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Dual effects of Tween 80 on protein stability

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Abstract

In this paper, we used IL-2 mutein as a model protein and evaluated the effect of Tween 80, a non-ionic surfactant. In summary, we found that the dual effects of Tween 80 on the stability of IL-2SA, such as that shaking-induced aggregation of IL-2 mutein was significantly inhibited in the presence of Tween 80. However, this surfactant adversely affected the stability of IL-2 mutein in solution during storage in terms of both oxidation and aggregation. These adverse effects are strongly temperature and formulation-dependent. Data particularly showed that IL-2 mutein in solution forms soluble aggregates to a different degree in different formulations during storage at 40 °C for 2 months. Aggregation was barely detectable during storage at 5 °C for 22 months. Addition of 0.1% Tween 80 significantly increased the rate of IL-2 mutein aggregation during storage. The IL-2 mutein aggregates are linked by both disulfide and non-disulfide bonds and their relative contribution is temperature-dependent. IL-2 mutein can be oxidized also to a different degree in different formulations during storage and the oxidation rate is strongly temperature-dependent with an activation energy between 21 and 25 kcal/mol. Addition of 0.1% Tween 80 not only increased the rate of oxidation in general but also altered the temperature-dependency of IL-2 mutein oxidation.
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Keywords: Surfactant; Tween 80; Protein; Stability

1. Introduction

An important class of non-ionic surfactants widely used in the pharmaceutical industry is Tweens. Their popularity is largely due to their effectiveness at low concentrations and relative low toxicities. In addition, they usually do not interact, or at least not strongly, with active ingredients. These characteristics have made them almost indispensable in the biotechnology industry in the past two decades. They have been extensively used to prevent/inhibit protein surface adsorption (Gombotz et al., 1996) and aggregation under various processing conditions, such as refolding (Bam et al., 1996), mixing (Katakam et al., 1995a,b), freeze–thawing (Chang et al., 1996), freeze-drying (Sarciaux et al., 1999), and reconstitution (Zhang et al., 1995; Zhang et al., 1996). As a result, several protein pharmaceutical products, both in liquid and solid dosage forms, contain Tweens as inactive pharmaceutical ingredients, including Actimmune, Activase, Intron A, and Recombinate (PDR, 1999).

However, one issue in using Tweens in protein preparations is their potential adverse effect on protein stability, which has not been widely reported. One of the adverse effects is the oxidative damage of the residual peroxides in Tweens, which are generated through an autoxidation process during processing or storage (Donbrow et al., 1978; Jaeger et al., 1994; Ha et al., 2002). This can be a serious problem as proteins are generally sensitive to oxidative degradation and often formulated at relatively low concentrations. Examples include oxidative degradation of recombinant human ciliary neurotrophic factor (rhCNTF) in solution (Knepp et al., 1996) and recombinant human granulocyte colony-stimulating factor (rhG-CSF) in solution during storage (Herman et al., 1996) by the residual peroxides in Tween 80 (a single oleic acid ester of polyoxyethylene sorbitan). We recently demonstrated that the residual peroxide in Tween 80

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could oxidize IL-2 mutein easily both in solution and solid states (Ha et al., 2002).

Another potential adverse effect of using Tweens in protein preparations is their direct interaction with proteins (Randolph and Jones, 2002). Such interactions have been reported between Tweens and rhGH (Bam et al., 1995; Bam et al., 1998) or LDH (Hillgren et al., 2002). Since most proteins have very limited conformational stability in solution, such direct interactions may potentially disrupt the delicate balance of all the folding forces responsible for the conformational stability and adversely affect the protein stability. Indeed, Tween 20 has been shown to enhance the aggregation of PEG-GCSF at 1 mg/mL in solution in a concentration dependent manner during storage at 29 °C (Treuheit et al., 2002). Although the instability mechanism was not clearly stated, it is likely due to an interaction between Tween 20 and the protein.

In this paper, we used IL-2 mutein as a model protein and evaluated the effect of Tween 80, a non-ionic surfactant. As expected, shaking-induced aggregation of IL-2 mutein was significantly inhibited in the presence of Tween 80. However, this surfactant adversely affected the stability of IL-2 mutein in solution during storage in terms of both oxidation and aggregation. These adverse effects are strongly temperature and formulation-dependent. The dual effects of Tween 80 on the stability of IL-2SA have not been reported and may have significant implications in future formulation of such a protein product.

2. Materials and methods

2.1. Materials

Recombinant human IL-2 mutein was expressed and produced in Chinese hamster ovary (CHO) cells as a mixture of *O*-glycosylated and non-glycosylated forms in house. The protein was then processed by several chromatographic steps to a purity level of greater than 99% by SDS-PAGE. The bulk protein was diafiltered to a protein concentration of approximately 5 mg/mL in a buffered solution at pH 5.5 and stored frozen at −70 °C before use. Low-peroxide Tween 80 (containing 20 μM/kg peroxides) was purchased from Sigma Chemicals (St. Louis, MO). USP-grade histidine, sucrose, and mannitol were purchased from Spectrum Chemicals (Gardena, CA). USP-grade glycine was purchased from J.T. Baker (Phillipsburg, NJ). Other chemicals were used as received.

2.2. Preparation of liquid IL-2 mutein stability samples

Frozen recombinant human IL-2 mutein at 5 mg/mL was thawed, dialyzed extensively overnight into four different formulations (A–D) buffered with histidine at pH 5.5. After dialysis, part of these solutions was spiked with Tween 80 and all solutions were then diluted to a protein concentration of 1 mg/mL with corresponding buffers. The final compositions of these formulations were listed in Table 1. These formulations were sterile filtered and 1.8 mL of each solution was aliquoted under a laminar flow hood into 2-mL sterile polypropylene vials. The filled samples were incubated at 40, 25, and 5 °C and removed periodically for analysis.

