Effects of Buffer Composition and Processing Conditions on Aggregation of Bovine IgG during Freeze-Drying

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Received September 21, 1998. Accepted for publication September 15, 1999.

Abstract □ The objective of this study was to identify critical formulation and processing variables affecting aggregation of bovine IgG during freeze-drying when no lyoprotective solute is used. Parameters examined were phosphate buffer concentration and counterion (Na versus K phosphate), added salts, cooling rate, IgG concentration, residual moisture level, and presence of a surfactant. No soluble aggregates were detected in any formulation after either freezing/thawing or freeze-drying. No insoluble aggregates were detected in any formulation after freezing, but insoluble aggregate levels were always detectable after freeze-drying. The data are consistent with a mechanism of aggregate formation involving denaturation of IgG at the ice/freeze–concentrate interface which is reversible upon freeze–thawing, but becomes irreversible after freeze-drying and reconstitution. Rapid cooling (by quenching in liquid nitrogen) results in more and larger aggregates than slow cooling on the shelf of the freeze-dryer. This observation is consistent with surface area measurements and environmental electron microscopic data showing a higher surface area of freeze-dried solids after fast cooling. Annealing of rapidly cooled solutions results in significantly less aggregation in reconstituted freeze-dried solids than in nonannealed controls, with a corresponding decrease in specific surface area of the freeze-dried, annealed system. Increasing the concentration of IgG significantly improves the stability of IgG against freeze-drying-induced aggregation, which may be explained by a smaller percentage of the protein residing at the ice/freeze–concentrate interface as IgG concentration is increased. A sodium phosphate buffer system consistently results in more turbid reconstituted solids than a potassium phosphate buffer system at the same concentration, but this effect is not attributable to a pH shift during freezing. Added salts such as NaCl or KCl contribute markedly to insoluble aggregate formation. Both sodium and potassium chloride contribute more to turbidity of the reconstituted solid than either sodium or potassium phosphate buffers at similar ionic strength, with sodium chloride resulting in a substantially higher level of aggregates than potassium chloride. At a given cooling rate, the specific surface area of dried solids is approximately a factor of 2 higher for the formulation containing sodium chloride than the formulation containing potassium chloride. Turbidity is also influenced by the extent of secondary drying, which underscores the importance of minimizing secondary drying of this system. Including a surfactant such as polysorbate 80, either in the formulation or in the water used for reconstitution, decreased, but did not eliminate, insoluble aggregates. There was no correlation between pharmaceutically acceptability of the freeze-dried cake and insoluble aggregate levels in the reconstituted product.

Introduction

Freeze-drying is usually the method of choice for manufacture of protein pharmaceutical and diagnostic agents which are physically or chemically unstable in aqueous solution and may be used in protein purification for concentration of proteins and to store the process intermediate in a relatively nongrowth promotive state. However, a major challenge in development and manufacture of such products is minimization of damage to proteins due to stresses arising from the freeze-drying process itself. Much of the published literature on freeze-drying of proteins deals with the use of protective solutes, particularly disaccharides, to minimize freeze-drying-induced damage.[1−4] There is only a small body of literature on the effects of processing conditions on protein integrity in the absence of protective solutes.[5−7]

Immunoglobulins represent a class of proteins which are important both as pharmaceuticals and as diagnostic agents. Immunoglobulins are also prone to formation of aggregates during freezing[8] and freeze-drying.[3,9] While protective solutes have been shown to be effective in minimizing aggregation, the use of sucrose, for example, as an excipient should be approached with caution for IgG formulations intended to be administered in large doses due to reported acute renal toxicity.[10] In addition, some immunoglobulins for which the final formulation is a sterile solution are freeze-dried as a process intermediate, where it may be desireable to minimize added solutes.

Within the limited body of published literature on aggregation of immunoglobulins in response to freeze-drying, variation in the immunoglobulin used as well as experimental conditions (protein concentration, buffer composition, added salts, and specific freezing and freeze-drying conditions) makes the formulation of general conclusions regarding the relative impact of these variables on aggregate formation uncertain. For example, Hansson[8] reported that high levels of insoluble aggregates (42%) were formed after multiple freeze−thaw cycles at −20 °C as measured by ultracentrifugation. In this study, IgG was dissolved in 0.05 M phosphate buffer and 0.5 N NaCl at pH 7. The buffer salt used (sodium or potassium) was not specified, and the buffer contained an unusually high level of added salts. Paborji et al.,[9] using 0.05 M sodium phosphate buffer and 0.5 N NaCl, investigated the impact of multiple freeze/thaw cycles on aggregation of a chimeric

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10.1021/js980383n CCC: $18.00
Published on Web 10/22/1999

L6 mouse−human monoclonal IgG_1 antibody by size exclusion HPLC (HPSEC). Loss of monomer and the formation of soluble dimers (2.5−16%) were observed under these conditions. Higher buffer concentration favored dimer formation, but the effect of pH change during freezing on dimer formation was uncertain. IgG concentration in the range of 1−10 mg/mL was reported not to have a significant effect on aggregation.

Identification of critical solution attributes and processing factors is essential in order to minimize empiricism in the development of formulation and processing conditions for protein pharmaceuticals. The broad objective of this project was to develop a better understanding of the role of formulation and process parameters in development of aggregates during freezing and freeze-drying of bovine IgG when no lyoprotectant is present. The specific objectives were to determine the relative importance of the following factors in aggregate formation after freeze/thaw and freeze-drying of bovine IgG formulations: IgG concentration, phosphate buffer composition, added salt, cooling rate, residual moisture, and the presence of surfactants typically used to minimize surface denaturation.

