Techniques for Assessing the Effects of Pharmaceutical Excipients on the Aggregation of Porcine Growth Hormone[1]

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Three denaturing techniques have been evaluated for their ability to induce irreversible aggregation and precipitation of recombinant porcine growth hormone (pGH). The denaturing stimuli included thermal denaturation, interfacial denaturation through the introduction of a high air/water interface by vortex agitation, and a guanidine (Gdn) HCl technique which involved rapid dilution of a partially unfolded state of pGH to nondenaturing conditions. Soluble and insoluble pGH fractions were evaluated for the presence of covalently modified species and soluble aggregates by size exclusion chromatography (SEC), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and isoelectric focusing (IEF). In each of the three denaturation methods, precipitation was found to be irreversible, as the precipitated pellet could not be solubilized upon resuspending in buffer. The soluble pGH fractions consisted of only monomeric material and the insoluble protein pellet could be completely solubilized with Gdn HCl or SDS. There was no evidence of detectable covalent modifications in the precipitated protein pellet following any of the three denaturation techniques. Three excipients, Tween 20, hydroxypropyl-β-cyclodextrin (HPCD), and sorbitol were evaluated for their stabilizing ability using each of the three denaturation methods and the degree of stabilization was found to be dependent upon the denaturing stimulus incorporated. Tween 20 was found to be highly effective in preventing pGH precipitation using the interfacial and Gdn techniques and was moderately effective using the thermal denaturation method. Inclusion of HPCD in the sample buffer significantly reduced precipitation using the thermal and interfacial methods but was ineffective in the Gdn technique. In contrast, sorbitol was ineffective in the interfacial technique and only moderately effective at high concentrations in reducing Gdn- and thermally-induced precipitation. These studies demonstrate the need to consider the nature of the denaturing stimulus when evaluating the potential protein-stabilizing properties of different pharmaceutical excipients.

KEY WORDS: porcine growth hormone; protein stability; protein aggregation; excipient effects.

INTRODUCTION

The aggregation of proteins in solution is a frequently encountered problem during refolding, purification, and formulation development. In terms of protein stability, aggregation is one of the least well-characterized aspects due primarily to the difficulties associated with quantitatively investigating the characteristics of the aggregated material. It is generally accepted that noncovalent aggregation occurs subsequent to denaturation and involves intermolecular interactions which are concentration dependent (1). Denaturation of globular proteins results in exposure of the more hydrophobic regions of the molecule (normally buried in the interior of the folded protein) to a relatively hydrophilic environment, thereby presenting the protein in a thermodynamically unstable state (2–4). It has been proposed that intermolecular protein interactions (i.e., aggregation) compete kinetically with intramolecular interactions (i.e., refolding) during protein renaturation accounting for the poor yields of native protein which are often observed (5,6).

Protein denaturation and aggregation can be induced by a variety of stress conditions, many of which are commonly encountered during purification procedures and formulation development. Principal factors contributing to protein denaturation in solution include exposure to extremes in temperature and pH, introduction of a high air/water or solid/water interface, and addition of organic or chaotropic solvents and certain additives. It is important to note that the aggregation of proteins in solution will not necessarily lead to precipitation, as the presence of insoluble aggregates will depend largely on the solubilizing properties of the solvent and the nature of the aggregate formed.

Numerous excipients have been cited in the patent and scientific literature for their ability to improve the physical stability of protein preparations by reducing aggregation (7,8). However, there are limited data regarding the mechanisms by which excipients interact with protein/solvent systems to reduce irreversible aggregation. If these data were readily available, it would provide a more rational scientific basis for the inclusion of excipients to prevent aggregation. Timasheff, Arakawa and co-workers have examined in detail the effects of selected solvent additives on the conformational stability of a number of globular proteins under conditions of reversible denaturation where aggregation was prevented through the use of low protein concentrations or nondenaturing concentrations of chaotropic solvents (for reviews see Refs. 9 and 10). These studies indicated that stabilization of the native protein structure by many of the additives tested occurred primarily through preferential hydration of the protein (i.e., preferential exclusion of the additive from the immediate solvent domain of the protein). While native-state structure stabilization would be expected to reduce the formation of aggregates and precipitates, other additional mechanisms could also account for the ability of excipients to reduce irreversible protein precipitation. In addition to stabilization of native protein structure, excipients might act by reducing the tendency for protein–protein interactions or by solubilizing unfolded species or protein aggregates.

The present investigation describes the characterization of three methods to induce aggregation and precipitation of a model protein (recombinant porcine growth hormone, pGH) as a prelude to the mechanistic investigation of the stabilizing effects of excipients under nonreversible conditions.

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MATERIALS AND METHODS

Chemicals

Lyophilized recombinant DNA-derived methionyl-pGH was prepared and generously supplied by Bresatec Ltd. (Adelaide, Australia). N-Terminal sequence analysis (Applied Biosystems Inc., Foster City, CA, Model 470A Protein Sequencer) confirmed a homogeneous product with an N-terminal sequence of Met-Phe-Pro-Ala-. Hydroxypropyl-β-cyclodextrin (HPCD) was a generous gift from Pharmatech Inc. (Alachua, FL). Tween 20, sodium dodecyl sulfate (SDS) (Sigma Chemicals, St. Louis, MO), sorbitol (Ajax Chemicals, New South Wales, Australia), and guanidine (Gdn) HCl (Mallinckrodt, Victoria, Australia) were of analytical grade quality. Acetonitrile (ACN) (Mallinckrodt) and trifluoroacetic acid (TFA) (Pierce, Rockford, IL) were of HPLC grade and were used as received. All other reagents were of at least analytical grade quality. Water was from a Milli-Q (Millipore, Milford, MA) water purification system. Cellulose acetate (0.22-μm) microcentrifuge filters were from Lida Corp. (Kenosha, WI).