Table 1
Compositions of IL-2 mutein formulations

	Formulations							
	A	B	C	D	A′	B′	C′	D′
IL-2 mutein (mg/mL)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Glycine (%)	2	0	0	0	2	0	0	0
Sucrose (%)	1	10	10	0	1	10	10	0
Mannitol (%)	0	0	0	5	0	0	0	5
Histidine (mM)	20	20	10	10	20	20	10	10
Tween 80 (%)	0	0	0	0	0.1	0.1	0.1	0.1

2.3. Shaking studies

Protein samples were prepared as described above except that the volume of protein solution in these vials was only 0.6 mL. A lower volume was filled so that air-induced protein aggregation during shaking could be facilitated by a large headspace. During shaking, samples were placed horizontally on an Orbital Shaker (VWR Scientific, Model #57018–754) and shaken continuously at 350 circles/minute for 24 h at room temperature. A set of control samples were also placed at room temperature to compare the stability of the protein under quiescent condition. Samples were then analyzed immediately after shaking.

2.4. SDS-PAGE

SDS-PAGE was performed using 4–12% Bis–Tris NuPAGE gels (Novex, San Diego, CA). IL-2SA stability samples were first treated in the vendor-provided sample buffer at 95 °C for 5 min. About 10–15 μg of the treated protein sample was loaded onto the gel. Mark 12™ (Novex, San Diego, CA) was used as the molecular weight standard without further treatment. Gels were run in MES buffer (Novex, San Diego, CA) at 200 V and stained with Colloidal Blue Stain Kit (Novex, San Diego, CA) or silver.

2.5. Determination of insoluble protein aggregates

Formation of insoluble aggregates in all the stability samples was monitored by measuring the optical density at both 280 and 350 nm. The aggregation index (AI), in percentage, a relative measurement of the degree of protein aggregation, was calculated using the following equation:

$$\mathrm{AI} = \left(\frac{100 \times \mathrm{OD}_{350}}{\mathrm{OD}_{280} - \mathrm{OD}_{350}}\right)$$

Since proteins exhibit negligible UV absorption at 350 nm, this index reflects the relative light-scattering intensity of the protein aggregates. It should be noted that the denominator in the equation does not correct any light-scattering contribution due to change in wavelength.

2.6. Reverse phase-high performance liquid chromatography (RP-HPLC)

This assay was performed on an Agilent HP 1100 series HPLC having a temperature-controlled autosampler. Briefly, the method requires two mobile phases—one containing 0.2% trifluoroacetic acid in HPLC-grade water (A) and the other containing 0.15% trifluoroacetic acid in acetonitrile (B). A C4 reversed phase HPLC column was used (Vydac 214TP5415, 5 μm particles of 300 Å pore size; 150 mm × 4.6 mm) and the column temperature was controlled at 30 °C. The elution program is to maintain B at 40% for 5 min, to increase B to 76% in 15 min, to increased B to 90% in 1 min, to maintain B at 90% for 3 min, to decrease B to 40% in 1 min, and to maintain B at 40% for 10 min. This assay also monitors the oxidized IL-2 mutein (at Met residues), which has a shorter retention time than the intact IL-2 mutein (Ha et al., 2002). The shorter retention time is likely due to the addition of an oxygen atom(s), which increased the polarity of the molecule. Similar behaviors were also observed for other proteins (Nguyen et al., 1993; Van Patten et al., 1999). The oxidation level in IL-2 mutein samples was calculated as the ratio of the peak area of the oxidized IL-2 mutein to the total peak area of both intact and oxidized IL-2 mutein, assuming the extinction coefficient of the oxidized form is the same as that of the intact protein.

3. Results

In this study, we examined the effect of 0.1% Tween 80 on the stability of IL-2 mutein both during vigorous shaking and during quiescent storage.

3.1. Effect of Tween 80 on aggregation of IL-2 mutein during shaking

IL-2 mutein aggregated readily upon shaking. After shaking the samples for 24 h at room temperature, those without Tween 80 were slightly hazy with very fine particles evenly distributed in the solution. Control samples stored at the same temperature for 24 h without shaking were clear without any visible particles. In the presence of 0.1% Tween 80, the protein solutions remain clear under the same shaking condition. The effect of shaking and Tween 80 was also evaluated by determination of the UV aggregation index as a parameter of clarity. As shown in Fig. 1, shaking induced a significant increase in aggregation index for all samples without Tween 80. It was noted that formulation D seemed to be more resistant to shaking-induced aggregation relative to the rest of the formulations. In the presence of 0.1% Tween 80, shaking-induced aggregation of IL-2 mutein was significantly reduced but the degree of inhibition was formulation-dependent. The effect of Tween 80 was strongest in formulation A, followed by formulations B, D, and C.

