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Research Article

Effect of Freezing on Aggregation of Human Growth Hormone

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The effect of freezing on formation of soluble and insoluble aggregates of human growth hormone (hGH) was studied. The amount of soluble aggregates was affected very little by freezing regardless of the cooling rate. In contrast, the formation of insoluble aggregates (particulates), as determined by light scattering in the 340- to 360-nm range, was found to increase sharply with increasing cooling rates. The amount of these particulates was also dependent on the pH of the solution. Freezing hGH solutions formulated at pH 7.4 resulted in highly scattering solutions, whereas pH 7.8 formulations showed significantly less scattering. These results emphasize the importance of understanding the freezing phenomenon for protein solutions and suggest that the formation of soluble aggregates and insoluble particulates may have different mechanisms.

KEY WORDS: human growth hormone (hGH); freezing; particulates; aggregation; light scattering.

INTRODUCTION

The formation of protein aggregates is often encountered in developing protein formulations for pharmaceutical use, e.g., upon adverse handling of bulk protein solutions and/or upon reconstitution of lyophilized material. Aggregates are of concern for three reasons: They may display reduced bioactivity as has been shown to be the case for human growth hormone (1). They have also been implicated in immunogenic reactions (2–6). Finally, visible protein aggregates may result in unacceptable physical characteristics such as opalescence.

Empirically, two classes of protein aggregates have been considered. The first is soluble aggregates, consisting of dimers, trimers, and higher oligomers, which may be covalently and/or noncovalently linked. These aggregates are freely soluble, contribute relatively little light scattering (7), and can be accurately quantitated by various chromatographic or gel permeation techniques. The second type of protein aggregate consists of much larger and essentially insoluble particulates. These materials may also contain either covalent and/or noncovalent linkages, are the cause of most of the measurable light scattering (7), and may ultimately lead to visible opalescence or even precipitates. These particulates may be evaluated by light scattering and recovered by filtration or centrifugation. Little is presently known about the relationship between these two classes of aggregates or even at what point the soluble forms may become insoluble particulates. Indeed, it is by no means entirely clear that protein particulates arise from the soluble forms.

The freezing of protein solutions has been an important research subject for many years, focusing mainly on quick freezing of enzymes in liquid nitrogen in order to preserve enzymatic activity (7–11). More recent work by Douzou, Franks, and others has emphasized the importance of low-temperature studies in order to understand the so-called "cold denaturation" of proteins (12–17). In addition, freezing of biological products has been extensively studied in combination with optimizing the lyophilization procedure of various proteins and several studies have investigated the water crystallization process therein (18–22).

In 1980, Schwartz *et al.* published their results on aggregation of ^{125}I-labeled human growth hormone (hGH)[4] in response to freezing (5). They demonstrated the conversion of significant amounts of protein monomer to high molecular weight species, especially upon storage at −20°C. Rapid freezing in liquid nitrogen failed to prevent this conversion. The authors concluded from their studies that freezing might be the critical step in producing aggregates during lyophilization. This speculation was supported by various other reports indicating increases in heterogeneity of hGH populations after freeze-drying (2–6).

The present work examines the freezing process in more detail in order to understand better the different phenomena occurring during the first step in the freeze-drying of protein solutions. Several freeze/thaw experiments were designed using hGH as a model and focusing on the effect of cooling rate and formulation on the formation of both soluble and insoluble aggregates.

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[4] Abbreviations used: hGH, human growth hormone; LS, light scattering; OD, optical density; IA, insoluble protein aggregates; SA, soluble protein aggregates; TM, total monomer; SE-HPLC, size exclusion-high performance liquid chromatography.

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MATERIALS AND METHODS

Preparation of Human Growth Hormone Formulations

Recombinant methionyl human growth hormone (hGH) was solvent-exchanged into different formulations as follows. The initial protein solution, containing 2 mg hGH/ml, 88 m*M* mannitol, 5 m*M* phosphate at pH 7.8, was first concentrated at 5°C with Amicon Centriprep 10 concentrators (No. 4304) to a protein concentration of about 30 mg/ml. Aliquots of this stock solution were then diluted with various formulation buffers back to approximately 2.5 mg protein/ml and dialyzed for 24 hr at 5°C against these same buffers using SpectraPor 7 dialysis tubing.

