A Specific Molar Ratio of Stabilizer to Protein is Required for Storage Stability of a Lyophilized Monoclonal Antibody

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Received 22 June 2000; revised 25 September 2000; accepted 26 September 2000

ABSTRACT: The selection of the appropriate excipient and the amount of excipient required to achieve a 2-year shelf-life is often done by using iso-osmotic concentrations of excipients such as sugars (e.g., 275 mM sucrose or trehalose) and salts. Excipients used for freeze-dried protein formulations are selected for their ability to prevent protein denaturation during the freeze–drying process as well as during storage. Using a model recombinant humanized monoclonal antibody (rhuMAb HER2), we assessed the impact of lyoprotectants, sucrose, and trehalose, alone or in combination with mannitol, on the storage stability at 40°C. Molar ratios of sugar to protein were used, and the stability of the resulting lyophilized formulations was determined by measuring aggregation, deamidation, and oxidation of the reconstituted protein and by infrared (IR) spectroscopy (secondary structure) of the dried protein. A 360:1 molar ratio of lyoprotectant to protein was required for storage stability of the protein, and the sugar concentration was 3–4-fold below the iso-osmotic concentration typically used in formulations. Formulations with combinations of sucrose (20 mM) or trehalose (20 mM) and mannitol (40 mM) had comparable stability to those with sucrose or trehalose alone at 60 mM concentration. A formulation with 60 mM mannitol alone provided slightly less protection during storage than 60 mM sucrose or trehalose. The disaccharide/mannitol formulations also inhibited deamidation during storage to a greater extent than the lyoprotectant formulations alone. The reduction in aggregation and deamidation during storage correlated directly with inhibition of unfolding during lyophilization, as assessed by IR spectroscopy. Thus, it appears that the protein must be retained in its native-like state during freeze–drying to assure storage stability in the dried solid. Long-term studies (23–54 months) performed at 40°C revealed that the appropriate molar ratio of sugar to protein stabilized against aggregation and deamidation for up to 33 months. Therefore, long-term storage at room temperature or above may be achieved by proper selection of the molar ratio and sugar mixture. Overall, a specific sugar/protein molar ratio was sufficient to provide storage stability of rhuMAb HER2.
 J Pharm Sci 90:310–321, 2001
Keywords: protein stability; lyophilization; monoclonal antibody; aggregation; sugars

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Journal of Pharmaceutical Sciences, Vol. 90, 310–321 (2001)

INTRODUCTION

The successful development of therapeutic proteins depends on the ability to deliver them in

their intact native state. Proteins are often formulated in aqueous solution to allow ease of use, but several degradation processes may be facilitated by the aqueous environment.[1] To obtain the desired stability during storage for up to 2 years, proteins are often dried to reduce the rate of chemical and physical degradation.[2,3]

There have been numerous reports of the ability of sugars, such as sucrose and trehalose, to stabilize proteins during lyophilization and storage in the dried solid.[4–8] Recent studies have also shown the utility of these sugars in producing freeze-dried,[9–11] spray freeze-dried, or spray-dried antibodies.[12,13] The mechanism of stabilization by these sugars during drying has been proposed to occur by producing a glassy matrix to restrict mobility and/or acting as a water substitute (reviewed by Carpenter et al.[14–16]). Several recent cases have shown that a glassy matrix alone is not sufficient to inhibit protein unfolding during dehydration and that hydrogen bonding of the sugar to the dried protein (water substitution) is a requisite for inhibition of lyophilization-induced protein unfolding.[5–7,17,18]

The removal of water in the absence of a stabilizing agent, such as a sugar, often results in a non-native protein structure in the dry state and may lead to aggregation during reconstitution.[4,17,19] Furthermore, it has also been shown with a few proteins that retention of the native protein structure in the initial lyophilized cake is important for optimal long-term storage stability in the dried solid (reviewed by Allison et al.[8] and Carpenter et al.[15,16]). In addition, it has been found that sugars provide a glassy matrix that is required to reduce the rate of degradative reactions during storage in the dried solid.[6,7,9,16,17]

Many stability studies are performed using isoosmotic concentrations of sugars (e.g., 275 mM sugar) and do not determine the minimum concentration required for optimal long-term stability. In the current investigation, to determine the optimum sugar content for protein stability, we studied the effect of a wide range of sugar-to-protein molar ratios, as well as different sugars, on the retention of native protein structure in the dried solid and prevention of degradation during storage. To assess the relationship between protein structure in the dried state and long-term storage stability, protein secondary structure was analyzed by infrared (IR) spectroscopy and the levels of aggregated and deamidated protein were determined in reconstituted samples.

The model protein selected for these studies was the recombinant humanized monoclonal antibody against the human epidermal growth factor receptor-2 (rhuMAb HER2), which has recently been approved by the U.S. Food and Drug Administration for treatment of metastatic breast cancer. This antibody has a molecular weight of ~150 kDa, and is composed of a human Fc domain of the subtype IgG_1 with humanized framework and complementary determining regions (CDRs) designed to provide comparable binding affinity to the murine form (see Carter et al.[20] for details on the humanization process). Previous studies by our group[21] and others[22] have shown that this antibody is susceptible to oxidation at Met-255 and Met-431 in the Fc domain. Deamidation and formation of isoaspartate and succinimide may also occur at Asn30 in the light chain and Asn102 in the heavy chain.[23] These reactions may be slowed or eliminated by the removal of water to produce a freeze-dried product. However, the removal of water may foster denaturation leading to another degradation route, aggregation. A successful formulation of rhuMAb HER2 must therefore provide protection against aggregation during lyophilization and storage, as well inhibition of chemical degradation. In the studies described herein, we have used storage at elevated temperature (40°C) to determine the relative stability of several formulations. We have also investigated conditions that are hypotonic (<275 mM) and may be used for formulations that require dilution into intravenous (iv) saline bags or reconstitution with volumes that are less than the fill volume yielding a more concentrated solution.

