Effect of Excipients on the Stability and Structure of Lyophilized Recombinant Human Growth Hormone

HENRY R. COSTANTINO,*,† KAREN G. CARRASQUILLO,‡ ROCIO A. CORDERO,‡ MARCO MUMENTHALER,† CHUNG C. HSU,† AND KAI GRIEBENOW‡

Contribution from *Pharmaceutical Research and Development, Genentech, Inc., 1 DNA Way, South San Francisco, California 94080 and Department of Chemistry, University of Puerto Rico, Rio Piedras Campus, San Juan, Puerto Rico 00931-3346.*

Received February 27, 1998. Accepted for publication April 9, 1998.

Abstract ☐ We have investigated the effect of mannitol, sorbitol, methyl α-D-mannopyranoside, lactose, trehalose, and cellobiose on the stability and structure of the pharmaceutical protein recombinant human growth hormone (rhGH) in the lyophilized state. All excipients afforded significant protection of the protein against aggregation, particularly at levels to potentially satisfy water-binding sites on the protein in the dried state (i.e., 131:1 excipient-to-protein molar ratio). At higher excipient-to-protein ratios, X-ray diffraction studies showed that mannitol and sorbitol were prone to crystallization and afforded somewhat less stabilization than at lower ratios where the excipient remained in the amorphous, protein-containing phase. The secondary structure of rhGH was determined using Fourier transform infrared (FTIR) spectroscopy. rhGH exhibited a decrease in α-helix and increase in β-sheet structures upon drying. Addition of excipient stabilized the secondary structure upon lyophilization to a varying extent depending on the formulation. Samples with a significant degree of structural conservation, as indicated by the α-helix content, generally exhibited reduced aggregation. In addition, prevention of protein–protein interactions (indicated by reduced β-sheet formation) also tended to result in lower rates of aggregation. Therefore, in addition to preserving the protein structure, bulk additives that do not crystallize easily and remain amorphous in the solid state can be used to increase protein–protein distance and thus prevent aggregation.

Introduction

In the development of a lyophilized pharmaceutical protein, sugars (saccharides and polyols) are often added to the formulation in order to improve stability and increase the shelf life.[1] Some specific examples of FDA-approved lyophilized protein formulations include sucrose in various human immunoglobulins and toxoid vaccines, lactose in glucagon and haemophilus b conjugate vaccine, and mannitol in urokinase and recombinant human growth hormone.[2] Despite the success shown by such examples, the mechanism regarding how sugars protect lyophilized proteins is still not fully understood.

Several factors are likely to play a role. For example, it has been shown that the secondary structure of some proteins may be altered upon lyophilization, and that sugars can help preserve the native conformation.[3,4] It is postulated that the hydroxyl groups in sugars form hydrogen-bonds with polar groups in proteins in the solid state,[5] substituting for water molecules which play a role in the structure. In this fashion, sugar molecules may "replace" water molecules in the solid state.[5] Others have proposed alternative views, for example in the case of the lyoprotectant trehalose.[6] Recent moisture sorption studies have revealed that sugars can indeed interact with proteins in such a fashion as to satisfy protein water-binding sites.[7,8]

Several mechanisms can cause a protein to undergo aggregation in the solid state. For various proteins it has been established that dehydration-induced structural alterations exposing reactive groups (in particular disulfide bonds)[9,10] is the initial step leading to covalent protein aggregation.[11] Such structural alterations are also expected to promote noncovalent aggregation of proteins. It follows that structural conservation will improve solid protein stability if the mechanism of deterioration is conformation-dependent. But even when the degradation occurs without conformational change, a sugar excipient may still provide stability for lyophilized proteins. For example, the therapeutic protein recombinant humanized monoclonal immunogloulin G, which largely retains its native secondary structure upon spray drying, is stabilized against solid-state aggregation by the addition of lactose.[12] An alternate view to explain this is that sugar excipients can serve to "dilute" proteins in the solid state, decreasing protein−protein contacts and preventing intermolecular degradation reactions such as aggregation.[9,13] Yet another conception is that sugar excipients provide a glassy matrix wherein protein mobility and hence reactivity are minimized.[14,15] In all of these views of the mechanism of solid-state stabilization, it is critical that the sugar remains in the amorphous, protein-containing phase. Various environmental factors, such as increased temperature and moisture, can induce sugar crystallization.[8,16,17]

In the present study we have investigated the effect of various sugar excipients on the solid-state stability of a model pharmaceutical protein, recombinant human growth hormone (rhGH). Growth hormone, or somatotropin, is susceptible to various deterioration pathways in the solid state, predominantly aggregation.[18,19] This protein is an FDA-approved drug for the long-term treatment of children with growth failure, currently available in both liquid and lyophilized forms containing mannitol.[2] In the present investigation, we have examined the protective effect of various excipients on aggregate formation during incubation at an elevated storage temperature.