Varying amounts of mannitol and phosphate are common excipients in currently marketed hGH formulations with a pH ranging from 7.4 to 7.8. Hence, the effect of pH and various concentrations of mannitol was investigated by choosing the following formulations:

(A) 5 m*M* phosphate, pH 7.4;
(B) 5 m*M* phosphate, pH 7.8;
(C) 88 m*M* mannitol, 5 m*M* phosphate, pH 7.4;
(D) 88 m*M* mannitol, 5 m*M* phosphate, pH 7.8;
(E) 250 m*M* mannitol, 5 m*M* phosphate, pH 7.4; and
(F) 88 m*M* mannitol, 5 m*M* phosphate, pH 7.8 (untreated control).

Formulation D is identical to formulation F (the starting material), except that formulation D was concentrated, diluted, and dialyzed as described above for the other formulations in order to assess independently the effect of the treatment procedure.

Finally, the various protein solutions were sterile filtered using a Nalgene Filter unit type TA/CA, low protein binding, 0.2 μm, and the hGH concentration was adjusted to 2.0 mg/ml based on a spectrophotometric determination as described below. The formulations were filled into 5-ml borosilicate glass vials, USP Type I (Wheaton), and closed with gray, siliconized butyl rubber stoppers (West). The fill volume was 2.5 ml per vial.

All other reagents used in these studies were of analytical grade.

Freeze/Thaw Experiments

The freeze/thaw studies were carried out in a Planer Biomed Kryo 10 instrument. The cooling rate was varied from 0.5 to 5°C/min, down to a temperature of −55°C. The fastest cooling rate (approx. 50°C/min) was achieved by first inserting the vials into a dry ice/isopropanol bath at about −80°C and then transferring them to the precooled Planer Biomed (−55°C). All samples were kept at −55°C for 30 min. Thawing back to +5°C was initiated with a constant heating rate of 5°C/min.

All vials were examined for their visual appearance within 2 hr after the freeze/thaw cycle according to the guidelines of the European Pharmacopoeia (23).

UV Spectroscopy

UV spectroscopy was carried out using a Kontron Uvikon 860 double-beam spectrophotometer to determine both the amount of UV light scattering (LS) and the protein concentration. The samples were placed in a rectangular quartz cell with a pathlength of 1 cm. Optical density (OD) was measured against a solvent-matched reference from 400 to 240 nm, using a sampling interval and a spectral bandwidth of 1 nm. In order to determine the degree of opalescence, the mean OD was calculated over the 340- to 360-nm range. The mean OD of the sample was then compared to the mean OD of the European Pharmacopoeia reference suspensions, which allowed placement of the sample into the appropriate category of opalescence as described previously (23,24). The mean OD of the 340- to 360-nm range has been shown to be indicative of the amount of insoluble aggregates present in hGH samples (24).

In addition, the OD at the absorption maximum (λ_{max}, ~277 nm) was corrected for light scattering by determining a least-squares fit of the log OD versus log wavelength (λ) values from 325 to 360 nm (25–29). The OD due to scattering at λ_{max} was extrapolated from this fit, based on an assumed linear dependence on wavelength in the log–log format. This scattering value was subtracted from the total OD at λ_{max} to calculate the corrected protein concentration, using an absorptivity of 0.75 ml/(mg · cm) for met-hGH.

The samples were then centrifuged for 5 min at 13,000 rpm (~7200*g*) at 20°C in a Micro-Centaur APO-5760 tabletop centrifuge. The supernatants were filtered through a Millex-GV_4 0.22-μm, sterile low protein binding filter and rescanned as detailed above. The percentage of insoluble aggregates (%IA) was calculated from the difference in protein concentration before and after centrifugation/filtration.

Size Exclusion-High Performance Liquid Chromatography (SE-HPLC)

The amount of soluble protein aggregates (%SA) was determined by size exclusion-high performance liquid chromatography (SE-HPLC) because this type of aggregate cannot be quantitated by the light-scattering correction described above. A silica-based TSK G2000SWXL column, 7.8-mm i.d. × 30.0-cm length with a pore size of 5 μm, was used.

Typically, 5 μl of the filtered protein samples (concentration, ~2 mg/ml), corresponding to ~10 μg of protein, was loaded onto the column and eluted with 50 m*M* sodium phosphate, 150 m*M* sodium chloride (PBS buffer), pH 7.25, at a flow rate of 1 ml/min. The detector wavelength was set to 214 nm. The retention time for the monomer under these conditions is typically 9 ± 0.5 min, all aggregates eluting earlier. The assignment of higher molecular weight forms as dimers, trimers, etc., was based on their retention times relative to those of molecular weight standards. Previous mass balance experiments with aggregate-containing hGH samples indicate that the absorptivities at 214 nm of the monomeric and various higher molecular weight forms of hGH are essentially identical (data not shown).