MATERIALS AND METHODS

Materials

Recombinant humanized monoclonal antibody against the human epidermal growth factor receptor 2 (rhuMAb HER2) was expressed in Chinese hamster ovary (CHO) cells and purified at Genentech, Inc. The purification was performed using Protein A affinity chromatography followed by cation exchange to remove aggregates and charged forms of the antibody. The final purified antibody was provided in 5 mM sodium histidine, pH 6 by Genentech's Product Recovery Operations.

Methods

Size Exclusion Chromatography

Distribution of intact protein, soluble aggregate and/or fragments of rhuMAb HER2 was determined using size exclusion chromatography (SEC). A high-performance liquid chromatography (HPLC) system (Hewlett Packard 1090L) was used with a TSK 3000 SWXL (Toso Haas Corporation) column at a flow rate of 1 mL/min using pH 7.2 phosphate-buffered saline (PBS) as the mobile phase. A 25-μg sample of protein was injected onto the column and detected by absorbance at 280 nm. Standards of known protein concentration were run as controls for each set of samples. Samples were run twice, and the average value was used to determine the fraction of aggregated protein in the sample [coefficient of variation (CV) $\pm$ 5%]. The fractions of aggregate, intact protein, and protein fragments were determined by assessing the relative peak areas of each species. For SEC, ion-exchange, and hydrophobic interaction methods, three vials were assayed for each time point and the results were reported as a mean and standard deviation (SD). All chromatographic methods yielded complete protein recovery (no protein loss) when compared with appropriate controls.

Cation-Exchange Chromatography

The amounts of deamidated cyclic imide and intact rhuMAb HER2 were quantitatively determined by cation-exchange chromatography (IEX). A wide-pore carboxy sulfon column was heated to 40°C, and chromatography was performed at a flow rate of 1 mL/min. Separation of various charged species was achieved through a NaCl gradient in 20 mM Na phosphate buffer (pH 6.9). Samples were diluted to 1 mg/mL protein, and then 75 μg of protein was injected onto the column and detected by absorbance at 214 nm. All samples were analyzed in duplicate along with appropriate standards (CV $\pm$ 5%).

Hydrophobic Interaction Chromatography

The detection of rhuMAb HER2 oxidized species was achieved by digestion with carboxypeptidase B and papain. The resulting Fab and Fc fragments were analyzed by hydrophobic interaction chromatography (HIC) using a TSK Butyl-NPR column (4.6 $\times$ 35 mm, Tosohaas) and a gradient from 0 to 2 M ammonium sulfate as previously described.[21]

Residual Moisture of Lyophilized Cakes

Residual moisture of lyophilized rhuMAb HER2 was determined by the Karl Fisher (Brinkmann, model 658) titration method. Measurements were made by an electrochemical technique that utilizes the conductive state of a hydranal composite/methanol solution. Samples for residual moisture analysis were prepared by dispersing the lyophilized cake to a powder in the vials. The percentage by weight of residual water moisture present was determined for sample weights ranging from 40 to 70 mg. A mean titer standard (CV $\pm$ 5%) derived from ten 5-μL water samples was used to calculate the percentage by weight of residual water present in the samples. Two or three moisture measurements were conducted per vial depending on the availability of material. Samples for excipient screening all had residual moisture contents of $\leq$ 2% w/w (g water/100 g solid).

pH of Frozen Protein Solution

The pH of rhuMAb HER2 formulated at 25 mg/mL in 60 mM trehalose, 5 mM histidine, pH 6.3, and 0.01% polysorbate 20 was determined at -20°C (frozen) using a combined pH electrode (Mettler Toledo, Wilmington, MA) as described previously.[24] The probe was calibrated at 25, 10, and 0°C with buffer standards at pH 4, 7, and 10 (Baxter Diagnostics), and the SD of the measurements were $\pm$ 0.10 pH units.

Infrared Spectroscopy

The IR spectra of native aqueous protein and protein in lyophilized formulations were acquired and processed as previously described using a Bomem Prota Spectrometer and software.[6–8,18]

Differential Scanning Calorimetry

Differential scanning calorimetric (DSC) analysis of lyophilized formulations was conducted as previously described[6–8,18,25] with a Perkin Elmer DSC-7, and the percentages of crystalline mannitol were determined as detailed previously.[25]