The shaking-induced aggregation of IL-2 mutein reduced the concentration of protein monomers. The HPLC results showed that the respective concentration of IL-2 mutein in formulation A through D was only 46%, 48%, 37%, and 59% of the initial value after shaking. These samples were also analyzed by SDS-

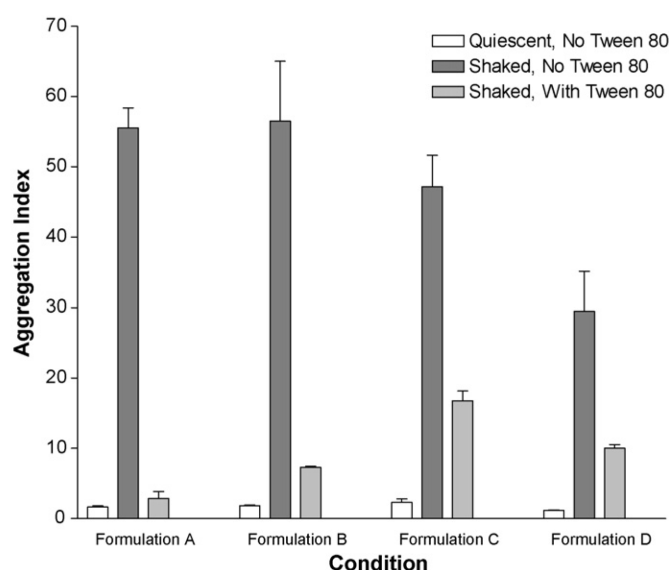


Fig. 1. Effect of Tween 80 at 0.1% on shaking-induced aggregation of IL-2 mutein. Samples were shaken for 24 h on a rotary shaker before UV measurement. Detailed description and the calculation of aggregation index were described under Section 2.

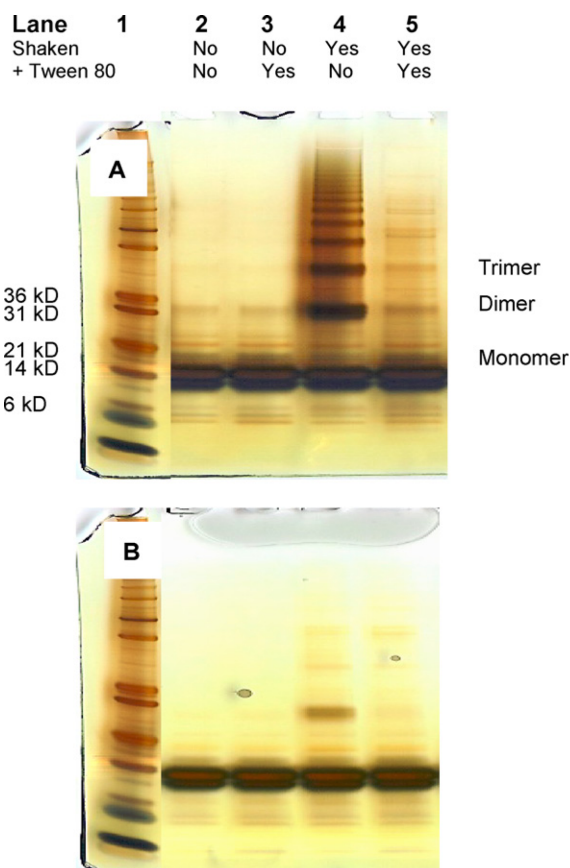


Fig. 2. Effect of 0.1% Tween on the stability of IL-2 mutein during shaking under non-reduced conditions (A) and reduced conditions (B). Lane 1 is the molecular weight (Mark 12). Lanes 2–3 are the initial stability samples in the absence and presence of 0.1% Tween 80. Lanes 4–5 are the samples shaken for 24 h in the absence and presence of 0.1% Tween 80.

PAGE both under non-reduced and reduced conditions. Fig. 2 shows the results of representative samples. IL-2 mutein was stained as two unresolved monomer bands with an apparent molecular weight of about 16 and 15 kDa, representing glycosylated and unglycosylated forms. Other minor amounts of aggregates and fragments were also present. Shaking induced significant formation of aggregates, which formed a clear ladder. The aggregate ladder matched the sizes of IL-2 mutein dimers, trimers, and oligomers. The decreasing intensity of the ladder with increasing molecular weight suggests sequential addition of monomers. Under reduced condition, the ladder largely disappeared, suggesting that the aggregates contain both reducible and non-reducible oligomers. Tween 80 was very effective in inhibiting formation of these aggregates. All four formulations have a similar gel pattern (data not shown).

3.2. *Effect of Tween 80 on aggregation of IL-2 mutein during storage*

The effect of Tween 80 was also examined on the storage stability of the protein. The SDS-PAGE results under non-reduced conditions are shown in Fig. 3A and B, where the initial and 2-month stability samples were compared in the absence (control) and presence of 0.1% Tween-80. The initial samples of the four control formulations do not contain any detectable dimers or polymers on the gel. Addition of 0.1% Tween 80 did not cause any formation of aggregates or fragments, either. After incubation at 40 °C for 2 months, only a week dimer band appeared on the gel for control formulations B and C. In contrast, a clear aggregate ladder was seen on the gel for all the samples containing 0.1% Tween 80 and the band intensity for formulations

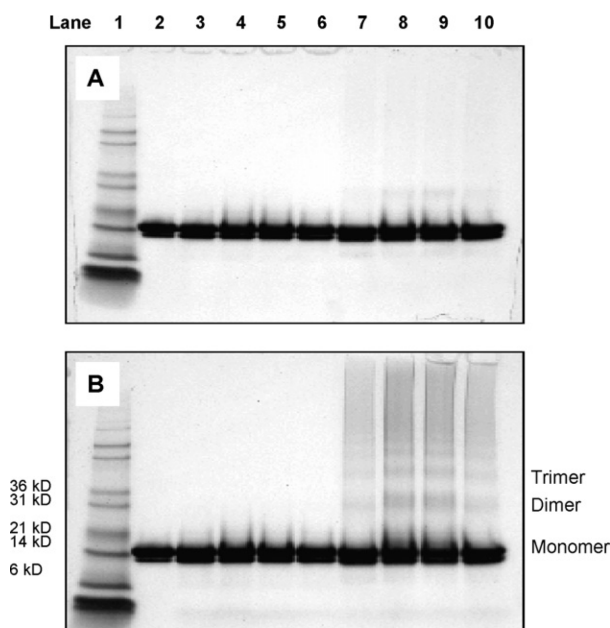


Fig. 3. SDS-PAGE analysis of IL-2 mutein stability samples under non-reduced conditions. Lanes 1 and 2 are the molecular weight (Mark 12) and IL-2 mutein reference. Lanes 3–6 are the initial stability samples and lanes 7–10 are the 2-month stability samples at 40 °C for formulations A, B, C, and D in the absence (A) and presence (B) of 0.1% Tween 80.