Experimental Section

Materials—Bovine IgG was obtained from Calbiochem-Novabiochem Corporation (La Jolla, CA) as a solution of concentrated bovine IgG in 0.01 M sodium phosphate buffer (pH 7.2) containing 0.15 N NaCl. The IgG solution was dialyzed against various buffer compositions using a dialysis cassette (Slide-A-Lyzer, Pierce Chemical Company, Rockford, IL) for at least 12 h at 4 °C with gentle stirring. IgG concentration was determined at 280 nm using a molar absorption coefficient of 1.3 mg^{-1} mL cm^{-1} (obtained from Calbiochem-Novabiochem Corporation). Disodium, monosodium, dipotassium, monopotassium phosphate, potassium chloride, and sodium chloride salts were obtained from Mallinckrodt Chemical (Paris, KY). Urea and sodium n-dodecyl sulfate (SDS) were obtained from Sigma Chemical Company (St. Louis, MO) and Calbiochem-Novabiochem Corporation (La Jolla, CA), respectively. Polysorbate 80 was obtained from Sigma Chemical Co (St Louis, MO). All materials were reagent grade or better.

Methods—The pH was adjusted at 25 °C with 0.1 N KOH, NaOH, or HCl to 7.1 for solutions of IgG in the presence of 0.22 N KCl and 0.22 N NaCl. The concentration of IgG was 1 mg/mL except where noted.

Freezing and Freeze-Drying—IgG solutions (1.5 mL fill volume) were placed in 5 mL serum vials (Baxter Scientific, McGaw Park, IL). Two freezing methods were investigated. Fast cooling was carried out by dipping vials in liquid nitrogen before transferring to a freeze-dryer shelf precooled to −45 °C. Slow cooling was accomplished by placing vials directly on the shelf of the lyophilizer at room temperature and decreasing the shelf temperature at a rate of 2 °C/min. For freeze−thaw experiments, thawing was carried out by allowing frozen solutions to thaw at room temperature.

Frozen solutions were held at −45 °C for at least 6 h before beginning freeze-drying (Lyoflex 08, BOC Edwards Calumatic, Tonawanda, NY or Dura-Stop, FTS Systems, Inc., Stone Ridge, NY). The system was evacuated to a pressure of 100 mTorr at −40 °C and held for 26 h (primary drying). The shelf temperature was then increased to 25 °C at a heating rate of 0.1 °C/min and held for a period of 24 h. Type T thermocouples were placed in the bottom center of two different vials to monitor product temperature. A sample thief was used to withdraw vials at different times during drying without disturbing the cycle. At the end of secondary drying, the samples were stoppered under vacuum and analyzed within 24 h.

Annealing experiments were carried out by rapidly cooling vials containing solutions of IgG in 0.01 M potassium phosphate buffer with 0.22 N NaCl (pH 7.1) by dipping in liquid nitrogen, followed by placing on the freeze-dryer shelves at −45 °C. Vials were then warmed to −15 °C at a rate of 2 °C/min for 3 h, followed by decreasing the shelf temperature to −45 °C. Rapidly cooled vials that were not annealed were used as a control. Freeze-drying was carried out as described above.

Measurement of pH Changes during Freezing—A method similar to that described by Gomez[11] was used to measure pH in frozen solutions. A special combination pH electrode (Ingold, Mettler-Toledo, Wilmington, MA) using a reference solution consisting of a mixture of glycerol and potassium chloride permits measurement of pH at temperatures as low as −30 °C. Calibration was done at room temperature with buffers of pH 4, 7, and 10 (Baxter Diagnostics, Inc., Deerfield, IL). A universal pH indicator solution (Fisher Scientific, NJ) was also used to visually verify the results, particularly for quench-cooling in liquid nitrogen. A 5 mL serum vial containing 4 mL of the appropriate formulation, two type T thermocouples (32 gauge), and the pH electrode was placed on the lyophilizer shelf at room temperature. The shelf temperature was decreased from 25 °C to −30 °C at a rate of 2 °C/min and held at −30 °C for at least 4 h. The results of pH changes obtained under these conditions were reproducible (standard deviation of ±0.17 at −30 °C).

Determination of Insoluble Aggregates—Absorbance was measured at 350 nm by a UV/visible spectrophotometer (Shimadzu Model 160U, Kyoto, Japan). All measurements were carried out in triplicate, and the appropriate buffer solution without IgG was used as a blank. To study the effect of bovine IgG concentration on aggregation, concentrations ranging from 0.5 to 8 mg/mL in the same buffer system were freeze-dried as described above and reconstituted with distilled water. A limited number of studies were also carried out where reconstituted freeze-dried solids were filtered and the absorbance measured at 280 nm in order to compare the two methods.

Size Exclusion Chromatography—High performance size exclusion chromatography (HPSEC) was used to measure soluble aggregates. The system consisted of a solvent pump, injector, UV detector and integrator (Waters Inc, Milford, MA). A Bio-Sil TSK-400 column (300 × 7.8 mm) from Biorad (Hercules, CA) was used with detection at 280 nm. The mobile phase consisted of 0.1 M Na phosphate buffer containing 0.01 M sodium azide and 150 mM NaCl at a final pH of 6.8. The flow rate was 1 mL/min and analysis time was approximately 20 min. The column was calibrated with molecular weight standards (Biorad Inc, Hercules, CA), including thyroglobulin (670 000), bovine γ globulin (158 000), chicken ovalbumin (44 000), horse myoglobulin (17 000), and vitamin B-12 (1350). Before injection, samples were filtered through a 0.22 mm filter (Acrodisc LC, Waters Corporation, Ann Arbor, MI). The injection volume was 100 μL.

