from Fig. 6, both fusion proteins could be purified to homogeneity (purity > 98%) by the same protein purification process composed of cation exchange chromatography, hydrophobic interaction chromatography (HIC), anion exchange chromatography and gel filtration. Since IFN-α2b–HSA(C34S) was less prone to aggregation, its recovery rate (35%) was higher than that of IFN-α2b–HSA (25%).

Antiviral assay using human amniotic cell line (WISH) and vesicular stomatitis virus (VSV) showed that the EC_{50} of IFN-α2b–HSA and IFN-α2b–HSA(C34S) were 132 ± 15.2 ng/ml and 128 ± 13.1 ng/ml, respectively.

3.6. Stability of IFN-α2b–HSA(C34S) under mechanical stress

As shown in Figs. 1 and 2, IFN-α2b–HSA(C34S) was less susceptible to agitation-induced aggregation as compared to IFN-α2b–HSA. After 72 h of continuous agitation, more than 90% of IFN-α2b–HSA(C34S) remained monomeric.

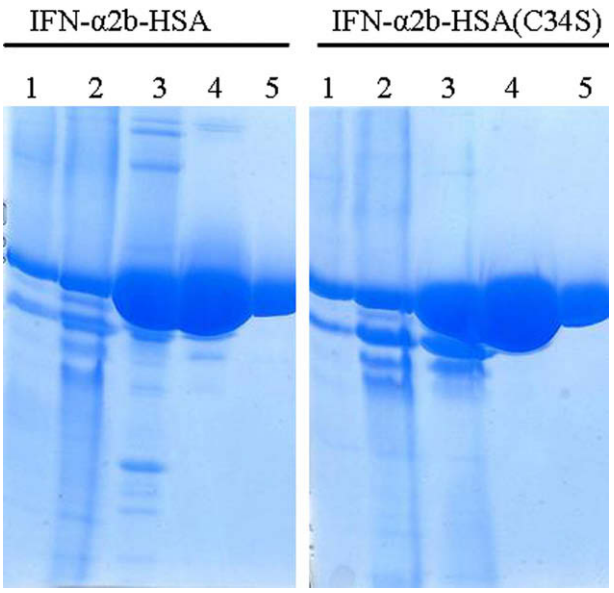

Fig. 6. Expression and purification of IFN-α2b–HSA and IFN-α2b–HSA(C34S). Lane 1: 10 μl bioreactor supernatant; lane 2: 2 μl eluant from SP Sepharose FF; lane 3: 10 μl eluant from phenyl Sepharose HP; lane 4: 10 μl eluant from Q Sepharose HP; lane 5: 10 μl eluant from Superdex 75.

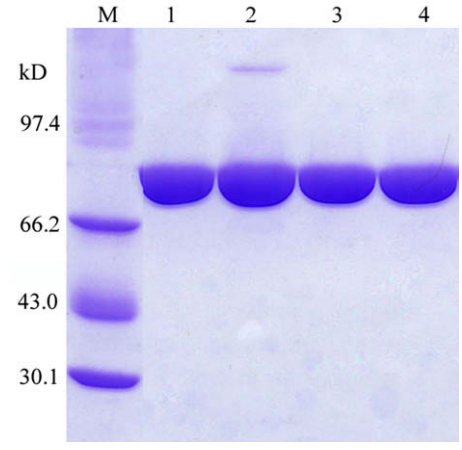

Fig. 5. Effects of Tween 80 on the aggregation of IFN-α2b–HSA and IFN-α2b–HSA(C34S) during quiescent storage (30 °C, 10 days). Lane 1: IFN-α2b–HSA without Tween 80; lane 2: IFN-α2b–HSA with 0.05% Tween 80; lane 3: IFN-α2b–HSA(C34S) without Tween 80; lane 4: IFN-α2b–HSA(C34S) with 0.05% Tween 80.

3.7. Stability of IFN-α2b–HSA(C34S) under thermal stress

As can be seen from Figs. 3 and 4, IFN-α2b–HSA(C34S) demonstrated much higher stability than IFN-α2b–HSA under thermal stress. After being incubated at 60 °C for 2 h, the level of monomeric protein was still above 95%.