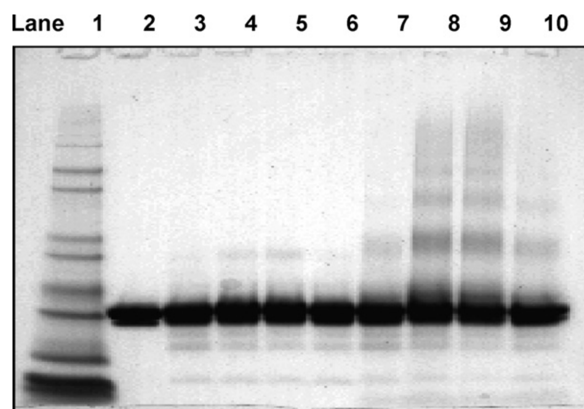


Fig. 4. SDS-PAGE analysis of the 2-month IL-2 mutein stability samples at 40 °C under reduced conditions. Lane 1 and 2 are the molecular weight (Mark 12) and IL-2 mutein reference. Lanes 3–6 are formulations A, B, C, and D; lanes 7–10 are formulations A′, B′, C′, and D′.

B and C again seem to be stronger than that for formulations A and D. Also, the aggregate ladder matched the sizes of IL-2 mutein dimers, trimers, and oligomers. Thus, the extent of IL-2 mutein aggregation with and without 0.1% Tween 80 is formulation-dependent. These results indicate that Tween 80 promoted aggregation of IL-2 mutein during storage at 40 °C.

To determine whether the aggregates were disulfide-linked, the 2-month stability samples at 40 °C were analyzed by SDS-PAGE under reduced conditions (Fig. 4). The weak dimer peak in the control samples did not disappear, suggesting that these dimers were not disulfide-linked. However, part of the aggregate bands disappeared in samples containing Tween 80, suggesting that Tween 80 promoted formation of both disulfide-linked and non-disulfide-linked oligomerization/aggregation. In addition, there were a few weak IL-2 mutein fragment bands under reduced condition for all formulations. This suggests that there are aggregates that are too large to enter the gel under non-reduced conditions, and are made of disulfide-linked IL-2 mutein fragments.

Although Tween 80 promoted formation of IL-2 mutein aggregates during storage at 40 °C, the aggregation index by UV measurement did not increase significantly (Table 1). In fact, the index decreased for formulations A, B and D. Since the aggregation index was calculated based on the difference in optical density between the sample and the placebo, the smaller values of the AI suggest that the change in the UV response of the placebo samples might not reflect accurately what happened of non-protein components in the protein samples. In any case, the insignificant change in aggregation index suggests that the Tween-80-induced aggregates were mostly, if not completely, soluble.

In comparison, samples that were stored at 5 °C for 22 months were also analyzed by SDS-PAGE. To increase the sensitivity of detecting degradation products, the samples were stained by silver. Under non-reduced conditions, there were no detectable dimer or other aggregate bands for the control formulations stored at 5 °C for 22 months (Fig. 5). Similarly, addition of 0.1% Tween 80 led to formation of IL-2 mutein aggregates. However, the relative distribution and size of the aggregate bands in these

Fig. 5. SDS-PAGE analysis of 22-month IL-2 mutein stability samples at 5 °C under non-reduced conditions. Lanes 1 and 10 are the molecular weight (Mark 12) and IL-2 mutein reference. Lanes 3–6 are formulations A, B, C, and D; and lanes 7–10 are formulations A′, B′, C′, and D′.