The percentage of total monomer (TM) was calculated from the amount of soluble (SA) and insoluble aggregates (IA) as follows:

$$\% \text{ TM} = [(100 - \% \text{ SA}) \times (100 - \% \text{ IA})]/100$$

RESULTS

Figure 1 presents the LS data in the 340- to 360-nm range after freezing at three cooling rates. Figure 2 presents soluble aggregate data from the SE-HPLC assay performed after one freeze/thaw cycle. In every case, the only aggregate species observed was dimer. Figure 3 summarizes the total monomer content of all formulations after freeze/thawing.

Effect of Cooling Rate

The mean OD values of the unfrozen samples in the 340- to 360-nm range were very comparable, varying between 0.006 and 0.011 OD unit for all formulations (Fig. 1). Freezing did result in an increase in uncorrected OD for some formulations and was most pronounced with the fastest cooling rate of 50°C/min. The difference between the two slower cooling rates, i.e., 0.5 and 5°C/min, was not significant, however. Upon comparison of the mean OD (340–360 nm) to the corresponding OD of the European Pharmacopoeia reference suspensions (23), the categories of opalescence were found to vary from clear (OD, <0.014) to very opalescent (OD, 0.087–0.144).

In general, the rate of freezing did not seem to influence greatly the formation of soluble aggregates (Fig. 2). Only the fastest cooling rate induced slightly more aggregates with formulations B (5 m*M* phosphate, pH 7.8) and D (88 m*M* mannitol, 5 m*M* phosphate, pH 7.8), the values being 1.3 and 1.5%, respectively.

With increasing cooling rate, the total monomer content decreased for all samples, but to various extents (Fig. 3).

Effect of pH

The lower pH (7.4) resulted in higher OD values, under all cooling rate and formulation conditions (Fig. 1). The most dramatic effect was observed with formulations A (5 m*M* phosphate, pH 7.4) and E (250 m*M* mannitol, 5 m*M* phosphate, pH 7.4), both displaying about 0.13 OD unit after the fastest cooling and a visually detectable high opalescence level.

Within standard error, there appeared to be no significant difference between the pH 7.4 and the pH 7.8 formulations with regard to soluble aggregate formation (Fig. 2). A slight tendency of the pH 7.8 formulations to display higher soluble aggregate levels was noted, however.

Although the data from the unfrozen samples already indicated a lower total monomer content for the pH 7.4 formulations (A, C, E) as compared to the pH 7.8 formulations (B, D, F), this effect became much more pronounced after freezing and was most significant at the fastest cooling rate of 50°C/min (Figure 3). These values are due to the formation

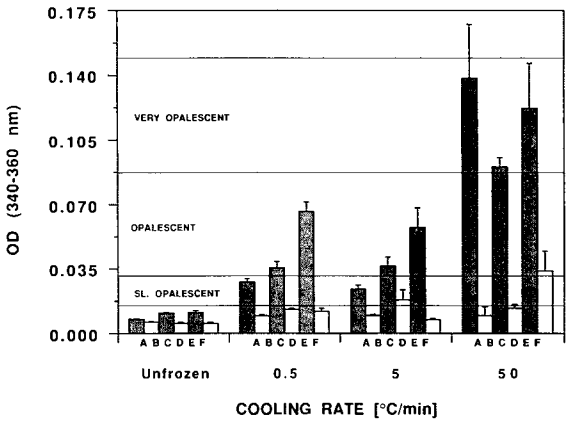

Fig. 1. Effect of cooling rate on light scattering, expressed as the mean optical density in the 340- to 360-nm range (±SE; $n = 5$) after freezing of various 2 mg/ml hGH formulations. (▨) A (5 m*M* phosphate, pH 7.4); (□) B (5 m*M* phosphate, pH 7.8); (▨) C (88 m*M* mannitol, 5 m*M* phosphate, pH 7.4); (□) D (88 m*M* mannitol, 5 m*M* phosphate, pH 7.8); (▨) E (250 m*M* mannitol, 5 m*M* phosphate, pH 7.4); (□) F (88 m*M* mannitol, 5 m*M* phosphate, pH 7.8), control.