Materials and Methods

Protein—Recombinant human growth hormone (rhGH) was produced at Genentech, Inc. (South San Francisco). The protein bulk containing 2 mg/mL rhGH, 88 mM mannitol, and 5 mM sodium phosphate, pH 7.8, was buffer-exchanged into a 100 mM ammonium bicarbonate solution and lyophilized to form excipient-free protein.[20] Samples were lyophilized in a Leybold (Germany)

* Corresponding author: Phone: 650-225-4710; Fax 650-225-3191.
† Genentech, Inc.
‡ University of Puerto Rico.

model GT20 unit at a chamber pressure of 150 μmHg and a shelf temperature of −35 °C for 48 h (primary drying) followed by a shelf temperature of 30 °C for about 10 h (secondary drying). All dried samples were stoppered under dry N_2 when the vacuum pressure was <127 mmHg. The lyophilized material was sealed in glass vials and stored at 2−8 °C until use. The lyophilization and cold-temperature storage did not adversely affect protein quality, particularly with regard to aggregation and clipping.

Excipients—Mannitol, sorbitol, methyl α-D-mannopyranoside, lactose, trehalose, and cellobiose, all analytical grade, were obtained from Sigma Chemical Co. (St. Louis, MO) and were used as supplied.

Ratios of Excipients to rhGH in Lyophilized Samples—Lyophilized samples containing different excipient-to-protein ratios were prepared by adding an appropriate amount of concentrated excipient solution to the excipient-free protein solution prior to lyophilization. The ratios selected were based on the molar amount of various strongly and weakly water-binding sites in the rhGH molecule, as described elsewhere.[21] The molar ratios chosen for our study were 31:1 (lyoprotectant-to-rhGH; representing 50% of strongly water-binding sites present in the rhGH molecule), 131:1 (100% of strongly and weakly water-binding sites), 300:1 and 1000:1 (excess beyond the total of all water-binding sites).

Preparation of Lyophilized Excipient/rhGH Samples—Lyophilized excipient-free rhGH was reconstituted with deionized water to form a stock solution containing 20 mg/mL protein. Protein concentration was confirmed by UV absorption at 278 nm. Concentrated excipient stock solutions were also prepared and combined with rhGH stock solutions to obtain the precise excipient-to-rhGH ratio desired. Solutions were filtered (0.22 μm), and the protein concentration was again confirmed by UV absorption. Aliquots of 4 mL of the excipient-to-protein solution were filled into 10-cc glass vials and lyophilized as described above for excipient-free protein. The sample of 1000:1 methyl α-D-mannopyranoside:rhGH exhibited "collapse" upon lyophilization and was not assayed for solid-state stability.

Residual Moisture Content—The residual moisture content was assayed by the Karl Fischer titration method.[21] All samples were found to contain a residual moisture of approximately 2−3% (w/w).

Glass Transition Temperature—The glass transition (T_g) for the excipients was measured by differential scanning calorimetry (DSC) using a Perkin-Elmer Model 7 unit. Approximately 10 mg of each sample was loaded into an aluminum sample pan, sealed, and placed in the calorimeter. An empty pan was used as a reference. Following an equilibration of 10 min at 30 °C, samples with sorbitol were cooled at a rate of 20 °C/min to −20 °C, whereas all other samples were heated at a constant rate of 10 °C/min to 120 °C. T_g was taken as the midpoint in the thermogram as measured from extensions of the pre- and posttransition baselines, using Perkin-Elmer software provided with the calorimeter.

X-ray Powder Diffraction (XRD)—XRD of the lyophilized excipient:protein samples was conducted as described elsewhere.[22]

Solid-State Stability Studies—Lyophilized excipient:rhGH samples were stored for up to 28 days at 50 °C. At selected timepoints, two vials at each excipient:protein ratio were reconstituted with sterile water-for-injection (WFI) to 1 mg/mL initial protein and assayed as follows. The amount of insoluble aggregates was determined by measuring the protein concentration (UV detection at 278 nm) of the reconstituted sample after centrifugation (3000 rpm for 30 min) and filtration (0.22 μm). Insoluble aggregates smaller than 0.22 μm were not necessarily removed by the filtration. The amount of soluble aggregates was determined by size-exclusion HPLC (UV detection at 214 nm) on a Tosoh TSK20000SWXL column (7.8 mm i.d. × 30 cm length, particle size 5 μm). Typically, 10 μL of each filtered, reconstituted sample was loaded onto the column at a flow rate of 0.5 mL/min. The mobile phase consisted of 150 mM sodium chloride, 50 mM sodium phosphate, pH 7.2.