Experimental Conditions

The protein was formulated at 25 mg/mL in either 5 mM succinate, pH 5.0 or 5 mM histidine, pH 6

with 0–250 mM trehalose and 0.01–0.2% w/v polysorbate 20. Additional studies were performed within this range of polysorbate 20 concentrations, and no effect was observed on the stability of rhuMAb HER2 following storage at 40°C for 6 months in formulations containing 60 mM trehalose and 5 mM histidine, pH 6 (data not shown). Additional solutions were prepared at the same protein concentration in 5 mM histidine, pH 6 with 0–250 mM sucrose and 0.1% polysorbate 20. To determine the effects of mannitol on stability of rhuMAb HER2, the protein was also formulated at 25 mg/mL in 5 mM histidine, pH 6 and 0.1% polysorbate 20 with 60 mM mannitol, and with combinations of mannitol and sucrose (55 mM mannitol/5 mM sucrose; 50 mM mannitol/10 mM sucrose; 40 mM mannitol/20 mM sucrose). The effect of protein concentration on stability was determined by formulation of rhuMAb HER2 at 25 or 50 mg/mL in either 60 mM trehalose, 20 mM trehalose/40 mM mannitol, or 20 mM sucrose/40 mM mannitol with 5 mM histidine, pH 6 and 0.1% polysorbate 20. Long-term stability of the lyophilized protein at 40°C was also determined for formulations containing trehalose alone or the mannitol/lyoprotectant combinations at 5 mg/mL rhuMAb HER2 in 5 mM histidine, pH 6 and 0.01% polysorbate 20. Each study was performed on a single lot of each formulation.

Lyophilization

For each formulation, 18 mL of solution was filled into depyrogenated, sterile 50-cc vials and lyophilized in a GT20 freeze-dryer. Primary drying was conducted at shelf temperatures of -10–5°C for 40–68 h (greater time for lower temperatures) and secondary drying was performed for 10–12 h at a shelf temperature of 20°C. For residual moisture studies, vials were removed at different times during secondary drying using a special access port to allow maintenance of vacuum. The chamber pressure was maintained constant at 150 mTorr throughout primary and secondary drying. Vials were capped under partial vacuum. Reconstitution was performed using 20 mL of Sterile Bacteriostatic Water for Injection (SBWFI) containing 1.1% benzyl alcohol. SBWFI was chosen to allow multiple withdrawals of the protein from the vial for repeated administration from a single vial (multidose vial). A formulation consisting of 25 mg/mL rhuMAb HER2, 60 mM trehalose, 5 mM histidine, pH 6, and 0.01% polysorbate 20 was placed at 40°C for 6 months, and vials were reconstituted with either sterile water for injection (SWFI) or SBWFI. This study demonstrated that reconstitution with SBWFI or SWFI yielded equivalent stability.

Stability Assessment

Control samples of each formulation before lyophilization were retained (stored frozen) and analyzed at the same time as lyophilized samples. Each formulation was reconstituted and tested immediately after lyophilization. Each formulation was stored at 40°C for 1 and 3 months in the lyophilized state and then reconstituted and analyzed. Long-term stability (23–55 months) was determined for selected formulations.

RESULTS AND DISCUSSION

A variety of different sugars and molar ratios of sugar to protein were used to produce lyophilized rhuMAb HER2 formulations. As noted in Table 1, after lyophilization and immediate reconstitution (no storage), even in the absence of sugars, there was only a slight increase in aggregate formation. However, on storage of these lyophilized formulations at 40°C, the extent of aggregation increased dramatically (Figures 1 and 2). Without sugars, the extent of rhuMAb HER2 aggregation increased from $\sim$1% to $>$10% after 3 months at 40°C. In the absence of sugar, a greater extent of aggregation was observed in the histidine formulation than in the succinate formulation after 3 months at 40°C (Figure 1). Previous studies had indicated the potential for histidine to provide stabilizing effects during lyophilization and storage,[26] but this stabilization was not observed with rhuMAb HER2.

The addition of sucrose or trehalose in the formulations reduced the amount of aggregation noted after storage and reconstitution (Figures 1 and 2). There was no apparent difference in the stabilizing affect of trehalose or sucrose (Figure 2b). The amount of aggregated protein decreased with increasing sugar concentration up to 100 mM, and only minor reductions in the amount of aggregate over 3 months of storage at 40°C were observed for formulations containing $>$60 mM sugar. At 60 mM sugar, the molar ratio of sugar to protein was 360. Similar stoichiometric amounts of carbohydrates were required to stabilize another recombinant antibody during spray drying and subsequent storage.[12]

Table 1. Effect of Lyophilization on the Aggregation of rhuMAb HER2[a]

Formulation	% Aggregate	
	Before Lyophilization	After Lyophilization
5 mM Succinate, pH 5	0.2 ± 0.1	1.4 ± 0.2
+ 60 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 100 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 150 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 200 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 250 mM trehalose	0.7 ± 0.2	0.9 ± 0.3
5 mM Histidine, pH 6	0.4 ± 0.2	1.1 ± 0.3
+ 30 mM trehalose	0.3 ± 0.1	0.5 ± 0.2
+ 60 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 100 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 150 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 200 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
+ 250 mM trehalose	0.0 ± 0.0	0.0 ± 0.0
5 mM Histidine, pH 6	—	—
+ 60 mM sucrose	0.5 ± 0.1	1.0 ± 0.1
+ 100 mM sucrose	0.4 ± 0.2	0.3 ± 0.1
+ 150 mM sucrose	0.2 ± 0.1	0.6 ± 0.2
+ 200 mM sucrose	0.7 ± 0.1	0.4 ± 0.2
+ 250 mM sucrose	0.6 ± 0.2	0.5 ± 0.2
5 mM Histidine, pH 6		
+ 60 mM mannitol	0.5 ± 0.2	0.4 ± 0.1
+ 55 mM mannitol/5 mM sucrose	0.4 ± 0.3	0.9 ± 0.2
+ 50 mM mannitol/10 mM sucrose	0.0 ± 0.0	0.5 ± 0.1
+ 40 mM mannitol/20 mM sucrose	0.0 ± 0.0	0.0 ± 0.0

[a] All formulations consisted of 25 mg/mL protein and were lyophilized as described in the MATERIALS AND METHODS section.