Other Methods—Water content was determined by Karl Fischer (KF) analysis with coulometric end point detection (Model 150, Denver Instruments, Arvada, CO). All measurements were carried out in triplicate. Dynamic light scattering studies on reconstituted IgG solutions were done by Protein Solutions, Inc. (Model SEM F60, Charlottesville, VA) using a He Ne laser at 633 nm. Scattering was measured at 60° from the incident beam.

The specific surface area of freeze-dried powders was measured by nitrogen adsorption (Model ASAP 2010 Micromeritics, Norcross, GA). Five data points were used to determine the adsorption isotherm. Lyophilized samples were outgassed at 30 °C for at least 48 h to remove moisture. Four replicates were determined for each sample. Environmental scanning electron microscopy was used to examine lyophilized powders (Model 2020, Philips, Inc., Wilmington, MA). Ten milligrams of the appropriate lyophilized powder was spread on a conductive carbon tape and placed on an aluminum mount. The accelerating voltage was 15 kV at a chamber pressure of 5 Torr.

Results and Discussion

Characterization and Quantitation of Aggregates—Absorbance at wavelengths ranging from 350 to 650 nm[7,12−14] is commonly used as a convenient measure of relative levels of insoluble aggregates in protein formulations, particularly at low levels of aggregation.[15] Absorbance vs wavelength data are presented in Figure 1 for IgG at 1 mg/mL in 0.01 M potassium phosphate buffer containing 0.22 N NaCl after freeze−thawing and after freeze-drying and rehydration following both slow and fast cooling. Note that absorbance is negligible for freeze/thawed samples in the range of 325−500 nm. A sharp increase in optical density due to scattering from insoluble

Table 1—Summary of Absorbance at 350 nm, Percent Aggregate after Filtration, and Surface Area of Freeze-Dried Solids

buffer formulations with 1 mg/ml IgG	freezing conditions	absorbance (350 nm)	% aggregate	surface area (m^2/g)
0.01 M KPB + 0.22 N NaCl	slow	0.71 ± 0.04	20.0 ± 1.9	6.6 ± 1.4
0.01 M KPB + 0.22 N NaCl	quench cooled	0.81 ± 0.06	29.9 ± 0.7	10.9 ± 1.3
0.01 M KPB + 0.22 N KCl	slow	0.36 ± 0.02	10.0 ± 2.0	3.6 ± 0.2
0.01 M KPB + 0.22 N KCl	quench cooled	0.5 ± 0.03	14.0 ± 2.0	5.3 ± 0.1
0.01 M KPB + 0.22 N NaCl	quench cooled and then annealed 3 h at –15 °C	0.55 ± 0.02	12.2 ± 1.0	7.7 ± 1.3
0.01 M KPB + 0.22 N NaCl	quench cooled	1.0 ± 0.01	32.7 ± 1.7	17.6 ± 0.5

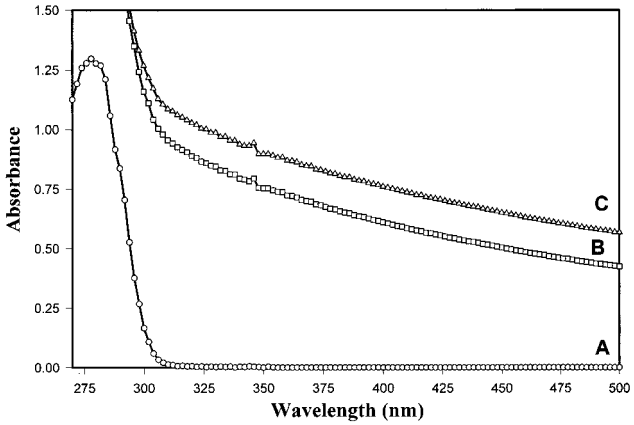

Figure 1—Absorbance vs wavelength of samples containing IgG (1 mg/ml) in a 0.01 M potassium phosphate buffer containing 0.22 N NaCl: (A) after freezing/thawing, (B) after freeze-drying following cooling at 2 °C/min, (C) after freeze-drying following quench-cooling in liquid nitrogen.

aggregates is observed after freeze-drying and, as expected, the extent of scattering increases with decreasing wavelength. Freeze-drying following rapid cooling consistently results in higher absorbance compared with slow cooling. On the basis of these data, 350 nm provides the best sensitivity for measuring turbidity while avoiding absorption due to the protein itself. This wavelength was used in all subsequent studies. A good rank order correlation was observed between absorbance at 350 nm and the quantity of protein remaining after filtration of reconstituted freeze-dried solids (Table 1).

HPSEC chromatograms of the formulation represented in Figure 1 are shown in Figure 2, where the peak at 9.8 min represents the IgG monomer. No soluble aggregates are observed after either freeze–thawing or freeze-drying. The monomer peak area is the same after freeze–thawing as that for the unfrozen solution, but decreases after freeze-drying because of formation of insoluble aggregates which are filtered out during sample preparation. Again, rapid cooling results in higher levels of insoluble aggregates than slow freezing, as reflected by the smaller peak area of the rapidly cooled, freeze-dried material. A peak arising (12.5 min retention time) from an IgG fragment with an apparent molecular weight less than about 6000 was always observed in chromatograms of freeze-dried IgG, but was not characterized further in this study.

The insoluble aggregates were completely dissolved by either 6 M urea or by 0.4% (w/v) sodium dodecyl sulfate (SDS). Only one peak, corresponding to the IgG monomer, was observed in the HPSEC chromatogram of solubilized aggregated material (data not shown). These observations support the conclusion that only noncovalent aggregates are observable under the experimental conditions reported here.