FTIR Spectroscopy—FTIR studies were conducted with a Nicolet Magna-IR System 560 optical bench as described previously.[4] A total of 256 scans at 2 cm^{-1} resolution using Happ-Ganzel apodization were averaged to obtain each spectrum. For all experiments involving aqueous solutions, a Spectra Tech liquid cell equipped with CaF_2 windows and 15-μm thick spacers was used. Lyophilized protein powders were measured as KBr pellets (1 mg of protein per 200 mg of KBr).[4,23,24] Each protein sample was measured at least five times. When necessary, spectra were corrected for the solvent background in an interactive manner using the Nicolet OMNIC 3.1 software[4,23,24] to obtain the protein vibrational spectra. We have confirmed that this procedure is reliable for water background subtraction when using 15 μm thick spacers.[23] Prior to further analysis, small water vapor bands present were eliminated from the spectra.

Table 1—Infrared Band Positions, Areas, and Assignments in the Amide I Region for Various Formulations of rhGH[a]

	band position (cm^{-1})			
sample	SD[b]	Gaussian curve-fitting[c]	area (%)	assignment
aqueous solution (excipient-free)	1686	1687 ± 1	6 ± 1	unordered[d]
	1682	1678 ± 1	10 ± 1	unordered
	1670	1670 ± 1	6 ± 1	unordered
	1655	1655 ± 1	57 ± 3	α-helix
	1639	1640 ± 1	14 ± 2	unordered
	1633	1634 ± 1	7 ± 3	β-sheet
lyophilized (excipient-free)	1692	1696 ± 2	14 ± 1	β-sheet
	1682	1683 ± 3	15 ± 1	unordered
	1672	1670 ± 1	20 ± 1	unordered
	1655	1655 ± 1	29 ± 3	α-helix
	1639	1640 ± 1	12 ± 2	unordered
	1631	1629 ± 1	10 ± 2	β-sheet
co-lyophilized with 131:1 mannitol:protein	1690	1694 ± 2	7 ± 3	β-sheet
	1683	1682 ± 2	18 ± 3	unordered
	1675	1670 ± 1	15 ± 2	unordered
	1654	1655 ± 0	39 ± 3	α-helix
	1637	1640 ± 1	12 ± 1	unordered
	1629	1630 ± 0	9 ± 1	β-sheet
co-lyophilized with 131:1 lactose:protein	1690	1691 ± 3	8 ± 3	β-sheet
	1681	1682 ± 1	12 ± 2	unordered
	1671	1671 ± 1	16 ± 1	unordered
	1656	1656 ± 0	48 ± 2	α-helix
	1640	1641 ± 1	5 ± 1	unordered
	1631	1632 ± 1	11 ± 2	β-sheet

[a] Data are the average and standard deviation of four to five independent determinations. [b] Second derivative. [c] Gaussian curve-fitting was performed of Fourier self-deconvoluted amide I spectra. [d] Unordered structures include random coil, extended chains and turns.

FTIR Data Analysis—*Second Derivatization*—All spectra were analyzed by second derivatization in the amide I and amide III regions for their component composition.[4,23–26] Second derivative spectra were smoothed with an 11-point smoothing function (10.6 cm^{-1}).[4]

Fourier Self-Deconvolution (FSD)—FSD[27–29] was applied to the unsmoothed spectra to enable quantification of the secondary structure in the amide I region by Gaussian curve-fitting[24,30] using the program OMNIC 3.1. The parameters chosen, a value of 24 for the full width at half-maximum (fwhm) and $k = 2.4$ for the enhancement factor, are in the range of those published.[30–33] Note that FSD alters the band shapes, but preserves the integrated band intensities when over-deconvolution is avoided.[27,31] The values chosen for FSD in our analyses were checked for the risk of such over-deconvolution (which could result in distorted band areas)[28,30] by employing the strategy outlined by Griebenow and Klibanov.[23]

Gaussian Curve-Fitting—The frequencies of the band centers found in the second derivative spectra in the amide I region were used as starting parameters for the Gaussian curve-fitting (performed using the program GRAMS/386 from Galactic Industries, Inc.). The secondary structure contents were calculated from the areas of the individual assigned bands and their fraction of the total area in the amide I region.[23,24,30] Gaussian curve-fitting was performed in the amide I region after band-narrowing of the protein vibrational spectra by FSD.[30,33] In all cases, a linear baseline was fitted in addition to the Gaussian bands. In most cases, the discrepancies between component frequencies obtained by second derivatization and the Gaussian curve-fitting were below 4 cm^{-1} (Table 1). The secondary structure content is reported as the average and standard deviation of the value calculated for at least four independently obtained spectra.