To determine if this stabilizing phenomenon was specific to sucrose and trehalose, mannitol was used in the formulation buffer at 60 mM (the apparent minimum sugar concentration required for stability). Although storage of the mannitol formulation at 40°C for 3 months resulted in less aggregation than the formulations without sugar, the mannitol formulation provided slightly less protection against aggregation than the same concentration of sucrose or trehalose (Figures 1–3). Previous studies have indicated that mannitol may crystallize during lyophilization, forming a separate phase from the protein.[6,27–19] If this phase separation had occurred, lyophilization-induced unfolding may not be inhibited (vide infra) and the amount of aggregated protein in the mannitol formulations should be comparable to or greater than that in the formulations without sugar. However, this low concentration of mannitol may remain amorphous and in the same phase as the protein during drying, providing protection to the protein. In support of this suggestion, DSC analysis (cf., Carpenter et al.[25]) determined that mannitol in the lyophilized 60 mM mannitol rhuMAb HER2 formulation was only 7% crystalline (data not shown).

Furthermore, the presence of sugars that remain amorphous during lyophilization (e.g., sucrose) in mannitol formulations can further inhibit mannitol crystallization.[30] Several studies have also shown that combinations of mannitol and an amorphous excipient improve protein stability after lyophilization and storage relative to that noted with mannitol alone.[31–33] To test for this effect, sucrose was added to the formulation to yield a range of sucrose and mannitol/protein molar ratios. The use of 20 mM sucrose (sucrose/protein molar ratio of 120:1), which was not optimum for stability when used alone, and 40 mM mannitol was sufficient to provide stabilization against aggregation after storage and reconstitution, yielding comparable stability to 60 mM

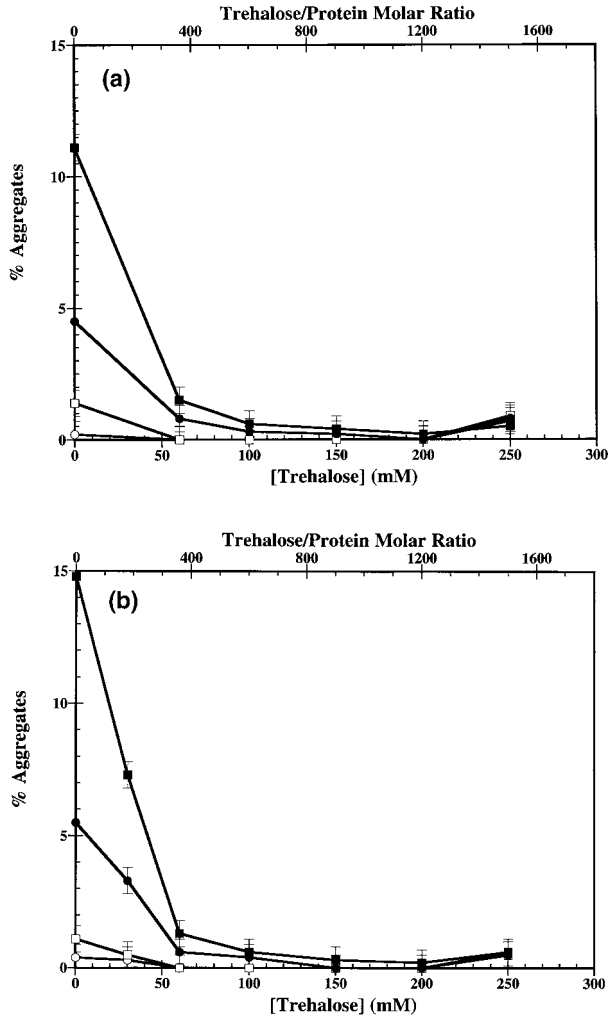

Figure 1. The effect of trehalose concentration on the fraction of aggregated protein after reconstitution of lyophilized formulations was measured by SEC. rhuMAb HER2 was formulated at 25 mg/mL in either 5 mM succinate, pH 5 (A) or 5 mM histidine, pH 6 (B). Samples were analyzed prior to lyophilization (open circles), immediately after lyophilization (open squares), or after storage of the lyophilized protein for 1 (closed circles) or 3 (closed squares) months at 40°C.