Dynamic light scattering data for insoluble aggregates formed by both fast and slow cooling are shown in Figure 3. Rapid freezing consistently results in larger aggregates, with an average particle size of about 1 μm, as compared

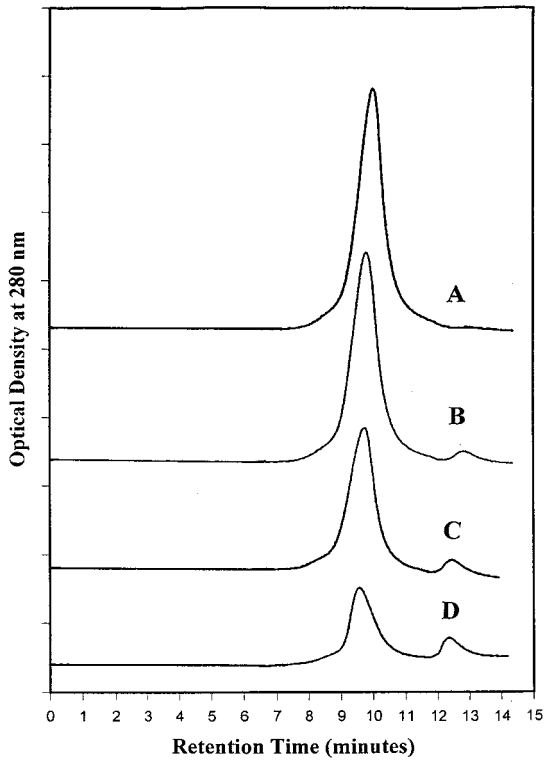

Figure 2—HPSEC chromatograms of bovine IgG in 0.01 M potassium phosphate buffer containing 0.22 N NaCl: (A) solution before any treatment, (B) after freezing/thawing, (C) after freeze-drying (slow cooling at 2 °C/min), (D) after freeze-drying (quench-cooling in liquid nitrogen).

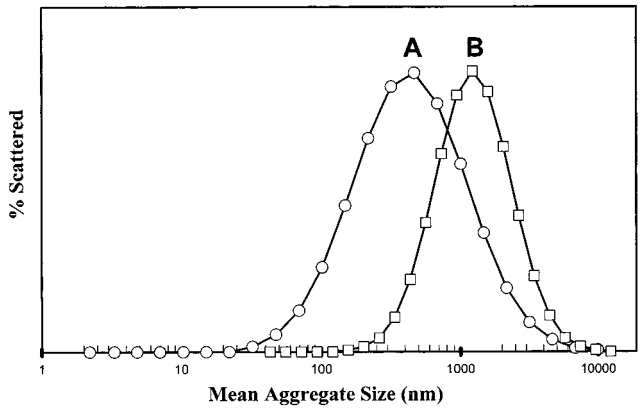

Figure 3—Effect of freezing rate on mean aggregate size of IgG, determined by dynamic light scattering: (A) slow cooling, (B) quench-cooling in liquid nitrogen.

with an average particle size of about 400 nm for slow cooling. The reason for the difference in average particle size is not known at present.

The data in Figures 1–3 demonstrate that fast cooling consistently results in higher levels of aggregated protein in a buffer containing 0.01 M potassium phosphate (pH 7.1)

with 0.22 N NaCl as compared with slow cooling. Similar results were obtained with 0.01 M potassium phosphate buffer (pH 7.1) and 0.22 N KCl (absorbance values of 0.36 vs 0.50 for slow and rapid cooling, respectively). These data add to the growing body of published data supporting the conclusion that rapid freezing can be detrimental to protein integrity. Eckhardt et al.,[14] using several formulations of human growth hormone and an apparatus for controlling cooling rate, demonstrated that the level of insoluble aggregate after freeze/thawing is directly related to the freezing rate over the range of 0.5 °C to 50 °C/min. Hsu et al.,[12] using formulations of tissue plasminogen activator (tPA), reported a direct relationship between freezing rate and development of turbidity in reconstituted samples during storage. These investigators postulated that denaturation takes place at the solid/void interface, which results in aggregate formation upon reconstitution. Chang and co-workers,[7] using multiple freeze−thaw cycles of phosphofructokinase, lactate dehydrogenase, glutamate dehydrogenase, interleukin-1 receptor antagonist, tumor necrosis factor, and ciliary neurotropic factor demonstrated that slow freezing resulted in less turbidity in thawed samples than freezing in liquid nitrogen. Jiang and Nail,[6] using lactate dehydrogenase and β-galactosidase, also reported that rapid freezing in liquid nitrogen resulted in lower recovery of enzyme activity after freeze−thawing compared with freezing in a freeze-dryer.

Strambini and Gabellieri[16] have proposed a mechanism for freezing-induced damage to proteins involving partial unfolding of proteins after adsorption to the ice surface. If this mechanism is operative, then more rapid freezing would be expected to result in a higher ice/freeze-concentrate interfacial area, a greater extent of protein adsorption, and more damage to the protein. While no aggregation is detected in bovine IgG after freeze/thawing under the conditions reported here, our data are consistent with this mechanism, where partial denaturation following adsorption to the ice surface is reversible after freeze/thawing. However, subsequent drying would result in a partially denatured protein in the solid state, which forms aggregates upon reconstitution.

To test the hypothesis that rapid freezing inhibits sodium phosphate precipitation, a pH universal indicator was used. No difference was observed after slow vs fast cooling (both showing an acidic pH shift) despite a significant difference in the amount of aggregates after freeze-drying and reconstitution.

Environmental SEM photomicrographs (Figure 4) show distinctly different morphologies for the freeze-dried powders following cooling at different rates. The slowly cooled specimen appears as relatively large filaments, whereas the rapidly cooled material appears to be more finely divided.