Band Assignments—The band assignment in the amide I region followed those in the literature and is summarized for some typical

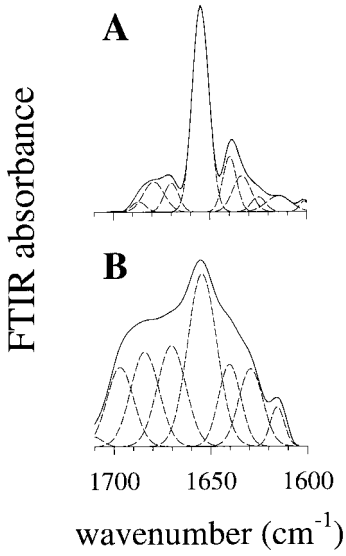

Figure 1—FTIR spectra of rhGH (A) in aqueous solution at pH 7.8 and (B) the lyophilized powder. The solid lines represent the superimposed FSD and the curve-fit, and the dashed curves represent the individual Gaussian bands.

samples in Table 1.[23,24,33,34] Shown are the results for the excipient-free aqueous and lyophilized protein, in addition to lyophilized formulations containing a molar ratio of excipient-to-protein of 131:1 lactose and mannitol, representing samples with the greatest and least degree of structural conservation, respectively. For the aqueous solution, the main band at 1655 cm^{-1} was assigned to α-helices (Figure 1A) and a band at 1634 cm^{-1} to β-sheets. All other bands were assigned to unordered structural elements (β-turns, random coil, extended chains). The secondary structure content determined by Gaussian curve-fitting in the amide I region using these assignments (57 ± 3% α-helix and 7 ± 2% β-sheet) were the same, within the error limits, as those determined by others utilizing the amide III spectral region[35] and also agrees well with the X-ray crystal structure (60% α-helix).[36]

When analyzing the spectra of lyophilized rhGH (Figure 1B), a new band at ca. 1696 cm^{-1} was assigned to β-sheets. Bands at such frequencies are often assigned to intermolecular β-sheets.[33,37] Otherwise, the frequencies of the Gaussian bands found for lyophilized rhGH were similar as for the aqueous solution and assigned the same.

Results and Discussion

Solid-State Aggregation of rhGH and the Effect of Excipients—In the dried state, intermolecular pathways predominate as the main degradation mode for the growth hormone molecule.[18] Thus, to assess the stability of lyophilized rhGH, we monitored the solid-state formation of soluble and insoluble aggregates. Samples of rhGH were co-lyophilized with various excipients at excipient:protein (mol:mol) ratios of 31:1, 131:1, 300:1, and 1000:1. Protein aggregation in the various samples was monitored following incubation at the accelerated storage condition of 50 °C.

First, we tested the stability of rhGH over time in the absence of any excipients. The formation of soluble aggregates was slight, increasing from about 2% upon lyophilization to about 4% following four-week storage (Figure 2A). The formation of insoluble aggregates was much more dramatic; whereas virtually no insoluble aggregates were formed upon lyophilization, more than half of the protein had formed insoluble aggregates after the four-week incubation (Figure 2B). Aggregate formation in human growth hormone is detrimental since it may lead to reduced bioactivity[38] and increased immunogenicity.[39]

Next, we tested various excipients for their potency in stabilizing rhGH against solid-state aggregation. Figure

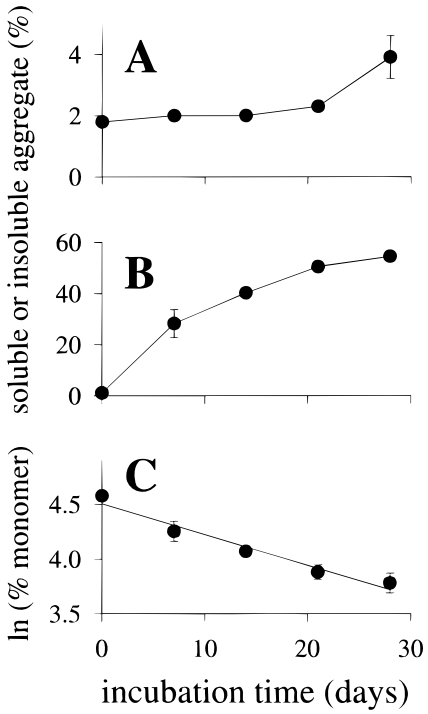

Figure 2—Solid-state stability of excipient-free rhGH. (A) Formation of soluble aggregates. (B) Formation of insoluble aggregates. (C) Loss of monomeric rhGH modeled as a pseudo first-order deterioration (calculated rate constant of 4.5 ± 0.1 day^{-1}).