Preparation of pGH Solutions

All experiments were conducted using 50 m*M* ammonium bicarbonate buffer adjusted to pH 8.50 following the addition of either Gdn or excipients. Filtration of pGH solutions was performed by microcentrifugation at 1700*g* for 5 min. Lyophilized pGH was reconstituted in buffer and filtered prior to use, and the concentration determined by UV absorbance (Cary 2290 Spectrophotometer, Varian Instruments, Victoria, Australia) at 278 nm using an absorptivity of 0.71 mL mg^{-1} cm^{-1} (15,714 M^{-1} cm^{-1}), which was determined (S. Bastiras, unpublished results) according to the method of Bewley (11). The UV absorbance of pGH was linearly related to concentration in the range 0.1 to 1 mg/mL.

Methods to Induce Aggregation

Thermal Denaturation. Samples of pGH (0.5 mg/mL) were heated in a thermostated water bath and the effects of temperature (25–70°C) and treatment time (15–120 min) on precipitation were determined. The concentration dependence of pGH precipitation was assessed by treating samples (0.25–1.0 mg/mL) for 60 min at 63°C. Following heat treatment, precipitation was allowed to proceed at room temperature for 30 min, after which the optical density due to light scattering was assessed at 450 nm using a UV-visible spectrophotometer (Cary 2290). Samples were then filtered to remove precipitated material and the UV absorbance of the filtrate was measured at 278 nm. Absorbance values for the treated samples were expressed relative to a control (an identical pGH solution receiving the same incubation time and filtration but no treatment) to obtain an estimate of the percentage of pGH remaining in solution.

Interfacial Denaturation. A high air/water interface was introduced into samples by vortexing (Lab-Line Instruments, Melrose Park, IL) 0.4-mL samples in 74 × 11-mm (i.d.) tubes. The effects of treatment time (15–120 sec) and pGH concentration (0.25–1.0 mg/mL) on precipitation were assessed. Following treatment, samples were equilibrated for 30 min at room temperature, after which the optical density and the filtrate UV absorbance were measured as described above.

Guanidine Dilution. The guanidine dilution technique involved a two-step dilution procedure and was an adaptation of a previously described method (12). In the first step, samples of pGH were prepared in Gdn buffers to give a protein concentration of 2 mg/mL and different Gdn concentrations ranging between 0 and 6 *M*. Samples were then equilibrated for 30 min at room temperature. In the second step, samples were diluted to produce a final pGH concentration of 0.2 mg/mL and a final Gdn concentration of 0.8 *M*, after which precipitation was allowed to proceed for 30 min at room temperature. Control solutions, incorporating the same pGH dilution procedure (i.e., 2 mg/mL diluted to 0.2 mg/mL) in the absence of Gdn, were included in all experiments.

The presence of precipitated pGH was monitored by the optical density at 450 nm. Samples were then filtered and analyzed by gradient elution HPLC (Beckman Instruments Inc., San Ramon, CA, Model 126 pump and 166 UV detector) using a 3 cm × 2.1-mm (i.d.) BU300 C4 column (Applied Biosystems Inc., Foster City, CA). The mobile phase solvents consisted of 0.11% TFA (solvent A) and 0.1% TFA in 70% ACN (solvent B) and elution of pGH was carried out using a linear gradient from 50 to 85% solvent B over 14 min and a flow rate of 0.6 mL/min. Detection was by UV absorbance at 220 nm. The percentage of pGH remaining in solution was calculated from the ratio of the peak area values for treated samples relative to the control solution.

Characterization of Soluble and Insoluble pGH

Following treatment procedures as described above, the soluble pGH fractions were analyzed for the presence of soluble aggregates and chemically modified pGH-related species using size exclusion chromatography (SEC), isoelectric focusing (IEF), and SDS–polyacrylamide gel electrophoresis (SDS-PAGE). The insoluble protein pellets, obtained by centrifuging the treated samples at 1600*g* for 15 min and decanting the supernatant, were solubilized using either 6 *M* Gdn for SEC analysis or 2.5% (w/v) SDS for SDS-PAGE evaluation.

Size Exclusion Chromatography. SEC was performed using a Superose 12 column (Pharmacia, Uppsala, Sweden) with a flow rate of 0.5 mL/min. The mobile phase was 100 m*M* ammonium bicarbonate (pH 8.50) for the soluble pGH fraction and 6 *M* Gdn HCl in 100 m*M* ammonium bicarbonate (pH 8.50) for the solubilized pellet fraction. The detection wavelength was either 220 or 278 nm using the bicarbonate mobile phase and 278 nm using the Gdn mobile phase. The injection volume was 20 μL for 220-nm detection and 100 μL for 278-nm detection.

Electrophoresis. Electrophoresis was conducted using the Phast system (Pharmacia, Uppsala, Sweden). Isoelectric focusing was performed using IEF pH 3–9 gels with application of 0.25 μg pGH. Gels were stained with 0.4% (w/v) silver nitrate according to the manufacturer's instructions. The isoelectric point of pGH was determined by comparison to a calibration curve using protein standards. SDS-PAGE was carried out under reducing (β-mercaptoethanol) and nonreducing conditions using gradient 8–25% polyacryl-

amide gels with the application of 0.2 μg pGH and staining with Coomassie blue R350. The molecular weight of pGH was estimated by comparison to a calibration curve using low molecular weight (14.4- to 94-kD) protein standards.