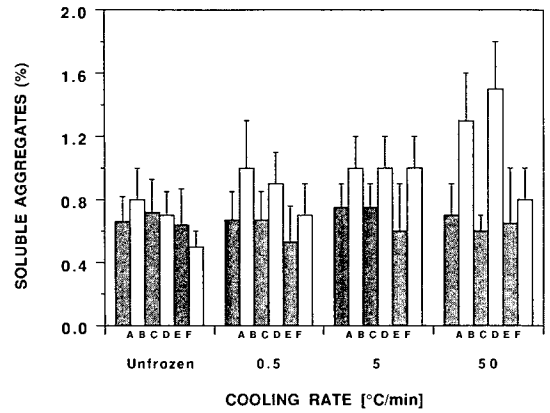

Fig. 2. Effect of cooling rate on soluble aggregate formation due to freezing of various 2 mg/ml hGH formulations. The amount of soluble aggregate is expressed as percentage of total area determined by SE-HPLC ± SD ($n = 3$). (▨) A (5 m*M* phosphate, pH 7.4); (□) B (5 m*M* phosphate, pH 7.8); (▨) C (88 m*M* mannitol, 5 m*M* phosphate, pH 7.4); (□) D (88 m*M* mannitol, 5 m*M* phosphate, pH 7.8); (▨) E (250 m*M* mannitol, 5 m*M* phosphate, pH 7.4); (□) F (88 m*M* mannitol, 5 m*M* phosphate, pH 7.8), control.

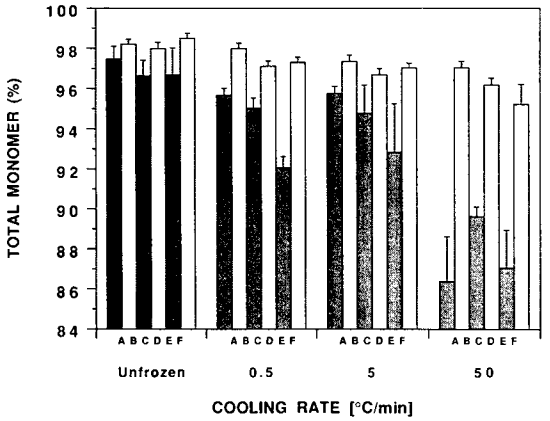

Fig. 3. Effect of cooling rate on total monomer content after freezing of various 2 mg/ml hGH formulations. The total monomer content was determined from the amount of insoluble and soluble aggregates as described under Materials and Methods ± SD ($n = 5$). (▨) A (5 m*M* phosphate, pH 7.4); (□) B (5 m*M* phosphate, pH 7.8); (▨) C (88 m*M* mannitol, 5 m*M* phosphate, pH 7.4); (□) D (88 m*M* mannitol, 5 m*M* phosphate, pH 7.8); (▨) E (250 m*M* mannitol, 5 m*M* phosphate, pH 7.4); (□) F (88 m*M* mannitol, 5 m*M* phosphate, pH 7.8), control.

of insoluble aggregates rather than the formation of soluble aggregates.

Effect of Mannitol

The addition of 88 m*M* mannitol appeared to increase LS slightly compared to phosphate alone at the same pH for slower cooling rates (Fig. 1, C vs A and D vs B). This trend was reversed, however, for the pH 7.4 formulations (A, C) at the fastest cooling rate. Formulation E contained the highest amount of mannitol (250 m*M*) and still showed an OD of 0.066 unit despite freezing at the lowest cooling rate of 0.5°C/min. It is therefore the least acceptable formulation in this study.

The addition of mannitol did not seem to promote the formation of soluble aggregates, regardless of the cooling rate (Fig. 2).

Adding 88 m*M* mannitol to the formulation could not be correlated with a decrease in total monomer content (Fig. 3). Adjusting the pH to 7.8 seemed to be a more important factor, independent of the presence of mannitol. Therefore, increasing the pH to 7.8 for the high-mannitol formulation (E) might well reduce the amount of particulates seen, purely from the observed pH effect.

Effect of Preparation Procedure

Although formulations F and D are very similar, there appeared to be a slight, albeit significant difference in LS after freezing at the fastest cooling rate (Fig. 1). The untreated control (F) showed slightly higher LS than the treated control (D). This observation might be due to the fact that D was filtered prior to freezing, whereas F was not.

The treated control (D) and the untreated control (F) were significantly different in content of soluble aggregates after freezing at the fastest cooling rate (Fig. 2). Formulation D contained about twice the amount of soluble aggregates of formulation F. Apparently, the preparation procedure did produce an unfavorable effect on aggregation of the protein, although the aggregates thus generated remained soluble. Interestingly, this effect is not seen in the same samples before the freeze/thaw cycle, within standard error.

The results measuring total monomer indicate that the amounts of soluble and insoluble aggregates encountered, even under the most adverse conditions employed in this study, are small, accounting for less than 10% of untreated control in most cases (Fig. 3).