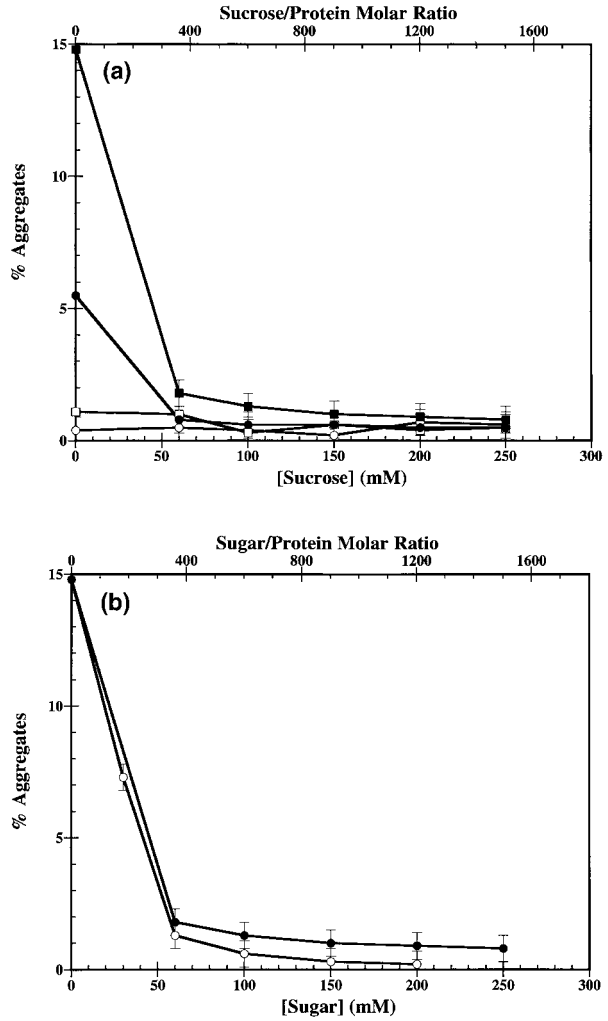

Figure 2. The effect of sucrose concentration on the fraction of aggregated protein after reconstitution of lyophilized formulations was measured by SEC. rhuMAb HER2 was formulated at 25 mg/mL in 5 mM histidine, pH 6. (A) Samples were analyzed prior to lyophilization (open circles), immediately after lyophilization (open squares), or after storage of the lyophilized protein for 1 (closed circles) or 3 (closed squares) months at 40°C. (B) Sucrose (closed circles) and trehalose (open circles, 5 mM histidine, pH 6) formulations provided comparable protection against aggregation upon stored at 40°C for 3 months.

sucrose alone (Figures 2 and 3). Stabilization of lyophilized proteins formulated with mannitol in combination with glycine,[31,34] sucrose, or trehalose[33] have been previously reported and indicate that mannitol provides some protection if it remains in the amorphous form.[32] These studies suggest that mannitol may either facilitate sucrose stabilization of the protein or directly stabilize the protein when maintained in the protein phase during drying (amorphous form). With the lyophilized 20:40 mM sucrose/mannitol rhuMAb HER2 formulation, DSC analysis indicated that only 4% of the mannitol was crystalline.

The effects observed in these experiments may have resulted from the overall concentration of the sugars used in these studies and not the specific molar ratio of sugar to protein. Different rhuMAb HER2 concentrations were therefore

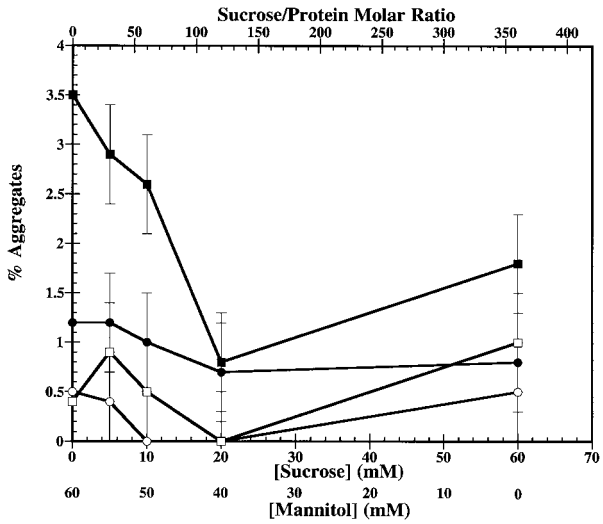

Figure 3. The stability of rhuMAb HER2 formulated in mannitol or mannitol/sucrose formulations was determined by measuring aggregation by SEC after reconstitution of lyophilized formulations. rhuMAb HER2 was formulated at 25 mg/mL in 5 mM histidine, pH 6. Samples were analyzed prior to lyophilization (open circles), immediately after lyophilization (open squares), or after storage of the lyophilized protein for 1 (closed circles) or 3 (closed squares) months at 40°C.

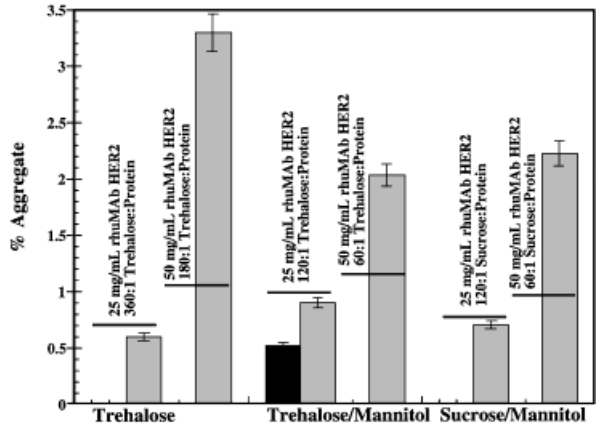

Figure 4. Effect of protein concentration on aggregation of rhuMAb HER2 in lyophilized formulations was assessed after reconstitution either immediately after lyophilization (solid bars) or after storage of the lyophilized protein for 1 month at 40°C.

studied to assess the mechanism of sugar stabilization. Maintaining the trehalose concentration at 60 mM and increasing the protein concentration from 25 to 50 mg/mL resulted in a significant increase in aggregation on storage for only 1 month at 40°C (Figure 4). As a result of this increase in protein concentration, the molar ratio of sugar to protein decreased from 360 to 180. The extent of aggregation at a molar ratio of sugar to protein of 180 was comparable to that observed for 30 mM trehalose and 25 mg/mL rhuMAb HER2 (molar ratio of 180, Figure 1), suggesting that the molar ratio is critical for stabilization of the protein during storage. The mixed sugar systems of 20:40 mM trehalose/mannitol and 20:40 mM sucrose/mannitol yielded similar results where a reduction from a molar ratio of trehalose or sucrose to protein from 120 to 60 yielded a marked increase in aggregation after 1 month of storage at 40°C. Overall, these data revealed that the molar ratio of sugar to protein appears to be a critical variable for protein stabilization during lyophilization and storage.