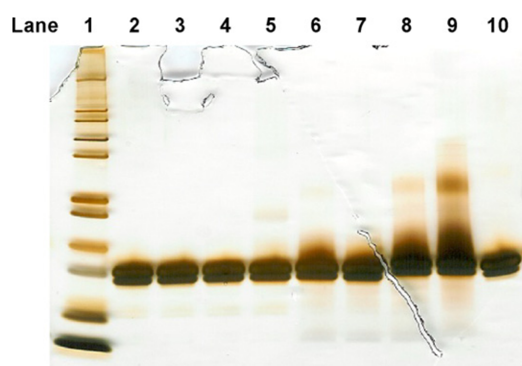


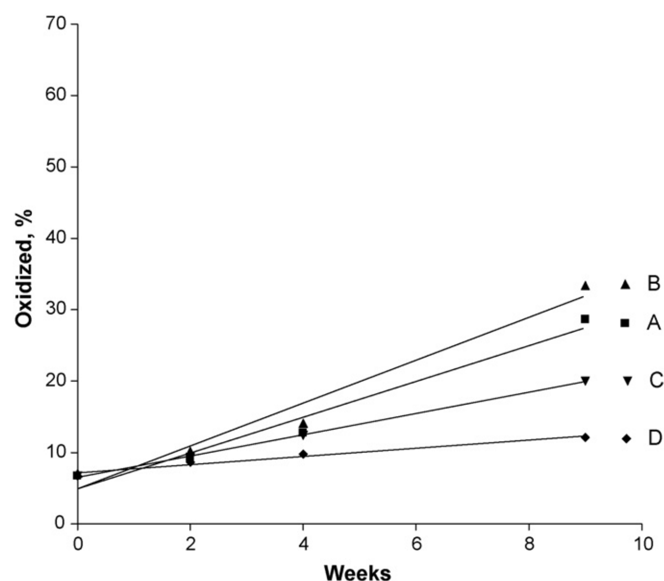
Fig. 6. SDS-PAGE analysis of 22-month IL-2 mutein stability samples at 5 °C under reduced conditions. Lanes 1 and 10 are the molecular weight (Mark 12) and IL-2 mutein reference. Lanes 3–6 are formulations A, B, C, and D; lanes 7–10 are formulations A′, B′, C′, and D′.

samples are different from those stored at 40 °C. The relative intensity of the aggregates in samples containing Tween 80 was formulation D > C > A > B, a different order from that at 40 °C.

Under reduced conditions, a week dimer band appeared for control formulation D (Fig. 6). This suggests that the dimer was in an aggregated form under non-reduced condition but too large to enter the gel. The smear of the aggregate bands under reduced conditions for the samples containing Tween 80 was slightly reduced, indicating that these aggregate bands are mostly non-disulfide-bonded (Fig. 6). The appearance of some fragment bands in all the formulations under reduced conditions suggests that there are aggregates that are too large to enter the gel under non-reduced conditions, and are made of disulfide-linked IL-2 mutein fragments, similar to that observed for samples stored at 40 °C (Table 2).

Table 2
Summary of aggregation index

	Formulations							
	A	B	C	D	A′	B′	C′	D′
Initial	3.9	3.8	3.2	7.0	2.6	3.4	3.1	4.1
2 months at 40 °C	3.5	5.2	<1.0	5.6	<1.0	<1.0	3.8	<1.0
4 months at 5 °C	4.2	2.4	3.6	5.0	3.7	4.9	3.0	<1.0



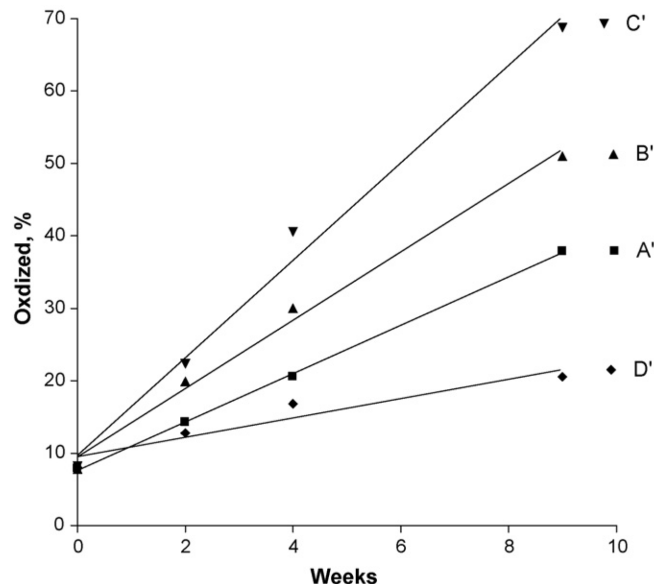


Fig. 7. Oxidation of IL-2 mutein during storage at 40 °C in the absence (A) and presence (B) of 0.1% Tween 80. Samples were analyzed by RP-HPLC.

3.3. *Effect of Tween 80 on oxidation of IL-2 mutein during storage*

Through RP-HPLC analysis, the relative amount of oxidized IL-2 mutein was determined. Fig. 7 shows the oxidation time courses of IL-2 mutein during storage at 40 °C. In the absence of 0.1% Tween 80, the oxidation level of IL-2 mutein increased linearly with time for formulations C and D but formulations A and B showed an slightly upward trend with time. The overall oxidation rate, as determined by linear regression, was formulation B > A > C > D (Table 3). In the presence of 0.1% Tween 80, oxidation level also increased linearly with time. However,

Table 3
Oxidation Rates of IL-2 Mutein during Storage

	Formulations							
	A	B	C	D	A′ (ratio)	B′ (ratio)	C′ (ratio)	D′ (ratio)
40 °C	10.9	12.9	6.39	2.45	14.3 (1.31)	20.3 (1.57)	28.9 (4.52)	5.71 (2.33)
25 °C	3.49	3.19	1.35	0.21	Not done	Not done	Not done	Not done
5 °C	0.17	0.085	0.048	0.016	2.55 (15.0)	1.35 (15.9)	2.77 (57.7)	4.05 (253)

the oxidation rate for formulations A, B, C, and D was, respectively, 1.3, 1.6, 4.5, and 2.3 times higher than the corresponding control formulations and relative order was formulation C > B > A > D.

The oxidation rates of the four control formulations were also obtained at 25 and 5 °C while those containing 0.1% Tween 80 were obtained only at 5 °C (Table 3). In the absence of Tween 80, the relative order of IL-2 mutein oxidation was formulation A > B > C > D at both 25 and 5 °C, which is different from that at 40 °C. As expected, the oxidation of IL-2 mutein was significantly increased in the presence of 0.1% Tween 80 at 5 °C. The increase in the rate of oxidation by Tween 80 for formulations A, B, C, and D was, respectively, 15.0, 15.9, 57.9, and 253 times at 5 °C, compared with a maximum of 4.5 times difference at 40 °C (Table 3). In addition, the order of IL-2 mutein oxidation in the presence of 0.1% Tween 80 was formulation D > C > B > A, which is also different from that at 40 °C.