Table 1 summarizes specific surface area data for freeze-dried solids from 1 mg/mL bovine IgG in potassium phosphate buffer containing both NaCl and KCl. The specific surface area of freeze-dried solids is significantly higher for quench-cooled relative to slowly cooled solutions. An unexpected result is that the specific surface area of freeze-dried solids from solutions containing NaCl is significantly higher than those containing KCl. The reason for this is not known at present, but the reduced specific surface area of solids containing KCl correlates with a lower degree of aggregation in the KCl system. This is further support for an aggregation mechanism involving partial denaturation at the ice/freeze−concentrate interface.

Effect of Annealing—If protein denaturation, and subsequent aggregation upon reconstitution, is promoted by a high ice/freeze−concentrate interfacial area following freezing, then annealing the system after fast freezing should decrease aggregation by decreasing the interfacial area. Results of an experiment to test this hypothesis are shown in Table 1. Note that the decrease in specific surface area of the annealed material relative to the quench-cooled control (17.6 vs 7.7 m^2/g) is accompanied by a decrease in percent aggregates in the reconstituted solids from 32% to about 12%. These data lend further support to an aggregation mechansim involving denaturation at the ice/freeze-concentrate interface that becomes irreversible upon drying.

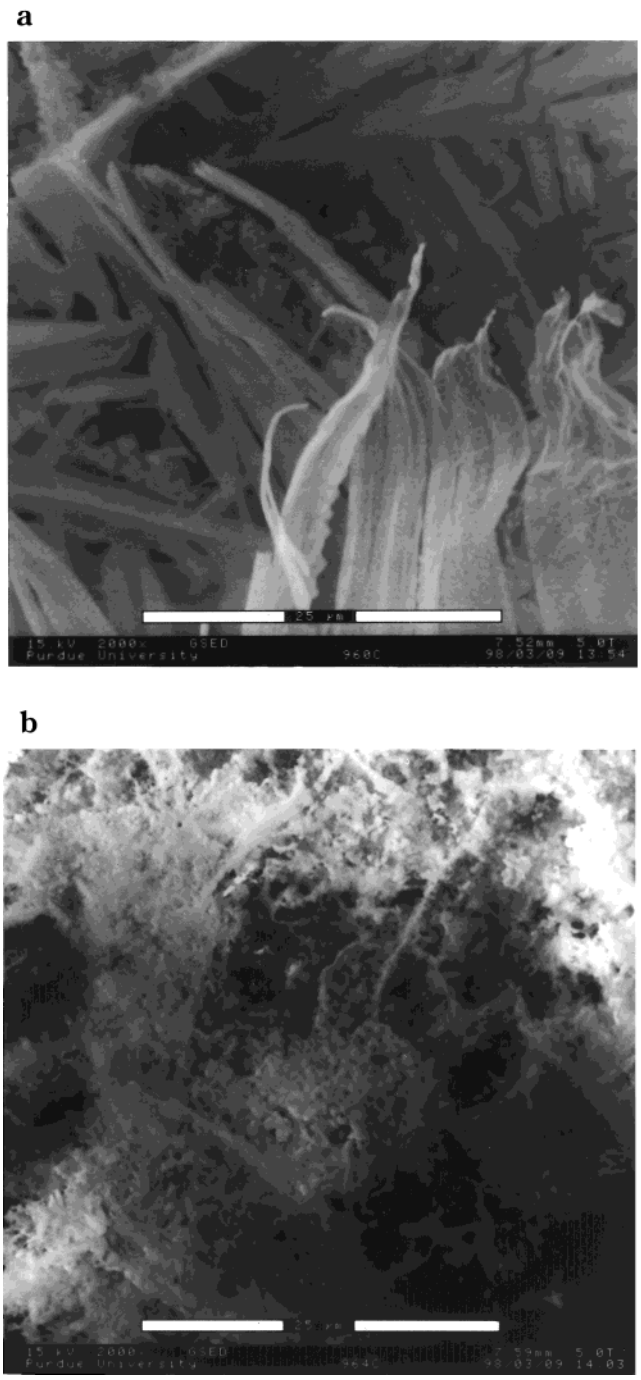

Figure 4—ESEM photomicrographs of freeze-dried IgG following (A) slow cooling, and (B) quench-cooling in liquid nitrogen. Magnification 2000×.

Effect of IgG Concentration—The effect of IgG concentration on both absorbance at 350 nm and on percent aggregate as determined by measuring the absorbance after filtration of the reconstituted solution after freeze-drying is shown in Figure 5 for solutions in 0.01 M

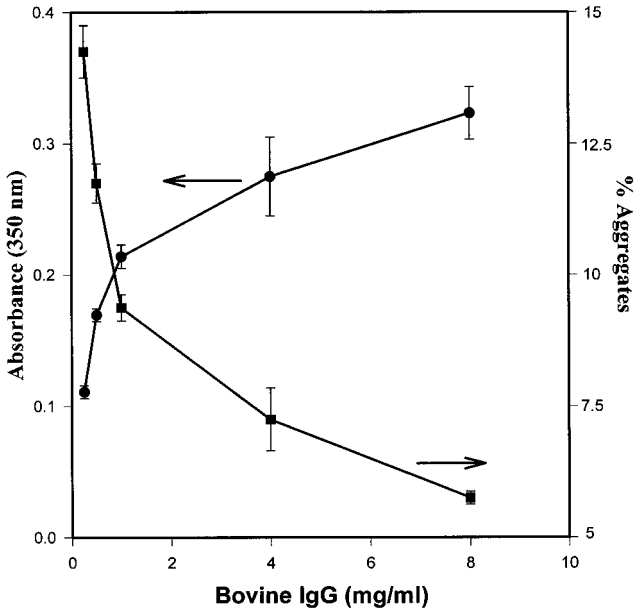

Figure 5—Effect of IgG concentration on absorbance at 350 nm and percent aggregated IgG after lyophilization. Buffer system was 0.01 M potassium phosphate with 0.22 N NaCl, frozen at a cooling rate of 2 °C/min. Error bars represent standard deviation of triplicate measurements from three separate vials.