Long-term protein storage at high temperatures (40°C) was performed on selected samples to further test the impact of the sugar/protein molar ratio on protein stability (Table 2). At very high sugar/protein molar ratios (600–1800:1), only a small increase in aggregation was observed after 33 months at 40°C. At the intermediate molar ratio of 360:1, there was an increase to ~10% aggregation after > 50 months. The rate of aggregation at 40°C for these formulations was ~0.2% per month at 40°C, assuming zero-order kinetics. For a requirement of > 95% monomer at the end of shelf-life, this formulation may be stored at 40°C for 18 months. Decreasing the molar ratio to 180:1 caused a dramatic increase in aggregation (29.9%) after 44 months at 40°C. At the appropriate molar ratio, trehalose and sucrose or their combinations with mannitol may provide long-term, room-temperature stability, and perhaps even formulations that do not require refrigeration even at relatively high ambient temperatures.

Although the initial protein formulations used in the studies just described contained ≤ 2% residual moisture, the impact of 1–8.4% residual moisture was studied for the formulation containing 60 mM trehalose, 5 mM histidine, pH 6, and 0.01% polysorbate 20 to assess the impact of moisture on protein stability. There was no effect of residual moisture over this range on the rate of aggregation during storage at 2–8 or 40°C for 12 months. These data suggest that moisture level differences (1–2% moisture in all formulations) between the formulations did not affect aggregation during storage.

Table 2. Effect of Excipients on the Long-Term Stability of rhuMAb HER2 at 40°C[a]

[Protein] (mg/mL)	Excipient	[Excipient] mM	Molar ratio Excipient: Protein	Storage Time (mo.)	SEC Analysis		IEX Analysis
					% Aggregate	% Monomer	% Main Peak
5	Sucrose	20	600	33	1.1	98.9	80.8
	Mannitol	40	1200				
5	Trehalose	20	600	33	1.7	98.9	81.7
	Mannitol	40	1200				
5	Trehalose	60	1800	33	0.8	99.2	80.1
50	Trehalose	100	300	23	9.7	90.3	76.5
50	Trehalose	60	180	44	29.9	70.1	74.0
25	Trehalose	60	360	51	8.5	91.6	79.8
25	Trehalose[b]	60	360	54	11.0	89.0	76.6
25	Trehalose[c]	60	360	—	0.1	99.9	80.5

[a] All formulations contained 5 mM histidine, pH 6.0, unless otherwise noted. Because of sample limitations, single vials were assayed in duplicate and the average values are reported (CV ± 4%).
[b] Formulation was prepared in 5 mM succinate, pH 5.0.
[c] Reference standard stored at 2–8°C.

Secondary Structure of Lyophilized Protein

To determine the relationship between protein structure in the dried solid and protein stability during storage, the effects of excipients on the secondary structure of lyophilized rhuMAb HER2 (25-mg/mL formulations) were investigated with second-derivative IR spectroscopic analysis. As expected for a monoclonal antibody, the spectra for the aqueous native protein, in the conformationally sensitive amide I region, was dominated by bands at 1640 and 1690 cm^{-1}, which are due to the β-sheet structure (Figure 5). Bands were also observed at 1665 and 1680 cm^{-1}, which are due to the turn structure. When the protein was lyophilized in the absence of sugar, the resulting IR spectra in the dried solid indicated a substantial perturbation of secondary structure, which can be seen as a large decrease in the intensity, a broadening of the band at 1640 cm^{-1}, and a shift in the high frequency β-sheet band to 1695 cm^{-1} and an increase its area (Figure 5). In the presence of 60 mM sucrose, these transitions were partially inhibited, as indicated by the increase in intensity at 1640 cm^{-1}, a shift in the high-frequency β-sheet band to 1693 cm^{-1}, and a decrease in area of this band. In the presence of 60 mM mannitol, the degree of structural protection was slightly less than that noted for 60 mM sucrose (Figure 5). The sample with 20 mM sucrose and 40 mM mannitol had a spectrum that was also very similar to that for the sample with 60 mM sucrose (data not shown). With sucrose alone, the maximal degree of structural protection was obtained with a 100 mM concentration, based on absorbance at 1640 cm^{-1} (Figure 5). Increasing sucrose concentrations to 200 mM did not result in further increases in structural protection (data not shown). Similar results were noted with trehalose, where the maximal degree of retention of native structure also occurred at 60 mM initial sugar concentration (data not shown).