Effect of Excipients on Aggregation/Precipitation of pGH

Buffers (50 m*M* ammonium bicarbonate) containing selected excipients were prepared and the final pH was adjusted to 8.50. For thermal denaturation in the presence of excipients, samples (0.5 mg/mL pGH) were treated at 63°C for 1 hr. Interfacial denaturation was carried out by vortexing 0.5 mg/mL pGH samples for 60 sec. Guanidine dilution was performed in the presence of excipients using initial Gdn and pGH concentrations of 3.5 *M* and 2 mg/mL, respectively, with the final conditions being 0.8 *M* Gdn and 0.2 mg/mL pGH. The excipient concentration was the same in both the initial and the final Gdn solutions. Solutions of pGH in buffer (without excipient) were included in all experiments and subjected to the same treatment procedure as samples containing excipients.

All pGH solutions containing excipients and denatured by either the thermal or the interfacial methods were monitored by UV absorbance in the wavelength range of 240–400 nm prior to treatment and following treatment/filtration. The absorbance of the filtrate at 278 nm was compared to the absorbance of the same solution prior to treatment to calculate the percentage of pGH remaining in solution. Solutions of pGH containing excipients and denatured using the Gdn dilution method were analyzed by HPLC. The percentage of pGH remaining in solution was calculated as the ratio of peak areas for the treated samples relative to a control solution.

RESULTS AND DISCUSSION

Recombinant pGH is a 22-kD single-domain protein consisting of four antiparallel α-helices (13) and was used as a model protein in these studies due to its propensity to aggregate. Porcine GH displays 91% sequence homology to bovine growth hormone (bGH) (13) and spectroscopic studies have demonstrated that the equilibrium unfolding characteristics of pGH (14) are very similar to those previously described for bGH (15). In an analogous manner to bGH (12), there is the potential for involvement of an equilibrium unfolding intermediate of pGH in the aggregation process.

In the present investigation, different methods were assessed for their ability to induce aggregation of pGH under nonreversible conditions, and three potential excipients with differing physicochemical properties were evaluated for their ability to prevent aggregation and precipitation.

Methods to Induce Aggregation

Thermal Denaturation. Thermal denaturation is commonly used to study the thermodynamics of reversible protein unfolding under conditions where aggregation is avoided by the use of low protein concentrations or solubilizing (but nondenaturing) solvents (2,16,17). In the present study, heat was used as a means to induce the denaturation and subsequent aggregation/precipitation of pGH. Two potential caveats associated with thermally induced denaturation and precipitation are (i) that the formation of a precipitate may represent a process whereby the reversible equilibrium solubility of the native protein is exceeded under the conditions of study and (ii) that covalent chemical modifications may occur (in addition to noncovalent interactions), leading to formation of a precipitate. Covalent modifications at elevated temperature have been investigated for a number of proteins and are generally linked to β-elimination of cystine residues, disulfide interchange, and/or deamidation of asparagine/glutamine residues (18–20).

Figure 1 represents the precipitation profile following treatment of pGH (0.5 mg/mL) at different temperatures for 1 hr. The percentage of pGH remaining in solution following treatment decreased sharply between 50 and 70°C and a corresponding increase in the optical density at 450 nm was observed. The effect of treatment time on the extent of precipitation was investigated at 63°C (which represented approximately 50–60% protein loss following 1-hr treatment) and a pGH concentration of 0.5 mg/mL (Table IA). The percentage of pGH remaining in solution decreased with increasing treatment time (15–60 min), with no additional loss being apparent following 2 hr of treatment.

The relationship between the initial pGH concentration and the extent of precipitation following treatment (63°C for 1 hr) is presented in Table IB. Under these conditions, the extent of precipitation increased with increasing pGH concentration, suggesting that multimolecular processes played a role in the formation of precipitates. The concentration dependence of precipitation did not appear to be a simple function of the saturated solubility of pGH. In addition to an increase in the amount of precipitate formed following heat treatment, the concentration of pGH remaining in solution also increased with increasing initial concentration (Table IB), suggesting that the saturated solubility was not exceeded under the conditions of the study. Previous reports in the literature have indicated that the solubility of pGH (pH 8.0 Tris buffer, μ = 0.12, 37°C) is greater than 100 mg/mL (21). The concentration dependence of pGH precipitation upon heating is consistent with recent results reported for

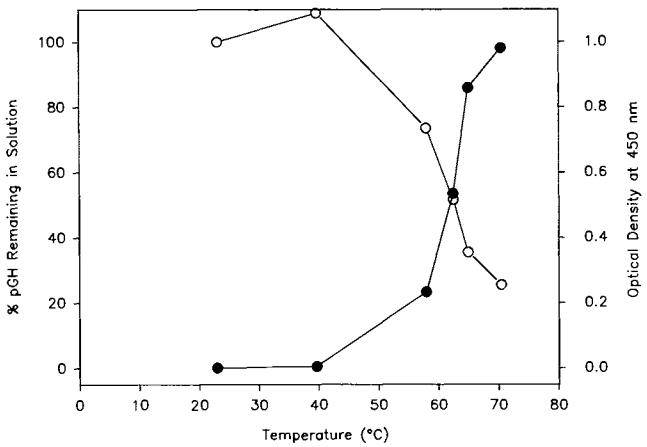

Fig. 1. Effect of temperature on the precipitation of pGH (0.5 mg/mL in pH 8.5 buffer) following a 1-hr treatment period. Data represent the optical density (450 nm) of unfiltered solutions (●——●) and the percentage of pGH remaining in solution (relative to an untreated control) after filtration (○——○). Refer to Materials and Methods for details of the procedure.