Table 2—Residual Moisture vs IgG Concentration

bovine IgG concentration (mg/mL) before lyophilization	residual moisture
0.5	1.25 ± 0.13
1	1.32 ± 0.14
2	1.4 ± 0.2
4	2.5 ± 0.02
8	2.5 ± 0.2

potassium phosphate buffer containing 0.22 N NaCl at pH 7.1. Even though absorbance at 350 nm increases as protein concentration increases from 0.5 to 8 mg/mL, the level of insoluble aggregates decreases by more than half. This direct effect of protein concentration on stabilization against damage caused by freeze-drying has been reported by other investigators. Hansson[17] reported that the quantity of aggregate formed in human IgG after multiple freeze−thaw cycles decreased with increasing IgG concentration until, at 50 mg/mL IgG, no aggregation was observed after multiple freeze−thaw cycles. Chang et al.[7] reported that increasing the concentration of interleukin-1 receptor antagonist (from 1 to 100 mg/mL) diminished the level of aggregates from 50% to 0.01%. This effect has also been reported for β-galactosidase[6,18] and lactate dehydrogenase.[6,19] However, to our knowledge, this concentration effect has not been explained.

Residual moisture level at each concentration was measured in order to test the hypothesis that higher concentrations of protein, under the same process conditions, will result in higher residual moisture levels and prevent damage caused by "overdrying". Data are summarized in Table 2. The data do indicate that residual moisture increases with increasing concentration. However, the range of residual moisture levels is only from 1.25% to 2.5%. In the range of 4 to 8 mg/mL IgG, the residual moisture level is approximately constant, but the amount of insoluble aggregate decreases significantly. It appears that residual moisture is, at most, only a partial explanation for the concentration effect.

Anchordoquy and Carpenter[20] reported that dissociation of lactate dehydrogenase subunits, and subsequent loss of activity, was due to the large pH decrease associated with the phosphate buffer during freezing. This effect was inhibited by protective levels of polymers such as bovine serum albumin and poly(vinylpyrrolidone). To test the hypothesis that higher protein concentration inhibits crystallization of buffer salts, pH shift during freezing was measured for the lowest and highest protein concentrations used in this study. Minimal difference in pH shift was observed between the lowest and highest concentrations (4.0 vs 4.2 for 0.5 mg/mL and 8 mg/mL, respectively). Therefore, pH change during freezing does not appear to be a likely explanation for the concentration effect. The data need to be interpreted with some caution, however, since the freezing conditions were different for the experiments in which the apparent pH during freezing was measured (freezing to −30 °C) versus the freezing conditions prior to freeze-drying (freezing to −45 °C).

Increasing IgG concentration was associated with an increase in specific surface area of the freeze-dried solids (from 6.6 m^2/g at 1 mg/mL to 9.0 m^2/g at 8 mg/mL) over the concentration range studied. Combined with the absorbance data in Figure 5, these data suggest that the concentration effect may be another manifestation of aggregation as an interfacial phenomenon. The increased specific surface area of freeze-dried solids with increased concentration is accompanied by increased absorbance at 350 nm of the reconstituted solution, suggesting that the increased turbidity arises from a larger amount of protein at the ice/freeze−concentrate interface during freezing. However, the fractional amount of protein at the interface must decrease with increasing concentration, given that the specific surface area increases by a factor of about 1.5 while the concentration increases by a factor of 8. The lower percentage of protein at the interface would explain the decrease in percent aggregated protein with increased concentration.

Another explanation for the protein concentration effect that was considered is the influence of protein concentration on T_g' (the glass transition of the maximally freeze-concentrated solute), where increased protein concentration would be expected to increase T_g'. However, as with many protein formulations not containing a lyoprotectant, thermal analysis of the frozen systems did not reveal a glass transition (data not shown). The freeze-drying conditions used for the study (primary drying at −40 °C) represent the approximate lower temperature limit for production scale freeze-drying. While the low primary drying temperature should minimize collapse during freeze-drying, the potassium phosphate buffer system without added salt did exhibit collapse. As discussed below, we observed no effect of collapse during freeze-drying on level of aggregation upon reconstitution. We conclude that any protein concentration effect on T_g' would not explain the observations reported here.

Effect of Buffer Composition on Aggregation—The effect of systematic variation in solution composition at a constant IgG concentration and nearly constant ionic strength on turbidity of the reconstituted solid is shown in Figure 6. The effect of the phosphate buffer (Na vs K salt) at two concentrations (0.1 M vs 0.01 M) is examined where ionic strength is adjusted with either NaCl or KCl. At a constant buffer concentration, the potassium salt consistently results in a significantly lower level of aggregate relative to sodium phosphate ($P < 0.001$). Adding either NaCl or KCl results in increased aggregation relative to buffer alone, and sodium chloride always results in more aggregation than the same concentration of potassium chloride.

pH was measured during the freezing process in order

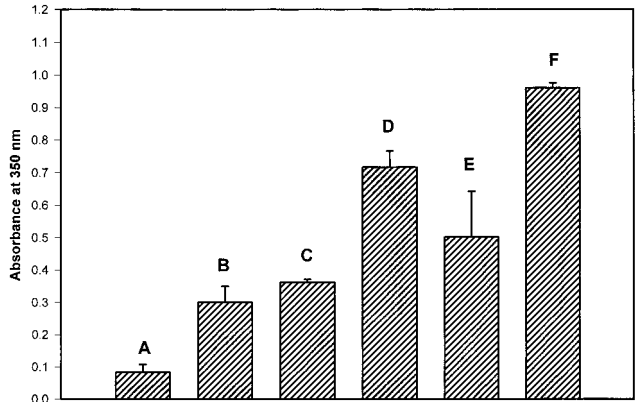

Figure 6—Effect of solution composition at similar ionic strength ($I = 0.24$ M) on IgG aggregation after freeze-drying in (A) 0.1 M potassium phosphate, (B) 0.1 M sodium phosphate, (C) 0.01 M potassium phosphate containing 0.22 N KCl, (D) 0.01 M potassium phosphate containing 0.22 N NaCl, (E) 0.22 N KCl, and (F) 0.22 N NaCl. Error bars represent standard deviation based on measurements from three separate vials.