Fig. 8 shows the relationship between oxidation rates of IL-2 mutein and temperature (1/*T*). Through linear regression analysis, the activation energy was calculated for all the control formulations. Linear regression was not performed on the Tween-containing formulations because data were obtained only at two temperatures −40 and 5 °C. In the absence of 0.1% Tween 80, the activation energy for formulations C through D was very consistent between 24.2 and 25.2 kcal/mol but that for formulation A was slightly lower (20.8 kcal/mol).

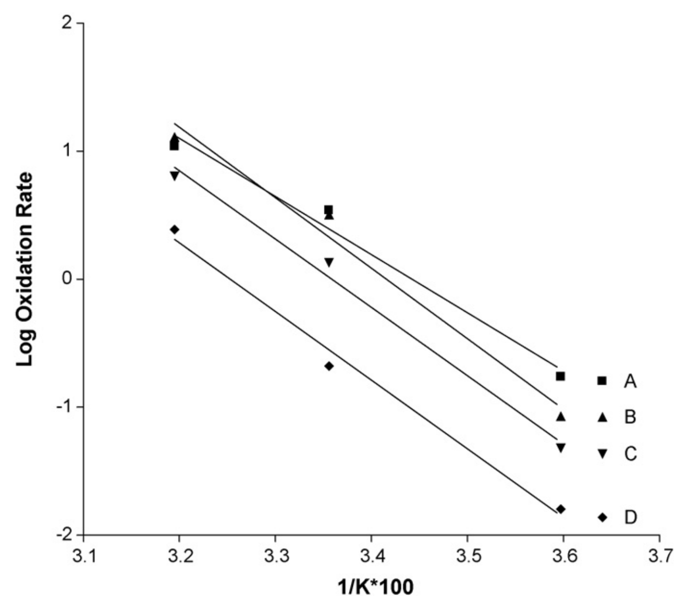


Fig. 8. Effect of temperature on oxidation of IL-2 mutein during storage in the absence of 0.1% Tween 80.

4. Discussion

4.1. Effect of Tween 80 on shaking-induced aggregation of IL-2 mutein

In this study, we demonstrated that IL-2 mutein aggregated readily upon shaking and Tween 80 at 0.1% significantly inhibited the aggregation. Both processes are formulation-dependent. The shaking-induced aggregation of IL-2 mutein was expected as this protein is relatively hydrophobic. In a similar study, rotation of IL-2 solution in a phosphate buffered (pH 7.0) saline at 250 RPM at 4 °C for 8 h reduced the activity by 67% and addition of 0.5% poloxamer 407 completely blocked the loss of activity (Wang and Johnston, 1993). A Other structurally similar proteins, including GCSF and the human growth hormone, also readily aggregate upon shaking and the presence of surfactants inhibited the aggregation of both proteins (Katakam et al., 1995a,b; Maa and Hsu, 1997; Bam et al., 1998; Treuheit et al., 2002).

Shaking increases the area of air/water interface in solution. Since this interface is hydrophobic, proteins will have to reorientate themselves at the interface and expose the hydrophobic regions in order to maximize their interaction with the interface (Damodaran and Song, 1988). Exposure of the hydrophobic regions in proteins would increase the chance of inter-molecular protein–protein interaction and hence, the chance of protein aggregation. A surfactant may occupy this interface competitively and thus inhibit the protein aggregation. In addition, a surfactant may interact with the protein form a less hydrophobic entity and inhibit protein aggregation (Randolph and Jones, 2002).

4.2. Tween 80-induced aggregation of IL-2 mutein during storage

In this study we demonstrated that storage of IL-2 mutein in solution in the absence of Tween 80 generated aggregates linked by both disulfide and non non-disulfide bonds. The disulfide-linked aggregates are mostly made of IL-2 mutein fragments. It is likely that hydrolysis of IL-2 mutein generated fragments, which have exposed –SH groups for disulfide bond formation and aggregation.

Addition of 0.1% Tween 80 promoted IL-2 mutein aggregation during storage. Both disulfide and non-disulfide chemical linkages were formed. Since we are not aware of any catalytic effect of Tween 80, the increased chemical bond formation in IL-2 mutein in the presence of Tween 80 is likely due to its

interaction with the protein, resulting in an increased exposure of hidden and less reactive groups. GCSF, a protein sharing the same structural fold with IL-2 mutein, was also shown to be adversely influenced by Tweens and addition of 0.01% Tween 20 increased the amount of protein aggregates from 1.2 to 5.9% in solution during storage at 29 °C for 59 weeks (Treuheit et al., 2002).

Indeed, direct interactions have been demonstrated between Tween 20 and rhGH (Bam et al., 1995; Bam et al., 1998), and between Tween 80 and lactate dehydrogenase (LDH) (Hillgren et al., 2002). PEG 6000, a molecule sharing the same structure with part of the Tween compounds, was shown to interact strongly with LDH molecules, creating hydrophobic zones in an aqueous solution (Hillgren and Alden, 2002). The surfactant–protein interaction is hydrophobic in nature as proteins with more hydrophobicity bind more surfactants (Bam et al., 1995; Bam et al., 1998). In a more recent report, a weak interaction was demonstrated between Tween 80 and the hydrophobic zones of LDH in an aqueous solution (Hillgren et al., 2002). IL-2 mutein is relatively hydrophobic and we propose that Tween 80 interact with IL-2 mutein in solution. Such a hydrophobic interaction(s) would occupy part of the hydrophobic area in the protein and cause a reduction of the hydrophobic area in the protein, resulting in a weaker intra-protein hydrophobic interaction, and leading to a looser protein fold with more exposure of reactive sites.