Table I. Effect of Treatment Time and Initial Protein Concentration on pGH Precipitation Following Thermal Denaturation

(A) Effect of treatment time on thermally induced precipitation[a]

pGH concentration (mg/mL)	Treatment time (min)	OD_{450}[b]	% pGH remaining[c]
0.5	0	0.009	100
0.5	15	0.533	60.3
0.5	30	0.568	54.9
0.5	60	0.711	49.5
0.5	120	0.770	49.5

(B) Effect of initial pGH concentration on thermally induced precipitation[a]

pGH concentration (mg/mL)	Treatment time (hr)	OD_{450}[b]	% pGH remaining[c]	mg/mL pGH remaining[d]
0.25	1	0.167	61.3	0.153
0.50	1	0.699	41.2	0.206
0.80	1	0.908	31.2	0.250
1.00	1	0.954	27.1	0.271

[a]Treatment involved heating samples at 63°C.
[b]Optical density at 450 nm used to monitor precipitation.
[c] Percentage remaining in solution calculated as the ratio of the absorbance (278 nm) of the treated sample filtrate to the absorbance of the filtrate for the untreated control.
[d]Concentration of pGH remaining in solution following treatment.

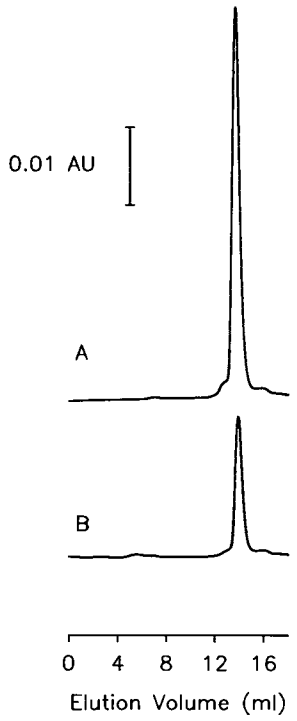

Fig. 2. Size exclusion chromatographic analysis of an untreated pGH control (0.5 mg/mL) solution (A) and the soluble pGH fraction from a sample heated at 63°C for 1 hr (B). Chromatography was conducted using a Superose 12 column, a 0.1 *M* ammonium bicarbonate, pH 8.5, mobile phase, a flow rate of 0.5 mL/min, detection at 278 nm, and an injection volume of 100 μL.

bGH, in which both the rate and extent of precipitate formation at 55°C were found to be highly concentration dependent (21).

The thermal denaturation/precipitation conditions which were used in all subsequent experiments (±excipients) included heating 0.5 mg/mL pGH samples at 63°C for 1 hr. Under these conditions, the method was reproducible, with the percentage of pGH remaining in solution being 45.9 ± 7.9% (mean ± SD; $n = 12$). The reversibility of precipitation following heat treatment was investigated by resuspending the precipitated material in fresh buffer, incubating at room temperature for 24 hr, and assaying the supernatant for pGH by HPLC. No pGH was detected in the supernatant of the pellet suspended in buffer, indicating that the precipitation was irreversible under the conditions of the study. Solubilization of the pellet in buffer containing 6 *M* Gdn HCl and analysis by SEC (6 *M* Gdn HCl in 0.1 *M* ammonium bicarbonate as the mobile phase) revealed that approximately 40–45% of the original pGH could be recovered in the precipitated material. There was no light scattering (OD at 450 nm) associated with the solubilized pellet fraction, suggesting that all of the precipitated material was solubilized with 6 *M* Gdn.

Figure 2 represents SEC chromatograms for an untreated control solution (A) and for the soluble pGH fraction remaining following heat treatment (B), both obtained using a detection wavelength of 278 nm. Under these conditions, the lower limit of detection of pGH was approximately 25 μg/mL (or 5% of the total pGH concentration). The chromatogram for the soluble pGH fraction indicated the presence of only monomeric protein and the absence of soluble aggregates, cleaved protein fragments, or covalently cross-linked species. While the 278-nm detection wavelength would detect only species containing aromatic residues, the same trend was also evident when the samples were analyzed using a detection wavelength of 220 nm, which would detect most peptide-related species. The slight shoulder which eluted just prior to the main peak in the untreated control chromatogram may represent a small percentage of covalently modified pGH. Interestingly, the shoulder was not apparent in the chromatogram of the soluble pGH fraction after heat treatment, indicating precipitation of the material in response to heat.

Figure 3 illustrates chromatograms for an untreated control solution (A) and the precipitated protein pellet solubilized in 6 *M* Gdn HCl (B), both obtained using 6 *M* Gdn HCl as the mobile phase and a detection wavelength of 278 nm. The small peak which eluted prior to the main peak in the control chromatogram again suggested the presence of a minor amount of covalently modified material which was not disrupted by 6 *M* Gdn. Importantly, the area of this peak was consistent in both the control chromatogram (Fig. 3A) and the chromatogram for the solubilized pellet (Fig. 3B), indicating that the material quantitatively precipitated upon heat treatment but was not formed as a direct result of the heat treatment. The precipitated protein solubilized in Gdn was found to consist primarily of denatured pGH, as the retention time of the major peak was identical to that for the untreated control solution.