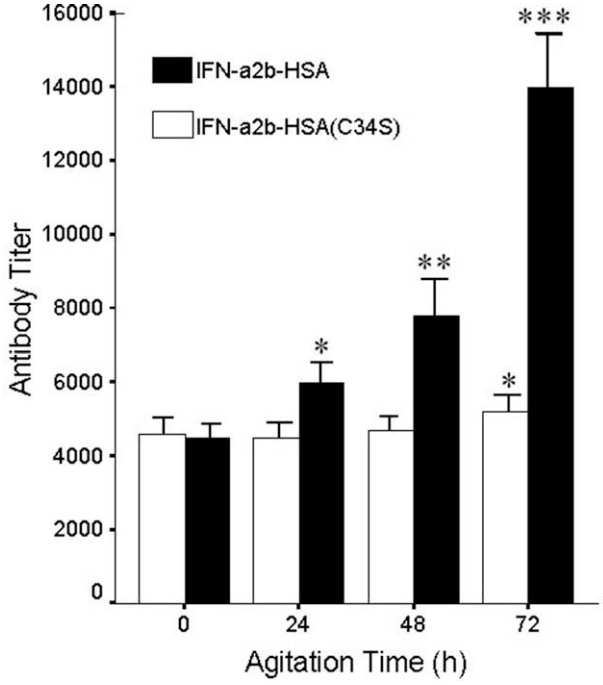

Fig. 7. Immunogenicity studies of IFN-α2b–HSA and IFN-α2b–HSA(C34S) agitated for 0, 24, 48, 72 h. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to unagitated fusion protein.

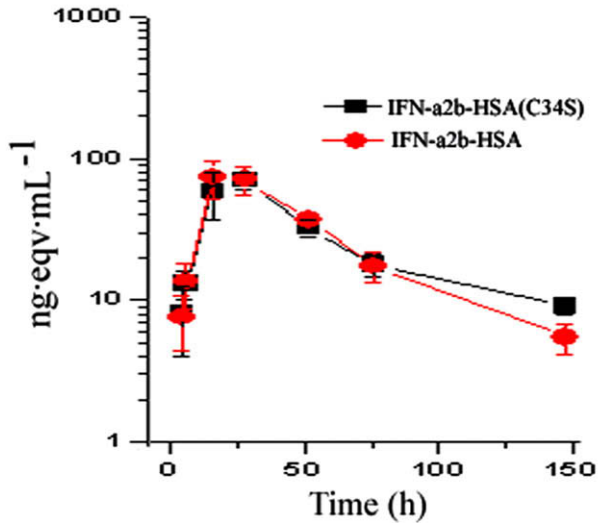

Fig. 8. Pharmacokinetics studies of IFN-α2b–HSA and IFN-α2b–HSA(C34S).

Table 1
Pharmacokinetic parameters of IFN-α2b–HSA and IFN-α2b–HSA(C34S) following single s.c. injection in rats.

Parameter	IFN-α2b–HSA	IFN-α2b–HSA(C34S)
$AUC_{(0-150\,h)}$ (h ng/ml)	3955 ± 656	4362 ± 671
MRT (h)	45.8 ± 4.4	48 ± 4.8
CL (L/h/kg)	7.5 ± 1.4	6.6 ± 1.0
V_{SS} (L/kg)	347 ± 93	320 ± 78
$T_{1/2}$ (h)	46 ± 8.4	51 ± 10
C_{max} (ng/ml)	73.2 ± 17.1	78 ± 19
T_{max} (h)	19.2 ± 6.6	19 ± 5.6
F (%)	43.8 ± 5.1	46.7 ± 4.2

Abbreviations: $AUC_{(0-150\,h)}$, area under the plasma drug concentration–time curve from time 0 to 150 h; MRT, mean residence time; CL, clearance; V_{SS}, volume of distribution at steady-state; $T_{1/2}$, terminal half-life; C_{max}, maximum concentration; T_{max}, time of maximum plasma concentration; *F*, bioavailability.

3.8. Immunogenicity studies

The formation of aggregates was associated with an increase in the immunogenicity of IFN-α2b–HSA in mice (Fig. 7). Consistent with its higher stability, the immunogenicity of IFN-α2b–HSA(C34S) increased less significantly upon agitation (Fig. 7). Moreover, it was worth noting that unagitated IFN-α2b–HSA and IFN-α2b–HSA(C34S) were interchangeable as coating antigen during ELISA, which indicated that before agitation, these two proteins were immunogenically equivalent.