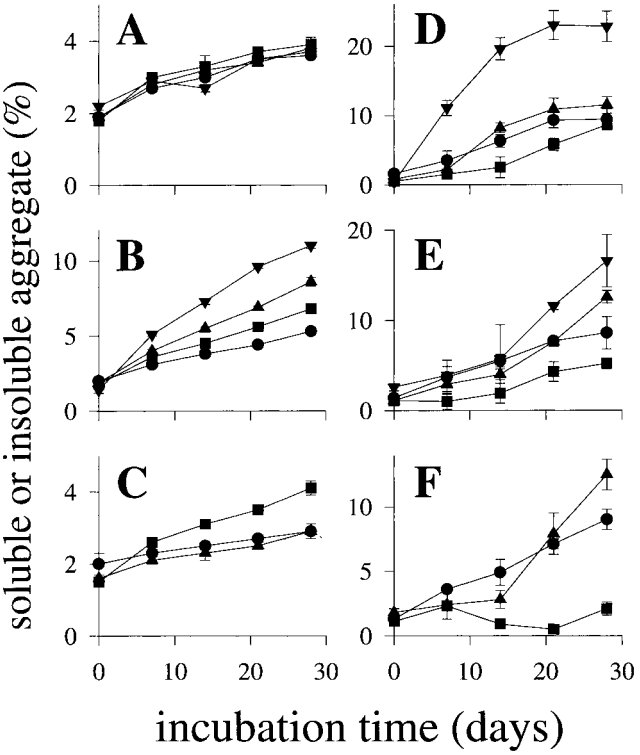

Figure 3—Soluble aggregate formation of rhGH co-lyophilized with (A) mannitol, (B) sorbitol, and (C) methyl α-D-mannopyranoside. Insoluble aggregate formation of rhGH co-lyophilized with (D) mannitol, (E) sorbitol, and (F) methyl α-D-mannopyranoside. Ratios of excipient-to-protein (mol:mol) were 31:1 (●), 131:1 (■), 300:1 (▲) and 1000:1 (▼).

3 shows both soluble and insoluble aggregation data for rhGH co-lyophilized with varying amounts of the straight-chain polyols mannitol and sorbitol and the more hydrophobic sugar, methyl α-D-mannopyranoside. No clear conclusions can be drawn regarding these excipients' ability

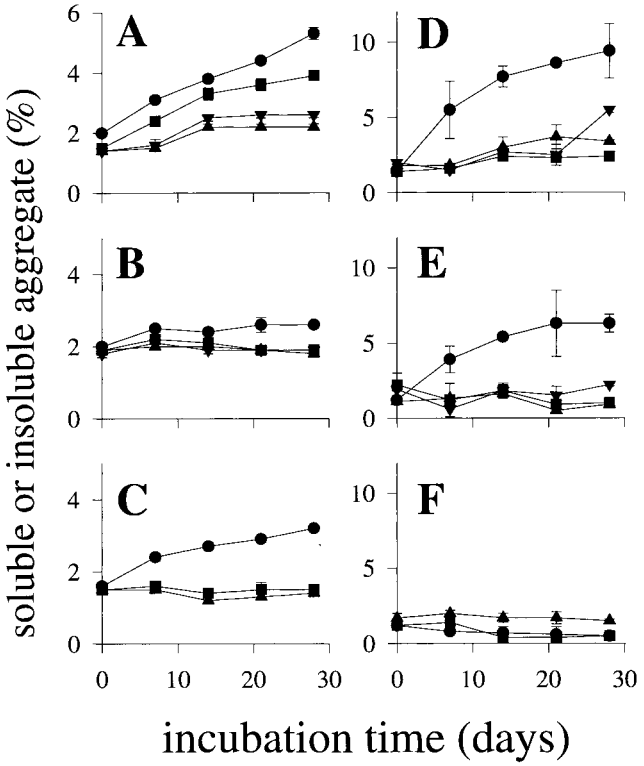

Figure 4—Soluble aggregate formation of rhGH co-lyophilized with (A) lactose, (B) trehalose, and (C) cellobiose. Insoluble aggregate formation of rhGH co-lyophilized with (D) lactose, (E) trehalose and (F) cellobiose. Ratios of excipient-to-protein (mol:mol) were 31:1 (●), 131:1 (■), 300:1 (▲) and 1000:1 (▼).