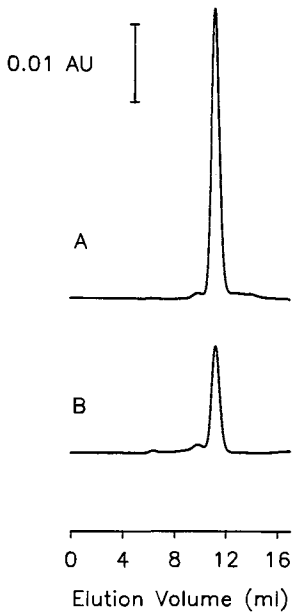

Fig. 3. Size exclusion chromatographic analysis of an untreated pGH control (0.5 mg/mL) solution (A) and the pGH pellet (solubilized with 6 *M* Gdn HCl) from a sample heated at 63°C for 1 hr (B). Chromatography was conducted as described in the legend to Fig. 2 using 0.1 *M* ammonium bicarbonate containing 6 *M* Gdn HCl, pH 8.5, as the mobile phase.

Analysis of the soluble pGH fraction by SDS-PAGE under reducing and nonreducing conditions demonstrated that the protein remaining following treatment had an apparent molecular weight which was consistent with that for an untreated control with no additional bands being detected. The soluble pGH fraction was further characterized using IEF, where it was observed that the p*I* was indistinguishable from that of an untreated control solution, indicating no significant changes in the concentrations of deamidated material. The precipitated protein pellet, solubilized with SDS, was also analyzed by reducing and nonreducing SDS-PAGE but neither technique detected the presence of high or low molecular weight species. Under the conditions employed for SDS-PAGE, the lowest level of detection was approximately 20 ng, or 10% by weight of the total pGH load.

The SEC and electrophoretic characterization of the soluble and insoluble pGH fractions indicated the absence of measurable concentrations of covalently modified species following heat treatment. The mass balance indicated that approximately 90% of the original material could be accounted for in the soluble fraction and the precipitated pellet. The remaining 10% could be associated with a dilution error in recovering the precipitated material, as no attempts were made to dry the pelleted fraction. While it is not possible to rule out the possibility of intrachain disulfide scrambling or β-elimination as the cause of pGH precipitation given the available data, the concentration dependence of precipitation infers that the process is polymolecular and consistent with aggregation being the principal source of precipitation. In addition, solubilization of the precipitate with Gdn HCl is indicative of the disruption of noncovalent associations and has been previously used as a test for covalent versus noncovalent thermoinactivation of enzymes (18).

Interfacial Denaturation. The surface activity of proteins has long been recognized and has been studied through the application of adsorbed monolayers (22). Protein adsorption at a water interface can lower the interfacial tension, leading to a reduction in the overall free energy and frequently results in protein denaturation (23,24). Aggregation can occur at the interface if the protein concentration in the adsorbed layer is sufficiently high or in the bulk solution upon desorption of the denatured protein (22).

The introduction of a high air/water interface, through agitation by vortexing, was evaluated as a means of inducing pGH denaturation and aggregation. This method had the advantage over agitation by shaking in that smaller sample volumes were required to obtain reproducible results. The influence of treatment time on the extent of precipitation using an initial pGH concentration of 0.5 mg/mL is presented in Fig. 4. The percentage of pGH remaining in solution decreased markedly with an increase in treatment time (0–60 sec) and was accompanied by a significant increase in the optical density at 450 nm. The extent of precipitation (expressed on a percentage basis) following exposure to the high air/water interface was independent of the initial pGH concentration in the range of 0.25 to 1 mg/mL. This may indicate that the interface formed upon vortexing was not saturated over the concentration range investigated, such that a constant fraction of pGH was exposed to interfacial denaturation. Conditions utilized in all subsequent experiments included vortexing 0.5 mg/mL samples for 60 sec. Under these conditions in the presence of buffer alone, the percentage of pGH remaining was 32.8 ± 6.6% (mean ± SD; $n = 9$).

No pGH was found in the supernatant of the precipitated pellet resuspended in fresh buffer and incubated for 24 hr, indicating that precipitation was irreversible. The soluble pGH fraction remaining in solution following treatment was characterized by SEC and found to be monomeric, whereas the pellet, solubilized in 6 *M* Gdn HCl buffer and analyzed by SEC, contained only denatured pGH and accounted for approximately 65–70% of the original pGH present.

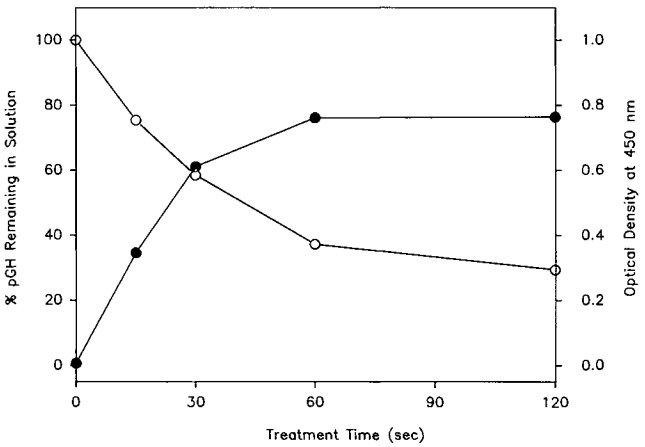

Fig. 4. Effect of treatment time on the precipitation of pGH (0.5 mg/mL in pH 8.5 buffer) following vortex agitation at a constant speed. Data represent the optical density (450 nm) of unfiltered solutions (●——●) and the percentage of pGH remaining in solution (relative to an untreated control) after filtration (○——○). Refer to Materials and Methods for details of the procedure.