3.9. Pharmacokinetics studies

The pharmacokinetics of IFN-α2b–HSA and IFN-α2b–HSA(C34S) were compared in rats after a single subcutaneous (s.c.) injection. As shown in Fig. 8, both fusion proteins had similar pharmacokinetic behavior. Both IFN-α2b–HSA and IFN-α2b–HSA(C34S) were absorbed slowly after s.c. injection. Plasma concentration reached maximum value in about 20 h after injection, then slowly decreased with a half-life of about 50 h. The detailed pharmacokinetic parameters were shown in Table 1.

4. Discussion

Protein pharmaceuticals are becoming increasingly important to the modern pharmaceutical industry [1]. Unfortunately, many protein pharmaceuticals have limited stability, particularly in solutions [2]. The instability of protein originated from intrinsic factors such as the large size and complex structure, but it was also affected by extrinsic factors such as mechanical and thermal stresses [3]. For some therapeutic proteins, aggregates formation is the prevalent physical instability reaction in liquid protein formulation [3,4]. Various techniques have been used to identify and quantify soluble and insoluble aggregates, including polyacrylamide gel electrophoresis (PAGE), size-exclusion chromatography (SEC), asymmetrical flow field-flow-fractionation, IR spectroscopy, UV spectroscopy (turbidimetric or nephelometric method), dynamic light scattering analysis, light blockage test, analytical centrifugation and visual inspection [3]. Different techniques can be employed to detect different types of aggregates. For example, turbidity assay can detect insoluble aggregates, SEC can detect soluble aggregates, reducing and non-reducing SDS–PAGE can detect disulfide-linked covalent aggregates. In this study, two of the most frequently used techniques (SDS–PAGE and SEC) were employed to identify and quantify the aggregates of IFN-α2b and HSA fusion protein. These two techniques were also required by the drug regulatory authorities in China.

Preliminary formulation studies revealed that IFN-α2b–HSA, a promising protein pharmaceutical for the treatment of hepatitis C, was susceptible to disulfide-linked aggregation under mechanical and thermal stresses. The poor stability of IFN-α2b–HSA posed an obstacle in the development of stable liquid formulation for this important therapeutic protein candidate. In this study, two different strategies: addition of excipients and protein engineering methodology, were employed to enable a liquid formulation for IFN-α2b–HSA.

The addition of non-ionic surfactant Tween 80 increased its stability during agitation, but its thermal stability was not affected. The effect of Tween and other surfactants on protein aggregation under agitation is sometimes more likely linked to the coating of surfaces by the surfactant (with the following prevention of adsorption to the surfaces) than to an interaction with the protein. Moreover, one issue in using Tween 80 in protein preparations is that it can contain a low level of residual peroxide, which can cause oxidative damage to protein pharmaceuticals [28]. As shown in this study, the addition of Tween 80 prompted the aggregation of IFN-α2b–HSA during quiescent storage. Thus, it is more desirable to elucidate the molecular mechanism of the instability of IFN-α2b–HSA, and increase its intrinsic stability by protein engineering methodology.

Cys34 was the only unpaired cysteine in albumin, and it was also the only unpaired cysteine in IFN-α2b–HSA fusion protein. In order to maintain Cys34 in a reduced state in the majority of albumin molecules, the reactivity of Cys34 must be finely controlled by the protein environment [29]. Crystallographic studies of albumin showed that Cys34 is buried in a shallow crevice on the surface of the protein [23]. However, it had been demonstrated that, under mechanical or thermal stress, Cys34 of albumin can open up and trigger thiol–disulfide interchange reaction, which may result in the formation of complex and heterogeneous intermolecular aggregates [25,26]. The covalent nature of IFN-α2b–HSA aggregates prompted us to postulate that the free sulfhydryl group which originated from the unpaired Cys34 in the HSA moiety should also be responsible for the instability of IFN-α2b–HSA. It was shown in this study that when the free sulfhydryl group in the HSA moiety of IFN-α2b–HSA fusion protein was eliminated by site-directed mutagenesis, it failed to trigger thiol–disulfide interchange reaction, and the resultant fusion protein had much higher stability against mechanical and thermal stresses. Moreover, the elimination of the free sulfhydryl group offered IFN-α2b–HSA fusion protein protection during the whole production

process, and IFN-α2b–HSA(C34S) was purified to homogeneity with higher yield.