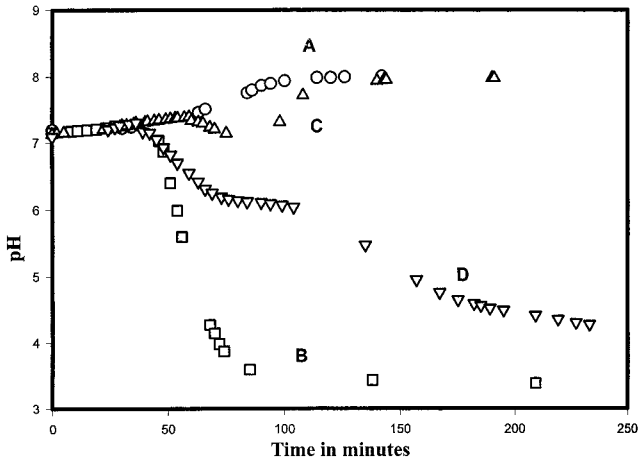

Figure 7—Apparent pH during freezing for (A) 0.1 M potassium phosphate buffer, (B) 0.1 M sodium phosphate buffer, (C) 0.01 M potassium phosphate buffer containing 0.22 N KCl, and (D) 0.01 M potassium phosphate buffer containing 0.22 N NaCl.

to examine the relationship between aggregation and pH shift during freezing. The data are summarized in Figure 7. As expected, pH increases somewhat (from 7.2 to about 8) during freezing for the potassium phosphate buffer, whereas pH decreases for the sodium phosphate buffer system (from 7.2 to about 3.5). The effect of added salt is to delay crystallization of both monobasic potassium phosphate and dibasic sodium phosphate. For potassium phosphate containing KCl, the pH of the frozen system reaches a similar pH to that of potassium phosphate buffer alone after about 100 min. For sodium phosphate buffer alone, the apparent pH reaches its equilibrium value after 100 min, but the sodium phosphate buffer containing NaCl is still slowly decreasing after 4 h. These data are in agreement with data reported by Gomez,[11] where an inhibition of disodium phosphate dodecahydrate precipitation was observed in the presence of 8 mM Na phosphate buffer containing 0.15 N NaCl.

As mentioned above, comparison of the data in Figures 6 and 7 should be made with some caution, because the pH measurements were made at −30 °C, whereas freezing of the IgG solutions was carried out at −45 °C. However, the data in Figure 7 support the conclusion that pH shift during freezing does not affect aggregation. Comparing curves B and D in Figure 7 with corresponding data in Figure 6, the level of aggregate is considerably greater for the solution containing NaCl relative to that containing sodium phosphate alone. Even if it is assumed that the solution containing sodium chloride eventually reaches the same pH during freezing as that containing buffer alone, the aggregate levels would be expected to be the same for the two systems if the pH shift during freezing were driving the aggregation process. Curves A and C in Figure 7 show that the two potassium phosphate buffer solutions reach the same apparent pH after about 2 h, but, again, the solution containing added salt results in a significantly higher level of aggregated protein. The absence of an effect of pH shift upon freezing on IgG aggregation is further illustrated in Figure 6, where 0.22 N KCl or NaCl alone (pH adjusted to 7.1) is compared to the corresponding solutions containing 0.01M potassium phosphate. Aggregate levels are significantly greater for solutions containing salt alone, where the apparent pH at −30 °C after 4 h is 6.4 and 7.0 for 0.22 N NaCl and KCl, respectively.

In studying the effect of formulation variables on the stability of freeze-dried human growth hormone, Pikal and co-workers[21] noted that the destabilizing effect of added NaCl could be attributed to either ionic strength or a specific ion effect of Na^+ or Cl^- and concluded that Cl^- is the most likely cause of the adverse effect. Our data support the conclusion that the effect of buffer solution composition on IgG aggregation is neither a pH shift effect nor an ionic strength effect, but rather a specific ion effect. Further work is in progress to test the hypothesis that the effects of added salts on IgG aggregation resulting from freeze-drying is predicted by the Hofmeister series, where destabilizing ions promote aggregation.

The freeze-dried cakes resulting from 0.1 M potassium phosphate buffer were collapsed, whereas freeze-dried solids resulting from 0.1 M sodium phosphate buffer retained their macroscopic structure. For potassium buffer systems containing NaCl or KCl, no collapse was observed. Besides illustrating that collapse during freeze-drying does not necessarily correlate with decreased integrity of a freeze-dried protein, this observation suggests that an alternative explanation for the data in Figure 6 is that residual moisture levels are different for the formulations, and that this difference in residual moisture level influences aggregation. Residual moisture levels were significantly different (2.8 ± 0.3% for 0.1 M potassium phosphate vs 1.5 ± 0.4% for 0.1 M sodium phosphate alone). To study this effect further, two formulations containing 0.01 M potassium phosphate (pH 7.1) and either 0.22 N KCl or NaCl, were compared with respect to both residual moisture level and insoluble aggregate levels by using a sample thief to remove samples during secondary drying. All samples retained their macroscopic structure. Data are summarized in Figure 8. The top panel shows that the difference in absorbance at 350 nm between the two buffer solutions is not distinguishable until the 49 h sample, at which time the absorbance of the sodium chloride system is significantly higher and remains so throughout the drying cycle. Although the residual moisture level in the potassium chloride formulation is higher than the sodium chloride formulation early in secondary drying, the residual moisture levels in the two formulations are not significantly different by the end of the cycle. The data in Figure 8 support the conclusion that the salt effect becomes evident only as the residual moisture level is decreased to 2−5%. Below this level, there is a significant effect of the added salt which is not explained by differences in residual moisture. No difference in cake appearance was observed between time 1, time 2, and samples taken at the end of the cycle (Figure 9). These data are consistent those of Hsu et al.[22] for tPA, where protein integrity is lost by overdrying.