Since hydrophobic interaction increases with increasing temperature, a stronger effect would be expected at high temperatures. This seems to be the case in this study, where significant disulfide-bonded aggregates were observed at 40 °C in 2 months but hardly any at 5 °C in 22 months. The temperature-dependent hydrophobic interaction may also explain why nonionic surfactants destabilize certain proteins, specifically at higher temperatures or close to the denaturation temperature. For examples, poloxamer 407 at 1% significantly increases the stability of urease at 0.33 mg/ml in phosphate buffer (pH 7.0) at 37 or 50 °C but decreases its stability at 75 °C (Wang and Johnston, 1993). Either Pluoronic F68 at 0.1 mg/ml or Tween 80 at 1 mg/ml reduces the T_m of IL-1R (Remmele et al., 1998). Tween 80, Tween 40 or Tween 20 reduces the T_m of rhGH at 3 mg/ml at a surfactant:rhGH molar ratio of 10:1 (Bam et al., 1998). Tween 20 at 0.03% also reduces the unfolding free energy of rhIFN-γ (Webb et al., 2002). Like IL-2 mutein, rhGH, rhIFN-γ and GCSF are all helix-rich proteins. It remains to be seen whether such proteins are particularly sensitive to protein–surfactant interactions.

4.3. Tween 80-induced oxidation of IL-2 mutein during storage

We demonstrated that IL-2 mutein was oxidized during storage in a formulation- and temperature-dependent manner. Addition of 0.1% Tween 80 promoted oxidation of IL-2 mutein. One obvious reason for the increased oxidation is the presence of residual peroxides in Tween 80. In our previous report, we demonstrated that aged Tween 80 accelerated oxidation of IL-2 mutein both in liquid and solid states (Ha et al., 2002). In this study, we specifically used low-peroxide Tween 80. The peroxide level in the raw Tween 80 was 20 μM/kg, which translates to a peroxide level of 20 nM in the stability samples containing 0.1% Tween 80. Assuming that all the peroxides could be consumed completely by reacting with IL-2 mutein during the storage period, the amount of peroxide-induced oxidized IL-2 mutein would be less than 1%. However, the increase of oxidized IL-2 mutein in the presence of 0.1% Tween 80 after storage was between 12–60% at 40 °C and 29–84% at 5 °C. In comparison, the increase of oxidized IL-2 mutein in the control formulations during storage was 5–26% at 40 °C and 0.4–4% at 5 °C. Therefore, the net increase due to the presence of 0.1% Tween 80 was 7–34% at 40 °C and 28–80% at 5 °C, much higher than that predicted due to peroxide-induced oxidation of IL-2 mutein. The higher-than-predicted amount of oxidized IL-2 mutein in the presence of 0.1% Tween 80 could be attributed to two possible causes: (1) formation of additional peroxides in Tween 80 during storage, and (2) more exposure of protein, resulting in more reactive oxidation site(s). One interesting observation is that more oxidation occurred at 5 °C in 22 months than at 40 °C in 2 months in the presence of 0.1% Tween 80 while the control formulations showed the opposite trend. This may suggest that Tween 80-induced oxidation of IL-2 mutein at 5 °C is likely to be caused, at least partially, by peroxides formed during storage. This is because peroxides can decompose with time and are more stable at a lower storage temperature (Ha et al., 2002).

4.4. Formulation and temperature effect on IL-2 mutein aggregation and oxidation

In this report, we demonstrated that both formulation and temperature have a strong effect on both aggregation and oxidation of IL-2 mutein. Changing the storage temperature not only changed the extent of IL-2 mutein aggregation in a formulation-dependent manner but also changed the mechanism(s) of IL-2 mutein aggregation, especially in the presence of 0.1% Tween 80. This indicates that an aggregation mechanism for a protein defined at one storage temperature may not be extrapolated to a different storage temperature.

The rate of oxidation of IL-2 mutein at 40 °C was approximately 63–153 times that at 5 °C in the control formulations, suggesting a strong temperature-dependent process. Changing the storage temperature also changed the rate order of IL-2 mutein oxidation in the four formulations with and without 0.1% Tween 80. This indicates that a relatively stable formulation at one storage temperature may not be stable at a different storage temperature.

The activation energy of IL-2 mutein in the control formulations was 20.8–25.2 kcal/mol, which is comparable to 22.7 kcal/mol reported for oxidation of methionine in human Insulin-like Growth Factor I (hIGF-I)1 in an aqueous solution (Fransson et al., 1996). The variation in the activation energy and frequency factor between the four formulations indicates a strong dependency of IL-2 mutein oxidation on the formulation composition.

5. Conclusions

IL-2 mutein in solution easily forms soluble and insoluble aggregates during shaking in a formulation-dependent manner. Tween 80 at 0.1% effectively inhibited the aggregation process. IL-2 muntein also forms soluble aggregates to a different degree in different formulations during storage at 40 °C for 2 months but aggregation was barely detectable during storage at 5 °C for 22 months. Addition of 0.1% Tween 80 significantly increased the rate of IL-2 mutein aggregation during storage. The IL-2 mutein aggregates are linked by both disulfide and non-disulfide bonds and their relative contribution is temperature-dependent. IL-2 mutein can be oxidized also to a different degree in different formulations during storage and the oxidation rate is strongly temperature-dependent with an activation energy between 21–25 kcal/mol. Addition of 0.1% Tween 80 significantly increased the rate of oxidation in general.