It is worth noting that such minor alteration of IFN-α2b–HSA fusion protein will reduce the concern of immunogenicity. In fact, it had been calculated that of the 55 protein pharmaceuticals approved for human pharmaceutical use in the USA at the end of 2000, 30 were with alterations from the native sequence [30]. More relevantly, cysteine to serine mutation had been successfully employed to improve the stability of two protein pharmaceuticals (Proleukin (human interleukin-2) and Betaseron (human IFN-β1b)) without increasing their immunogenicity [30]. It had been demonstrated that conjugation of Cys34 of human serum albumin with maleimide derivatives of human insulin [31], growth hormone-releasing factor (GRF) 1–29 [32], glucagon-like peptide-1 (GLP-1) 7–36 [33] and atrial natriuretic peptide [34] had no impact on the structure of HSA, and did not increase its immunogenicity. In this study, it was observed that unagitated IFN-α2b–HSA and IFN-α2b–HSA(C34S) were interchangeable as coating antigen during ELISA, which indicated that no new epitope had been created by cysteine to serine mutation. Activity assay in our laboratory also revealed that Cys34Ser mutation did not compromise the antiviral activity of IFN-α2b–HSA fusion protein.

Although Cys34 has important physiological functions: it is the binding site for a variety of small molecules and provides antioxidant activity [35], results presented in this study and elsewhere showed that it was irrelevant to the long circulating half-life of albumin or albumin fusion protein [36]. Thus, under the circumstance where only the long half-life of HSA is to be exploited (such as in the case of HSA fusion technology), stabilized HSA mutant without free sulfhydryl group was more desirable, as it can increase production yields and facilitate the formulation development process. The results presented in this study indicated that protein engineering methodology could be successfully incorporated to facilitate the development of protein pharmaceuticals.