to stabilize rhGH against soluble aggregate formation (Figures 3A−C); indeed, it seems that the addition of sorbitol may destabilize the protein (Figure 3B). However, the insoluble aggregate data (Figures 3D−F) reveal some interesting trends. For instance, at a mannitol-to-protein ratio of 31:1, rhGH is significantly more stable toward insoluble aggregate formation, with less than 10% insoluble aggregates formed after four-week storage, compared to the case of excipient-free protein (stability of rhGH:mannitol and excipient-free protein shown in Figure 3D and Figure 2B, respectively). As the excipient content is increased to 131:1 mannitol:rhGH, the stability is further improved. However, at the higher ratios of 300:1 and 1000:1, rhGH shows increased insoluble aggregation. Similarly to the case of mannitol, the data for sorbitol (Figure 3E) and methyl α-D-mannopyranoside (Figure 3F) also suggest that a 131:1 level of excipient-to-protein is optimal in stabilizing the protein against solid-state insoluble aggregate formation. The significance of this ratio is further discussed below.

Figure 4 depicts soluble and insoluble aggregate formation for rhGH co-lyophilized with the disaccharides lactose, trehalose, and cellobiose. All three of these excipients show a dramatic protective effect against both soluble and insoluble aggregate formation. For example, lactose and trehalose afforded essentially complete stabilization to the protein with regards to insoluble aggregation when present at 131:1 excipient:rhGH and above (Figures 4D and 4E). Cellobiose was perhaps even more potent, with essentially no insoluble aggregates formed even at the lowest level tested, 31:1 excipient:rhGH (Figure 4F).

It is important to note that lactose, a disaccharide of glucose and galactose, and cellobiose, which is comprised of two glucose units, contain $\beta 1 \rightarrow 4$ linkages and are thus both reducing sugars. The two glucosyl units of trehalose are bonded via an $\alpha 1 \rightarrow 1$ linkage; trehalose is not a reducing sugar. Reducing sugars have the potential to react with amino groups in proteins via the Maillard reaction.[40,41]

Table 2—Formation of Non-Native Monomeric rhGH in Various Lyophilized Formulations[a]

excipient:rhGH (mol:mol)	non-native monomer (%)[b] 1 week	2 weeks	3 weeks	4 weeks
methyl α-D-mannopyranoside:rhGH				
31:1	–[c]	–	–	–
131:1	–	1.0 ± 0.1	1.4 ± 0.1	1.9 ± 0.2
300:1	1.2 ± 0.1	1.5 ± 0.1	1.2 ± 0.1	1.5 ± 0.2
lactose:rhGH				
31:1	–	–	–	–
131:1	–	11.8 ± 0.1	13.5 ± 0.6	14.7 ± 0.5
300:1	–	13.6 ± 0.7	15.7 ± 0.3	16.7 ± 0.3
1000:1[d]	12.4 ± 0.1	15.8 ± 0.3	19.3 ± 0.5	22.3 ± 0.3
cellobiose:rhGH				
31:1	–	19.8 ± 1.3	25.0 ± 0.8	26.2 ± 0.6
131:1	–	12.9 ± 1.0	16.5 ± 0.3	17.8 ± 0.7
300:1	–	12.9 ± 0.3	15.4 ± 0.4	19.8 ± 1.3
1000:1	5.6 ± 0.3	14.4 ± 0.4	16.3 ± 0.3	18.6 ± 0.4

[a] Species evidenced by HPLC with a retention near the native monomer, e.g., shoulder in the main peak. This species was not seen in rhGH co-lyophilized with the nonreducing sugar trehalose, mannitol, or sorbitol, and is probably a result of reaction with excipient, i.e., glycosylation. [b] Incubation at 50 °C. [c] Not detected. [d] Formation of non-native monomer was 8.3 ± 0.3% upon lyophilization.