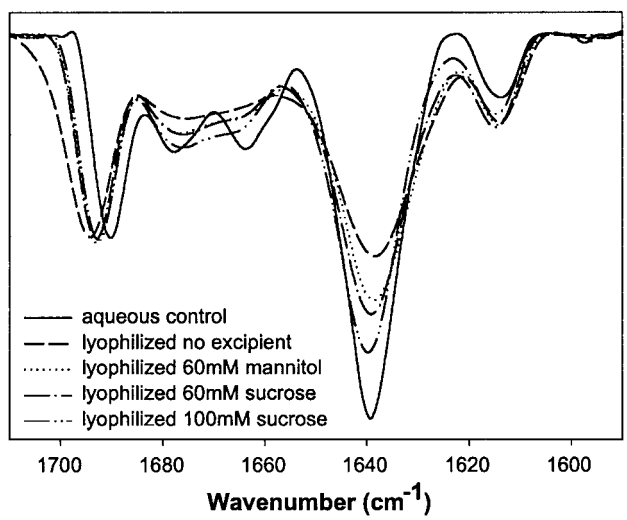

Figure 5. Second-derivative IR spectra were obtained for rhuMAb HER2 formulations: liquid control (5 mM histidine, pH 6, 60 mM trehalose), and lyophilized solids (5 mM histidine, pH 6 without sugar or with 60 mM sucrose, 100 mM sucrose, or 60 mM mannitol).

The spectra of all the formulations taken after 3 months of storage at 40°C, had only slightly reduced absorbance at 1640 cm^{-1} relative to that noted in the respective formulations immediately after lyophilization (data not shown). Also, in the spectra for the stored samples, as was the case for the spectra of samples immediately after lyophilization, bands indicative of intermolecular β-sheet structure (e.g., 1622 cm^{-1}) were not present. Similar results were found with the long-term stored samples that were analyzed by IR spectroscopy (data not shown), which included all formulations shown in Table 2, except the trehalose sample with an excipient/protein molar ratio of 180:1. Unfortunately, the single vial of this sample, which had the highest level of aggregation noted in the current study, was reconstituted before investigation of the dried solid by IR spectroscopy. Taken together, the IR spectroscopic and aggregation results document that either aggregation was not occurring in the dried solid and/or that the level of intermolecular β-sheet present in samples containing up to 15% aggregated rhuMAb HER2 is not sufficient to be detected by solid-state IR spectroscopy.

The capacities of the sugars to inhibit protein unfolding during lyophilization and aggregation during storage/rehydration were almost maximal at 60 mM initial sugar concentration; only a slight improvement is noted by increasing concentration to 100 mM sucrose concentration (Figures 2b and 5). Thus, it appears that optimal storage stability noted with a minimal excipient/protein molar ratio of 360 is due in large part to the retention of native protein structure in the initial lyophilized solid.

Another physical factor that has been found to be important in determining the stability of lyophilized protein formulations during long-term storage is the glass transition temperature (T_g) of the amorphous phase containing the protein.[6–9,14–17] Storage of samples above T_g can lead to rapid degradation.[6–9,14–17] The T_g values for a limited number of the formulations, for which stability results are presented in Figures 1 and 2, were determined by DSC. In all cases, the measured T_g values were >60°C, which far exceeds the storage temperature of 40°C used in the current study.

Chemical Stability of Lyophilized Protein

Another important degradation route for rhuMAb HER2, deamidation and succinimide formation, may be affected by lyophilization in different sugars. Both of these reactions may be catalyzed by water and, therefore, studies were performed with a range of residual moisture levels (1–8.4% w/w) for lyophilized rhuMAb HER2 formulated at 25 mg/mL in 60 mM trehalose, 5 mM histidine, pH 6, and 0.01% polysorbate 20. During storage for 12 months at 40°C, residual moistures between 1 and 4% w/w did not have an impact on the rate or extent of loss in rhuMAb HER2 main peak (only 2% loss in main peak), as measured by IEX. At 8.4% w/w residual moisture, the rate of degradation was increased, yielding a 19% decrease in main peak after 12 months at 40°C. This deamidation may be the result of water catalysis or a reduction in the T_g of the lyophilized cake. Measurements of the frozen pH of the same formulation indicated that the pH changed from 6.3 (solution) to 6.7 on freezing to -20°C. However, the short exposure of the protein to this small pH shift is not sufficient to increase the rate of rhuMAb HER2 deamidation in the lyophilized cake.

When sugars were not present in the formulation, a loss in rhuMAb HER2 main peak in the IEX chromatogram was observed immediately after lyophilization and additional losses were observed on incubation of the lyophilized cake at 40°C (Figure 6). The addition of sucrose (Figure 6a) or trehalose (Figure 6b) reduced the rate of loss in rhuMAb HER2 main peak, and a 60 mM concentration of either was sufficient for maximal protection. Interestingly, when the sucrose/mannitol formulations were tested at these conditions, the 20 mM sucrose/40 mM mannitol formulation prevented a decrease in main peak over 3 months of storage at 40°C compared with ~1% or 5% decrease in main peak for the 60 mM sucrose or mannitol, respectively. The rate of deamidation in the 60 mM sucrose and 20:40 mM sucrose/mannitol formulations are similar, and 60 mM of sucrose or trehalose is sufficient for maximum inhibition of deamidation. Given that these formulations have a similar of retention of native protein structure in the initial lyophilized formulations, the stabilization against deamidation may be caused by maintenance of the protein in the native state.