Effect of a Surfactant on Aggregation—Nonionic

Table 3—Effect of Polysorbate 80 on IgG Aggregation

sample information	medium of reconstitution	absorbance (350 nm)
0.01 M KPB + 0.22 N NaCl, pH 7.1 containing 1 mg/mL bovine IgG	distilled water	0.54 ± 0.06
0.01 M KPB + 0.22 N NaCl, pH 7.1 containing 1 mg/mL bovine IgG and 0.02% w/w Tween 80	distilled water	0.18 ± 0.02
0.01 M KPB + 0.22 N NaCl, pH 7.1 (25 °C) containing 1 mg/mL bovine IgG and 0.1% w/w Tween 80	distilled water	0.18 ± 0.05
0.01 M KPB + 0.22 N NaCl, pH 7.1 containing 1 mg/mL bovine IgG	0.02% w/w Tween 80	0.15 ± 0.01

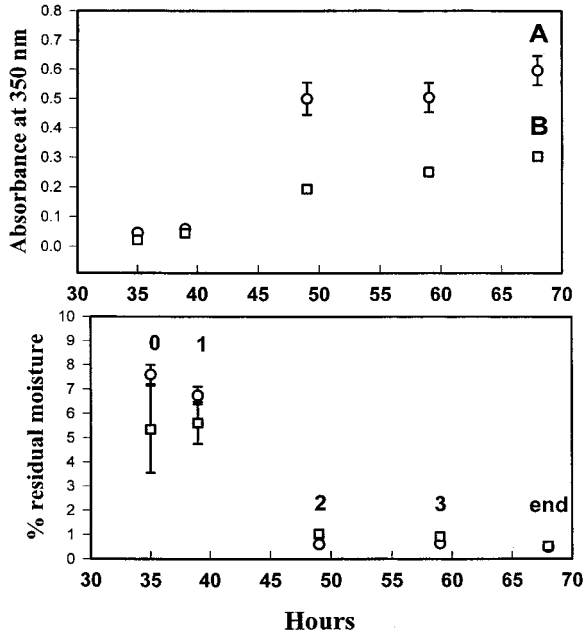

Figure 8—Effect of secondary drying time on absorbance at 350 nm and residual moisture: (A) 0.01 M potassium phosphate containing 0.22 N NaCl, (B) 0.01 M potassium phosphate containing 0.22 N KCl. Error bars represent the standard deviation of triplicate measurements from three separate vials.

Figure 9—Reconstituted vials of lyophilized bovine IgG in 0.01 M potassium phosphate with 0.22 N NaCl removed during secondary drying, showing development of turbidity in the reconstituted product.

surfactants are commonly used in formulations to prevent protein adsorption to surfaces and to minimize interfacial denaturation by preferentially adsorbing to hydrophobic surfaces and to hydrophobic regions of proteins. Table 3 summarizes absorbance data at 350 nm obtained for reconstituted solids with and without polysorbate 80 in the medium of reconstitution or in the formulation before freeze-drying. Use of a surfactant—either in the reconstitution medium or in the formulation—significantly decreases, but does not eliminate, aggregation. No difference is observed in aggregation between a formulation containing 0.02% or 0.1% w/w polysorbate 80. No significant difference was observed between incorporation of the surfactant in the formulation and incorporation in the medium of reconstitution. These data contrast with those of Chang and co-workers,[7] who reported essentially complete protection of six different proteins by incorporation of 0.01% polysorbate 80 into the formulation prior to freezing. Incorporation of surfactant into the reconstitution medium instead of the formulation was less effective in the case of interleukin-1 receptor antagonist, but still reduced the quantity of aggregate relative to reconstitution with water alone (23% vs 50% aggregate, respectively).

Practical Considerations—The data reported here are consistent with the body of literature pointing to protein aggregation resulting from freezing and freeze-drying as an interfacial phenomenon. While further investigation is warranted, particularly in the area of specific ion effects, several broad conclusions may serve as guidelines for development of protein formulations and processing conditions. First, keep the protein concentration as high as practical. Second, minimize the buffer concentration and do not use additional salts unless necessary. From the standpoint of aggregation, potassium phosphate buffer appears to be preferable to sodium phosphate buffer, and potassium chloride is preferable to sodium chloride. Identification of critical process parameters should include thermal history of freezing, since a number of reports suggest that rapid freezing should be avoided. Particular attention should be given to establishment of residual moisture specifications, particularly since the conventional wisdom that "the dryer, the better," is not always true.

References and Notes

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Acknowledgments

The authors acknowledge Dr. John Carpenter for helpful criticism of the manuscript, and Robert Verberig for photographic assistance. The analytical support of Protein Solutions, Inc. for particle size determination is gratefully acknowledged. Financial support (fellowships for J.M. Sarciaux) provided by Astra, Pfizer, and Pharmacia&Upjohn, Inc., is also greatly appreciated.

JS980383N