Table 3—Psuedo First-Order Rate Constants for Deterioration of Monomeric rhGH When Lyophilized in the Presence of Various Excipients[a]

excipient	k (×10^{-3} days^{-1}) at an excipient:rhGH ratio of: 31:1	131:1	300:1	1000:1
mannitol	3.9 ± 0.5	3.8 ± 0.4	5.2 ± 0.7	9.9 ± 2.0
sorbitol	3.9 ± 0.2	3.5 ± 0.3	6.7 ± 0.7	9.2 ± 0.7
methyl α-D-mannopyranoside	3.1 ± 0.1	0.9 ± 0.5	4.6 ± 1.0	
lactose	4.1 ± 0.6	1.3 ± 0.2	1.1 ± 0.3	1.7 ± 0.5
trehalose	2.1 ± 0.5	0.4 ± 0.2	0 ± 0.2[b]	0.2 ± 0.2
cellobiose	0.3 ± 0.1	0 ± 0.2	0 ± 0.1	0 ± 0.1

[a] The negative of the slope of the plot of ln[monomer] vs time. [b] In cases where a slight positive slope was observed, the rate constant was taken as zero (within the error of the fit).

In the case of lactose- and cellobiose-containing samples, we found that some 10−20% of soluble rhGH was in the form of an altered monomer over the four-week incubation at the accelerated stability condition, as distinguished by size-exclusion HPLC (Table 2). The presence of this species did not interfere with the quantitative analysis of soluble and insoluble aggregation. It is probable that this species is a glycosylated form the rhGH monomer. A very small amount (1−2%) of a similar species was also seen in rhGH co-lyophilized with methyl α-D-mannopyranoside. Although the latter is not a reducing sugar, it is possible that it contained a reducing sugar impurity, e.g., mannose, which reacted with rhGH upon solid-state storage. As discussed elsewhere,[41] reducing sugars should be avoided in biopharmaceutical formulations, even though they may be potent stabilizing excipients.

To more quantitatively compare the difference in stability of the various rhGH formulations, we analyzed the aggregation as a pseudo first-order process with respect to monomer (an example plot is shown in Figure 2C for excipient-free rhGH). A summary of the rate constants for all excipient:rhGH samples is listed in Table 3.

The data show that all of the excipients employed in our study imparted significant stabilization to rhGH, particularly when present at a level of 131:1 excipient:protein and higher (Figure 5). The straight-chain polyols mannitol and

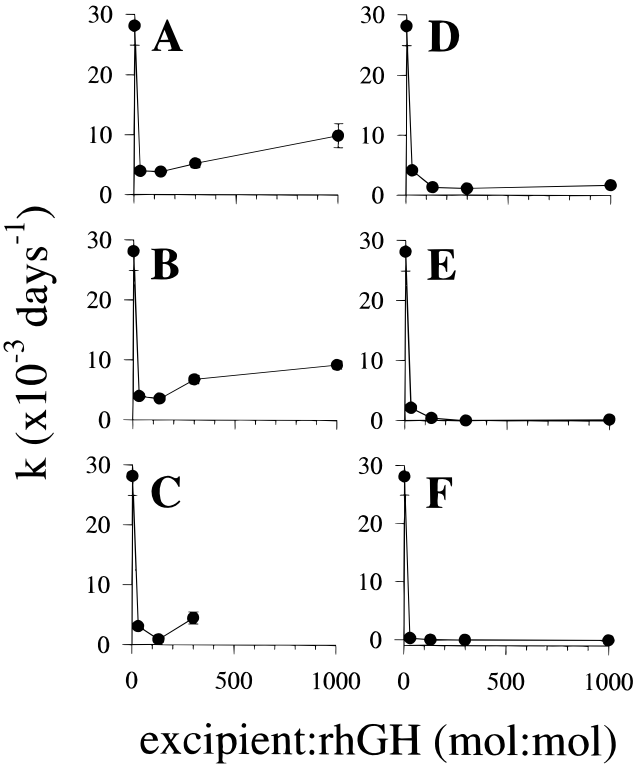

Figure 5—Psuedo first-order constants for the deterioration of rhGH co-lyophilized with various amounts of (A) mannitol, (B) sorbitol, (C) methyl α-D-mannopyranoside, (D) lactose, (E) trehalose, and (F) cellobiose.

sorbitol and methyl α-D-mannopyranoside were not quite as potent as the disaccharides tested, and exhibited an optimum stabilization at 1:131 excipient:rhGH. Lactose, trehalose, and cellobiose imparted a maximum stabilization effect when present at 131:1 excipient:protein and above.

It is interesting that the 131:1 ratio represents the level of all potential strongly and weakly water-binding sites in the rhGH molecule.[21] It is possible that the excipient molecules afford stability to the protein by replacing water in the solid state, which would be consistent with the observed stability data (Figure 5). This conclusion is also supported by recent moisture sorption data showing that addition of sugars to rhGH decreases the accessibility of water-binding sites in a humidified atmosphere.[7,8] These interactions may also have a relation to rhGH's stability against lyophilization-induced structural alteration, as described below.