References

[1] A.K. Pavlou, J.M. Reichert, Recombinant protein therapeutics—success rates, market trends and values to 2010, Nat. Biotechnol. 22 (2004) 1513–1519.
[2] S. Frokjaer, D.E. Otzen, Protein drug stability: a formulation challenge, Nat. Rev. Drug Discov. 4 (2005) 298–306.
[3] H.C. Mahler, R. Müller, W. Friess, A. Delille, S. Matheus, Induction and analysis of aggregates in a liquid IgG1-antibody formulation, Eur. J. Pharm. Biopharm. 59 (2005) 407–417.
[4] A.S. Rosenberg, Effects of protein aggregates: an immunologic perspective, AAPS J. 8 (2006) E501–E507.
[5] S. Hermeling, D.J. Crommelin, H. Schellekens, W. Jiskoot, Structure–immunogenicity relationships of therapeutic proteins, Pharm. Res. 21 (2004) 897–903.
[6] S. Hermeling, L. Aranha, J.M. Damen, M. Slijper, H. Schellekens, D.J. Crommelin, W. Jiskoot, Structural characterization and immunogenicity in wild-type and immune tolerant mice of degraded recombinant human interferon-alpha2b, Pharm. Res. 22 (2005) 1997–2006.
[7] S. Hermeling, H. Schellekens, C. Maas, M.F. Gebbink, D.J. Crommelin, W. Jiskoot, Antibody response to aggregated human interferon alpha2b in wild-type and transgenic immune tolerant mice depends on type and level of aggregation, J. Pharm. Sci. 95 (2006) 1084–1096.
[8] V.T. Chuang, U. Kragh-Hansen, M. Otagiri, Pharmaceutical strategies utilizing recombinant human serum albumin, Pharm. Res. 19 (2002) 569–577.
[9] G.M. Subramanian, M. Fiscella, A. Lamousé-Smith, S. Zeuzem, J.G. McHutchison, Albinterferon alpha-2b: a genetic fusion protein for the treatment of chronic hepatitis C, Nat. Biotechnol. 25 (2007) 1411–1419.
[10] R.J. Melder, B.L. Osborn, T. Riccobene, P. Kanakaraj, P. Wei, G. Chen, D. Stolow, W.G. Halpern, T.S. Migone, Q. Wang, K.J. Grzegorzewski, G. Gallant, Pharmacokinetics and in vitro and in vivo anti-tumor response of an interleukin-2–human serum albumin fusion protein in mice, Cancer Immunol. Immunother. 54 (2005) 535–547.
[11] W. Halpern, T.A. Riccobene, H. Agostini, K. Baker, D. Stolow, M.L. Gu, J. Hirsch, A. Mahoney, J. Carrell, E. Boyd, K.J. Grzegorzewski, Albugranin, a recombinant human granulocyte colony stimulating factor (G-CSF) genetically fused to recombinant human albumin induces prolonged myelopoietic effects in mice and monkeys, Pharm. Res. 19 (2002) 1720–1729.
[12] B.L. Osborn, L. Sekut, M. Corcoran, C. Poortman, B. Sturm, G. Chen, D. Mather, H.L. Lin, T.J. Parry, Albutropin: a growth hormone-albumin fusion with improved pharmacokinetics and pharmacodynamics in rats and monkeys, Eur. J. Pharmacol. 456 (2002) 149–158.
[13] A. Duttaroy, P. Kanakaraj, B.L. Osborn, H. Schneider, O.K. Pickeral, C. Chen, G. Zhang, S. Kaithamana, M. Singh, R. Schulingkamp, D. Crossan, J. Bock, T.E. Kaufman, P. Reavey, M. Carey-Barber, S.R. Krishnan, A. Garcia, K. Murphy, J.K. Siskind, M.A. McLean, S. Cheng, S. Ruben, C.E. Birse, O. Blondel, Development of a long-acting insulin analog using albumin fusion technology, Diabetes 54 (2005) 251–258.
[14] L.L. Baggio, Q. Huang, T.J. Brown, D.J. Drucker, A recombinant human glucagon-like peptide (GLP)-1-albumin protein (albugon) mimics peptidergic activation of GLP-1 receptor-dependent pathways coupled with satiety, gastrointestinal motility, and glucose homeostasis, Diabetes 53 (2004) 2492–2500.
[15] D.K. Chou, R. Krishnamurthy, T.W. Randolph, J.F. Carpenter, M.C. Manning, Effects of Tween 20 and Tween 80 on the stability of Albutropin during agitation, J. Pharm. Sci. 94 (2005) 1368–1381.
[16] H.L. Zhao, C. Xue, Y. Wang, X.Y. Li, X.H. Xiong, X.Q. Yao, Z.M. Liu, Circumventing the heterogeneity and instability of human serum albumin–interferon-alpha2b fusion protein by altering its orientation, J. Biotechnol. 131 (2007) 245–252.
[17] V.G. Bain, K.D. Kaita, E.M. Yoshida, M.G. Swain, E.J. Heathcote, A.U. Neumann, M. Fiscella, R. Yu, B.L. Osborn, P.W. Cronin, W.W. Freimuth, J.G. McHutchison, G.M. Subramanian, A phase 2 study to evaluate the antiviral activity, safety, and pharmacokinetics of recombinant human albumin–interferon alfa fusion protein in genotype 1 chronic hepatitis C patients, J. Hepatol. 44 (2006) 671–678.