The formation of insoluble precipitates following agitation or filtration of protein solutions has often been attributed to shear forces, however, it has been suggested that the increase in the air/water interface during agitation and filtration is the principal cause of denaturation and aggregation (25). A recent investigation with human growth hormone (hGH) evaluated aggregation at shear forces comparable to that encountered during mechanical shaking but avoided the introduction of an air/water interface by pumping solutions through a capillary tube (26). In these studies, aggregation was not observed when samples were subjected to shear only (shear rate − exposure time product of 8×10^3), indicating the likely involvement of the air/water interface in the denaturation and aggregation of hGH upon agitation. While the shear introduced into the pGH solutions by vortexing was most likely higher than that associated with mechanical shaking, the low exposure time would be expected to minimize the contribution of shear to the denaturation process.

Guanidine Dilution. The guanidine denaturation method was adapted from previous investigations with bGH conducted by Brems and co-workers (12). In these studies, a partially unfolded intermediate form of bGH, populated with intermediate concentrations of Gdn HCl, was much more susceptible to precipitation upon rapid dilution to nondenaturing conditions (i.e., low Gdn HCl concentrations) than were either the folded or the completely unfolded forms of bGH. The tendency of partially unfolded species to undergo precipitation in a manner similar to that reported for bGH has since been described for several other small globular proteins and appears to be consistent with aggregation of the intermediate states (27,28).

The equilibrium guanidine unfolding profile of pGH displays similar characteristics to that reported for bGH when monitored by spectroscopic techniques (14). The guanidine denaturation and dilution procedure described for bGH was investigated as a further means of inducing the aggregation and precipitation of pGH. Precipitation was accomplished by diluting pGH solutions containing variable initial Gdn concentrations into nondenaturing (low-Gdn concentration) buffers. As shown in Fig. 5, precipitation of pGH was maximal when the initial Gdn concentration was in the range of 2.5 to 3.5 *M*. The percentage remaining in solution when samples were diluted from 2 mg/mL pGH and 3.5 *M* Gdn to 0.2 mg/mL pGH and 0.8 *M* Gdn was 6.6 ± 2.2% (mean ± SD; $n = 5$). The profile of pGH precipitation depicted in Fig. 5 is consistent with the reported studies for bGH, as well as other globular proteins, in that the partially unfolded form is more susceptible to aggregation and precipitation than either the folded or the unfolded species.

Only monomeric material was observed when the soluble pGH fraction remaining following the dilution procedure was assessed by SEC. Solubilization of the precipitated pellet with 6 *M* Gdn HCl and analysis by SEC revealed the presence of only denatured pGH and accounted for approximately 80% of the original material.

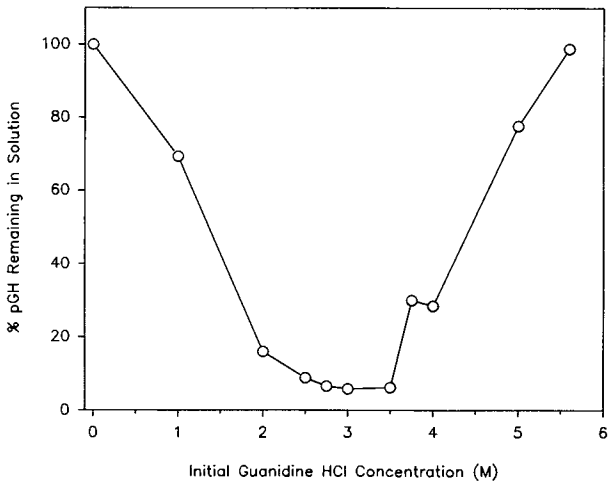

Fig. 5. Effect of the initial Gdn HCl concentration on the precipitation of a 2 mg/mL pGH solution when diluted to a final pGH concentration of 0.2 mg/mL and a Gdn HCl concentration of 0.8 *M*. The data are expressed as a percentage relative to control pGH solutions which did not contain Gdn HCl. Refer to Materials and Methods for details of the procedure.

Excipient Effects on Aggregation/Precipitation of pGH

From the wide range of excipients reported as protein stabilizers in the patent and scientific literature (for reviews see Refs. 7 and 8), three excipients with different physicochemical properties were evaluated for their effects on the aggregation and precipitation of pGH using the three methods described above. The excipients investigated included (i) Tween 20, which has been cited for its ability to reduce aggregation and precipitation (29), (ii) HPCD, which has been shown to increase solubility and decrease aggregation of selected proteins (30,31), and (iii) sorbitol, which has documented utility in stabilizing native protein structure (32–34).

Figure 6 presents the effect of different Tween 20 concentrations on the precipitation of pGH from solutions which were subjected to either heat, interfacial, or Gdn-induced denaturation and precipitation. All of the concentrations of Tween 20 studied were above the reported CMC value of 0.006% (w/v) (35). The nonionic surfactant was effective in preventing pGH precipitation in both the Gdn and the inter-

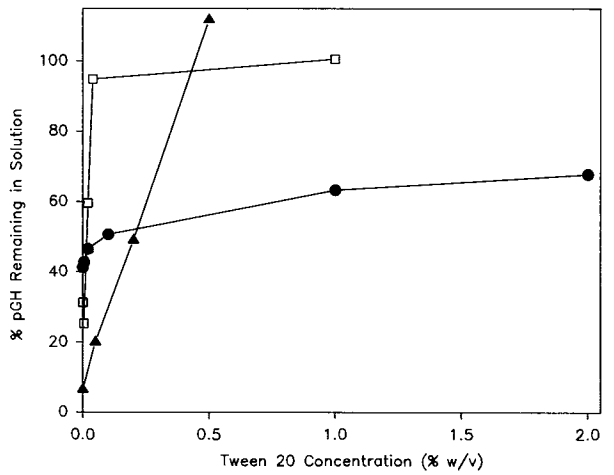

Fig. 6. Effect of Tween 20 (%, w/v) on the percentage of pGH remaining in solution after being subjected to either thermal (●——●), interfacial (□——□) or Gdn-induced (▲——▲) denaturation and precipitation. Refer to Materials and Methods for details of the procedures.