References

Bam, L., Cleland, J.L., Randolph, T.W., 1996. Molten globule intermediate of recombinant human growth hormone: stabilization with surfactants. Biotechnol. Prog. 12, 801–809.

Bam, N.B., Cleland, J.L., et al., 1998. Tween protects recombinant human growth hormone against agitation-induced damage via hydrophobic interactions. J. Pharm. Sci. 87, 1554–1559.

Bam, N.B., Randolph, T.W., et al., 1995. Stability of protein formulations: investigation of surfactant effects by a novel EPR spectroscopic technique. Pharm. Res. 12, 2–11.

Chang, B.S., Kendrick, B.S., Carpenter, J.F., 1996. Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. J. Pharm. Sci. 85, 1325–1330.

Damodaran, S., Song, K.B., 1988. Kinetics of adsorption of proteins at interfaces: role of protein conformation in diffusional adsorption. Biochim. Biophys. Acta 954, 253–264.

Donbrow, M., Azaz, E., Pillersdorf, A., 1978. Autoxidation of polysorbates. J. Pharm. Sci. 67, 1676–1681.

Fransson, J., Florin-Robertsson, E., et al., 1996. Oxidation of human insulin-like growth factor I in formulation studies: kinetics of methionine oxidation in aqueous solution and in solid state. Pharm. Res. 13, 1252–1257.

Gombotz, W.R., Pankey, S.C., Bouchard, L.S., Phan, D.H., MacKenzie, A.P., 1996. Stability, Characterization, Formulation and Delivery System Development for Transforming Growth Factor-beta1. Plenum Press, New York.

Ha, E., Wang, W., et al., 2002. Peroxide formation in polysorbate 80 and protein stability. J. Pharm. Sci. 91, 2252–2264.

Herman, A.C., Boone, T.C., Lu, H.S., 1996. Characterization, Formulation, and Stability of Neupogen (Filgrastim) a Recombinant Human Granulocty-colony Stimulating Factor. Plenum Press, New York.

Hillgren, A., Alden, M., 2002. A comparison between the protection of LDH during freeze–thawing by PEG 6000 and Brij 35 at low concentrations. Int. J. Pharm. 244, 137–149.

Hillgren, A., Evertsson, H., et al., 2002. Interaction between lactate dehydrogenase and Tween 80 in aqueous solution. Pharm. Res. 19, 504–510.

Jaeger, J., Sorensen, K., Wolff, S.P., 1994. Peroxide accumulation in detergents. J. Biochem. Biophys. Methods 29, 77–81.

Katakam, M., Bell, L.N., et al., 1995a. Effect of surfactants on the physical stability of recombinant human growth hormone. J. Pharm. Sci. 84, 713–716.

Katakam, M., Bell, L.N., Banga, A.K., 1995b. Effect of surfactants on the physical stability of recombinant human growth hormone. J. Pharm. Sci. 84, 713–716.

Knepp, V.M., Whatley, J.L., Muchnik, A., Calderwood, T.S., 1996. Identification of antioxidants for prevention of peroxide-mediated oxidation of recombination human ciliary neurotrophic factor and recombinant human nerve growth factor. J. Pharm. Sci. Technol. 50, 163–171.

Maa, Y.-F., Hsu, C.C., 1997. Protein denaturation by combined effect of shear and air–liquid interface. Biotechnol. Bioeng. 54.

Nguyen, T.H., Burnier, J., et al., 1993. The kinetics of relaxin oxidation by hydrogen peroxide. Pharm. Res. 10, 1563–1571.

PDR, 1999. Physician's Desk Reference. Medical Economics Company, Montvale, NJ.

Randolph, T.W., Jones, L.S., 2002. Surfactant–protein interactions. Pharm. Biotechnol. 13, 159–175.

Remmele Jr., R.L., Nightlinger, N.S., et al., 1998. Interleukin-1 receptor (IL-1R) liquid formulation development using differential scanning calorimetry. Pharm. Res. 15, 200–208.

Sarciaux, J.M., Mansour, S., Hageman, M.J., Nail, S.L., 1999. Effects of buffer composition and processing conditions on aggregation of bovine IGG during freeze-drying. J. Pharm. Sci. 88, 1345–1361.

Treuheit, M.J., Kosky, A.A., et al., 2002. Inverse relationship of protein concentration and aggregation. Pharm. Res. 19, 511–516.

Van Patten, S.M., Hanson, E., et al., 1999. Oxidation of methionine residues in antithrombin. Effects on biological activity and heparin binding. J. Biol. Chem. 274, 10268.

Wang, P.L., Johnston, T.P., 1993. Thermal-induced denaturation of two model proteins: effect of poloxamer 407 on solution stability. Int. J. Pharm. 96, 41–49.

Webb, S.D., Cleland, J.L., et al., 2002. A new mechanism for decreasing aggregation of recombinant human interferon-gamma by a surfactant: slowed dissolution of lyophilized formulations in a solution containing 0.03% polysorbate 20. J. Pharm. Sci. 91, 543–558.

Zhang, M.Z., Pikal, K., Nguyen, T., Arakawa, T., Prestrelski, S.J., 1996. The effect of the reconstitution medium on aggregation of lyophilized recombinant interleukinl-2 and ribonuclease A. Pharm. Res. 13, 643–646.

Zhang, M.Z., Wen, J., Arakawa, T., Prestrelski, S.J., 1995. A new strategy for enhancing the stability of lyophilized protein: the effect of the reconstitution medium on keratinocyte growth factor. Pharm. Res. 12, 1447–1452.