Besides the potential of excipient−protein interactions to stabilize the protein, other factors may play a role, such as the excipient's ability to dilute protein molecules in the solid-state and retard intermolecular reactions.[12] Also, the excipient may provide a glassy matrix in which reactivity is retarded, and hence stability is improved.[14,15] For instance, Hancock and Zografi[15] have described how the amorphous state influences solid-state physical and chemical properties in pharmaceutical formulations. It is important for stability that the pharmaceuticals remain in the amorphous, glassy phase, below the glass transition temperature (T_g). The T_g is the temperature at which a material undergoes a change from a highly viscous glass to a viscoelastic rubber.

For globular proteins such as rhGH, it is difficult to obtain T_g values.[42] As discussed elsewhere, the difficulty in measuring T_g for proteins by standard techniques such as differential scanning calorimetry may be due to the large internal heterogeneity of domains and broad distribution of relaxation times.[43,44] Most dry proteins exhibit T_gs in the range of 150−200 °C.[40] The T_g of a lyophilized excipient:protein mixture may then be estimated as a contribution of individual T_gs.[15]

Table 4—Glass Transition Temperatures of Various Compounds[a]

excipient	T_g (°C)	source
mannitol	4[b]	Franks et al.[45]
sorbitol	−2	Franks et al.[45]
methyl α-D-mannopyranoside	70	this study
lactose	108	Saleki-Gerhardt and Zografi[16]
trehalose	115	Saleki-Gerhardt and Zografi[16]
cellobiose	77	this study
water	−133	Slade and Levine[46]

[a] The data for the T_g of sugar/polyol excipients from the literature are for rigorously dried samples; data for methyl α-D-mannopyranoside and cellobiose contain about 2–3% residual moisture. [b] Mannitol is difficult to prepare in the amorphous state following lyophilization. In the present study, mannitol tended to crystallize upon lyophilization and therefore no T_g was observed.

Therefore, the addition of a component with a lower T_g than rhGH will serve to lower the T_g of the system relative to pure protein. The excipients used in our study all exhibit T_gs lower than that expected for pure rhGH (Table 4). In addition, there was some residual water present in our lyophilized samples (in the range of 2−3% for all samples in our study) which also decreases the T_g of the system.[40] Therefore, it is expected that addition of the relatively low-molecular-weight polyol and saccharide excipients used in our study would lower the T_g relative to rhGH alone.[42] Even so, it was observed that the protein stability was improved in the presence of such excipients. Consequently, the stabilization afforded against solid-state aggregation effect cannot be explained solely in terms of the excipient's ability to impact mobility through changes in the T_g of the system. A similar conclusion was made for the effect of various excipients on stabilizing bovine growth hormone toward thermal unfolding in the lyophilized state.[42]

Nonetheless, the physical state of the system, i.e., amorphous or crystalline, is likely to impact stability and T_g is an important parameter.[15] Certainly it is required (but not necessarily sufficient) that the excipient remains in the amorphous, glassy phase with the protein at the storage condition. Above their T_g, amorphous polyols and sugars may be susceptible to crystallization at the crystallization temperature, T_c. If this occurs to an excipient in a pharmaceutical protein formulation, the stabilization effect may be lost as the excipient and protein phase separate. Also, in a closed system, crystallization may result in the release of water (e.g., formation of anhydrous crystals) which is then available for the protein, and may cause further destabilization, since water plays a key role in solid-state protein aggregation.[11,40]

We also examined the physical state of lyophilized excipient:rhGH samples by X-ray diffraction (XRD). All samples, except for 300:1 and 1000:1 mannitol:rhGH, were amorphous upon lyophilization, as evidenced by the lack of any distinguishing features in their diffractograms (data not shown). However, there was the possibility that the excipient in the formulation may crystallize during high-temperature storage, particularly in the case where the protein content is relatively low, since proteins have been shown to inhibit sugar crystallization in the solid state.[8,17,22]

To test this, we performed XRD on samples containing the highest levels of excipient, following their incubation for four weeks at 50 °C. The XRD data, depicted in Figure 6, show that 1000:1 lactose, trehalose, and cellobiose all remained amorphous following the high-temperature incubation. However, the data for 1000:1 sorbitol:rhGH (Figure 6B) and 300:1 methyl α-D-mannopyranoside:rhGH (Figure 6C) revealed that the excipient had undergone

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.