facial techniques, with near-quantitative "protection" at concentrations above 0.5% (w/v). In contrast, Tween 20 was only marginally effective in preventing the heat-induced denaturation and precipitation of pGH at concentrations up to 2% (w/v). When the effect of HPCD on pGH precipitation was studied in an analogous manner to that described for Tween 20, a different profile of excipient based effects was observed (Fig. 7). HPCD was very effective in preventing precipitation resulting from thermal and interfacial denaturation but ineffective when evaluated using the Gdn dilution procedure. Sorbitol was also evaluated in the three denaturation procedures and the data are presented in Fig. 8. In contrast to Tween 20 and HPCD, sorbitol offered no advantage against precipitation when evaluated in the interfacial procedure and was only marginally effective when included at high concentrations in the thermal and guanidine procedures.

The characteristics of the soluble pGH fractions remaining following the thermal, interfacial, and Gdn dilution procedures in the presence of each of the three excipients were examined using SEC. For samples which were subjected to either the thermal or the interfacial denaturation methods, the soluble pGH fractions in the presence of excipients had retention times which were indistinguishable from that of a control solution, indicating either that there was no interaction between the excipient and the pGH present in solution or that any interaction was reversible under the conditions used for the SEC. In comparison, pGH samples containing Tween 20 and subjected to the Gdn dilution method displayed no protein peaks when analyzed by SEC. This trend was evident even for samples containing 0.5% (w/v) Tween 20, which displayed very little pGH loss as analyzed by HPLC (Fig. 6). However, inclusion of 0.5% (w/v)Tween 20 in the mobile phase and subsequent analysis of the soluble fraction remaining following the Gdn dilution procedure revealed a peak for pGH with a retention time identical to that for an untreated control analyzed using the same SEC method.

The effects of the excipients on the full Gdn precipitation profile were investigated by HPLC and these data are

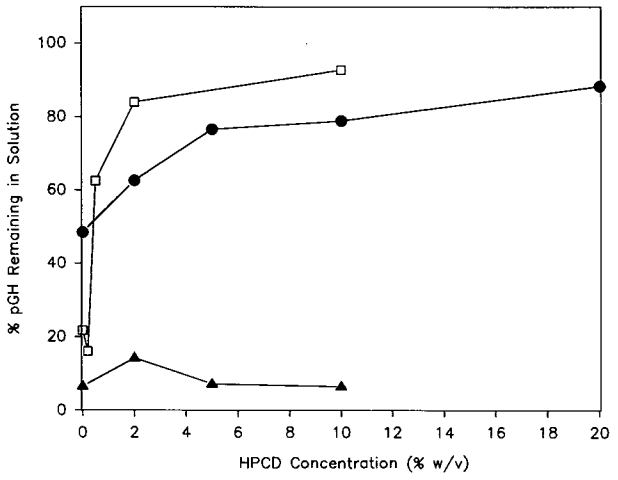

Fig. 7. Effect of HPCD (%, w/v) on the percentage of pGH remaining in solution after being subjected to either thermal (●——●), interfacial (□——□), or Gdn-induced (▲——▲) denaturation and precipitation. Refer to Materials and Methods for details of the procedures.

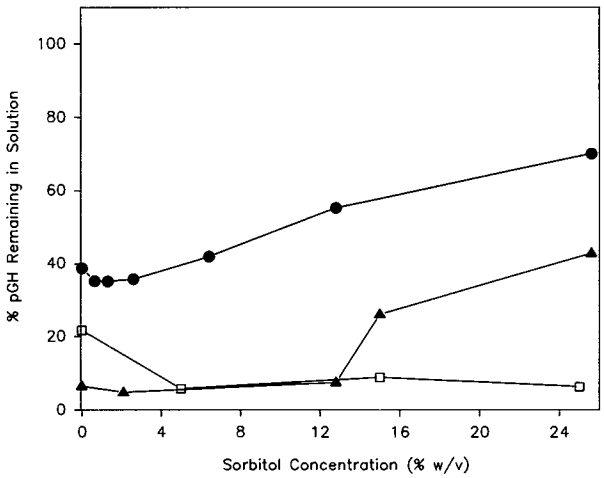

Fig. 8. Effect of sorbitol (%, w/v) on the percentage of pGH remaining in solution after being subjected to either thermal (●——●), interfacial (□——□), or Gdn-induced (▲——▲) denaturation and precipitation. Refer to Materials and Methods for details of the procedures.

presented in Fig. 9. At a concentration of 0.5% (w/v), Tween 20 was found to inhibit the precipitation of pGH at initial Gdn concentrations ranging between 0 and 6 *M*. Although HPCD and sorbitol were ineffective in preventing pGH precipitation from an initial Gdn concentration of 3.5 *M* (used to generate the corresponding data in Figs. 6–8), the pGH precipitation profiles in the presence of these excipients (2% w/v and 15% w/v, respectively) were displaced to higher initial Gdn concentrations relative to buffer alone.