DISCUSSION

This report outlines the effect of formulation and cooling rate on freezing of hGH solutions. In general, the lower pH (7.4) appears to promote the formation of insoluble aggregates, resulting in an increase in OD due to LS in the 340- to 360-nm range, whereas the higher pH (7.8) somewhat favors the formation of soluble aggregates. Presumably, the decreased solubility at lower pH largely results from moving closer to the pI of the protein (5.2), although the decreased solubility may also reflect an increase in the susceptibility of hGH to surface-induced denaturation through slight pH-induced conformational changes. The present data also suggest that the mechanisms of formation of soluble and insoluble aggregates may be different and that particulate matter is not always simply an extension in size or mass of soluble aggregate forms.

The results for formulations D (treated control) and F (untreated control) indicate a distinct sensitivity toward the formation of soluble aggregates as a result of prior sample handling and/or concentration changes. Although it is unclear if any one step is primarily responsible, the mechanical agitation of hGH during its concentration, transfer, dilution, and filtration and the exposure of hGH to various surfaces are likely causes for the increased propensity of surface-active hGH toward aggregation.

It is known that during freezing of any solution, the solutes (e.g., proteins, buffer salts, etc.) tend to concentrate due to formation of pure water ice crystals (18,30). This effect can be detrimental in protein solutions for two reasons. First, the salt concentration in the interstitial unfrozen liquid may increase to levels that could cause denaturation or salting out of the protein. In the case of buffer species present in the formulation, this effect might even result in dramatic pH changes and further denaturation of the protein. Second, the protein itself may increase in concentration, leading to aggregation of individual molecules (18,30).

What role the cooling rate plays in this freeze–concentration phenomenon has been described as follows: the slower the cooling rate, the larger are the ice crystals being formed. Conversely, fast freezing is believed to prevent extensive crystal growth and to substantially hinder the concentration and, hence, possible denaturation of the solutes (20). However, the present studies with hGH showed just the opposite tendency in terms of aggregate formation. An increase in cooling rate seemed to correlate with an increase in insoluble aggregates and a decrease in total content of monomeric hGH. Surface denaturation occurring at the relatively large ice–liquid interface between the protein and the tiny ice crystals that are formed upon rapid freezing may be a possible explanation. These crystals may act as sites for protein denaturation and particulate formation. This phenomenon may be less likely to play a major role with the slow freezing rate, in which case the pure ice crystals are much larger and present less surface area than that of smaller crystals.

It is also possible that the present observations might, in part, be due to the thawing as well as the freezing process. This hypothesis is supported by the fact that recrystallization is often observed during thawing of fast frozen solutions (31,32). In other words, the tiny ice crystals rearrange, which may further increase exposure of protein molecules to the ice–liquid interface and subsequent denaturation. The effect of varying heating rates on thawing of hGH solutions will be the subject of future studies.

The present studies suggest that very fast freezing of protein solutions, as is often recommended for certain enzymes (8–12), should be carried out with more caution. Although in many reported instances enzyme activity seemed to be maintained by instantaneous freezing, it is possible that the assays used to quantitate activity are simply not sensitive enough to detect minor changes due to aggregation of small (<5%) amounts of protein.

The variation of other critical parameters such as protein concentration and freezing profile, as well as investiga-

tion of the other phases of lyophilization (primary and secondary drying), will be the subject of future studies. In addition, the tendencies seen in these freeze/thaw studies need to be confirmed with results of more rigorous handling, such as shaking, in order to understand other factors that may contribute to aggregation/denaturation.

Combining the present results with future data will help in establishing guidelines for the freezing and thawing of hGH solutions and, hopefully, will provide some insight with regard to freezing mechanisms of protein solutions in general. This knowledge can be applied to freezing of bulk protein solutions, as is often required in holding or storage steps prior to final filling into vials, as well as to freezing being the initial step of lyophilization.

SUMMARY

The effect of freezing on aggregation of human growth hormone was studied with respect to cooling rate, excipients, and pH of the formulation. The content of soluble aggregates was largely unaffected by the cooling rate. However, increasing cooling rates were found to correlate with an increase in insoluble aggregates (particulates), measured as an increase in OD in the 340- to 360-nm range due to light scattering. This tendency was most pronounced with the fastest rate of 50°C/min.

The pH of the formulations was found to be critical also. Lower pH values (7.4) promoted the formation of particulates, thereby increasing light scattering of the protein solution. In contrast, at a higher pH (7.8) insoluble aggregates were not detected, even at the fastest cooling rate, although there was a slight increase in the amount of soluble aggregates.

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