Longer term studies at 40°C using a range of molar ratios also suggested that a specific molar ratio of sugar to protein inhibits the rate of deamidation. As shown in Table 2, high molar ratios of sugars to protein (600–1800:1) completely inhibited deamidation for 33 months at 40°C. At a 360:1 molar ratio of trehalose to protein, only a

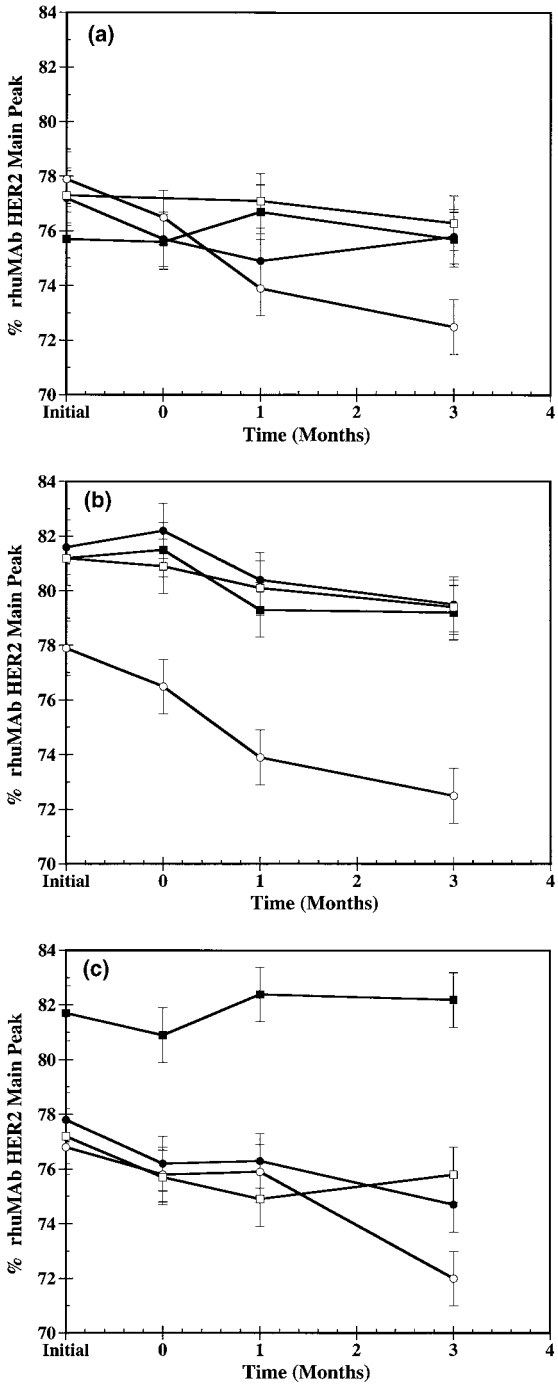

Figure 6. The rhuMAb HER2 main peak area was measured by IEX prior to lyophilization (initial) and after lyophilization with and without storage at 40°C. Formulations consisted of 5 mM histidine (pH 6) and 0.01% polysorbate 20 with 60 (closed circles), 100 (open squares), or 200 (closed squares) mM sucrose (A) or trehalose (B), or without sugar (open circles). (C) Protein in the same buffer with 60 mM mannitol (open circles), 10 mM sucrose/50 mM mannitol (closed circles), 20 mM sucrose/40 mM mannitol (closed squares), and 60 mM sucrose (open squares) was also tested.

slight decrease in main peak was observed after 51 months at 40°C. The lower molar ratio (180:1) resulted in a decrease in main peak of ~6%. The effect of sugars on stabilizing the protein against deamidation was not as significant as their effect on aggregation. Nevertheless, a specific molar ratio of sugar to protein did provide optimum long-term stability.

Each formulation studied in the aggregation and deamidation experiments was also studied for methionine oxidation, which has been previously observed for this protein.[21] After lyophilization and storage at 40°C for 3 months, there was no change in the amount of methionine oxidation (4% oxidized Fc domain), suggesting that this chemical degradation route is effectively inhibited in the lyophilized formulations.

CONCLUSIONS

These studies demonstrated that a minimal specific molar ratio of sugar to protein is sufficient to provide optimal stabilization against aggregation during storage of lyophilized rhuMAb HER2 formulations. A sugar-to-protein molar ratio of 360:1 was sufficient to inhibit aggregation and deamidation after 3 months of storage at 40°C. Both sucrose and trehalose provided equivalent protection during lyophilization and storage. Mannitol alone at the same molar ratio was only slightly less effective at preventing aggregation during storage at 40°C. Mannitol conferred stability most likely because it remained mostly amorphous during lyophilization. The addition of sucrose or trehalose to the mannitol formulations yielded equivalent storage stability compared with both sugars alone at the same total molar concentration (60 mM) and molar ratio (360:1 protein/sugar). Protein stability after storage for several years at 40°C was achieved at molar ratios of 360:1 or greater, indicating the potential for development of room-temperature stable formulations by selection of the appropriate sugar and molar ratio.

The reduction in aggregation and deamidation during storage correlated directly with inhibition of unfolding during lyophilization. Thus, it appears that the protein must be retained in its native-like state during freeze–drying to assure storage stability in the dried solid. This condition may be achieved by selecting the appropriate concentration (molar ratio) of stabilizing sugars for a particular protein.

ACKNOWLEDGMENTS

We gratefully acknowledge support from NSF Grant BES9816975. We thank Nina Le, Paul Sheehan, and Yong-sung Kim for technical assistance, and Dr. Tue Nguyen for his support of this work.

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