The nature of the effects of Tween 20, HPCD, and sorbitol on the aggregation of pGH is currently under investigation. Potential mechanisms for stabilization include (i) sta-

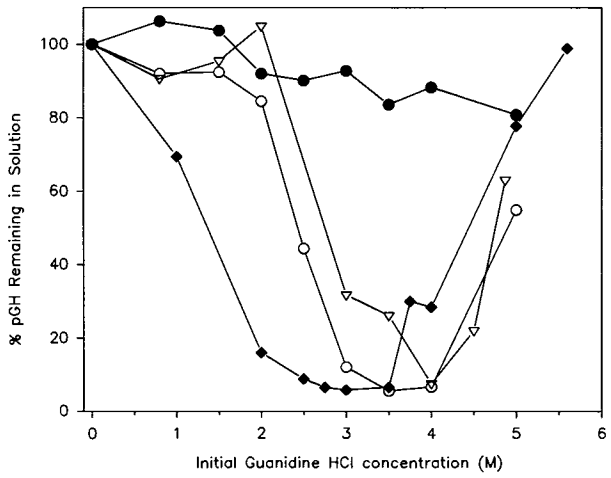

Fig. 9. Effect of excipients on the Gdn HCl precipitation profile of pGH determined by HPLC. Initial Gdn HCl solutions containing 2 mg/mL pGH were diluted to a final pGH concentration of 0.2 mg/mL and a Gdn HCl concentration of 0.8 *M*. Excipient concentrations were kept constant in the initial and final buffers. The solutions studied were pH 8.5 buffer (◆——◆), buffer containing 0.5% (w/v) Tween 20 (●——●), 2% (w/v) HPCD (○——○), and 15% (w/v) sorbitol (▽——▽). The data are expressed as a percentage relative to control pGH solutions which did not contain Gdn HCl. Refer to Materials and Methods for details of the procedure.

bilization of the native, folded structure of pGH, (ii) stabilization or solubilization of an unfolded (or partially unfolded) species, and (iii) alterations in properties of the solvent, thereby reducing protein–protein interactions. Solubilization of aggregated species is not considered a potential mechanism of stabilization with these excipients under the conditions used to induce precipitation, as there was no evidence of high molecular weight species upon analysis of the soluble fractions by SEC.

The effectiveness of Tween 20 in preventing interfacially induced denaturation and precipitation of pGH is most likely a function of its surface activity. At concentrations above the CMC, Tween lowers the surface tension of water by approximately 30 mN/m (35), which would be expected to decrease the driving force for protein adsorption. In addition, the presence of adsorbed Tween at the interface would reduce the available "sites" for protein adsorption.

The concentrations of Tween 20 necessary for preventing pGH precipitation using the Gdn dilution method were also above the CMC, which is consistent with a previous study of nonionic detergent-assisted refolding of rhodanese where concentrations of a similar magnitude were necessary to prevent aggregation (36). In the rhodanese studies, there was no evidence of a specific interaction between the enzyme and the nonionic surfactant micelles and the importance of the CMC was interpreted in terms of maximizing the monomeric concentration of surfactant. Although the effects of Tween 20 on the solution conformation of pGH have not yet been determined, previous reports describing the stabilizing effects of surfactants on insulin have indicated a possible role of nonmonomeric forms of surfactants in decreasing precipitate and aggregate formation (29).

Few reports exist in the literature describing the effects of HPCD on protein stability, and consequently the potential mechanisms of stabilization remain speculative. Recent reports have indicated that HPCD reduces agitation-induced aggregation of hGH (37), reduces aggregation of interleukin-2 (IL-2) during lyophilization/reconstitution, and stabilizes insulin solutions against aggregation during room temperature storage (31). The effectiveness of HPCD in reducing interfacially induced precipitation of pGH may be related to its surface activity in a manner analogous to that proposed for Tween 20 [the reported surface tension of a 0.1% (w/v) solution of HPCD is 61 mN/m, relative to 72 mN/m for water at 25°C (30)]. The shift in the Gdn precipitation profile (to higher Gdn concentrations) in the presence of 2% w/v HPCD could be indicative of either native-state structure stabilization or stabilization/solubilization of a partially unfolded species. Brewster and co-workers have indicated that the inclusion of HPCD in formulations of IL-2 does not alter bioactivity or conformation when assessed by fourth-derivative UV spectroscopy (31). These data suggest either that the interaction between HPCD and the protein is reversible or that the effect of HPCD arises through changes in the properties of the solvent.

The effects of sorbitol on the thermal- and Gdn-induced precipitation profiles of pGH are most likely related to native state structure stabilization of pGH consistent with the known effects of polyhydric alcohols on conformational stability (32–34). Stabilization of the native structure in the presence of sorbitol would require higher temperatures or Gdn concentrations to induce unfolding and likewise, to form a partially unfolded species. While sorbitol was ineffective in preventing precipitation in the Gdn method, the shift in the precipitation profile is consistent with stabilization of the folded state. Although the presence of the partially unfolded intermediate in the presence of sorbitol has not yet been confirmed, the similarity between the Gdn precipitation profiles in buffer and those in buffer containing sorbitol suggests similar mechanisms of precipitation.

In summary, this controlled study was designed to assess empirically the utility of three denaturing techniques to induce the noncovalent, irreversible aggregation and precipitation of pGH at low solution concentrations. The differing efficacy of these excipients clearly demonstrates the need to consider the nature of the denaturing stimulus when evaluating the stabilizing potential of pharmaceutical excipients. Investigations regarding the mechanisms of pGH stabilization with these excipients are ongoing.

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