[18] V.K. Sharma, D.S. Kalonia, Polyethylene glycol-induced precipitation of interferon alpha-2a followed by vacuum drying: development of a novel process for obtaining a dry, stable powder, AAPS J. 6 (2004) 31–44.
[19] C. Withers-Martinez, E.P. Carpenter, F. Hackett, B. Ely, M. Sajid, M. Grainger, M.J. Blackman, PCR-based gene synthesis as an efficient approach for expression of the A + T-rich malaria genome, Protein Eng. 12 (1999) 1113–1120.
[20] P.J. Fraker, J.C. Speck Jr., Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphrenylglycoluril, Biochem. Biophys. Res. Commun. 80 (1978) 849–857.
[21] E.N. Lee, Y.M. Kim, H.J. Lee, S.W. Park, H.Y. Jung, J.M. Lee, Y.H. Ahn, J. Kim, Stabilizing peptide fusion for solving the stability and solubility problems of therapeutic proteins, Pharm. Res. 22 (2005) 1735–1746.
[22] T. Nouchi, H. Mizokami, Y. Tajima, Y. Miyatsu, M. Sakaguchi, K. Yagi. Method for preparing human serum albumin containing heat-treatment, United States Patent No. 6908749, 2005.
[23] S. Sugio, A. Kashima, S. Mochizuki, M. Noda, K. Kobayashi, Crystal structure of human serum albumin at 2.5 Å resolution, Protein Eng. 12 (1999) 439–446.
[24] T. Peters Jr., All About Albumin: Biochemistry, Genetics, and Medical Applications, Academic Press, New York, 1995.
[25] T. Maruyama, S. Katoh, M. Nakajima, H. Nabetani, Mechanism of bovine serum albumin aggregation during ultrafiltration, Biotechnol. Bioeng. 75 (2001) 233–238.
[26] A. Oliva, A. Santoveña, M. Llabres, J.B. Fariña, Stability study of human serum albumin pharmaceutical preparations, J. Pharm. Pharmacol. 51 (1999) 385–392.
[27] Y. Ogasawara, T. Namai, T. Togawa, K. Ishii, Formation of albumin dimers induced by exposure to peroxides in human plasma: a possible biomarker for oxidative stress, Biochem. Biophys. Res. Commun. 340 (2006) 353–358.
[28] W. Wang, Y.J. Wang, D.Q. Wang, Dual effects of Tween 80 on protein stability, Int. J. Pharm. 347 (2008) 31–38.
[29] A.J. Stewart, C.A. Blindauer, S. Berezenko, D. Sleep, D. Tooth, P.J. Sadler, Role of Tyr84 in controlling the reactivity of Cys34 of human albumin, FEBS J. 272 (2005) 353–362.
[30] A.L. Kurtzman, S. Govindarajan, K. Vahle, J.T. Jones, V. Heinrichs, P.A. Patten, Advances in directed protein evolution by recursive genetic recombination: applications to therapeutic proteins, Curr. Opin. Biotechnol. 12 (2001) 361–370.
[31] K. Thibaudeau, R. Léger, X. Huang, M. Robitaille, O. Quraishi, C. Soucy, N. Bousquet-Gagnon, P. van Wyk, V. Paradis, J.P. Castaigne, D. Bridon, Synthesis and evaluation of insulin–human serum albumin conjugates, Bioconjug. Chem. 16 (2005) 1000–1008.
[32] L. Jetté, R. Léger, K. Thibaudeau, C. Benquet, M. Robitaille, I. Pellerin, V. Paradis, P. van Wyk, K. Pham, D.P. Bridon, Human growth hormone-releasing factor (hGRF)1-29-albumin bioconjugates activate the GRF receptor on the anterior pituitary in rats: identification of CJC-1295 as a long-lasting GRF analog, Endocrinology 146 (2005) 3052–3058.
[33] R. Léger, K. Thibaudeau, M. Robitaille, O. Quraishi, P. van Wyk, N. Bousquet-Gagnon, J. Carette, J.P. Castaigne, D.P. Bridon, Identification of CJC-1131-albumin bioconjugate as a stable and bioactive GLP-1(7–36) analog, Bioorg. Med. Chem. Lett. 14 (2004) 4395–4398.
[34] R. Léger, M. Robitaille, O. Quraishi, E. Denholm, C. Benquet, J. Carette, P. van Wyk, I. Pellerin, N. Bousquet-Gagnon, J.P. Castaigne, D. Bridon, Synthesis and in vitro analysis of atrial natriuretic peptide–albumin conjugates, Bioorg. Med. Chem. Lett. 13 (2003) 3571–3575.
[35] U. Kragh-Hansen, V.T. Chuang, M. Otagiri, Practical aspects of the ligand-binding and enzymatic properties of human serum albumin, Biol. Pharm. Bull. 25 (2002) 695–704.
[36] T.R. McCurdy, S. Gataiance, L.J. Eltringham-Smith, W.P. Sheffield, A covalently linked recombinant albumin dimer is more rapidly cleared in vivo than are wild-type and mutant C34A albumin, J. Lab. Clin. Med. 143 (2004) 115–124.